

UNIVERSIDADE FEDERAL DE MINAS GERAIS

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**COVID-19 E SAÚDE REPRODUTIVA MASCULINA: consequências da infecção e
métodos inovadores de diagnóstico**

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Marcelo Horta Furtado

COVID-19 E SAÚDE REPRODUTIVA MASCULINA: consequências da infecção e métodos inovadores de diagnóstico

Tese apresentada ao Programa de Pós-Graduação em Biologia Celular do Departamento de Morfologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial para a obtenção do título de Doutor em Ciências.

Orientador: Dr. Guilherme Mattos Jardim Costa

Coorientadora: Dra. Samyra Maria dos Santos Nassif Lacerda

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Às treze horas do dia **12 de dezembro de 2025**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora da Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho final intitulado: **"COVID-19 E SAÚDE REPRODUTIVA MASCULINA: CONSEQUÊNCIAS DA INFECÇÃO E MÉTODOS INOVADORES DE DIAGNÓSTICO"**, requisito final para obtenção do grau de Doutor em Biologia Celular. Abrindo a sessão, o Presidente da Comissão, **Dr. Guilherme Mattos Jardim Costa**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato, para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

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Pelas indicações, o candidato foi considerado: **Aprovado**

O resultado final foi comunicado publicamente ao candidato pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 12 de dezembro de 2025.**

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“Live as if you were to die tomorrow. Learn as if you were to live forever.” (Mahatma Gandhi)

RESUMO

O SARS-CoV-2 demonstrou capacidade de afetar órgãos extrapulmonares, incluindo o sistema reprodutor masculino, que apresenta alta expressão de receptores de entrada viral como o ACE2. Embora estudos iniciais tenham sugerido a vulnerabilidade dos testículos à infecção, a extensão real do envolvimento viral, sua persistência no trato genital masculino e suas implicações para a saúde reprodutiva permanecem pouco compreendidas. Este estudo teve como objetivo investigar se o SARS-CoV-2 infecta e se replica nos testículos, se persiste no sêmen após a recuperação clínica e se induz alterações funcionais na qualidade espermática, mesmo quando os parâmetros seminais convencionais permanecem normais. Para isso, conduzimos uma investigação multifacetada que incluiu a análise de tecido testicular de pacientes não vacinados falecidos por COVID-19, a avaliação longitudinal do sêmen de indivíduos recuperados de forma leve ou moderada da doença e a aplicação de modelos avançados de aprendizado de máquina em amostras seminais. Nos tecidos testiculares, RNA e proteínas virais foram detectados por RT-qPCR com *primers* específicos, por imunofluorescência, por nanosensores e por microscopia eletrônica de transmissão. Partículas virais e fábricas de replicação foram observadas principalmente em macrófagos e células espermatogoniais, acompanhadas de perda de células germinativas, ruptura da barreira de células de Sertoli, inflamação e fibrose. Em pacientes recuperados, o RNA viral foi identificado em 55% das amostras de sêmen coletadas até seis meses após a infecção, com infectividade confirmada por cultura celular em VERO e por visualização de partículas virais. Apesar de manter parâmetros seminais dentro dos critérios de normalidade da OMS, modelos de aprendizado de máquina aplicados a dados espermáticos e espectroscópicos distinguiram com alta acurácia as amostras de pacientes pós-COVID-19 em relação aos controles, com taxas de acerto de 77% e 90%, respectivamente. Esses achados demonstram que o SARS-CoV-2 pode infectar e replicar nos testículos, persistir no sêmen por longos períodos após a recuperação e induzir alterações subclínicas que não são detectadas por análises convencionais. Em conjunto, os dados indicam que o trato genital masculino pode atuar como alvo e reservatório viral, reforçando a importância do acompanhamento reprodutivo de longo prazo e da incorporação de ferramentas diagnósticas avançadas na avaliação da fertilidade masculina após a COVID-19.

Palavras-chave: COVID-19, SARS-CoV-2, sêmen, testículo, sistema reprodutor masculino

ABSTRACT

SARS-CoV-2 has been shown to affect extrapulmonary organs, including the male reproductive system, which expresses high levels of viral entry receptors such as ACE2. Although early studies suggested the testis might be vulnerable to infection, the true extent of viral involvement, its persistence in the male genital tract, and its implications for reproductive health remain uncertain. This study aimed to investigate whether SARS-CoV-2 infects and replicates in the testes, persists in semen after clinical recovery, and induces functional alterations in sperm quality, even when conventional semen parameters appear normal. To this end, we conducted a multifaceted investigation involving testicular tissue analysis from non-vaccinated deceased COVID-19 patients, longitudinal semen evaluation from recovered individuals with mild or moderate disease, and advanced machine learning analysis of semen samples. In testicular specimens, SARS-CoV-2 RNA and protein were detected using RT-qPCR with gene-specific primers, immunofluorescence, nanosensors, and transmission electron microscopy. Viral particles and replication factories were found primarily in macrophages and spermatogonial cells, accompanied by germ cell loss, disruption of the Sertoli cell barrier, inflammation, and fibrosis. In recovered patients, viral RNA was detected in 55% of semen samples collected up to 6 months post-infection, and viral infectivity was confirmed by VERO cell culture and visualization of viral particles. Despite normal semen profiles according to WHO criteria, machine learning models trained on sperm parameters and optical spectroscopy data successfully distinguished COVID-19-recovered samples from controls, achieving accuracies of 77% and 90%, respectively. These findings demonstrate that SARS-CoV-2 can infect and replicate in the testis, persist in semen long after recovery, and induce subclinical alterations that are not captured by standard analyses. Taken together, the data suggest the male genital tract may serve both as a target and a reservoir for SARS-CoV-2, reinforcing the need for long-term reproductive monitoring and the integration of advanced diagnostic tools into the evaluation of male fertility following COVID-19.

Keywords: COVID-19, SARS-CoV-2, semen, testicle, male reproductive tract

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1. INTRODUÇÃO E JUSTIFICATIVA

A pandemia de COVID-19, causada pelo coronavírus SARS-CoV-2, tornou-se um dos eventos mais disruptivos da saúde pública global em décadas, afetando profundamente sociedades, economias e sistemas de saúde em todo o mundo. Desde seu surgimento, em dezembro de 2019, a COVID-19 se alastrou rapidamente, infectando mais de 778 milhões de pessoas e resultando em mais de 7 milhões de mortes reportadas até outubro de 2025 ao redor do mundo [1]. Embora as campanhas de vacinação em massa, assim como a evolução natural do vírus, que resultou em variantes menos letais, tenham contribuído significativamente para reduzir a gravidade dos casos e o número de óbitos, a pandemia continua a representar um desafio. Além da fase aguda da doença, surgiram novos desdobramentos clínicos, como a COVID longa, uma condição crônica que pode persistir por meses ou até anos, afetando a qualidade de vida de inúmeros pacientes e gerando impactos duradouros nos sistemas de saúde e na economia global [2].

A COVID longa, também conhecida como "síndrome pós-COVID-19" ou "sequelas pós-agudas da infecção por SARS-CoV-2 (PASC)", é caracterizada por uma miríade de sintomas que variam desde fadiga extrema e dificuldades respiratórias até comprometimento cognitivo e disfunções em múltiplos órgãos [3]. Embora sua fisiopatologia exata ainda não seja plenamente compreendida, estudos apontam para a persistência viral em certos tecidos, disfunções imunológicas, inflamação crônica e alterações metabólicas e mitocondriais [4-13]. Esses fatores podem contribuir para a manutenção dos sintomas a longo prazo, mesmo em indivíduos que apresentaram uma forma leve da infecção aguda [4, 14]. Nesse contexto, o impacto da COVID-19 sobre o sistema reprodutivo masculino tornou-se um tema de crescente interesse científico, dada a alta prevalência do receptor ACE2 nos testículos, órgão vital para a produção de hormônios sexuais e espermatozoides [15].

O SARS-CoV-2 utiliza a enzima conversora de angiotensina 2 (ACE2) como porta de entrada para invadir as células hospedeiras [16]. A abundante presença desse receptor em diversas células testiculares, como as células de Leydig, Sertoli e espermatogônias, coloca os testículos entre os principais alvos extrapulmonares do vírus [17, 18]. Embora pesquisas preliminares já tenham mostrado que o SARS-CoV-2 pode causar danos a diversos órgãos, como os pulmões, coração e rins, os efeitos diretos sobre os testículos ainda são pouco

explorados e compreendidos [19, 20]. No entanto, é plausível que o vírus, assim como o SARS-CoV, que utiliza o mesmo receptor celular, possa causar orquites e inflamação testicular grave, prejudicando a produção de espermatozoides e de testosterona [21]. A extrapolação desses dados ao SARS-CoV-2 sugere que os testículos podem estar sujeitos a danos semelhantes, com potencial para afetar a fertilidade masculina, especialmente em homens não vacinados ou que tenham apresentado formas graves da infecção.

Estudos recentes sugerem que a infecção pelo SARS-CoV-2 pode levar à infiltração leucocitária nos testículos, causando danos às células de Leydig e ao epitélio seminífero, o que pode resultar em disfunção da síntese hormonal e da espermatogênese [22-25]. Além disso, o aumento de citocinas inflamatórias pode desencadear uma resposta autoimune nos túbulos seminíferos, comprometendo ainda mais a função testicular [26]. Esses possíveis danos reforçam a necessidade de investigar a fundo o papel do vírus no ambiente testicular e suas consequências a longo prazo sobre a fertilidade masculina, especialmente em homens não vacinados que foram gravemente acometidos pela COVID-19.

Um aspecto ainda mais alarmante refere-se à possível presença do vírus no sêmen. A presença do SARS-CoV-2 no sêmen tornou-se uma questão de grande relevância, especialmente devido à possibilidade de transmissão sexual. Diversos estudos sobre outros vírus, como o HIV, Zika, hepatite B e C, mostraram que eles podem permanecer viáveis no sêmen por longos períodos, mesmo após a infecção aguda ter sido resolvida em outros fluidos corporais [27-29]. Da mesma forma, há uma crescente preocupação de que o SARS-CoV-2 possa replicar-se no ambiente testicular e ser detectado no sêmen [30, 31]. No entanto, até o momento, os resultados dos estudos são conflitantes. Algumas pesquisas não conseguiram detectar o vírus no sêmen [32], enquanto outras o detectaram em quantidades significativas, especialmente em casos de infecção grave [33-38].

Essas discrepâncias podem ser atribuídas à variabilidade dos métodos de detecção empregados [39]. O RT-qPCR, amplamente utilizado para a detecção de COVID-19, pode não ser suficientemente sensível em amostras seminais, resultando em falsos negativos [40, 41]. Em estudos recentes, o SARS-CoV-2 foi detectado no parênquima testicular de pacientes que faleceram em decorrência da COVID-19 [22-25]. Esse achado reforça a hipótese de que o vírus pode ser eliminado no sêmen e, potencialmente, transmitido por via sexual, o que coloca em

risco não apenas parceiros sexuais, mas também profissionais de saúde, como urologistas e embriologistas que lidam com material seminal em procedimentos de reprodução assistida.

Entender a relação entre o SARS-CoV-2, o testículo e o sêmen não é apenas uma questão de ciência básica; tem implicações práticas cruciais para a saúde reprodutiva masculina e para a saúde pública em geral. A avaliação do sêmen como biomarcador da saúde masculina oferece uma nova janela para o diagnóstico de complicações pós-COVID-19. Vários trabalhos têm mostrado uma queda dos parâmetros espermáticos seminais, principalmente nos primeiros 3 meses após o início dos sintomas da COVID-19 [42-51]. Homens com parâmetros seminais alterados, além de apresentarem maior risco de infertilidade, também podem apresentar taxas mais elevadas de mortalidade a longo prazo, como demonstrado em estudos anteriores que correlacionam parâmetros seminais ruins com a saúde geral do homem [52-55]. Assim, a avaliação das características seminais em homens recuperados da COVID-19 pode fornecer insights importantes não apenas sobre a fertilidade, mas também sobre a saúde geral.

Nesse sentido, a inteligência artificial (IA) e o aprendizado de máquina (ML) emergem como ferramentas promissoras para melhorar o diagnóstico e o prognóstico na medicina reprodutiva [56]. Nos últimos anos, o uso de IA para modelagem preditiva na área da saúde tem apresentado resultados impressionantes, permitindo a identificação de padrões complexos e de informações ocultas que métodos estatísticos convencionais não conseguem revelar [57, 58]. No contexto da COVID-19, a aplicação do aprendizado de máquina ao sêmen pode ser uma ferramenta promissora para diferenciar amostras de pacientes infectados e não infectados, com potencial para prever desfechos reprodutivos e detectar alterações que não seriam percebidas em análises de rotina, de forma rápida e precisa.

Nosso grupo de pesquisa tem avançado nessa área ao utilizar a espectroscopia em combinação com o aprendizado de máquina para analisar amostras de sêmen de camundongos infectados pelo vírus Zika [59]. Por meio dessa abordagem, conseguimos distinguir, com alta precisão, amostras de espermatozoides de camundongos infectados e de camundongos saudáveis. Ao expandir essa metodologia para pacientes humanos, estamos explorando a capacidade da IA de diferenciar o sêmen de homens que nunca foram infectados pelo SARS-CoV-2 de aqueles que se recuperaram da infecção, com o objetivo de identificar alterações sutis, porém significativas, que possam indicar danos persistentes à fertilidade.

A integração de IA e espectroscopia não só proporciona diagnósticos rápidos e confiáveis, mas também representa um avanço tecnológico importante para a triagem de homens com COVID longa e para a compreensão dos impactos reprodutivos da infecção. Esse método inovador, além de ser eficiente e econômico, pode se tornar uma ferramenta de grande valor para urologistas e centros de reprodução, ajudando a identificar problemas de fertilidade de maneira menos invasiva e mais precisa do que as técnicas tradicionais.

Em suma, a interseção entre a infecção pelo SARS-CoV-2, a saúde reprodutiva masculina e o avanço das tecnologias de IA abre novas fronteiras na pesquisa científica. Entender como a COVID-19 afeta os testículos e o sêmen é essencial para o desenvolvimento de intervenções terapêuticas direcionadas, bem como para a prevenção de complicações de longo prazo à fertilidade. Ao integrar tecnologias inovadoras, como o aprendizado de máquina, à pesquisa biomédica, podemos não apenas aprimorar a compreensão da patologia da COVID-19, mas também oferecer soluções mais eficazes para mitigar seus impactos duradouros na saúde reprodutiva masculina, contribuindo para um futuro mais saudável no período pós-pandêmico.

2. OBJETIVOS

2.1 Objetivo geral

Avaliar a presença e persistência do vírus SARS-CoV-2 no testículo e sêmen e suas consequências para a saúde reprodutiva masculina.

2.2 Objetivos específicos

- Identificar a presença, a persistência e a infectividade do SARS-CoV-2 no testículo de pacientes que vieram a óbito por COVID-19 (amostras post-mortem) e no sêmen de pacientes que se recuperaram de COVID-19 (convalescentes);
- Otimizar a detecção do SARS-CoV-2 no parênquima testicular por meio de diferentes metodologias complementares e, em seguida, avaliar as consequências morfofisiológicas da COVID-19 no testículo humano;
- Realizar análise dos parâmetros seminais de pacientes que se recuperaram de um quadro clínico de COVID-19 leve ou moderada, com o intuito de identificar possíveis alterações no sêmen que possam impactar a fertilidade;
- Desenvolver e aplicar ferramentas de inteligência artificial para diferenciar padrões seminais de indivíduos que nunca contraíram COVID-19 de indivíduos que se recuperaram, a fim de melhorar o diagnóstico e o prognóstico do impacto da doença no sistema reprodutivo masculino.

3. RESULTADOS

3.1 Artigo 1

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RESEARCH ARTICLE

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High SARS-CoV-2 tropism and activation of immune cells in the testes of non-vaccinated deceased COVID-19 patients

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Abstract

Background Cellular entry of SARS-CoV-2 has been shown to rely on angiotensin-converting enzyme 2 (ACE2) receptors, whose expression in the testis is among the highest in the body. Additionally, the risk of mortality seems higher among male COVID-19 patients, and though much has been published since the first cases of COVID-19, there remain unanswered questions regarding SARS-CoV-2 impact on testes and potential consequences for reproductive health. We investigated testicular alterations in non-vaccinated deceased COVID-19-patients, the precise location of the virus, its replicative activity, and the immune, vascular, and molecular fluctuations involved in the pathogenesis.

Results We found that SARS-CoV-2 testicular tropism is higher than previously thought and that reliable viral detection in the testis requires sensitive nanosensors or RT-qPCR using a specific methodology. Through an in vitro experiment exposing VERO cells to testicular macerates, we observed viral content in all samples, and the subgenomic RNA's presence reinforced the replicative activity of SARS-CoV-2 in testes of the severe COVID-19 patients. The cellular structures and viral particles, observed by transmission electron microscopy, indicated that macrophages and spermatogonial cells are the main SARS-CoV-2 lodging sites, where new virions form inside the endoplasmic reticulum Golgi intermediate complex. Moreover, we showed infiltrative infected monocytes migrating into the testicular parenchyma. SARS-CoV-2 maintains its replicative and infective abilities long after the patient's infection. Further, we demonstrated high levels of angiotensin II and activated immune cells in the testes of deceased patients. The infected testes show thickening of the tunica propria, germ cell apoptosis, Sertoli cell barrier loss, evident hemorrhage, angiogenesis, Leydig cell inhibition, inflammation, and fibrosis.

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Conclusions Our findings indicate that high angiotensin II levels and activation of mast cells and macrophages may be critical for testicular pathogenesis. Importantly, our findings suggest that patients who become critically ill may exhibit severe alterations and harbor the active virus in the testes.

Keywords SARS-CoV-2 replication, Macrophages, Spermatogonia, Sertoli cell, Leydig cell, Renin-angiotensin system, Infertility, Spermatogenesis, Testosterone, Nanotechnology

Background

Since the testis displays one of the highest expressions of angiotensin-converting enzyme 2 (ACE2) receptors, which mediate the cellular entry of SARS-CoV-2, and current data suggest that men are more affected than women [1], deep testicular evaluations of patients affected by COVID-19 is imperative. Furthermore, previous studies present discordant results concerning SARS-CoV-2 detection in testicular parenchyma through RT-PCR [2–5] and the extent of testicular damage caused by the virus [5]. Although some testicular alterations promoted by SARS-CoV-2 infection were previously shown [2–5], many of the molecular players of testicular pathogenesis in COVID-19 remain unknown. Indeed, several questions about viral infection remain unexplored, such as the viral replication, route of infection, and the identity of infected cells.

Thus, studying the cellular, enzymatic, hormonal, and critical gene alterations in the testes of non-vaccinated COVID-19 patients should contribute to a better understanding of SARS-CoV-2 biology and its possible impact on testes and male fertility. Recently, Edenfield and Easley [6] published a perspective article in *Nature Reviews Urology* urging the field to unveil the potential mechanisms involving SARS-CoV-2's entry and pathophysiological effects on affected subjects.

In this study, we used different methods to detect SARS-CoV-2 in the testis parenchyma of patients deceased with COVID-19 and investigated the virus's infective and replicative capacities using testicular macerates in VERO cell culture. We also revealed the cellular and molecular alterations in human testicular pathophysiology and correlated the findings with the patient's clinical data, thus unveiling potential mechanisms underlying the observed alterations.

Results

Hypertension, diabetes, and obesity are the main comorbidities

All 11 patients studied herein were admitted to the Intensive Care Unit (ICU) of the Mater Dei Hospital (Belo Horizonte, Brazil) due to severe pulmonary symptoms. The mean age was 63.9 ± 13.11 years (range 46 to 88), and the mean body mass index was $32.18 \text{ Kg/m}^2 \pm 6.04$ (range 25.95–44.46). All patients had children, except

for patient #9. Table 1 summarizes the patients' clinical characteristics. The mean disease duration (from onset to death) was 23.73 ± 8.24 days (range 13–38), while the meantime of ICU admission to death was 15.81 ± 6.19 days (range 8–25). The most prevalent comorbidities were systemic arterial hypertension (nine cases), diabetes mellitus (six cases), and obesity (six cases).

SARS-CoV-2 reliable detection in testes

We first tested the testicular tissue using a conventional RT-qPCR protocol for SARS-CoV-2, but only patient #8 was positively detected (cycle threshold—CT=38). To improve detection and reduce interference of intrinsic tissue factors, we performed a cDNA synthesis using SARS-CoV-2 specific viral primers. The RT-qPCR revealed the virus's presence in 10 of 11 patients (Table 2).

To confirm the genetic data, we used a nano-designed sensor, which employs localized surface plasmon resonance—LSPR [7], to detect the SARS-CoV-2 Spike (S)-protein and the nucleocapsid (N)-protein (Additional file 1: Fig. S1a–q). S-protein was found in all patients, while N-protein was observed in nine patients (Table 2 and Additional file 1: Fig. S1m–q). In addition, prominent S-protein immunolabeling was evidenced in the testes of all COVID-19 patients (Additional file 1: Fig. S2a–p), especially in patient #8 (positively detected by all methodologies). Our data suggest that more sensitive techniques are required for the reliable detection of SARS-CoV-2 (even in a low viral titer) in testes.

Main infected cells in testis parenchyma

Several infected monocytes/macrophages (CD68+) were detected surrounding blood vessels and migrating to the parenchyma (IF and TEM data), suggesting that these cells might be delivering SARS-CoV-2 to the testes (Fig. 1a–c, arrows), and contributing to the infection of testicular cells. Infected macrophages were confirmed through double immunofluorescence and transmission electron microscopy (TEM) (Fig. 1d–g). Monocytes/macrophages were also found inside the tubular compartment, indicating a possible route for viral spreading inside the seminiferous tubules (Fig. 1h). Conversely, chymase-positive mast cells were not labeled for S-protein (Additional file 1: Fig. S3a–b).

Table 1 Clinical features of 11 COVID-19 patients

COVID-19 patient	Age (years)	Body-weight (Kg)	BMI	Smoking habit	Comorbidities	Previous testicular disorder	Fertility record (children)	Disease duration (days)	Hospital stay (days)	Body temp. (max)	Sign of Orchitis	Cause of death
#1	75	120	41.5	Yes	SAH, diabetes, obesity	No	Yes	26.00	21.0	38.7 °C	No	Multiple organ failure
#2	76	103	31.79	No	SAH, obesity	No	Yes	32.00	25.0	37.9 °C	No	Septic shock
#3	56	100	32.65	No	SAH, diabetes, obesity	No	Yes	31.00	20.0	38.4 °C (> 24 h)	No	Septic shock
#4	56	80	26.12	No	SAH, diabetes, obesity, hypothyroidism	No	Yes	26.00	16.0	39.3 °C (> 24 h)	No	Multiple organ failure
#5	88	88	30.4	No	SAH, diabetes, hypothyroidism, CRI	No	Yes	13.00	10.0	40.2 °C	No	Multiple organ failure
#6	76	85	29.41	No	Hypothyroidism	No	Yes	38.00	20.0	38.1 °C	No	Multiple organ failure
#7	50	100	34.6	No	SAH, obesity	No	Yes	19.00	10.0	41.5 °C (> 24 h)	No	Septic shock
#8	63	75	26.57	No	SAH, diabetes, hypothyroidism, CRI	No	Yes	18.00	11.0	38.4 °C	No	Multiple organ failure
#9	62	75	25.95	No	SAH	No	No	14.00	10.0	38.9 °C (> 24 h)	No	Multiple organ failure
#10	55	130	44.46	No	SAH, diabetes, obesity	No	Yes	28.00	23.0	37.9 °C (> 24 h)	No	Multiple organ failure
#11	46	99	30.56	No	No	No	Yes	16.00	8.0	40.9 °C (> 24 h)	No	Multiple organ failure

BMI body mass index, SAH systemic arterial hypertension, CRI chronic renal insufficiency, temp. temperature

Table 2 SARS-CoV-2 detection in testicular parenchyma

Patients	Genetic evaluations				Protein evaluations				Infection of VERO cells				
	E gene (RD) (CT)	RNP ctrl (CT)	N1 gene (SP) (CT)	β-actin ctrl (CT)	LSPR-nanosensor N shift (nm)	LSPR-nanosensor S shift (nm)	IF Spike (-/+)	1st day- N1 gene (CT)	2nd day- N1 gene (CT)	RNP ctrl (CT)	+ RNA (CT)	- RNA (CT)	IF Spike (-/+)
CTRL #1	-	20	-	18.55	0 (-)	0 (-)	-	-	-	20	-	-	-
CV-19 #1	-	23	32.78 (+)	24.59	31.5 (+)	51 (+)	+	34 (+)	33 (+)	29	29 (+)	28 (+)	+
CV-19 #2	-	27	36.99 (+)	18.50	13.2 (+)	18.9 (+)	+	34 (+)	34 (+)	27	32 (+)	33 (+)	-
CV-19 #3	-	22	36.34 (+)	6.36	1.2 (-)	17.5 (+)	+	34 (+)	31 (+)	30	26 (+)	25 (+)	+
CV-19 #4	-	20	35.96 (+)	19.25	11.2 (+)	17 (+)	+	34 (+)	32 (+)	29	29 (+)	28 (+)	+
CV-19 #5	-	20	36.14 (+)	20.03	9.9 (+)	20.9 (+)	+	32 (+)	27 (+)	32	23 (+)	25 (+)	+
CV-19 #6	-	21	36.22 (+)	10.30	13.2 (+)	15 (+)	+	34 (+)	32 (+)	29	27 (+)	27 (+)	-
CV-19 #7	-	20	35.37 (+)	17.67	4.5 (-)	12.4 (+)	+	34 (+)	32 (+)	25	-	-	+
CV-19 #8	38 (+)	20	35.95 (+)	19.34	17.5 (+)	17 (+)	+	34 (+)	34 (+)	26	-	-	+
CV-19 #9	-	21	-	19.81	12.9 (+)	17.5 (+)	+	35 (+)	34 (+)	27	32 (+)	35 (+)	-
CV-19 #10	-	23	34.03 (+)	20.71	7.2 (+)	13.6 (+)	+	34 (+)	32 (+)	30	-	-	-
CV-19 #11	-	23	35.65 (+)	16.27	8.7 (+)	16.5 (+)	+	34 (+)	33 (+)	30	29 (+)	33 (+)	-

CV-19 COVID-19 patient; RNP RNase P gene; RD routine diagnosis; SP specific primers; CT cycle threshold; LSPR localized surface plasmon resonance; IF immunofluorescence; Spike spike protein; (+), detection of viral content; (-), non-detection of viral content. Positivity in the RT-qPCR is detected when the CT is lower than 40, and in the LSPR nanosensor is when the shift is higher than 5 nm

Most S-protein labeling was identified inside the seminiferous tubules, mainly in germ cells (Fig. 1i–m). In some areas, spermatogonial cells displayed intense S-protein labeling compared to spermatocytes and spermatids (Fig. 1n, red arrowhead). SARS-CoV-2 was detected in different types of germ cells (Fig. 1o–p). Patients who died up to 20 days after ICU admission presented the highest mean fluorescence index in the testis (Additional file 1: Fig. S2a).

Some Sertoli and Leydig cells presented viral particles in their cytoplasm (TEM data), albeit with lower S-protein immunolabeling intensity (Fig. 2a–c', arrowheads and insets). However, many of these cells did not show obvious viral particles in the cytoplasm (TEM data) (Fig. 2d–i).

Viral replication and viability

TEM analyses identified a viral replication factory in macrophages and spermatogonial cells (Fig. 3a–f). In both cell types, we observed SARS-CoV-2 replication complexes with the formation of convoluted membranes (replication membranous webs, RMW) containing double-membrane vesicles (DMV) and endoplasmic reticulum Golgi intermediate complex (ERGIC) showing new virions (Fig. 3c–f).

In a biosafety level-3 (BSL-3) lab, we exposed VERO CCL-81 cell cultures to testicular homogenates from all patients (Fig. 3g–v). We found that SARS-CoV-2 was infective and replicating in these cells, diminishing the CT value (N1 gene) along with the culture time (Table 2). The cytopathic effect (patients #1, #3, #4, #5, #7, and #8; Fig. 3g–n), immunofluorescence (patients #1, #3, #4, #5, #7, and #8; Fig. 3o–v), immunoperoxidase (patient #1; Additional file 1: Fig. S2q–s), RT-qPCR (genes N1) (all samples; Table 2), and identification of the genomic and subgenomic SARS-CoV-2 RNA (patients #1, #2, #3, #4, #5, #6, #9, and #11; Table 2) in the infected VERO cell culture confirmed the viral replication. SARS-CoV-2 was detected in the testes of all patients who died either soon or long after

the onset of symptoms. The replicative data indicate we were not observing just fragments of viral particles. Our data also suggest that the virus remains viable within the testis of a severe COVID-19 patient for an extended period.

Clinical data and testicular alterations

Although we presented the data of all patients in a single group (COVID-19), the COVID-19 patients were further subdivided into groups according to the phenotype of the testes' parenchyma. Critically ill patients who maintained elongated spermatids in the seminiferous epithelium were categorized as phase I patients (patients #6, #7, #8, #9, and #11). Patients presenting primary spermatocytes as the most advanced germ cell type were categorized as phase II patients (patients #1, #3, #4, #5, and #10). Specifically, patient #2 was classified as phase III because his testis presented few spermatogonia inside the seminiferous tubules (Fig. 4a–d). A weak correlation (Spearman's $\rho = -0.33$; $p = 0.30$) was observed between the germ cell loss and the patient's age. Furthermore, we did not observe a strong correlation between the patient's age and the progression of the pathogeny (Spearman's $\rho = 0.33$; $p = 0.31$).

Remarkably, there was a positive correlation (Spearman's $\rho = 0.6862$; $p = 0.0197$) between the above-described phases and the time from ICU admission to death (Fig. 4e). All phase I patients died less than 20 days after ICU admission, with most deceasing well before 20 days. All phase II patients died 20–23 days after ICU admission. Patient #2 (phase III) died after 25 days. Our findings suggest that the more extended the severe condition (ICU stay), the lower the number of surviving germ cells. It is essential to mention that none of the patients presented scrotal symptoms or complaints during their hospital stay prior to ICU admission. Also, the clinical history did not reveal previous testicular disorders. All patients presented fever during hospitalization, six of them for longer than 24 h. However, we did not find evidence of a strong correlation between fever and the

(See figure on next page.)

Fig. 1 SARS-CoV-2 infection in monocytes, macrophages, and germ cells. **a** Immunofluorescence for S-protein in testicular parenchyma showing positive germ cells (red arrowhead), monocytes (white arrow), macrophages (orange arrowhead), and Leydig cells (green arrowhead); **a'** negative control omitting the primary antibody (scale bars = **a** 50 μ m; **a'** 40 μ m). **b, c** TEM images showing infected infiltrative monocytes (mono, arrows); yellow = disrupted cytoplasm of endothelial cells; red boxes = cytoplasmic areas replenished with viral particles; inserts = high magnification of viral particles (Scale bars = **g** 2 μ m; **h** 200 nm). **d–f** 3D reconstruction of double immunofluorescence for CD68 (w, red color) and S-protein (x, green color), depicting an infected macrophage (y, merge–yellow color) (scale bars = 10 μ m). **g** TEM image of an infected macrophage (m) showing a viral particle in its cytoplasm (insert). **h** Presence of macrophages (arrows) inside the seminiferous tubule (green arrow) and in the interstitial area (red arrow) (scale bar = 10 μ m). **i–l** Different types of infected germ cells: **i** spermatogonial cell (Spg); **j** pachytene spermatocytes (P); **k** round spermatids (R); **l** elongated spermatids (E) (scale bars = 15 μ m). **m–n** Immunofluorescence against the S-protein shows that spermatogonial cells (red arrowheads) are more intensely labeled than other germ cells (scale bars = **a** 30 μ m; **b** 10 μ m). **o–p** TEM images showing viral particles (in low and high magnification) in round spermatids (scale bars = **o** 2 μ m; **p** 200 nm). Immunofluorescence images in the testis of patient #8. TEM images in testes from patients #1, #7, and #8. Blue = dapi counterstaining; green staining = IgG-CFL 488; red staining = IgG-546. ST, seminiferous tubule cross-section; IC, intertubular compartment; BV, blood vessel

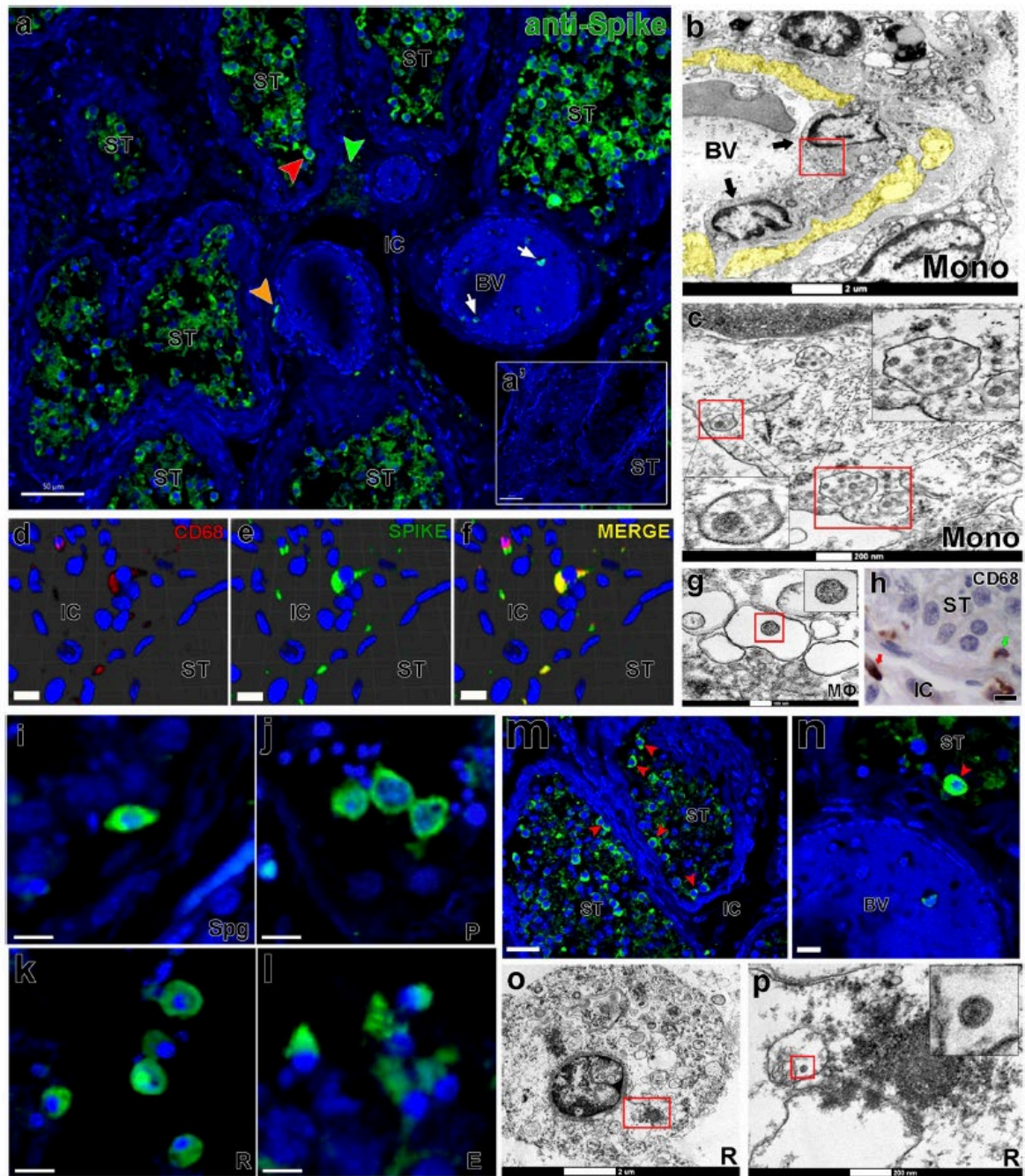


Fig. 1 (See legend on previous page.)

development of testicular pathogenesis (Spearman's $\rho = -0.195$; $p = 0.57$).

Imbalanced renin-angiotensin system

We found ACE2 protein in spermatogonial, Leydig, and Sertoli cells of Controls and COVID-19 patient testes (Fig. 4f). However, testicular cells of COVID-19 patients showed weak ACE2 staining intensity, especially inside the seminiferous tubules (Fig. 4g–i). qPCR revealed augmented ACE2 expression in the testis of phase I patients and diminished in Phase II and III patients (Fig. 4j). ACE protein expression reduced, whereas angiotensin II receptor type 1 (AGT1R) increased in all phases (Fig. 4j).

COVID-19 patients presented more intensely immunolabeled testicular cells for the AGT1R than controls, particularly in Leydig cells, macrophages, and peritubular myoid cells (Fig. 4k–n). Angiotensin II levels were higher in the testis parenchyma of COVID-19 patients than in Controls (Fig. 4o).

Mast cell and macrophage activation

Total protein was significantly higher in testes from COVID-19 patients than in controls (Additional file 1: Fig. S3c). Our morphological and molecular data suggest that mast cells and macrophages play a critical role in testicular pathogenesis. The number of mast cells found in the testis of COVID-19 patients was ten times higher than that found in controls (Fig. 5a–c). Immunohistochemistry revealed chymase-positive mast cells in the testes of the COVID-19-affected patients (Fig. 5d–g). Interestingly, mast cells were detected next to areas of the testicular parenchyma displaying a high concentration of immune cells, hemorrhagic areas, altered Leydig cells, interstitial fibrotic areas, damaged tunica propria, and thickened tubular basal membrane (Additional file 1: Fig. S3d–m). qPCR data showed that Control testes presented minimal expression of chymase (CMA1) and tryptase (TPSB2), whereas all testicular samples from the COVID-19 patients exhibited increased expression (Fig. 5h). In phase I patients, mast cells were highly prevalent in the testes' intertubular compartment. Phase II and III patients showed these cells inside the tubular and intertubular compartments (Additional file 1: Fig. S3j–m).

Testes of COVID-19 patients also presented elevated mRNA levels of histamine H4 receptor (HRH4), cyclooxygenase-2 (COX2) and monocyte chemoattractant

protein-1 (MCP1; 10x higher) (Fig. 5h). MCP1 is particularly unfavorable for testicular pathogenesis because it can attract more infected monocytes (Additional file 1: Fig. S3n–o). Macrophage (CD68+) numbers were augmented, and these cells were highly active (N-acetyl-BD-glucosaminidase (NAG) and periodic acid–Schiff (PAS) assays) in infected testis (Fig. 5i–q).

Taken together, our findings suggest a significant contribution of activated mast cells and macrophages to the pathophysiology of COVID-19 in the human testes, which could eventually induce harmful effects on the reproductive health of severely ill patients.

Lymphocytes (CD3+) were frequently observed in the testes of COVID-19 patients, suggesting a prolonged infection (Fig. 5r–v). Neutrophils were also identified in the testes of COVID-19 patients (Fig. 5x); however, their myeloperoxidase activity (MPO assay) was not altered when compared to Controls (Fig. 5w). These data suggest that macrophages are more active than neutrophils in the testes of COVID-19 patients.

Effects on the tubular compartment

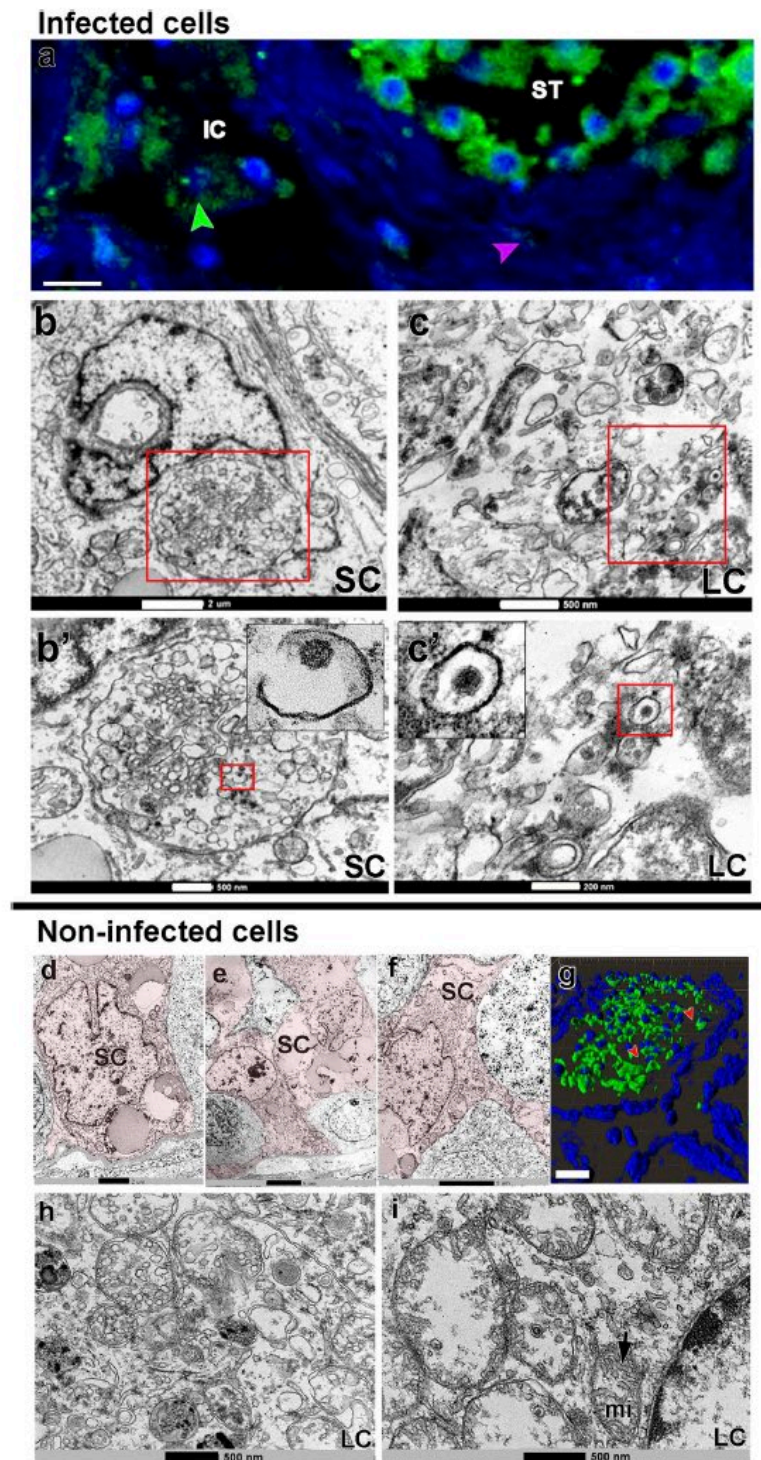
While the germ cell population and tubular diameter diminished in COVID-19 patients (Additional file 1: Fig. S4a–b), the tunica propria enlarged, reflecting a higher number of peritubular myoid cells and collagen fibers (Additional file 1: Fig. S4c–j). Interestingly, the tunica propria and the basal membrane (PAS+) thickened next to mast cells and macrophages and sometimes presented a corkscrew appearance (Additional file 1: Fig. S4k–n).

There were many caspase-3-positive germ cells in the testes of all COVID-19 patients (Fig. 6a–d). The rete testis area presented degenerating germ cells (Additional file 1: Fig. S4o), and BAD and BAX levels were augmented in nearly all COVID-19 patients (Fig. 6e). DAZL and TMPRSS2 genes, known to be highly expressed in germ cells, diminished as the phases progressed (Fig. 6e).

Tight junctions of the seminiferous tubules were also compromised in COVID-19 patients, with reduced levels of occludin and claudin-11 (Fig. 6e). Expressions of critical Sertoli cell genes (Sox9 and Gata4) were also reduced (Fig. 6e). Moreover, the seminiferous epithelium detached from the thickened tunica propria (Fig. 6f and Additional file 1: Fig. S4p–t), indicating downregulation of tubular junctional proteins.

(See figure on next page.)

Fig. 2 SARS-CoV-2 infection in testicular somatic cells. **a** Immunofluorescence against S-protein evidencing weak labeling in peritubular myoid (pink arrowhead) and Leydig cells (green arrowhead) (scale bar = 15 μ m). **b–c'** TEM images showing viral particles (in low and high magnification) in the Sertoli cell (SC) (scale bars = **b** 2 μ m; **b'** 500 nm) and Leydig cell (LC) (scale bars = **c** 500 nm; **c'** 200 nm). **d–f** TEM images of non-infected Sertoli cells (SC, pink) (scale bars = **e** 2 μ m; **f–g** 5 μ m). **g** 3D reconstruction of a seminiferous tubule cross-section showing non-labeled areas surrounding germ cells (red arrowheads) (Scale bar = 40 μ m). **h–i** high magnification of non-infected Leydig cells. Arrow = tubular crest of a mitochondrion (mi) (scale bars = 500 nm). Immunofluorescence images in the testis of patient #8. TEM images in testes from patients #1, #7, and #8



Leydig cells apoptosis and inhibition

There was a gradual volumetric reduction of Leydig cells (Fig. 6g) as the testicular pathogeny developed. Intratesticular testosterone levels measured in the testicular homogenates of COVID-19 patients were lower (~30 times) than the controls (Fig. 6h). Caspase-3 immunolabeling revealed that these cells were undergoing apoptosis (Fig. 6i–l). Additionally, their morphology was altered, presenting a vacuolated cytoplasm (with a degenerating content) (Fig. 6m–n and Additional file 1: Fig. S5a–d).

Reduction in the expression of LHR, STAR, 3BHSD, and 17BHSD in the testis of COVID-19 patients was consistent with the observed low number of Leydig cells (Fig. 6e). The levels of HRH4 were highly augmented in the affected patients (Fig. 5h). This data indicates that local histamine can inhibit steroidogenesis in the testes of COVID-19 patients.

Testis fibrosis and vascular alterations

Testicular fibrosis was commonly observed in the testis parenchyma of COVID-19 patients. Collagen fibers increased progressively (Fig. 6o–u), showing high amounts of type I and III collagen in testis parenchyma (Fig. 6t–u, Additional file 1: Fig. S5e–h). Further, the expression levels of Col3a were highly augmented in the testes of phase I patients (Fig. 6e).

Expressions of the angiogenic factors VEGF and PDGFA and small vessel volumetric proportion (CD31+) increased in the testis of COVID-19 patients (Fig. 6e). Immature blood vessels (TEM and Immunohistochemistry data; CD31+ cells) were observed inside the tunica propria, suggesting an angiogenic process (Fig. 6v and Additional file 1: Fig. S5i–n). Red blood cells outside the testicular vasculature were frequently observed in the testes of COVID-19 patients (Fig. 6w–α, arrows). Phase I patients presented many red blood cells inside the seminiferous tubule lumen and intratesticular rete testis, possibly due to a reduction in tight epithelial junctions (Additional file 1: Fig. S5o). Some thrombi were detected inside the vascular system (Additional file 1: Fig. S5p).

Furthermore, Endothelin 1/2/3, a protein involved in blood-vessel vasoconstriction, was highly expressed in the first phase patients but reduced in others (Additional file 1: Fig. S5q–t).

Discussion

Herein, we used different and sensitive methods to detect SARS-CoV-2 proteins, genomic and subgenomic RNA, and virus particles in the testis of deceased patients with COVID-19. In Fig. 7, we hypothesized the potential viral, cellular, and molecular mechanisms of infection, replication, and damage by SARS-CoV-2 in the testes of non-vaccinated patients who became severely ill, admitted to the ICU, and eventually died from the disease. Our data suggest a direct influence of SARS-CoV-2 in testicular cells, which might probably deregulate ACE2, elevating the levels of angiotensin II, a potent pro-inflammatory and angiogenic peptide. Consequently, angiogenic and inflammatory factors might induce the infiltration and activation of mast cells and macrophages in the testes of COVID-19 patients. Taken together, the deleterious effects observed in the testes of COVID-19 patients, i.e., fibrosis, vascular alteration, inflammation, tunica propria thickening, Sertoli cell barrier loss, germ cell apoptosis, and inhibition of Leydig cells, seem to be associated with elevated angiotensin II and activation of mast cells and macrophages (Fig. 7a). A protein-protein (or gene-gene) network (Fig. 7b) shows the interaction of factors associated with angiotensin II regulation, activation of mast cells and macrophages, and alterations in testes tubular and intertubular compartments.

Six methodologies, including two types of RT-qPCR, immunofluorescence, Vero cell culture, nanosensors, and TEM were used to clarify and evidence the presence of SARS-CoV-2 in the testes. These findings are critical because previous studies presented conflicting results regarding the detection of SARS-CoV-2 RNA in the testes [2–5]. The data also suggest that SARS-CoV-2 tropism for testes is higher than previously thought and that the conventional RT-qPCR protocol may only detect

(See figure on next page.)

Fig. 3 Infective SARS-CoV-2 particle formation and replication activity in Vero cells. **a–f** TEM images of testicular parenchyma (scale bars = **a** 5 μm; **b** 500 nm; **c** 200 nm; **d** 200 nm; **e** 200 nm; **f** 100 nm). **a** Altered seminiferous tubule cross-section, depicting a macrophage in tunica propria containing a replication membranous web (RMW; blue box) and a spermatogonial cell displaying an endoplasmic reticulum Golgi intermediate complex (ERGIC, white box). The pink box indicates a spermatogonial cell clone surrounded by the cytoplasm of a Sertoli cell (labeled in pink). **b**, **c** Higher magnifications of spermatogonial cells showing structures similar to new virions inside the ERGIC. Although very large, this structure also resembles a hijacked nuclear pore complex (present in situations of viral replication). **d** Higher magnification of the RMW from the macrophage depicted in “figure a,” evidencing the presence of double-membrane vesicles (DMV). **e–f** Images from an interstitial macrophage presenting RMW, DMV, and ERGIC filled with new virions (labeled in green). **g–n** The cytopathic effect of SARS-CoV-2 was evaluated in VERO CCL-81 cells (empty area, *). Scale bars = 30 μm. **g** Cell confluence of VERO CCL-81 cultures before exposition to testicular macerates. **h** VERO CCL-81 cells exposed to SARS-CoV-2 (positive control). **i–n** VERO CCL-81 cells exposed to testicular macerates of COVID-19 patients (empty area, *) (arrowheads indicate structures similar to a syncytium cell). Three wells were replicated per patient. **o–v** S-protein immunofluorescence in VERO CCL-81 cells (blue labeling: DAPI; green labeling: spike protein). Scale bars = 300 μm. **o** Negative control omitting the primary antibody. **p** Positive control using VERO CCL-81 cells infected with SARS-CoV-2. **q–v** Immunofluorescence in VERO CCL-81 cell culture after exposition to testicular homogenates

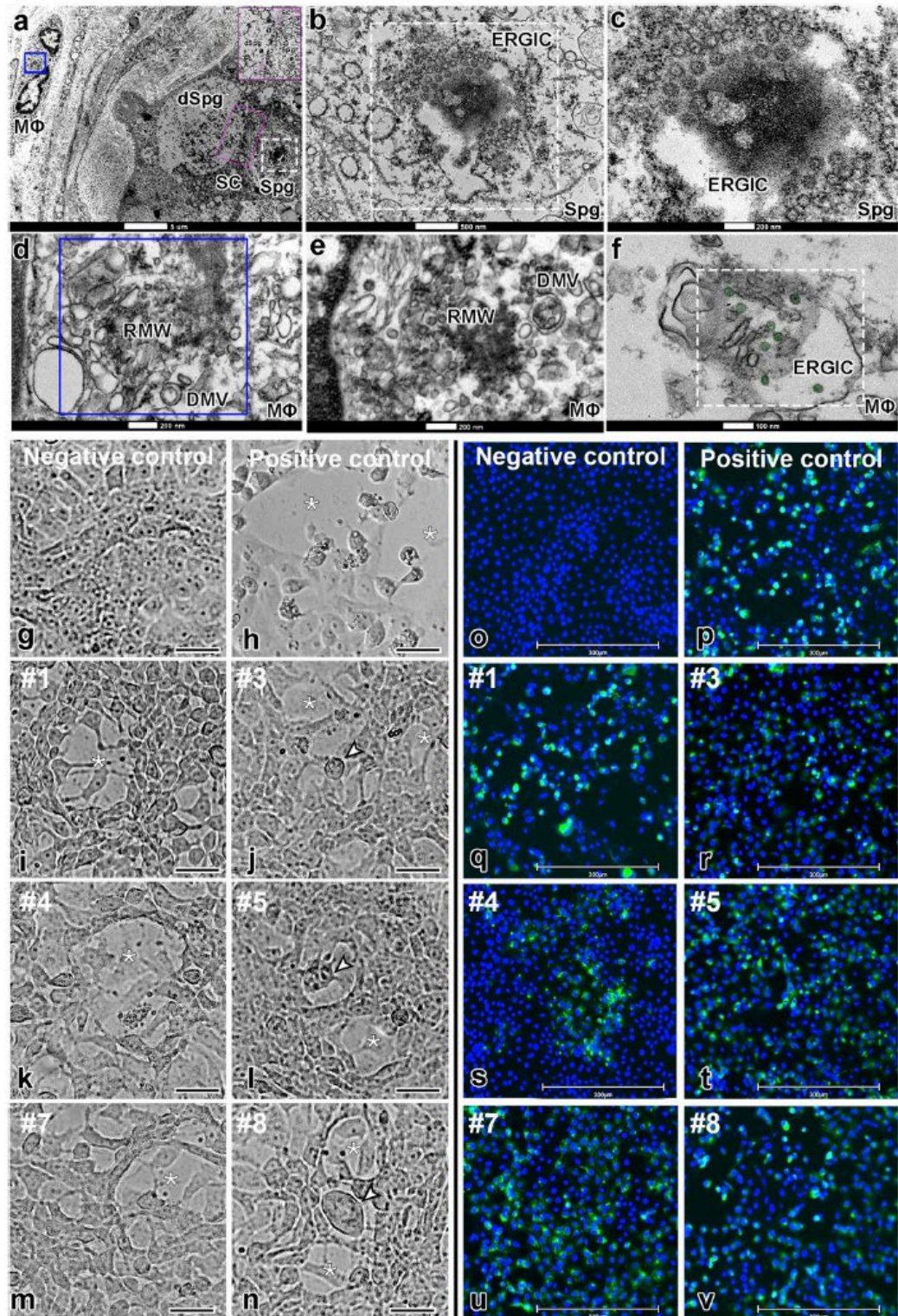


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infected testes with higher viral loads. The precise detection of nanosensors may be related to their capacity to detect femtograms of viral proteins, as shown in the present study. One can say that better viral genetic detection in VERO cell culture may be related to the enrichment of SARS-CoV-2 in the cell culture supernatant. An experimental study in monkeys revealed that SARS-CoV-2 have high tropism to the testes and that the viral signal was higher in the gonads than in the lung [8]. Accordingly, we observed many germ cells positive for the S-protein, and other authors also detected the N-protein in germ cells [4]. Our finding is reinforced by the detection of SARS-CoV-2 RNA in the semen of patients who suffered severe forms of COVID-19 [9]. However, further studies should be undertaken to examine this possibility thoroughly. In a systematic review [10], the authors indicated the low risk of SARS-CoV-2 detection in the semen of recovered men after collecting data from different studies.

We detected infected infiltrating monocytes in deceased patients with severe COVID-19 (non-vaccinated). It is possible that the “macrophage paradox” described for SARS-CoV-2-induced lung damages [11] may also apply to the testes. Thus, although macrophages combat viral infections, they can also act as “Trojan horses” [11], facilitating viral entrance and replication of the virus in the testis. While it is conceivable that the testis can be infected by direct invasion caused by viremia, our results suggest that infected monocytes/macrophages migrating (e.g., from the lungs) may also be actively transporting and disseminating the virus into the testes [12]. Furthermore, we revealed a high SARS-CoV-2 infection and replication (according to morphological features in the cell cytoplasm) [13] in spermatogonia and macrophages, which are known to express ACE2 and TMPRSS2 [6, 14].

Previous research indicated that SARS-CoV-2 negativity in RT-qPCR tests (at least in two consecutive assays) usually occurs between 6 and 12 days from the onset of symptoms [15]. However, we found that the virus remains infective after a long infection period in the testes. It is

well known that the testicular immune privilege prevents the autoimmune attack of haploid germ cells but allows viruses to escape immunosurveillance [16]. Thus, our findings suggest that the testes maintain infective viruses for extended periods in non-vaccinated severe COVID-19 patients. Furthermore, the testis environment has been related to delayed viral clearance in men compared to women [17]. Regarding these gender differences, it has been shown that male gonads express genes that increase SARS-CoV-2 susceptibility and severity [18]. In this context, the testes should not be neglected in evaluating the patient's clinical condition because they may be a potential site of viral replication and, consequently, a source of viral load. However, further research to clarify whether or not the testes may serve as a viral reservoir for SARS-CoV-2 should be thoroughly pursued due to its potential impact on public health. To this end, identifying SARS-CoV-2 access to the testes and its potential replication sites is relevant since testicular immune tolerance may hamper viral clearance from the human body, as observed for other viruses [16].

It is known that the interaction of SARS-CoV-2 and ACE2 promotes this enzyme's internalization, resulting in elevated levels of angiotensin II in the affected tissues [19]. Although overstimulation of the ANG II/AGT1R pathway underlies the damages observed in several tissues of COVID-19 patients [20], the present study is the first to show a role for this pathway in the testes. This molecular pathway was evidenced by the diminished expression of ACE2 (mainly in the tubular compartment), increased labeling of AGT1R in testicular cells, and elevated levels of angiotensin II. The influx of immune cells could have been mediated by angiotensin II, a potent pro-inflammatory molecule [21]. Furthermore, it is known that MCP1 and COX2 are stimulated by angiotensin II [22, 23].

Interestingly, an increased number of tryptase and chymase-positive mast cells and activated macrophages in the testes are associated with testicular inflammation and infertility in men [24, 25]. These cells can compromise

(See figure on next page.)

Fig. 4 Imbalanced renin-angiotensin system in the testis of COVID-19 critically ill patients. **a–d** Initial categorization of COVID-19 patients according to the most advanced germ cell in the seminiferous epithelium: phase 1 (P1), phase 2 (P2), and phase 3 (P3). Toluidine-blue counterstaining (scale bars = 50 μ m). **a** Control patients showing elongated spermatids (E). **b** COVID-19 patients showing elongated spermatids (E), spermatocytes (Spc), and spermatogonial cells (Spg). **c** COVID-19 patients displaying spermatocytes (Spc) and spermatogonial cells (Spg). **d** COVID-19 patients presenting spermatogonial cells (Spg) only. **e** Positive correlation between patient's phases and the time frame from ICU admission to death (Spearman's $\rho = 0.6862$; $p = 0.0197$). **f–i** Immunostaining of ACE2 in the testis of control (**f**) and COVID-19 patients (**g–i**) (scale bars = 50 μ m). **h'** Negative control, omitting the primary antibody. Red: Alexa-594; blue: dapi; green arrowheads: Leydig cell; red arrowheads: spermatogonial cell; blue arrowheads: Sertoli cell. **j** Heat map of the evaluated genes related to the renin-angiotensin system. The colors compare COVID-19 patients to controls. Green: lower expression than controls. Red: higher expression than controls. **k–n** Immunostaining of AGT1R in the testis of control (**k**) and COVID-19 patients (**l–n**). Purple arrowheads: peritubular myoid cells; green arrowheads: Leydig cells; orange arrowhead: macrophages (scale bars = 50 μ m). **o** ELISA assay for angiotensin II (t-test; two-tailed; infected groups vs control; * $p < 0.05$). P1, P2, P3: phases 1, 2, and 3 of the COVID-19 patients, respectively. ST, seminiferous tubule; IC, intertubular compartment. Individual values can be found at <https://doi.org/10.6084/m9.figshare.16777786.v8>

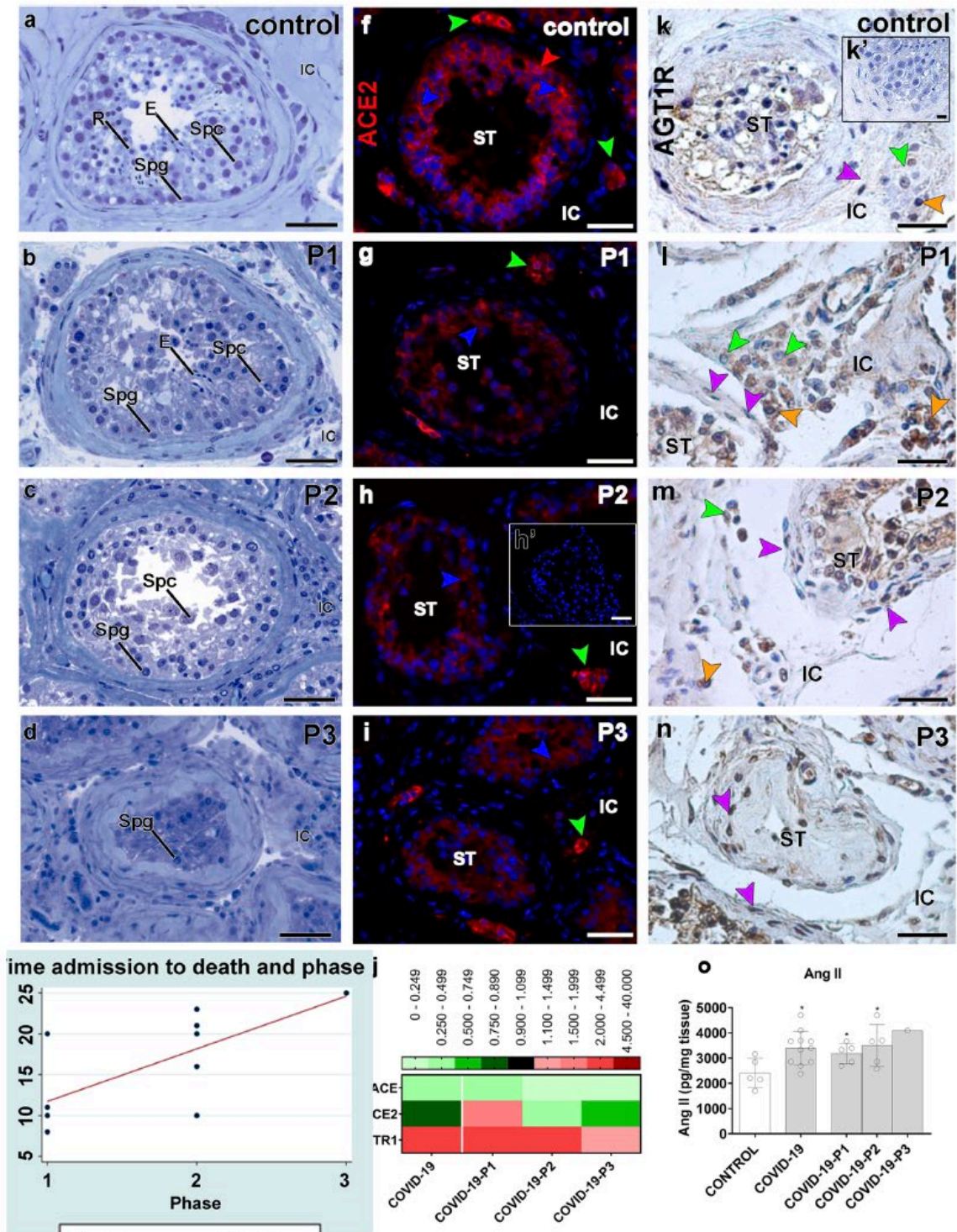


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the immune-privileged testicular environment, secreting pro-inflammatory cytokines, and interfering with testicular homeostasis [26]. Thus, the observed tubular damages may have occurred because of the high levels of angiotensin II [27], activated mast cells [28], and activated macrophages [29]. Furthermore, proteases and cytokines released by activated mast cells and macrophages are also known to disrupt tight epithelial junctions and deregulate the human blood-testis barrier [30].

In the intertubular compartment, the high levels of angiotensin II, mast cells producing histamines, and pro-inflammatory cytokines produced by macrophages can inhibit steroidogenesis [31, 32]. In line with Yang et al. [33], we showed the reduction in Leydig cell numbers and revealed, for the first time, that the intratesticular testosterone levels are 30 times reduced in the testes of patients with severe COVID-19. The low intratesticular testosterone levels observed herein may disturb the immune-privileged milieu [34] and compromise spermatogenesis [35]. In addition, we showed testis parenchyma's fibrosis and vascular alterations. It is known that angiotensin II, activated mast cells, and activated macrophages stimulate collagen synthesis by fibroblasts [36–38] and increase vascular permeability [39–41]. In light of our findings, it can be speculated that the mechanisms discussed above may also be taking place in the testes of the severely affected patients studied herein. Further studies (e.g., utilizing human cell lines or animal models) are necessary to expand the proposed mechanisms and evaluate the long-term effects on testis function.

In relation to the patient's clinical history, we did not observe a significant correlation between fever and testes damage. Recent data indicate that fever is not correlated with alterations in sperm quality following SARS-CoV-2 infection. We also showed that the more extended severe condition in ICU, the lower the number of surviving germ cells in the testes. Notably, the chronology of testicular pathology was based on the presence of advanced germ cells in the testis parenchyma and the time of ICU

admission. Although our histological and molecular alterations reinforced this conceptual classification, the data of all individuals were also presented in a single group. However, analyzing the data of other studies, we noted that the worse testicular phenotype also seemed to be associated with the period of infection [4, 5]. Recent clinical results have shown that gonadal status may serve as a biomarker to identify patients with worse clinical outcomes and that extended hospitalization is linked to lower levels of total and free testosterone [42].

It is essential to mention that this study showed several molecular fluctuations during SARS-CoV-2 testicular pathogenesis. We provided a general overview, comparing testes' cellular and molecular aspects from controls and deceased COVID-19 patients. We should point out the relevant limitations of the current study. Not all COVID-19 patients studied presented the same comorbidities. We observe the necessity of investigating the relationship between the comorbidities (e.g., diabetes and obesity) and the maintenance of the testicular immune privilege state. Although all patients in the present study had children (except for patient #9), we have no previous fertility data, such as hormonal and semen analyses. Controls were not submitted to the medications used for COVID-19 patients. On the other hand, we harvested and processed the testicles as soon as the patients died, in contrast with many cadaver studies showing long organ collection delays, which may have compromised the precise histology, detection of virion particles, and perception of peptides, mRNA, and hormonal fluctuations.

Finally, although some of the observations and proposed mechanisms might, at first, paint a dire scenario for severe COVID-19 disease, it is worth noticing that the present study only included non-vaccinated patients who died from COVID-19. Recent data on semen from mildly infected patients, who recovered from COVID-19, revealed that they reestablished their sperm quality after 3 months of the infection [43]. Nevertheless, our study reveals concerning aspects regarding the consequences

(See figure on next page.)

Fig. 5 Presence of activated mast cells and macrophages in the testis parenchyma of COVID-19 critically ill patients. **a** Toluidine blue staining showing an elevated number of mast cells (arrows) in testis parenchyma (scale bar = 50 μ m). **b** In some regions, mast cells were identified inside the seminiferous tubules (red arrow). **c** Number of mast cells counted in 15 fields of testis parenchyma at $\times 400$ magnification (*t*-test; two-tailed; infected groups vs control; $*p < 0.05$). **d–g** Immunostaining for chymase in testes of control and COVID-19 patients. Activated mast cells (arrows) were observed in COVID-19 patients only (scale bars = 50 μ m). **d'** Negative control (scale bars = 50 μ m). **h** Heat map of the evaluated genes related to the mast cells and macrophages. The colors compare COVID-19 patients to controls. Green: lower expression than controls. Red: higher expression than controls. **i–l** Immunostaining for CD68 in testes of control and COVID-19 patients, illustrating the high prevalence of this cell in the deceased patients (scale bars = 50 μ m). **i'** Negative control (scale bars = 50 μ m). **m–o** PAS-positive monocytes (red arrow) and macrophage (green arrow) indicating their activation (scale bars = 15 μ m). **o'** PAS-negative cells in the interstitial cells of a control patient (scale bar = 10 μ m). **p** Number of macrophages counted in 10 fields of testis parenchyma at $\times 400$ magnification (*t*-test; two-tailed; infected groups vs control; $*p < 0.05$). **q** N-acetylglucosaminidase (NAG) assay indicating the activation of macrophages in COVID-19 patients' testes (*t*-test; two-tailed; control vs infected group; $p > 0.05$). **r–v** Immunostaining for CD3 and lymphocyte number in testes of control and COVID-19 patients (*t*-test; two-tailed; infected groups vs control; $*p < 0.05$) (scale bars = 50 μ m). **r'** Negative control (scale bars = 50 μ m). **x–w** Neutrophils and their activity (s: MPO assay) in testis parenchyma (*t*-test; two-tailed; control vs infected group; $p > 0.05$) (scale bar = 10 μ m). Individual values can be found at <https://doi.org/10.6084/m9.figshare.16777786.v8>

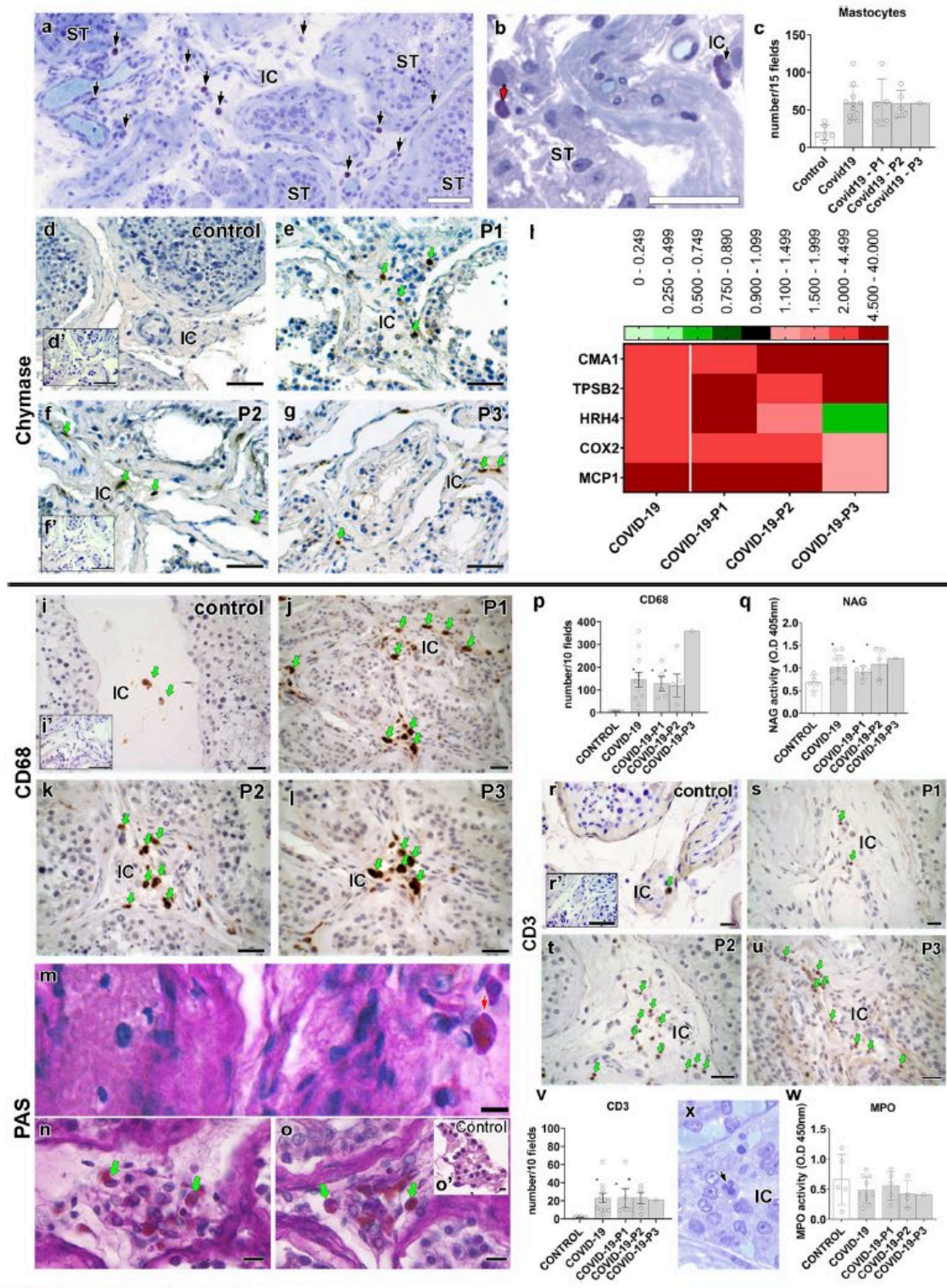


Fig. 5 (See legend on previous page.)

of SARS-CoV-2 invasion of testicles that warrant further investigation by the scientific community at large.

Conclusions

Our multidisciplinary findings might contribute to a better understanding of SARS-CoV-2 tropism, biology, and its impact on testes and male fertility. This is the first study that shows (1) the high SARS-CoV-2 tropism to the testis; (2) one mode of SARS-CoV-2 entrance in testes; (3) SARS-CoV-2 preferred infection and replication in spermatogonia and macrophages; (4) that the virus remains infective after a long infection period; (5) high levels of angiotensin II and activated mast cells and macrophages are critical players in promoting all testicular alterations; (6) the more extended and severe the disease, the lower the number of surviving germ cells; (7) fluctuation in the expression of several essential testicular genes; (8) that the intratesticular testosterone levels are 30 times reduced in testes of severely affected COVID-19 patients; (9) the prevalent types of collagen present in SARS-CoV-2 mediated testicular fibrosis; (10) the fluctuation of vasoconstrictive peptides in testes of non-vaccinated COVID-19 critically ill patients.

Methods

COVID-19 patients

From January 26 to March 4, 2021, we enrolled 11 non-vaccinated male patients deceased from COVID-19 complications, confirmed by SARS-CoV-2 RT-qPCR performed during their hospital stay in the Mater Dei Hospital Intensive Care Unit, Belo Horizonte, Brazil. All 11 patients were admitted to the ICU due to severe pulmonary symptoms (Table 1). The standard treatment at ICU included antibiotics, antimycotics, sedatives, muscular relaxants, analgesics, antihypertensives, inotropes, vasopressors agents, invasive mechanical ventilation, and hemodialysis. The Research Ethics Committee of

the Mater Dei Hospital and the National Research Ethics Committee (CONEP) approved this investigation under the number CAAE: 30999320.1.0000.5128.

Postmortem collection of both testicles was performed after a legally responsible family member signed an informed consent document.

Testicles were collected through an incision on the median raphe of the scrotum. Two authors (MHF and YLG) collected all testicles no later than 3 h after the patient's death. To perform viral and testicular genetic studies, fragments of testicular parenchyma were sampled and immersed into RNAlater® solution (Sigma-Aldrich). To investigate the viral replicative activity and testosterone and angiotensin levels, testis fragments were also sampled and then snap-frozen in liquid nitrogen. The remaining testicular halves were immersed in different fixatives, such as paraformaldehyde 4%, Bouin, methacarn, and glutaraldehyde 4%. Testis fragments were embedded in methacrylate, Epon 812 resin, and Paraplast® for histological, transmission electron microscopy, and immunohistochemistry analyses.

Control patients

The control group was composed of six patients who underwent orchiectomy due to prostate cancer suspicion. These patients did not go through any treatment at the time of orchiectomy. Moreover, they exhibited normal spermatogenesis in seminiferous tubules. The age and hormonal levels of these patients are presented in Additional file 2: Table S1. Testicular fragments were obtained after the study was approved by the Ethics Committee in Research of the Universidade Federal de Minas Gerais COEP/UFGM (COEP ETIC n°117/07). All patients signed the informed consent.

Testicular samples were placed in liquid nitrogen, embedded in methacrylate, Epon, paraplast®, and conserved in RNAlatter. These samples were used for TEM,

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Fig. 6 Main testicular dysfunctions following SARS-CoV-2 infection. **a–d** Immunostaining showing Caspase-3 positive germ cells (green arrows) in testes of controls and COVID-19 patients (scale bars = 50 µm). **a'** Negative control (scale bars = 50 µm). **e** Heat map of critical genes from the tubular and intertubular compartments. The colors compare COVID-19 patients to controls. Green: lower expression than controls. Red: higher expression than controls. **f** Measurement of the tunica propria thickness (t-test; two-tailed; infected groups vs control; * $p < 0.05$). **g** Leydig cell volume density in testes of controls and COVID-19 patients (t-test; two-tailed; infected groups vs control; * $p < 0.05$). **h** Measurement of intratesticular testosterone using RIA (t-test; two-tailed; infected groups vs control; *** $p < 0.001$; ** $p < 0.01$). **i–l** Immunostaining for caspase 3, evidencing positive labeling in Leydig cells (green arrows) (scale bars = 50 µm). **i'** Negative control (scale bars = 50 µm). **m–n** TEM showing the presence of several electron-dense vacuoles in Leydig cell cytoplasm (white arrows) (scale bars = **m** 2 µm; **n** 1 µm). **o–r** Trichrome Masson staining in the testis of controls and COVID-19 patients. In higher magnification, inserts (P1, P2, and P3) show the collagen deposition in the seminiferous tubules (scale bars = 200 µm). **s–u** Quantification of total collagen fibers, type I collagen, and type III collagen (t-test; two-tailed; infected groups vs control; * $p < 0.05$; COVID-19-P1 vs COVID-19-P2; # $p < 0.05$). **v** Quantification of newly formed blood vessels (CD31+) (t-test; two-tailed; infected groups vs control, * $p < 0.05$). **w** Quantification of testicular hemorrhage (t-test; two-tailed; infected groups vs control, * $p < 0.05$). Testicular hemorrhage was classified as normal (**x**) and as containing high numbers of red blood cells (arrows) in tubular and intertubular compartments (**y**), a vast number of red blood cells in the intertubular compartment (**z**), and few red blood cells in the intertubular compartment (**a**) (scale bars = 50 µm). P1, P2, P3: phases 1, 2, and 3 COVID-19 patients, respectively. BV, blood vessels; IC, intertubular compartment; ST, seminiferous tubule cross-section. Individual values can be found at <https://doi.org/10.6084/m9.figshare.16777786.v8>

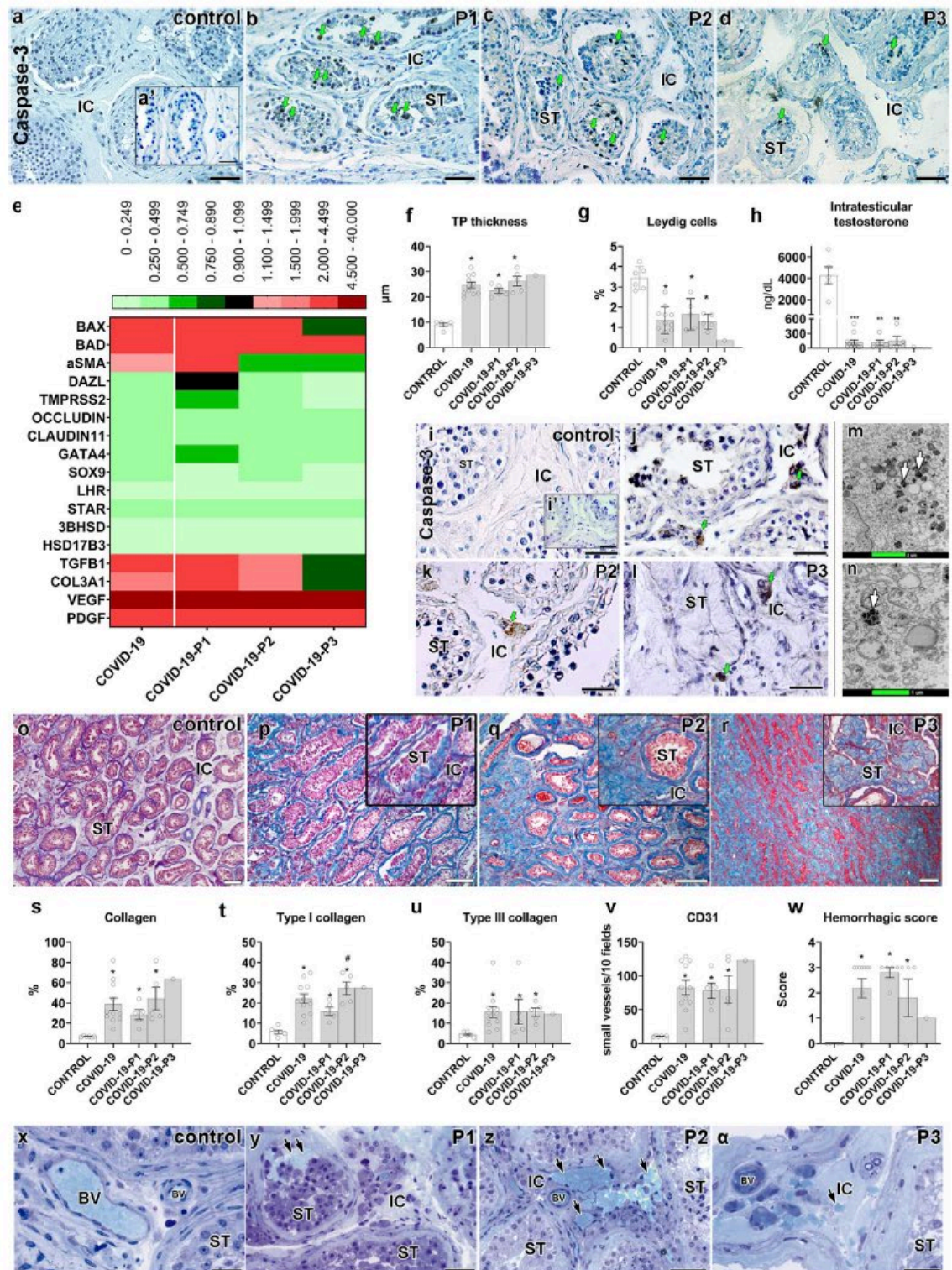


Fig. 6 (See legend on previous page.)

histological, hormonal, and molecular analyses. The mean age of patients was 58 years old, ranging from 46 to 65 years old.

Detection OF SARS-CoV-2 in testis tissue

Genetic assays

SARS-CoV-2 detection using standard RT-qPCR RNA of samples was extracted according to the protocol specified by the extraction kit (QIAamp® Viral RNA Mini Kit). The samples were stored in an ultra-freezer at -80°C . The collected samples were tested for the presence of SARS-CoV-2 viral RNA by RT-qPCR with primers to amplify the envelope (E) gene and the human transcript of the gene for RNaseP (RNP) as the endogenous control [44]. Samples were considered positive with a cycle threshold (CT) ≤ 40 .

RT-qPCR using specific viral primers Testes samples were macerated and submitted to RNA extraction using the Viral RNA Kit (Zymo Research, USA), following the manufacturer's protocol. A two-step RT-qPCR approach was performed to optimize the detection of the viral RNA in the tissue samples and to avoid the possible influence of host cellular RNA. The obtained RNA was first submitted to cDNA synthesis using the CDC's SARS-CoV-2 specific reverse primer 2019-nCoV_N1-R (TCT GGT TAC TGC CAG TTG AAT CTG) and the SuperScript™ III First-Strand Synthesis System (Invitrogen, Brazil).

The viral cDNAs were then amplified in a qPCR reaction using the GoTaq qPCR Master Mix (Promega, USA). We used both N1 primers from CDC's SARS-CoV-2 detection protocol (2019-nCoV_N1-F: GAC CCC AAA ATC AGC GAA AT; 2019-nCoV_N1-R: TCT GGT TAC TGC CAG TTG AAT CTG) and followed the cycling recommendation indicated by the enzyme's supplier in a QuantStudio 3 Real-Time PCR System (Applied Biosystems, USA). To normalize the results, the same process was performed to amplify the human β -actin control.

Protein assays

Nanosensor

Gold nanorods (GNRs) were synthesized by the seed-mediated growth method as previously described [45]. The nanoparticles contained an average aspect ratio of 10×38 nm and a light absorbance peak of 713 nm. They were covalently functionalized with the polyclonal antibody anti-Spike protein (Rhea Biotech, Brazil) and a polyclonal antibody anti-Nucleocapsid protein (CTVacinas, Brazil) through a carbodiimide-activated amidation reaction. The binding between the gold surface and the antibodies was mediated by adding a capping layer formed by α -lipoic acid. A 2 mM α -lipoic acid solution (LA; Sigma Aldrich, USA) in ethanol was added to the GNR suspension (0.039 mg/mL). These suspensions were exposed to an ultrasonic bath (UNIQUE model U5C1850, 154W, 25KHz) at 55°C for 30 min. The suspension was sonicated again for 2 h at 30°C and left to rest overnight at RT to stabilize the interaction. GNRs were then centrifuged at 5600 g for 10 min and suspended in an aqueous solution. The GNR-LA complexes were kept at 4°C in the dark. Next, the modified GNRs were re-dispersed in a 10 mM phosphate buffer containing 16 mM EDAC and 4 mM sulfo-NHS (30 min in an ice-bath under sonication). After another centrifugation step, GNR-LA suspensions were blocked with poly (ethylene glycol)-thiolate (5kD mPEG-SH, 10-4 mM, from Nanocs) for 10 min in an ice bath, under stirring (Additional file 1: Fig. S1a-b).

TEM images of the nanosensors were obtained on a 120 kV FEI Technai G2-12 (Spirit BioTwin, USA) microscope (Additional file 1: Fig. S1c). Samples were directly dripped onto a holey carbon film supported on a copper grid (400 mesh) (Pelco®, USA) without any further processing. Zeta potential was obtained with a Zetasizer Nano ZS90 analyzer from Malvern at an angle of 173° at RT, as previously described [7, 46, 47]. The nanoparticle's size and zeta potential were measured simultaneously three times and in triplicate (Additional file 1: Fig. S1d-e).

Samples' labeling and measurements were previously described [7]. Briefly, GNR-S were incubated with 1mg/mL of anti-S protein polyclonal antibody (Rhea

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Fig. 7 Hypothetical viral and molecular mechanisms of testis infection and damage by SARS-CoV-2. **a** SARS-CoV-2 (green color) was identified in spermatogonial cells (Spg), Sertoli cells (SC), Leydig cells (LC), infiltrative monocytes (Mono), macrophages (MΦ), spermatocytes (sptc), and spermatids (sptd). Note viral factories in macrophages and spermatogonial cells (green arrows). A direct influence of SARS-CoV-2 in testicular cells hampers ACE2 activity, while activation of mast cells (chymase positive) elevates the levels of angiotensin II (a potent pro-inflammatory molecule) (asterisks). Angiogenic and inflammatory factors can induce the infiltration and activation of mast cells. High levels of angiotensin II, activation of mast cells, and inflammatory factors can activate (polarize) macrophages. The testicular phenotype of COVID-19 patients (fibrosis, vascular alteration, inflammation, tunica propria thickening, Sertoli cell barrier loss, germ cell apoptosis, and inhibition of Leydig cells) can be linked to elevated angiotensin II and active mast cells and macrophages. **b** Genes network related to angiotensin II, activated mast cells, and macrophages (pink box) extracted from STRING (<https://string-db.org/>). These three elements upregulate the inflammatory, apoptotic, fibrotic, and vascular genes while downregulating critical seminiferous tubule and Leydig cell genes. Red arrows: upregulated genes; green arrows: downregulated genes; ~: genes upregulated and downregulated depending on the phase

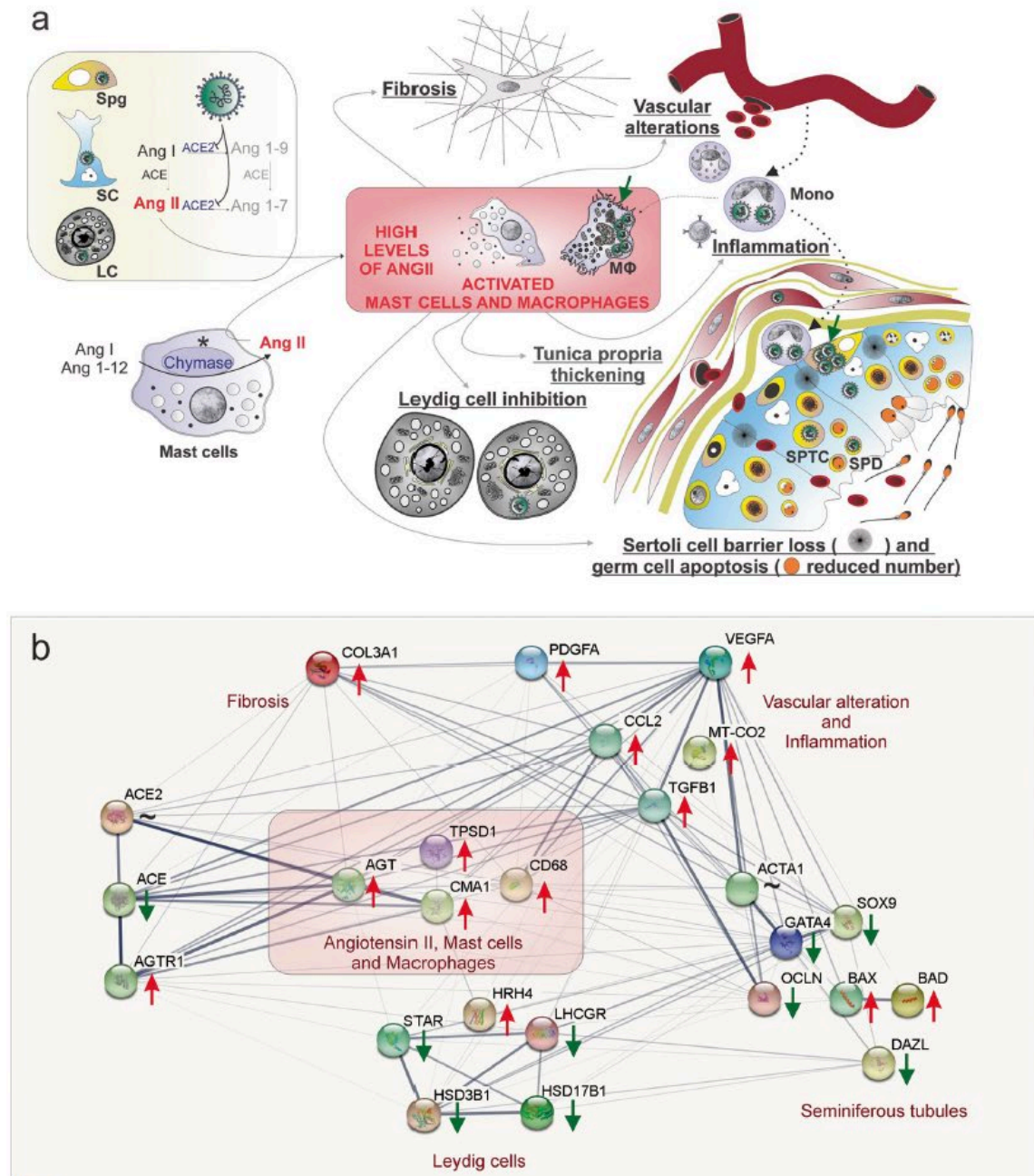


Fig. 7 (See legend on previous page.)

Biotech, Brazil). Next, the samples were labeled with goat anti-rabbit IgG-CFL 488, 100 µg/mL lex: 488 nm, λem: 520 nm (Santa Cruz Biotechnology, USA) (Additional file 1: Fig. S1f). For GNR-N, samples were incubated with 10 µg of a polyclonal antibody anti-protein

N for 60 min under axial shaking. Unbound antibodies were blocked with BSA (1%) for 30 min. Next, samples were labeled with Alexa Fluor® 546 goat anti-rabbit IgG H & L, 5 µg/mL lex: 540 nm, λem: 585 nm (Invitrogen, USA) and incubated under continuous shaking

in the dark and at RT for 30 min (Additional file 1: Fig. S1g). Samples were measured using the spectrophotometer Varioskan Flash spectral scanning multimode reader (Thermo Scientific, USA).

To determine the concentration of antibodies in the LSPR-nanosensor, an antibody curve was carried out ranging from 0.25 μg to 10 μg for anti-S and anti-N proteins, respectively (Additional file 1: Fig. S1h-l). The concentration chosen was 0.5 μg for both GNR-S and GNR-N nanosensors. Afterward, the nanosensors were exposed to purified S- and N-proteins, ranging from 10 μg to 10 fg, and their binding affinity was assessed by UV-Vis spectroscopy (Thermo Scientific, USA).

Finally, the GNR-S and GNR-N nanosensors were exposed to macerated testes samples (obtained from the 11 patients with COVID-19), diluted in PBS-1X, for 30 min at 4 °C under sonication, and their respective LSPR were measured using optical plates (Costar, USA), by UV-Vis spectroscopy (Thermo Scientific, USA). Spectra analyses were performed using OriginPro version 9.0. We normalized and smoothed the curves and measured the biosensing event through the X-axis intercept of the derivative of the Gaussian peak for each patient. We compared the COVID-19 patient curves to the GNR and negative control patient curves (Additional file 1: Fig. S1m-q). We focused on the red-shift that occurred at point zero (derivative axis) of the longitudinal peak. In this analysis, 5nm or below shifts were not considered significant.

Immunofluorescence against Spike protein

Immunofluorescence was performed using a validated primary anti-S protein antibody [48] (Rhea Biotech, Brazil) to detect and corroborate the viral presence in the testicular parenchyma (Additional file 2: Table S2). Reactions were visualized using Alexa-488 (Thermo Fisher Scientific, USA) conjugated secondary antibody, and images were acquired using a Nikon Eclipse Ti fluorescence microscope. As controls, we performed three different assays: (1) we used the testes of controls (prostate cancer patients); (2) we omitted the primary antibody in the testes of COVID-19 patients; and (3) we performed a negative control antigen, incubating the primary antibody with purified Spike protein (1:10; donated by CT-Vacinas-UFMG) before the immunofluorescence assay (Additional file 1: Fig. S2m-p).

SARS-CoV-2 activity and replication

VERO cell exposition to testicular macerates

In the BSL-3 laboratory, testis samples from the 11 COVID-19 patients were macerated using the tissue

Lyser (Tissuelyser II Retsch) and metallic beads (Qiagen, USA). All fragments were disrupted in 600 μL of DMEM without serum, and 300 μL was used for infection in vitro. VERO CCL-81 cells were seeded in a 6-well plate (Sarstedt, Germany) under 90% confluence. Culture media was discarded, and testis macerates were placed in the cell culture system for 1 h, at 37 °C and 5% CO_2 , with periodic homogenization. We did a negative control, exposing VERO cells to DMEM, and a positive control, exposing VERO cells to DMEM with SARS-CoV-2 (Wuhan strain SARS-CoV-2; MOI 0.01; donated by Dr. Edison Luiz Durigon).

After incubation, 2 mL of fresh supplemented DMEM was added, and cells were maintained (at 37 °C and 5% CO_2) for 48 h. Next, 300 μL of each sample were adsorbed in another 6-well plate containing Vero CCL-81 cells for 1 h, at 37°C and 5% CO_2 , with periodic homogenization. Afterward, 2 mL of fresh DMEM was added, and cells were incubated. In parallel, 200 μL of each supernatant was collected (1st and 2nd day; Table 2), inactivated in 200 μL of Isothiocyanate of Guanidine, and frozen at - 80 °C for the RT-qPCR analysis.

Viral detection in the supernatant

To confirm the isolation of SARS-CoV-2, we extracted the total RNA from the supernatant (200 μL) using a PureLink RNA Mini Kit (Invitrogen, USA), and the RT-qPCR reaction was performed using iTaq Universal Probes One-Step Kit (Bio-Rad). The SARS-CoV nucleocapsid RNA (N1) was measured with the following primer-probe set: F 5'-GACCCCAAAATCAGC GAAAT-3', R 5'-TCTGGTTACTGCCAGTTGAATCTG -3', and Probe 5'-FAM-ACCCCGCAT/ZEN/TACGTT TGGTGGACC-3IABkFQ-3'. The human transcript of the gene for RNaseP (RNP) was used as the endogenous control.

SARS-CoV-2 genomic and subgenomic RNA evaluation in VERO cells

Cytopathic effects were monitored, and the results were compared with a Mock-infection and concentrated SARS-CoV-2 virus as a positive control. After two passages, infected and control Vero CCL-81 cells were detached with Trypsin (Gibco, USA), spun down, and the concentrated pellets were kept frozen at - 80 °C for the RT-qPCR analysis. After cell lysis using Tissuelyser II Retsch and metallic beads (Qiagen, USA), 200 μL of each sample was added to 200 μL of lysis buffer with 2-mercaptoethanol. After homogenization, 200 μL of 70% ethanol was added. After washing with buffers, we added 40 μL of RNase-free water, and the samples were centrifuged for 1min 12000 $\times$ g to collect the total RNA.

SARS-CoV-2 genomic RNA was measured with the following primer-probe set: CoV-F3 (5'-TCCTGGTGA TTCTTCTTCAGGT-3'), CoV-R3 (5'-TCTGAGAGA GGGTCAAGTGC-3') and CoV-P3 (5'-AGCTGCAGC ACCAGCTGTCCA-3'). SARS-CoV-2 subgenomic RNA was measured with the following primer-probe set: CoV-sgRNA-F (5'-CGATCTCTTGTAGATCTGTTCTC-3'), CoV-sgRNA-R (5'-ATATTGCAGCAGTACGCACACA-3'), and CoV-sgRNA-P (5'-ACACTAGCCATCCTTACT GCGCTTCG-3').

Immunofluorescence and immunoperoxidase in Vero cells

To confirm viral isolation from the patient's testes, we performed an immunofluorescence assay using the anti-S protein commercial antibody (Abcam, USA). The cells were seeded in a 96-well Black plate with a transparent bottom (Costar, Corning Incorporated, USA), and 50 µL of each supernatant from the third passage were adsorbed for 1 h, at 37 °C and 5% CO₂, with periodic homogenization. Next, 50 µL of supplemented DMEM was added to each well and kept incubated for 4 h.

After the VERO infection, the contents were removed, and cell fixation was carried out using Paraformaldehyde 4% (BioRad, USA) for 15 min. The plates were washed with PBS 1X and blocked with a PBS solution containing 5% of Donkey Serum for 1h. The cells were then incubated with the anti-S antibody (Abcam, USA) for 1 h at 37°C. Afterward, cells were rinsed with PBS 1X and incubated with secondary antibody Alexa Fluor 488 anti-rabbit (Invitrogen, USA) and DAPI 300nM (dilution 1:1000) for 2 h at dark and room temperature. Finally, cells were washed with PBS 1X, and images were taken using EVOS 5000 ML (Invitrogen, USA).

We performed an immunohistochemistry assay using the anti-S commercial antibody (Rhea Biotech, Brazil). After fixation, the plates were blocked with a PBS solution containing 3% FBS for 15 min. Then, the cells were incubated overnight with the anti-S antibody (dilution 1:500) at RT. The cells were also incubated with an anti-rabbit IgG antibody conjugated to horseradish peroxidase (Promega, USA), diluted at 1:2500 at RT for 60 min. Immunoassay was revealed using the KPL TrueBlue Peroxidase Substrate (SeraCare, USA) for 10 min at RT, under gentle stir.

Histomorphometric analysis

All testicular slides were scanned using the Panoramic MIDI II slide scanner (3DHISTECH, Hungary). The histomorphometric analyses were performed using the

CaseViewer software (3DHISTECH, Hungary) and the Image J v.1.45s software (Image Processing and Analysis, in Java, USA).

Seminiferous epithelium cell composition

To describe the seminiferous epithelium integrity, at least 50 seminiferous tubule cross-sections were evaluated and classified according to the germ cell composition in the seminiferous epithelium. According to this classification, the patients were then categorized into three phases, as follows: phase 1, cross-sections containing all germ cell layers (spermatogonia, spermatocytes, and spermatids); phase 2, cross-sections containing spermatogonia and spermatocytes; phase 3, cross-sections containing only spermatogonia and/or degenerating/Sertoli cell-only seminiferous tubules. The results are presented as the percentage of seminiferous tubules in each category. After this analysis, we examined the clinical data of COVID-19 patients to understand the evolution of testicular pathology.

Seminiferous tubule measurements

Seminiferous tubules were analyzed using computer-assisted image analysis of 30 randomly chosen seminiferous tubules cross-sections per donor. To determine the seminiferous tubule diameter and tunica propria width, the measurements were taken at × 400 magnification, and the results were expressed in micrometers.

Leydig cell volume density

The volume densities (%) of testicular tissue components were obtained after counting 7200 points over testis parenchyma. The intersections that coincided with Leydig cells were counted in 15 randomly chosen fields by horizontal scanning of the histological sections at × 200 magnification [49].

Hemorrhagic scores

As red cell bleeding was a common finding, this pathology was measured in four scores, as follows:

1. Patients who presented many red blood cells inside the seminiferous tubule lumen and intertubular compartment (score 3);
2. Patients with vast areas of red blood cells bleeding in the intertubular compartment (score 2);
3. Patients with small areas of red blood cell bleeding in the intertubular compartment (score 1);
4. Patients without red blood cell bleeding (score 0).

Histochemistry techniques

Mast cell counts

Toluidine-blue staining was used to determine the number of mast cells. We investigated 15 testicular fields ($\times 20$ magnification), and the cells were quantified per patient.

PAS staining

A Schiffs kit (Sigma-Aldrich, USA) was used for PAS staining, as per the manufacturer's protocol. In brief, the sections were pre-treated with periodic acid for 5 min at RT, slowly rinsed in distilled water, and then stained with Schiff's solution for 10 min at RT in the dark. The nuclei were stained with hematoxylin for 5 min at RT, followed by six dips in 1% hydrochloric alcohol. After dehydration with 70, 90, and 100% graded alcohol, the sections were immersed twice in xylene for 10 min each. Then, the slides were mounted with a coverslip and sealed with Entellan resin (Sigma-Aldrich, USA). Images were captured using light Olympus microscopy (BX-60).

Masson's Trichrome and Picrosirius red

Tissue samples were stained with Masson's Trichrome and Picrosirius red to assess fibrosis and collagen types I and III. Images of the two techniques were captured in a Spot Insight Color digital camera adapted for Olympus BX-40, using the Spot software version 3.4.5. To determine the area of fibrosis and differentiate the types of collagens, images of three random areas of each patient were obtained at $\times 100$ magnification. Images were analyzed with Image J v. 1.53c software (National Institutes of Health, USA) using the Color Deconvolution tool, getting the average of the three areas evaluated [50].

Immunostaining

For immunostaining, deparaffinized sections (5 μ m thick) were dehydrated and submitted to heat-induced antigenic recovery (water bath) with buffered sodium citrate (pH 6.0) at 90 °C for 40 min. Then, the sections were immersed in BSA 3% (in PBS) solution to block non-specific antibody binding and incubated overnight at 4 °C with primary antibodies (Additional file 2: Table S2). Reactions were visualized using biotin-conjugated secondary antibodies (anti-goat: 1:100 dilution, Abcam, ab6740; anti-mouse: 1:200 dilution, Imuny, IC1M02; anti-rabbit: 1:200 dilution, Abcam, ab6720) combined with Elite ABC Kit (Vector Laboratories, USA). Signal detection was obtained via peroxidase substrate 3,3'-diaminobenzidine (DAB; Sigma Aldrich, USA) reaction and counterstaining with Mayer's hematoxylin (Merck, USA).

To identify targets of SARS-CoV-2 in the testis intertubular compartment, double-immunofluorescence anti-S with anti-CD-68 or anti-Chymase was performed.

Reactions were visualized using Alexa-488 (1:100 dilution), Alexa-546 (1:200 dilution), and Alexa-633 (1:200 dilution), all from Thermo Fisher Scientific (USA), and conjugated secondary antibody. For ACE2 evaluation, we used anti-ACE2 (Proteintech, USA) and Alexa-594 (1:100 dilution) from Jackson ImmunoResearch (USA). Images were acquired using the Nikon Eclipse Ti fluorescence microscope.

To analyze immunopositive macrophages and T lymphocytes, CD68 and CD3 positive cells were quantified by counting 10 testicular fields at $\times 400$ magnification. Only cells with visible nuclei, brown cytoplasm, and morphology compatible with the evaluated cells were counted. Microvessel density analysis was also performed in 10 fields at $\times 400$ magnification, and only CD31 immunopositive structures with or without lumen were counted (vessels containing muscle walls were not counted).

Transmission electron microscopy

Testes fragments were fixed by immersion in 4% glutaraldehyde (EMS, USA). Smaller pieces (1–2 mm thickness) were obtained and postfixed in reduced osmium (1% OsO₄ and 1.5% potassium ferrocyanide in distilled water) for 90 min, dehydrated in ethanol, and embedded in Araldite epoxy resin. Ultrathin sections (60 nm thick) were obtained using a diamond knife on a Leica EM UC6 ultramicrotome (Leica Microsystems) and mounted on 200 mesh copper grids (Ted Pella). The ultrathin sections were stained with lead citrate (Merck, USA) and analyzed using a transmission electron microscope (Tecnai G2-12 Thermo Fisher Scientific/FEI, USA). The viral particle was defined when presenting a round to oval shape, a membrane, an electron-dense interior, and within a membrane compartment [51].

Enzymatic and hormone measurements

The enzymatic activity of MPO and NAG and the total concentration of testosterone and angiotensin II were determined in human testis homogenates. For this purpose, 50 mg of snap-frozen testis from deceased COVID-19 patients and controls were homogenized in 450 μ L cold PBS supplemented with a protease inhibitor cocktail (Cat n° S8830, Sigma-Aldrich). After three freeze/thaw cycles in a liquid nitrogen/water bath (37 °C), the samples were centrifuged (14,000 g, 10 min, 4 °C), and the supernatants were collected.

For MPO and NAG measurements, 100 μ L of tissue homogenates were mixed 1:1 in MPO buffer assay (0.1M Na₃PO₄, 0.1% [w/v] HETAB, pH 5.0) or NAG buffer assay (0.2M citric acid, 0.2M Na₂HPO₄, pH 4.5), respectively, just prior the freeze/thaw step. The activity of MPO and NAG, which is an indirect estimation of the

abundance of neutrophils and macrophages, was measured in a colorimetric enzymatic assay. Intratesticular testosterone levels were determined in testis homogenates using a chemiluminometric immunoassay run on the Atellica IM Analyzer (Siemens Healthcare Diagnostics). The concentration of Angiotensin II was measured by ELISA, according to the procedures supplied by the manufacturer (MyBioSource, San Diego, CA, USA). The kit applied the sandwich ELISA technique. The sensitivity of the assay was 12 pg/mL.

Testicular gene expressions

Total RNA was isolated from testes using AurumTM Total RNA MiniKit[®] (BioRad, USA). A Nanodrop spectrophotometer (Thermo Fischer, USA) was used to measure the quantity and integrity of total RNA. RNA (2 µg/sample) was reverse transcribed using the iScript cDNA Synthesis Kit (BioRad, USA). cDNAs (10 ng) were amplified by qPCR with iTaq Universal SYBR Green Supermix (BioRad, USA) in Rotor-Gene Q (Qiagen, USA). The primer sequences used can be found in Additional file 2: Table S2. Relative levels of expression were determined by normalization to RPL19 e HPRT1 using the $\Delta\Delta CT$ method. The testicular gene expressions were displayed as Heat maps (Fig. 5 and Fig. 6) and individual graphs (Additional file 1: Fig. S6 and Additional file 1: Fig. S7).

Statistical analyses

Demographics and clinical characteristics were presented using descriptive statistics: mean and standard deviation (SD) for normally distributed continuous data, median and interquartile range (IQR) for non-normally distributed continuous data, and proportions and frequencies for categorical data. The presence and the strength of a linear relationship between demographics and clinical characteristics and the damage to the testicles were analyzed using Spearman correlation.

All quantitative data were tested for normality and homoscedasticity of the variances following Kolmogorov–Smirnov (Dallal–Wilkinson–Lilliefors) and Bartlett tests. Data from the fluorometry assay analyses were evaluated by one-way ANOVA for comparisons within groups, followed by Newman-Keuls (normal distribution). Histomorphometric and gene expression data were analyzed by unpaired Student's *t*-test, comparing COVID-19 groups to controls and COVID-19-P1 to COVID-19-P2 (COVID-19-P3 was not considered for statistics). The data obtained were represented as the mean $\pm$ SEM and geometric mean $\pm$ SD. Graphs and statistical analyses were conducted using GraphPad PRISM v6.0 (GraphPad Software, Inc.). Differences were

considered statistically significant at $p < 0.05$. The individual raw data are available online [52].

Abbreviations

17BHS	17 beta-hydroxysteroid dehydrogenase
3BHS	3 beta-hydroxysteroid dehydrogenase
ACE2	Angiotensin-converting enzyme 2
AGT1R	Angiotensin II receptor type 1
ANG II	Angiotensin II
BAD	BCL2 associated agonist of cell death
BAX	BCL2 associated X protein
BSL-3	Biosafety level 3
CMA1	Chymase 1
Col3a	Collagen type III alpha 1 chain
COVID-19	Coronavirus disease 2019
COX2	Cyclooxygenase-2
CT	Cycle threshold
DAZL	Deleted in azoospermia like
DMV	Double-membrane vesicles
E gene	Envelope gene
ERGIC	Endoplasmic reticulum Golgi intermediate complex
GNR	Gold nanorods
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HRH4	Histamine H4 receptor
ICU	Intensive care unit
LHR	Luteinizing hormone receptor
LSPR	Localized surface plasmon resonance
MCP1	Monocyte chemoattractant protein-1
MPO	Myeloperoxidase
NAG	N-acetyl-BD-glucosaminidase
N-Protein	Nucleocapsid protein
PAS	Periodic acid–Schiff
PDGFA	Platelet derived growth factor subunit A
RMW	Replication membranous webs
RNP	Ribonuclease P/ribonucleoprotein
RPL19	Ribosomal protein L19
S-protein	Spike protein
STAR	Steroidogenic acute regulatory protein
TEM	Transmission electron microscopy
TMPS2	Transmembrane serine protease 2
TPSB2	Tryptase beta 2
VEGF	Vascular endothelial growth factor

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12915-022-01497-8>.

Additional file 1: Fig. S1. COVID-19 nanosensor platforms. Fig. S2.

Immunolabeling against S-protein in testes of all COVID-19 patients and in VERO cells. Fig. S3. Histology of activated mast cells and infiltrative monocytes in COVID-19 patients. Fig. S4. Tubular compartment morphological alterations. Fig. S5. Leydig cell, collagen deposition, and blood vessel alterations in COVID-19 patients. Fig. S6. Transcript level of key genes related to high angiotensin II levels, immune cells and vascular system and fibrosis. Fig. S7. Relative expression of important genes associated with the tubular compartment and Leydig cells. Individual values can be found at <https://doi.org/10.6084/m9.figshare.16777786.v8>.

Additional file 2: Table S1. Clinical data from control patients. Table S2. Antibodies and Primers (qPCR) used in this study.

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Authors' contributions

This study counted on the help of several researchers due to the difficulties imposed by the pandemic in assessing the research areas. The research team was divided into seven groups, responsible for the sample collection, study design, experiments, and data analyses in each research area: (1) Reproductive Biology Group (G.M.J.C.; S.M.S.N.L., A.F.A.F., N.T.W., M.R.B., G.H.C.S., H.C.G., and A.C.B.); (2) Urology Group (M.H.F., A.K.Z., L.G.G.P., Y.L.G., B.P.M., M.S.P., and T.P.F.); (3) Pathology Group (G.D.C. and F.R.S.); (4) Virology Group (V.V.C., F.G.F., M.L.N., R.S.A., F.G.S., G.R.F.C., C.B., and P.R.); (5) Nanotechnology Group (A.F.V., L.M.A.; E.M.N.M., and L.A.M.); (6) Immunology Group (G.B.M. and M.M.A.); (7) Renin-Angiotensin/Male Fertility Group (L.M.K., A.M.M., M.B.E. (and K.E.O.). Coordination of the groups: G.M.J.C., G.D.C., A.K.Z., M.H.F. Technical and resource support: G.M.J.C., G.D.C., H.C.G., V.V.C., F.G.F., M.L.N., R.S.A., G.B.M., A.K.Z., M.H.F. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. The datasets generated and/or analyzed during the current study are also available in figshare: <https://doi.org/10.6084/m9.figshare.16777786.v8>

Declarations

Ethics approval and consent to participate

The Research Ethics Committee of the Mater Dei Hospital and the National Research Ethics Committee (CONEP) approved this investigation under the number CAAE: 30999320.1.0000.5128. Postmortem collection of both testicles was performed after a legally responsible family member signed an informed consent document.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Supplementary Information

Fig. S1: Recognition of S and N protein

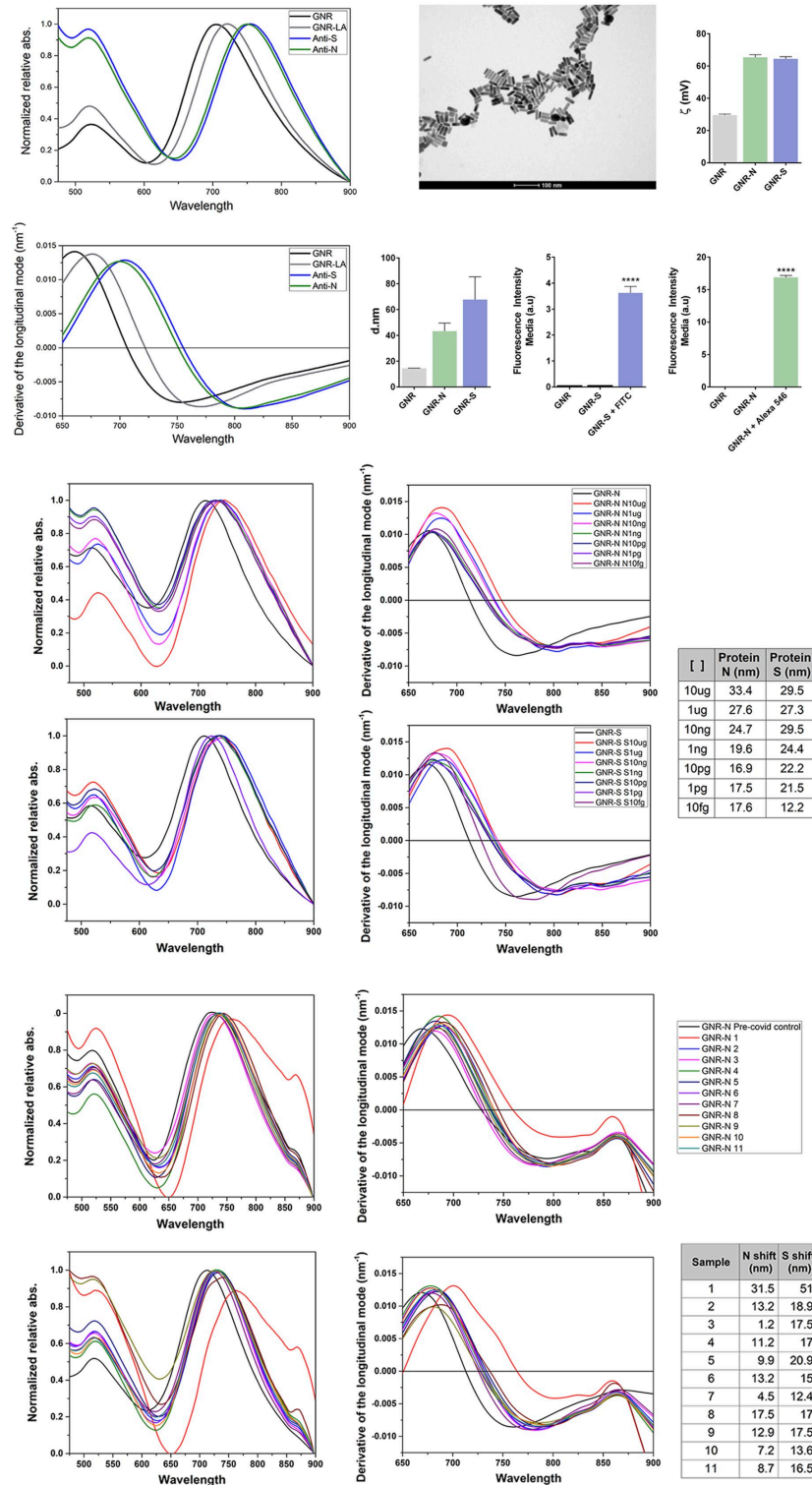


Fig. S1. COVID-19 nanosensor platforms. Gold nanorods (GNRs) were coated with anti-S and anti-N antibodies to construct a COVID-19 nanosensor and then characterized by different techniques. a) UV-Vis LSPR spectra of bare GNRs, lipoic-acid coated surface (GNR-LA) and GNR nanosensors recognizing S- and N-proteins, respectively. b) derivative curve of the observed light absorbance redshifts. c) TEM image of the GNRs. d) stability of both nanosensors by zeta potential measurements. e) the dynamic light scattering of both nanosensors and their respective hydrodynamic radius. f-g) fluorophores binding both nanosensors. h-k) Light absorbance spectra due to LSPR and respective derivative curves regarding the limits of detection of both nanosensors exposed to different concentrations of S- and N-proteins in means of nm shifting. m-p) Light absorbance spectra due to LSPR and respective derivative curves of the nanosensors recognizing the presence of SARS-CoV-2 in each sample compared to non-infected Control. q) the LSPR shift (nm) of each nanosensor after sample exposure. (mean $\pm$ SD, ****p<0.001)

Fig. S2

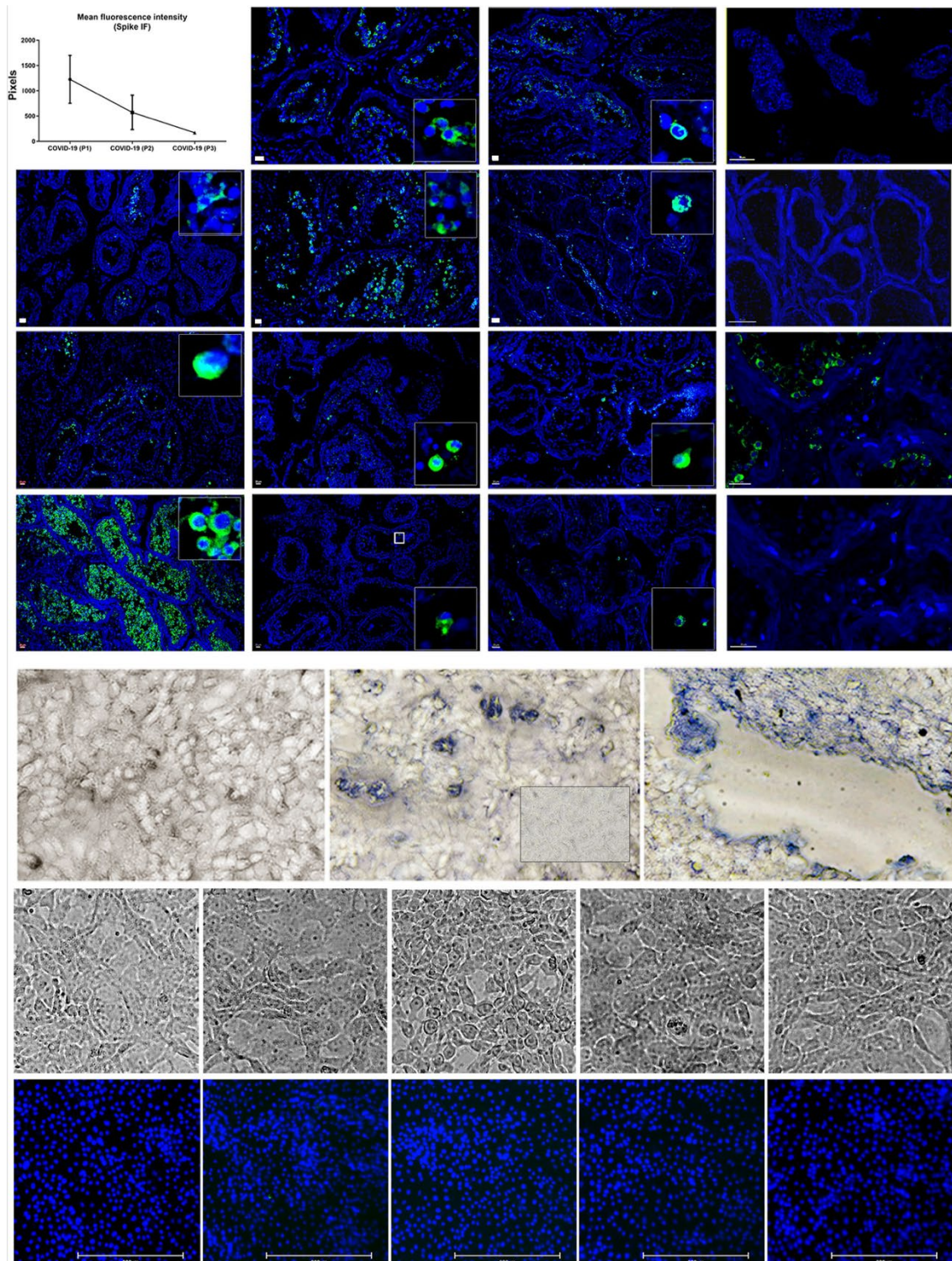
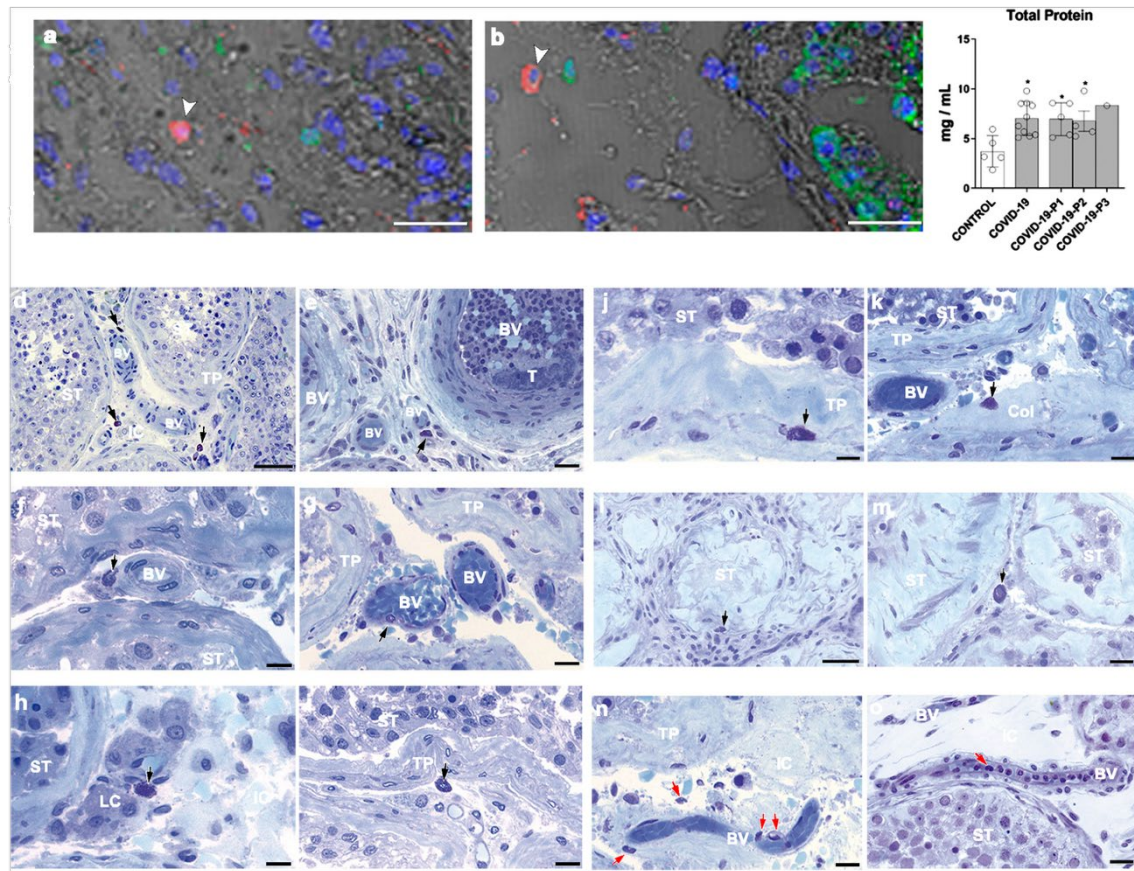


Fig. S2. Immunolabeling against S-protein in testes of all COVID-19 patients and in VERO cells. a) mean fluorescence index (in pixels) evaluating the large images of immunostainings against S-protein. b-l) images of testis parenchyma of COVID-19 patients. b-f) first phase COVID-19 patients. g-k) second phase COVID-19 patients. l) third phase COVID-19 patient. Inserts depict labeled germ cells in all patients. Scale bars = 20 μ m. m-o) Controls of the immunofluorescence reactions. m) negative image of Control patient #1 (Scale bar = 70 μ m). n) negative control, omitting the primary antibody. o-p) antigen control, comparing the tissue incubated with primary antibody (o) and primary antibody previously incubated with purified Spike protein. q-z') immunoperoxidase and immunofluorescence in VERO cells exposed to testicular macerates. q-s) VERO cells exposed to testicular macerate from patient #1. q) negative control. r) immunostaining (blue labeling) depicting S-protein in VERO cells. r') Negative control, omitting the primary antibody. s) image illustrating the cytopathic effect (*) in VERO cells. t-z) reduced cytopathic effect in VERO cells exposed to testicular macerates from patients #2, #6, #9, #10, and #11. Scale bars = 30 μ m. t'-z') no labeling for S-protein in VERO cells exposed to testicular macerates from patients #2, #6, #9, #10, and #11. Scale bars=300 μ m. Individual values can be found at: <https://doi.org/10.6084/m9.figshare.16777786.v8>.

Fig. S3**Fig. S3. Histology of activated mast cells and infiltrative monocytes in COVID-19 patients.**

a-b) activated mast cells (chymase +, white arrowheads) are not labeled for the S-protein (green). Blue = dapi staining. (Scale bars = 50 μ m). c) quantity of protein per milligram of tissue indicating an inflammatory process (t-test; two-tailed; infected groups vs control, * $p < 0.05$). d-m) a high number of mast cells in the testes of COVID-19 patients (arrows) (Scale bar = 50 μ m). d-e) mast cells near blood vessels (BV) filled with immune cells (T represents a thrombotic area) (Scale bar = 20 μ m). f-g) perivascular mast cells (arrows) near intact (f) and disrupted (g) blood vessels (Scale bars = 15 μ m). h) mast cell (arrow) near Leydig cells (Scale bar = 15 μ m). i) mast cell (arrow) near thickened tunica propria (Scale bar = 15 μ m). j) mast cell (arrow) inside the seminiferous tubule (Scale bar = 15 μ m). k) mast cell (arrow) near a collagen matrix (Col) in the intertubular space (Scale bar = 15 μ m). l) mast cell (arrow) inside a fibrotic seminiferous tubule

(Scale bar = 50 μm). m) mast cell in the intertubular area of a third-phase patient (Scale bar = 25 μm). n-o) a high number of infiltrative monocytes (red arrows) in the intertubular compartment (IC) (Scale bars = 20 μm). Individual values can be found at: <https://doi.org/10.6084/m9.figshare.16777786.v8>.

Fig. S4

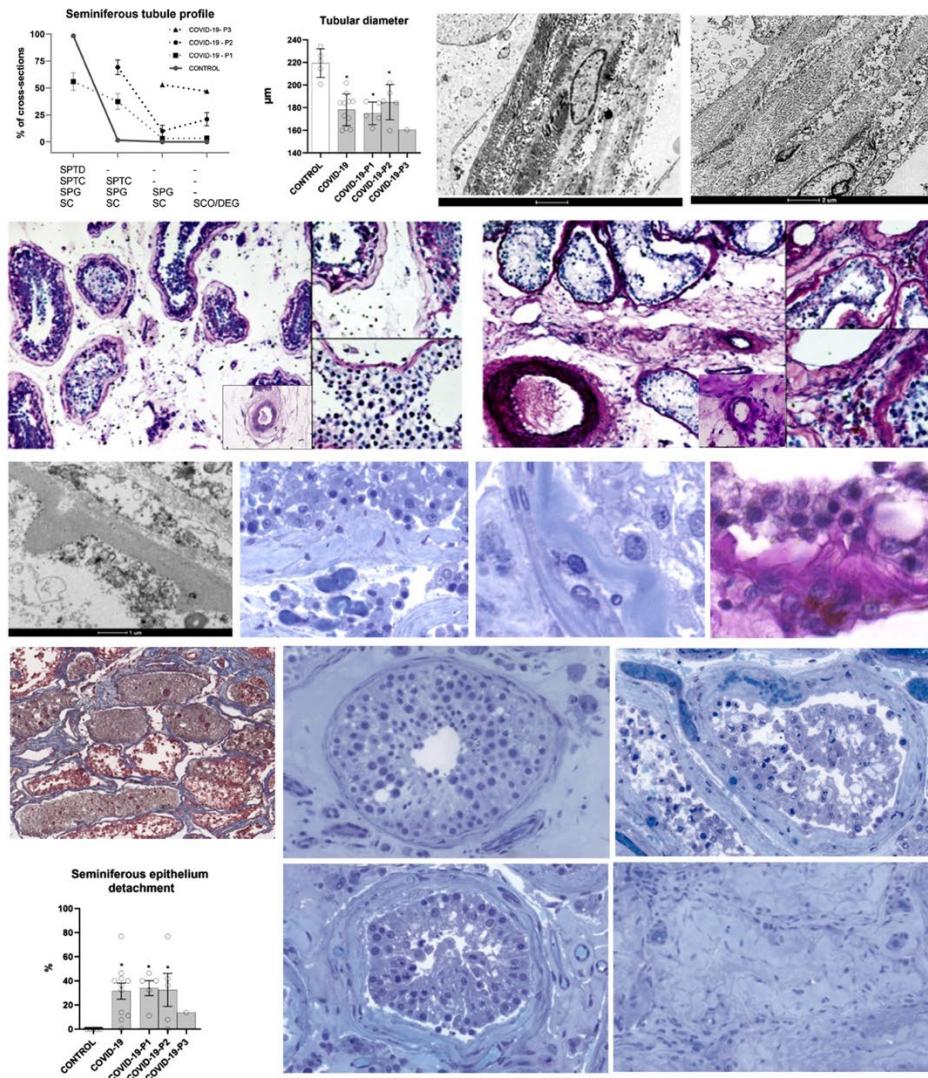


Fig. S4. Tubular compartment morphological alterations. a) quantification of the seminiferous tubule cross-sections profile (in %) in Controls and COVID-19 patients. b) seminiferous tubule diameter in Controls and COVID-19 patients (t-test; two-tailed; infected groups vs control, $*p<0.05$). c-d) a high number of collagen fibers (asterisks) and peritubular myoid cells (My, white arrows) in COVID-19 patients (Scale bars = 2 μ m). e-j) basement membrane (PAS+) in Controls (e-g) and COVID-19 patients (h-j) (Scale bars = c: 30 μ m). Asterisks denote high deposition of glycoproteins in the basement membrane and surrounding blood vessels. k-n) convoluted appearance of the basement membrane of COVID-19 patients (asterisks) (Scale bars = s: 1 μ m; t-v: 10 μ m). Mast cells (black arrows) and macrophages (green arrows) were observed near these areas. o) rete testis (RT) area filled with sloughing and apoptotic germ cells (green arrows) in COVID-19 patients (Scale bar = 50 μ m). p-t) seminiferous epithelium detachment (black arrows) from the tunica propria in Controls and COVID-19 patients (t-test; two-tailed; infected groups vs control, $*p<0.05$) (Scale bars = 50 μ m). Individual values can be found at: <https://doi.org/10.6084/m9.figshare.16777786.v8>.

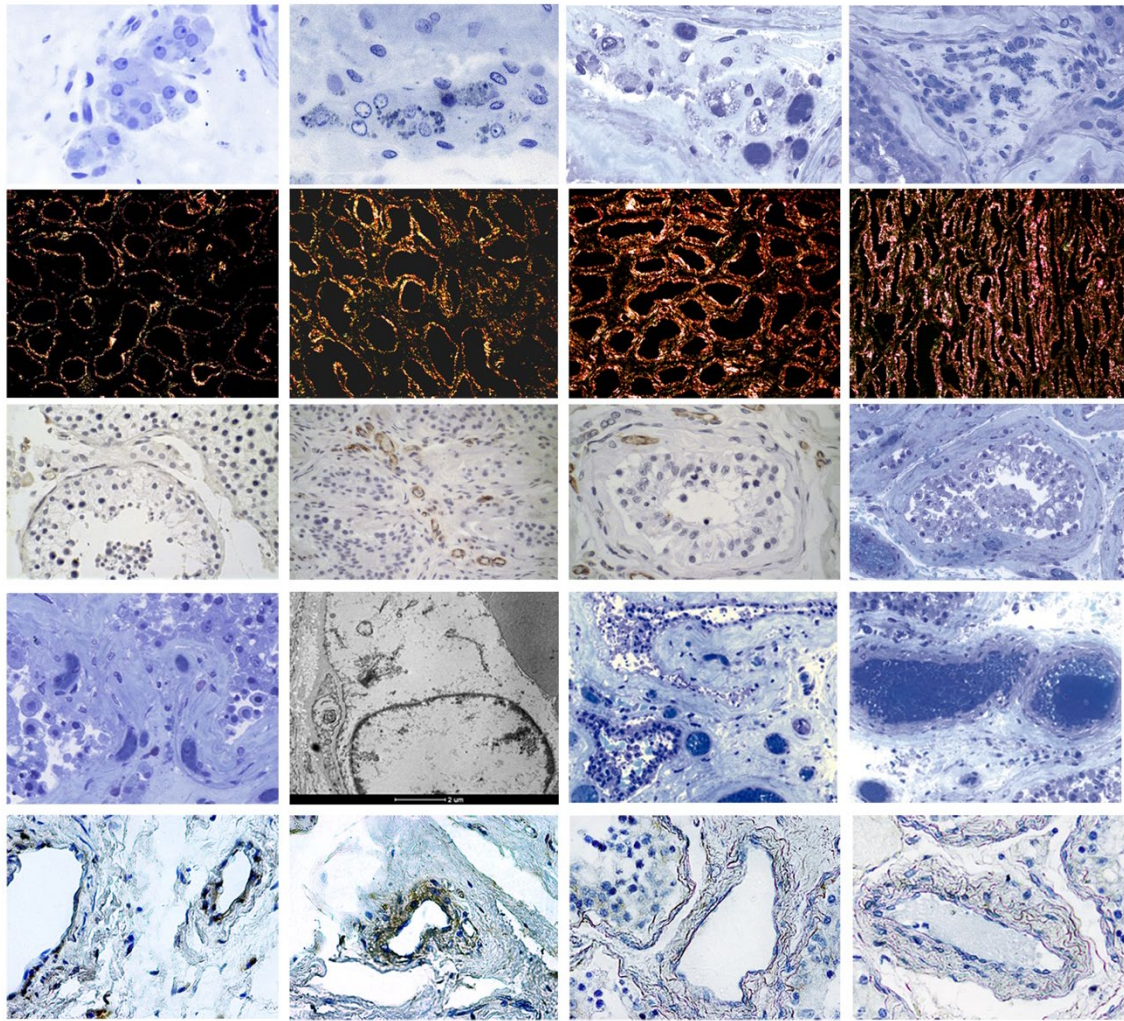
Fig. S5

Fig. S5. Leydig cell, collagen deposition, and blood vessel alterations in COVID-19 patients. a-d) Leydig cell morphology in Controls and COVID-19 patients (Scale bars =30 μ m). b-d) vacuoles and granules in the Leydig cell cytoplasm. e-h) Picrossirius staining demonstrating Birefringence in Yellow Orange (type I collagen) and green (type III collagen) (Scale bars = 100 μ m). i-k) CD31 immunolabeling in Controls (i) and COVID-19 patients (j-k) evidencing the newly formed blood vessels in testis parenchyma (Scale bar = 30 μ m). l-m) blood vessels inside tunica propria (arrows) of COVID-19 patients (Scale bars = 30 μ m). n) TEM image of an immature endothelial cell identified in tunica propria (Scale bar = 2 μ m). o) red blood cells (arrows) inside the rete testis (RT)lumen (Scale bar = 30 μ m). p) thrombus (*)

inside the blood vessel (Scale bar = 30 μ m). q-t) Endothelin1/2/3 immunolabeling in Controls (q) and COVID-19 phase one, phase two, and phase three (p1, p2, and p3) patients showing a higher intensity in phase one patients followed by weak labeling in phases two and three patients (Scale bars = 50 μ m).

Fig. S6

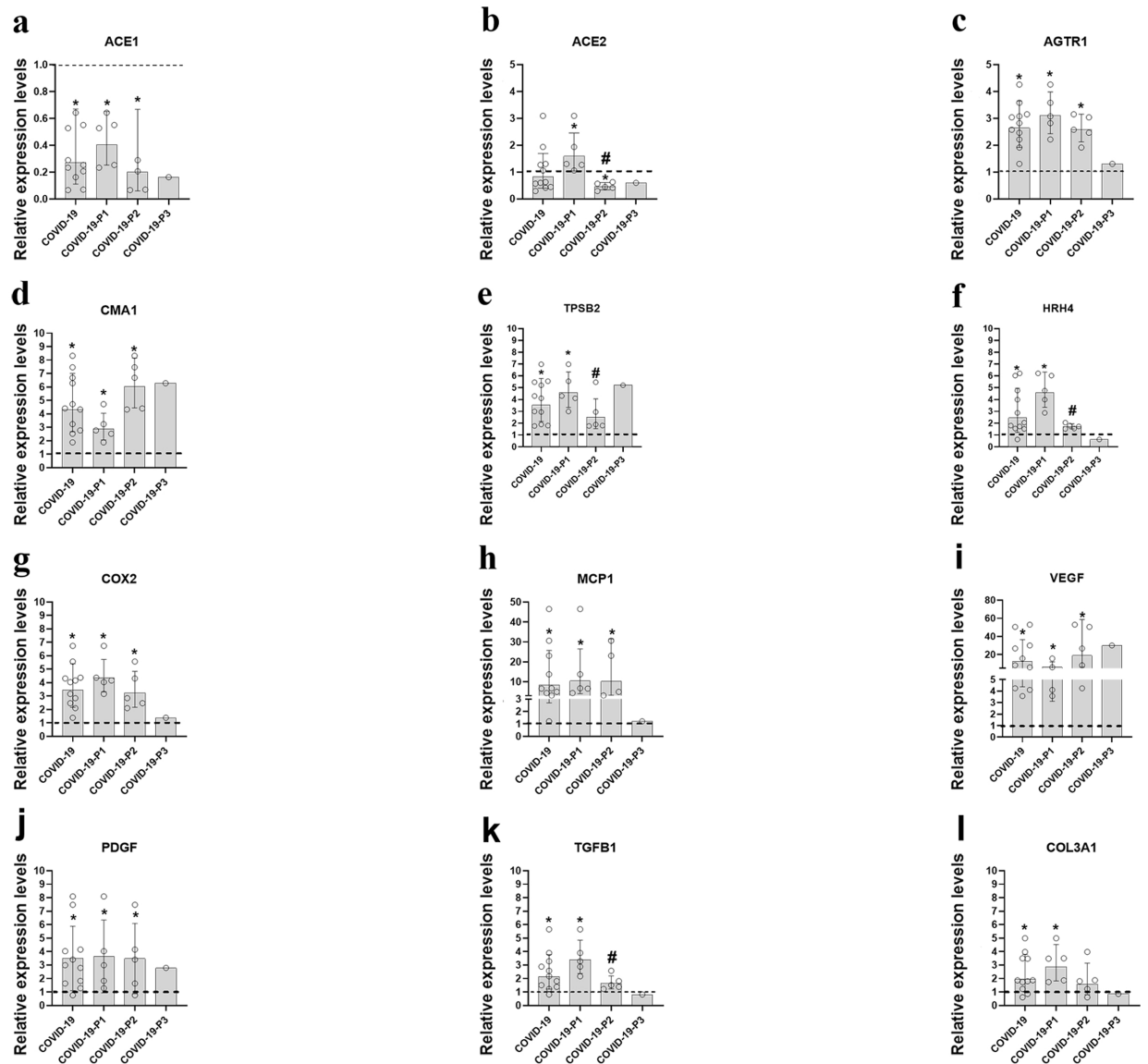


Fig. S6. Transcript level of key genes related to high angiotensin II levels, immune cells and vascular system, and fibrosis. a-c) Genes from the renin-angiotensin system: ACE1 (a), ACE2 (b), and AGT1R (c). d-e) mast cell genes: chymase (CMA1) (d), and tryptase (TPSB2) (e). f) Histamine receptor (HRH4). g) Cyclooxygenase-2 (COX-2). h) Monocyte chemoattractant protein 1 (MCP1). i) Vascular Endothelial Growth Factor (VEGF). j) Platelet-Derived Growth Factor (PDGF). k) Transforming Growth Factor Beta1 (TGFB1). l) Collagen Type III Alpha 1 Chain (COL3A1). Data are expressed as geometric mean $\pm$ SD. * $p < 0.05$ COVID-19 patients compared to the Control group (which is set at 1; dashed line). # indicates significant differences between COVID-19-P1 and COVID-19-P2 ($p < 0.05$); t-test (two-sided). Individual values can be found at: <https://doi.org/10.6084/m9.figshare.16777786.v8>.

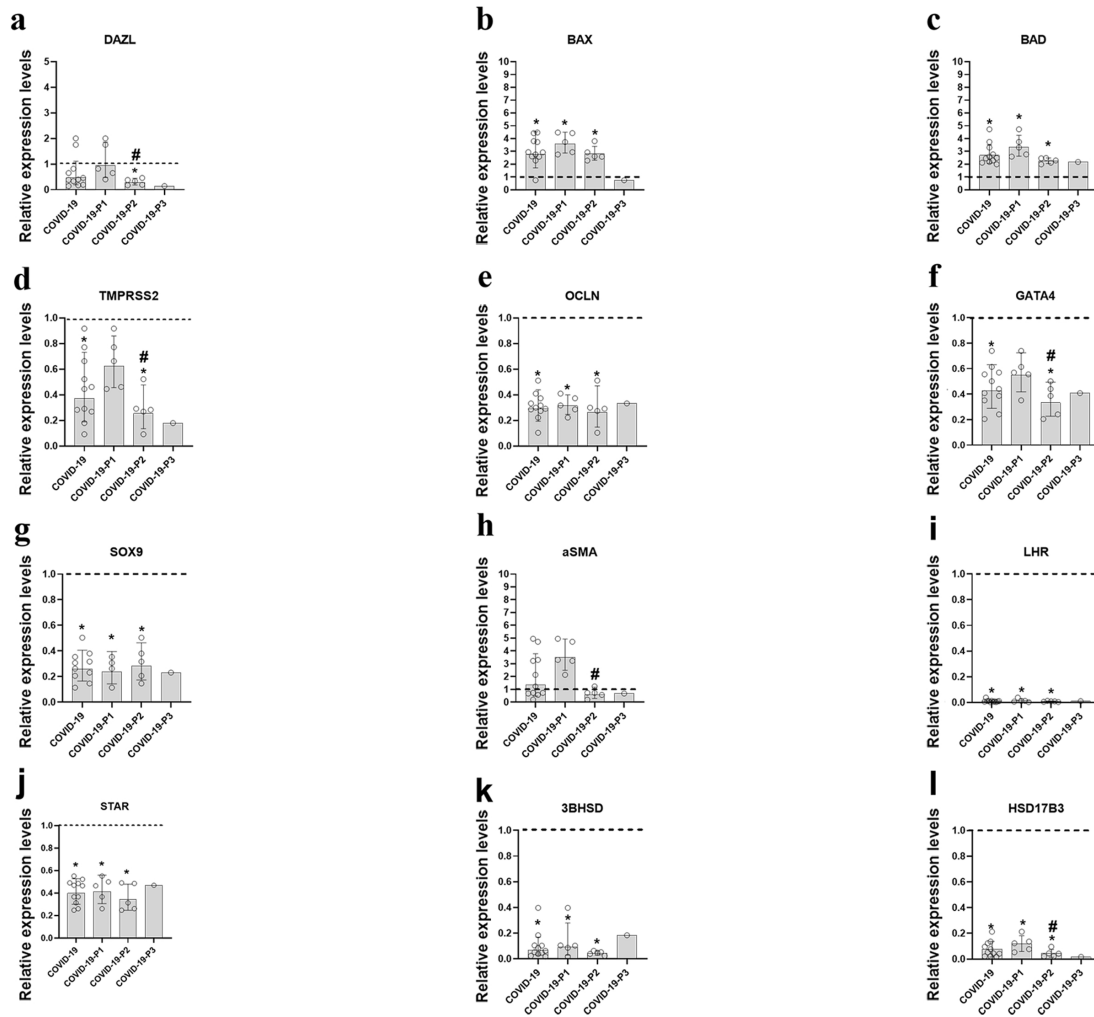
Fig. S7

Fig. S7. Relative expression of important genes associated with the tubular compartment and Leydig cells. a) Deleted In Azoospermia Like (DAZL). b) BCL2 Associated X Protein (BAX). c) BCL2 Associated Agonist Of Cell Death (BAD). d) Transmembrane Serine Protease 2 (TMPRSS2). e) Occludin (OCLN). f) GATA Binding Protein 4 (GATA4). g) SRY-Box Transcription Factor 9 (SOX9). h) Actin, Alpha Skeletal Muscle (aSMA). i-l) genes related to Leydig cell steroidogenesis: Luteinizing Hormone Receptor (i), Steroidogenic Acute Regulatory Protein (j), 3 Beta-Hydroxysteroid Dehydrogenase (k), Hydroxysteroid 17-Beta Dehydrogenase 3 (l). Data are expressed as geometric mean $\pm$ SD. * $p < 0.05$ COVID-19 patients compared to the Control group (which is set at 1; dashed line). # indicates significant differences

between COVID-19-P1 and COVID-19-P2 ($p < 0.05$); t-test (two-sided). Individual values can be found at: <https://doi.org/10.6084/m9.figshare.16777786.v8>.

3.2 Artigo 2

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Page 1 of 25

Journal of Medical Virology

Research Article

Detection of Infectious SARS-CoV-2 in Semen Months After COVID-19: A Potential Clue to Long COVID Pathophysiology

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Additional Information

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Manuscript JMV 1.docx	Main Document - MS Word	193.6 KB	Page 4
Figures (5).docx	Figure	4.4 MB	Page 22

Detection of Infectious SARS-CoV-2 in Semen Months After COVID-19: A Potential Clue to Long COVID Pathophysiology

Running title:

Seminal SARS-CoV-2 and Long COVID Clues

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45 *Abstract*

46

47 Viral persistence is considered one of the key mechanisms driving long COVID symptoms. The male
 48 genital tract, particularly the testis, is an immune-privileged site that may serve as a viral reservoir
 49 for SARS-CoV-2. However, the presence of SARS-CoV-2 in semen and its long-term infectivity
 50 remains controversial. This observational, prospective, cross-sectional study included 38 men treated
 51 at Mater Dei Hospital in Brazil who had recovered from mild to moderate COVID-19. Semen samples
 52 were collected from mildly symptomatic participants at 30 days, 3 months, and 6 months after
 53 symptom onset. The moderately symptomatic group provided a single sample approximately 6
 54 months post-infection. Semen analysis and SARS-CoV-2 detection were performed using RT-qPCR,
 55 followed by viral isolation in Vero CCL-81 cells. Infectivity was confirmed through
 56 immunofluorescence and transmission electron microscopy (TEM), and viral genotyping was
 57 conducted for strain classification. SARS-CoV-2 was detected by RT-qPCR in 21 of 38 patients
 58 (55%), with viral persistence observed for more than 6 months post-infection. Infective and
 59 replicating virus was confirmed in 17 of 24 RT-qPCR-positive samples using Vero cell culture,
 60 immunofluorescence, and TEM. Genotyping of the virus was feasible in only one sample due to low
 61 viral load in most of the samples. This study is the first to demonstrate the long-term persistence and
 62 infectivity of SARS-CoV-2 in semen, suggesting that the male genital tract may serve as a viral
 63 reservoir. These findings highlight the importance of semen analysis for detecting viral persistence
 64 and understanding long COVID pathophysiology.

65

66 *Keywords:*

67 Male genital tract, virus detection, viral infectivity, PASC, post-acute sequelae, coronavirus infection

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93 *Introduction*

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95 Long COVID is a chronic, multisystem condition that persists for at least three months after acute
96 SARS-CoV-2 infection. It may present as a continuous, relapsing, remitting, or progressive disease
97 and affects millions worldwide despite advances in vaccination and changes in viral virulence. The
98 enduring impact of long COVID continues to pose a substantial burden on healthcare systems and
99 economies globally.¹

100
101 Although the underlying mechanisms of long COVID remain incompletely understood, several
102 pathways have been implicated, including viral persistence, immune dysregulation, mitochondrial
103 and complement system dysfunction, prothrombotic inflammation, and alterations in the
104 microbiome.¹ Among these, viral persistence has emerged as a particularly compelling factor. This
105 phenomenon, documented across a range of viral infections, refers to the sustained presence of viral
106 genetic material and/or proteins in host tissues beyond the acute phase. Mounting evidence supports
107 an association between viral persistence and post-acute sequelae of SARS-CoV-2 (PASC),
108 suggesting it may contribute directly to the chronic symptoms characteristic of long COVID.¹

109
110 The testis is among several organs considered immune-privileged sites, along with the brain, the eye,
111 and the pregnant uterus.² While this status preserves testicular function by limiting immune
112 responses, it also creates a potential sanctuary for viral pathogens.² SARS-CoV-2 has demonstrated
113 tropism for testicular tissue, with viral RNA and particles identified in autopsy specimens from men
114 who died with COVID-19.³ The virus has been detected in multiple testicular cell types—including
115 Sertoli and Leydig cells, spermatogonia, macrophages, and fibroblasts—and even within the
116 seminiferous tubules lumen.³ Furthermore, the blood-testicular barrier may not constitute a perfect
117 barrier to viruses, especially in the presence of systemic or local inflammation. Notably, viral particles
118 have been detected within the seminiferous tubules, raising the possibility of SARS-CoV-2 shedding
119 in the semen.³

120
121 Given that at least 27 different viruses have been identified in human semen,⁴ the possibility of
122 SARS-CoV-2 shedding in semen has generated significant interest. While SARS-CoV-2 has been
123 detected in testicular tissue, its presence in semen remains controversial. Most studies, with few
124 exceptions,⁵⁻¹⁰ have not detected SARS-CoV-2 in semen. However, these studies often involved
125 limited follow-up and variable timing of sample collection, typically focusing on the acute or early
126 recovery phase. Indeed, most of these studies reported the persistence of SARS-CoV-2 based on the
127 detection of viral genomic RNA, without investigating viable, infectious viral particles in semen.

128
129 This study aimed to fill a critical knowledge gap by investigating the long-term presence and potential
130 infectivity of SARS-CoV-2 in semen, evaluating whether the virus can persist and remain viable in
131 the male genital tract (MGT) well beyond the acute phase of infection. We hypothesized that
132 infectious SARS-CoV-2 particles may persist in semen for extended periods following clinical
133 recovery.

134 *Methodology*

135 COVID-19 patients

136
137
138 This observational, prospective, cross-sectional study included adult men (18–50 years) diagnosed
139 with COVID-19 by RT-PCR from oropharyngeal swabs, who attended Hospital Mater Dei between
140 October 2020 and October 2021, and consented to participate. The only exclusion criterion was prior

141 vasectomy. Participants were divided into two groups based on symptom severity: mildly
142 symptomatic (not hospitalized) and moderately symptomatic (hospitalized).

143
144 All participants had clinically recovered from COVID-19. Clinical data were collected through a
145 questionnaire, and biological samples were obtained after obtaining informed consent. The study was
146 approved by the National Research Ethics Committee (CONEP) under CAAE:
147 36122420.5.0000.5128 and conducted in accordance with the Declaration of Helsinki. Access to
148 Brazilian genetic heritage was registered in the National System for the Management of Genetic
149 Heritage and Associated Traditional Knowledge (SISGEN), under registration number AC1D978.
150

151 Semen specimens

152 After completing a 15-day quarantine, mildly symptomatic patients provided semen samples at 15–
153 45 days, 3 months, and 6 months after symptom onset. Moderately symptomatic patients provided a
154 single sample 6 months after diagnosis. Samples were collected by masturbation, after 2–3 days of
155 abstinence, in a clinic laboratory room following strict hygiene protocols. Remaining raw semen from
156 both groups was frozen in liquid nitrogen (–196 °C) and stored in a dedicated biorepository at the MF
157 Male Fertility Clinic under the supervision of one of the authors (MHF) until analysis.
158

159 Semen analysis

160 Semen samples were analyzed following WHO guidelines for assessing semen volume,
161 concentration, motility, morphology, vitality, and leukocyte count. Volume was determined by
162 weighing the collection container. After liquefaction, concentration and motility were assessed under
163 a phase-contrast microscope (×200–400, OLYMPUS BX43) with standard dilutions applied when
164 necessary. Sperm motility was evaluated using a Makler chamber with a 10 µL aliquot, analyzing at
165 least 200 spermatozoa. Morphology was assessed using Tygerberg's strict criteria after Papanicolaou
166 staining, under oil immersion (×100). A single experienced technician performed all analyses.
167

168 Lymphocytic panel

169 We used flow cytometry (FACSCalibur, BD Biosciences, USA) to detect the presence of CD3, CD4,
170 CD8, and CD45 cells in semen. In brief, round cells were obtained by centrifuging the semen sample
171 in a 40% density gradient at 1200 RPM for 15 minutes. After centrifugation, the formed central halo
172 was separated from the gradient using a Pasteur pipette, washed twice in PBS 1X, and centrifuged at
173 1200 RPM for 7 minutes. Subsequently, the cells were quantified in a Neubauer chamber.
174

175 Cells were stained with anti-CD45, CD3, CD4, and CD8 monoclonal antibodies using the BD
176 Multitest™ CD3/CD8/CD45/CD4 kit. One million cells were incubated in the dark for 15 minutes at
177 room temperature, washed, and then resuspended in 500 µL of 1X PBS. Data acquisition (20,000
178 events) was performed on a BD FACSCalibur and analyzed using BD CellQuest software. Based on
179 FSC vs SSC profiles, T lymphocytes (CD3+) were identified within the CD45+ gate and
180 characterized as CD4+ or CD8+.
181

182 Detection of SARS-CoV-2 in semen

183 *SARS-CoV-2 detection using RT-qPCR*

184 We used two protocols for RT-qPCR. For the first 15 samples, total RNA was extracted using the
 185 QIAamp® Viral RNA Mini Kit (Qiagen), following the manufacturer's instructions. The samples
 186 were stored in an ultra-freezer at -80°C until RT-qPCR analysis. The presence of SARS-CoV-2 viral
 187 RNA was detected by RT-qPCR using primers to amplify the envelope (E) gene and the human
 188 transcript of the RNase P (RNP) gene as the endogenous control.

189
 190 For the remaining samples, we extracted the total RNA following the protocol outlined in the
 191 PureLink RNA Mini Kit (Invitrogen, USA). A water-only control (RNA Blank) was included with
 192 each round of extraction to check for RNA contamination. The RT-qPCR reaction was performed
 193 using iTaq Universal Probes One-Step Kit (Bio-Rad). The nucleocapsid gene's N1 locus of the
 194 SARS-CoV-2 RNA was measured with a high-precision primer-probe set (IDT, Coralville, USA).
 195 The human transcript of the 18S ribosomal RNA (18S rRNA) was used as the endogenous control,
 196 ensuring the reliability of the results.

197
 198 Reactions were run on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories,
 199 Hercules, CA) under the following conditions: reverse transcription at 50 °C for 10 min followed by
 200 a single cycle of 95 °C for 3 min, then 45 cycles of PCR with denaturation at 95 °C for 15 s, annealing
 201 at 55 °C for 30 s and extension at 60 °C for 30 s. Samples were considered positive with a cycle
 202 threshold (Ct) ≤ 40.

203

204 *SARS-CoV-2 isolation*

205 *VERO cell culture*

206 In a BSL-3 lab, 1×10⁵ VERO cells (ATCC®CCL-81) in 500 µL of DMEM were seeded into 24-well
 207 plates and incubated at 37°C with 5% CO₂ for 24 hours until 90% confluence. Only samples that
 208 tested positive for SARS-CoV-2 were used for inoculation. Prior to inoculation, all semen samples
 209 underwent RT-qPCR analysis. The samples were thawed and diluted 1:10 in DMEM. Subsequently,
 210 100 µL of the diluted semen was added to each well and incubated for 1 hour at 37°C with gentle
 211 intermittent mixing to facilitate viral adsorption.

212
 213 Following incubation, 1 mL of fresh DMEM was added, and the cells were maintained at 37°C and
 214 5% CO₂ for 72 hours. Supernatants (150 µL) were collected every 24 hours, mixed with lysis buffer
 215 plus beta-mercaptoethanol, and stored at -80 °C for subsequent RNA extraction and RT-qPCR. Each
 216 sample yielded three time-point specimens. Negative controls used DMEM alone, and positive
 217 controls used DMEM containing the SARS-CoV-2 Delta strain (MOI 0.01).

218

219 *Immunofluorescence in Vero cells*

220

221 Vero cell infection was confirmed via immunofluorescence using a commercial anti-S protein
 222 antibody (Abcam, USA). Cells were seeded in 96-well black plates with transparent bottoms and
 223 incubated with 50 µL of third-passage supernatant for 1 hour at 37°C and 5% CO₂ with
 224 homogenization. Then, 50 µL of DMEM was added and incubated for 24 hours.

225

226 Cells were fixed with 4% paraformaldehyde for 15 minutes, washed with 1X PBS, and permeabilized
 227 with 1% PBS-Triton for 15 minutes. Blocking was performed with PBS containing 5% Donkey

228 Serum for 1 hour. Cells were then incubated with rabbit anti-S protein antibody (1:1000) for 1 hour
 229 at 37 °C, followed by Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody (1:1000,
 230 Invitrogen) for 2 hours at room temperature in the dark. Nuclei were stained with DAPI (300 nM) for
 231 15 minutes.

232
 233 After washing, fluorescence images were captured using the BioTek Cytation 5 system. The signal
 234 was measured at an excitation of 495 nm and an emission of 520 nm. Negative controls included
 235 samples incubated with either the primary or the secondary antibody alone.

236
 237

238 *Transmission electron microscopy (TEM) in Vero Cells*

239

240 TEM was used to confirm SARS-CoV-2 infection in Vero cells. Cells were detached with trypsin,
 241 washed in 0.05 M phosphate buffer, and fixed in 4% glutaraldehyde for 4 hours. After rinsing, the
 242 pellets were post-fixed in reduced osmium (1% OsO₄ + 1.5% potassium ferrocyanide), dehydrated in
 243 ethanol, and embedded in Araldite resin.

244

245 Ultrathin sections (60 nm) were cut with a diamond knife on a Leica EM UC6 ultramicrotome (Leica
 246 Microsystems), mounted on 200-mesh copper grids (Ted Pella), stained with lead citrate (Merck,
 247 USA), and examined under a Tecnai G2-12 transmission electron microscope (Thermo Fisher/FEI).
 248 Viral particles were identified by their 50–70 nm round or oval shape, membrane envelope, electron-
 249 dense core, and localization within membrane-bound compartments.

250

251

252 *SARS-CoV-2 whole-genome sequencing*

253 Libraries were prepared using the QIAseq FX DNA Library Prep kit (QIAGEN, Hilden, NRW,
 254 Germany). Genomes were sequenced on the MiSeq platform (Illumina, San Diego, CA, USA) using
 255 v3 cartridges (600 cycles) according to the manufacturer's instructions. Three negative controls were
 256 used at all sample processing steps (cDNA synthesis, viral genome amplification, and library
 257 preparation) in each batch.

258

259 *Viral genome assembly and classifications*

260 Sequencing data were processed with a custom pipeline. First, a quality control step was performed
 261 with Trimmomatic v0.39, which removed adapter and primer sequences, short reads (<50
 262 nucleotides), and low-quality bases (Phred score < 30). Then, reads were mapped to the SARS-CoV-
 263 2 reference genome (GenBank accession: NC_045512) with Minimap2, and unmapped reads were
 264 discarded. Mapping files were then indexed and sorted with SAMtools v1.12, while the BCFtools
 265 v1.12, consensus option was used to estimate consensus genome sequences. Low-coverage sites were
 266 masked with BEDtools v2.30.0. Depth thresholds sites with less than 10-fold depth were masked.
 267 Consensus sequences were then classified using the Pangolin tool v.3.1.11 or the NextClade web
 268 application v.1.7.0.

269

270 *Statistical analysis*

271

272 Descriptive statistics were used: mean ± SD for normally distributed data; median and interquartile
 273 range (IQR) for non-normally distributed data; and frequencies for categorical variables. The
 274 Wilcoxon rank-sum test was used to compare medians between non-normal groups, and the Friedman

Q test was used for repeated non-parametric measures. Post hoc comparisons employed the Conover test with Bonferroni correction. Fisher's exact test was used to assess categorical variables. A p-value <0.05 was considered significant. Analyses were performed using Stata 17 (StataCorp, College Station, TX, USA).

Results

Patient's symptoms and sample collection

Of the 38 study participants, 27 had mild COVID-19, and 11 had moderate disease. Only one mild-case patient reported testicular symptoms, and four participants had infertility risk factors, including smoking, anabolic steroid use, previous varicocele, and cryptorchidism (Table 1).

In the mild group, 46 semen samples were collected. The first sample (27 patients) was obtained on average 27 days post-symptom onset. Follow-up collections occurred in 40.7% and 29.6% of patients, at approximately 129 and 221 days, respectively. All moderate-group patients provided one sample approximately 200 days after symptom onset (Table 1).

Seminal parameters and lymphocytic profile

In patients with mild COVID-19, there was a non-significant trend toward reduced sperm count, concentration, and total motility in the second sample, followed by recovery in the third. Notably, leukocyte concentration decreased in the third sample (Table 2). Lymphocyte markers (CD3, CD4, CD8, and CD45) tended to increase in the second sample and then declined below baseline in the third (Table 3).

Among the eight patients who provided all three samples, no significant changes were found, although the same trends were observed (Figure 1). Patients with moderate COVID-19 showed no abnormalities in semen parameters around 200 days post-symptom onset (Table 2).

RT-qPCR

RT-qPCR detected SARS-CoV-2 in 23 of 57 semen samples from 21 out of 38 patients, all of which had high Ct values (38–39). In the mild COVID-19 group, 11 of 27 patients had at least one positive sample. Two tested positive only in the second collection, while three were positive in the third, with two of them having no prior detection of SARS-CoV-2 RNA. (Figure 2).

In the moderate group, 91% (10/11) of patients had detectable SARS-CoV-2 in semen (Figure 2). Among 21 samples, most underwent replicate testing. Of the ten patients with positive results, seven showed discordant replicates, and three were concordant (two positive and one negative) (Figure 2).

Vero cell data

To assess SARS-CoV-2 viability, 24 RT-qPCR-positive raw semen samples were inoculated into VERO CCL-81 cell cultures under BSL-3 conditions. Viral replication was confirmed in 9 of 12 mild

and 8 of 12 moderate COVID-19 samples, as viral load increased over time (Figures 2 and 3). All 24 samples showed S protein expression by immunofluorescence. Three samples with the highest fluorescence intensity (from 30 days, 3 months, and 6 months post-infection) were further analyzed by TEM, revealing viral particles within endosomal vesicles in the Vero cell cytoplasm (Figure 4 and Table 4).

Genotyping

To distinguish reinfection from viral persistence, all RT-PCR-positive semen samples were genotyped and compared with circulating variants at the time of infection and at the time of semen collection. Twenty-four Vero cell-derived samples were sequenced due to their higher viral loads; however, none had a Ct value below 30, which limited amplification and sequencing depth. As a result, variant classification was not possible for most samples. Only sample 66 yielded sufficient coverage (65.2%) and depth (18.8%) and was identified as lineage 19A (clade B) by Nextclade; however, Pangolin could not assign a lineage.

Discussion

Here, we aimed to determine whether SARS-CoV-2 could be detected in the semen of mild and moderate COVID-19-recovered patients and to assess how long the virus persists, with follow-up up to 6 months after symptom onset. We found SARS-CoV-2 in semen from 11/27 mild COVID-19-recovered patients and 10/11 moderate ones. Furthermore, we could detect infectious viruses up to 8 months after the onset of symptoms using various techniques, including qRT-PCR in ejaculated semen, Vero cells culture with RT-qPCR, immunofluorescence, and TEM. These findings suggest that potential infectious SARS-CoV-2 viruses could persist for a prolonged period in the MGT, even in long-term COVID-19-recovered patients.

Our study shows a temporary decline in sperm quality after symptomatic COVID-19, with recovery between 3 and 6 months post-infection. This may be due to direct testicular injury, inflammation, immune or hormonal disruption, fever, medication, or stress—all known reversible factors affecting spermatogenesis. These factors may impair spermatogenesis individually or collectively, though such effects are typically reversible. Multiple studies report COVID-19-related declines in semen volume, concentration, motility, morphology, and viability, regardless of disease severity.^{9,11-33} Most show deterioration within 3 months and recovery by 6 months,^{11,17,19-21,24,28,29,32,33} consistent with our results. Others found no impact,³⁴⁻⁴³ and only Best et al. (2021) reported persistent impairment during a 91-day follow-up.¹⁴ Longitudinal data suggest recovery after 1 or 2 spermatogenic cycles, with semen parameters generally remaining above the 5th WHO percentile, including in our cohort. We also observed transient seminal inflammation, with CD3, CD4, CD8, and CD45+ cells peaking at 3 months and returning to normal by 6 months.^{9,11,28,44} This mirrors findings of increased seminal cytokines post-COVID-19, as reported by Martinez et al. (2023), who found inflammation lasting up to 3 months.²⁸

We detected SARS-CoV-2 in the semen of 21 out of 38 (55%) mildly to moderately ill recovered patients by RT-qPCR, with presence/persistence for over 6 months in some cases. Among 37 previous studies, only six reported viral presence in semen at lower rates (2–16%).⁵⁻¹⁰ Most of these studies evaluated patients during the acute or early recovery phases, with varying degrees of illness severity (mild, moderate, and severe). In contrast, our study focused solely on mild and moderate recovered

cases. Notably, Purpura et al. (2022) are an exception, detecting viruses in a similar recovery context.¹⁰

On the other hand, the remaining 31 studies did not detect SARS-CoV-2 in semen.^{11-21,34-40,43-55} Of these, 13 studies focused on patients in the acute phase of infection (≤ 15 days) and included asymptomatic and mildly, moderately, and severely symptomatic individuals.^{11,18,20,35,36,38,39,45,46,49,50,52,53} Even long-term studies, such as those conducted by Pavone et al. (2022) and Lauritsen et al. (2024), which followed patients for up to 30 days and 3–6 months, respectively, did not detect the virus in semen.^{20,53} The other 18 negative studies also included asymptomatic and symptomatic COVID-19 patients of varying severity,^{12-17,19,21,34,37,40,43,44,47,48,51,54,56} and 11 of these studies specifically tested semen samples collected 30 days or more after the onset of symptoms.^{13-17,34,40,43,44,48,51}

Interestingly, despite the lack of detectable virus in semen in the majority of the studies, one report identified viral particles in spermatozoa using TEM in 9 out of 13 patients (69%) up to 90 days after hospital discharge — corresponding to approximately 100 to 110 days post-infection.⁵⁴ In a separate study, viral RNA fragments were detected in 2 out of 12 patients (17%) using a capture-based probe hybridization method.⁴⁰ These findings suggest that, while infectious virus may not be consistently present in semen, viral components can persist in spermatozoa for extended periods after clinical recovery.

Several studies, including the one by Li et al. (2020),⁵ have faced criticism due to methodological shortcomings that may have contributed to false-positive RT-qPCR results in semen.⁵⁶ Their study included only severe COVID-19 cases, which are known to be associated with higher systemic viral loads and, potentially, a greater likelihood of viral dissemination to body fluids like semen.⁵⁶ Moreover, false positives in RT-qPCR testing can arise due to several factors: contamination, poor test specificity, and improper determination of the threshold at which a sample with a low viral load is considered truly positive.^{55,57} In fact, they did not report key parameters, such as Ct values, primers used, or the limit of detection (LOD), nor did they clarify whether aseptic techniques were used during sample collection.⁵ Subsequent positive studies, including ours, addressed these concerns by implementing stricter protocols to avoid semen contamination.^{6-8,10} Another potential source of semen contamination is residual viral shedding from the urinary tract.^{7,16,56} However, Saylam et al. (2021) mitigated this risk by collecting semen and urine samples at separate times to prevent cross-contamination. In their study, the only positive semen sample showed no viral presence in urine, confirming the specificity of their RT-qPCR results.⁷

A recurring concern in studies reporting positive RT-qPCR results in semen is the detection of SARS-CoV-2 at late-cycle thresholds (Ct 38–39), which some interpret as false positives. However, analytical validation of RT-qPCR assays ensures high specificity, and Ct values of 40 or less are widely accepted as positive.⁵⁸ In our study, we detected the virus at late Ct values (38–39 Ct) in all positive cases. These late Ct values likely reflect low viral RNA levels—especially near the assay's LOD—where test reproducibility is expected to be variable. A negative retest in such cases does not necessarily invalidate an initial positive result.⁵⁸ These considerations may explain some of the discrepancies observed in our study and the inconsistent results reported in other research.

Supporting this interpretation, Hallak et al. (2024) found virus-infected spermatozoa in nine of 13 patients using TEM despite negative RT-PCR results. This discrepancy was attributed to the very low viral concentration in semen, which is plausible considering that the testes contribute only about 5% of the ejaculate.⁵⁴ Additionally, variations in SARS-CoV-2 viral load have been reported depending on the viral variant.⁵⁸ Another possible explanation for their findings is the lack of a viable virus with only the RNA-free capsid present in the spermatozoa.

It is also important to acknowledge that RT-qPCR, although the gold standard, is not infallible. Its sensitivity can vary from 70% to 98% depending on the test used, and false negatives can occur—particularly in samples with low viral loads or under suboptimal pre-analytical conditions.^{59,60} False-negative rates have been reported to be as high as 58%.⁶¹ Factors such as improper sampling, RNA degradation, PCR inhibition, and sample mishandling (e.g., repeated freeze-thaw cycles) can significantly impact test performance, leading to late Ct values and false-negative results.⁵⁵

Differences in study outcomes may also stem from variations in disease stage, symptom severity, timing of sample collection, potential contamination, and methodological inconsistencies. Notably, there is considerable heterogeneity in the RT-PCR primers employed across studies. While most studies targeted common genomic regions (e.g., ORF1ab, N, S, E, RdRP), only 61% of the negative studies specified their primers,^{13,16-18,21,36,38-40,43-46,48,50-53,55} and less than half used high-sensitivity targets such as the N gene and ORF1ab.^{13,17,18,21,36,39,40,43,44,46,48,50,52,53} Furthermore, only six of these studies described strategies for preserving RNA.^{18,39,40,43,46,50} Such variability in primer selection and sample-handling protocols likely contributes to inconsistent SARS-CoV-2 detection rates in semen across studies.

Lastly, it is crucial to understand that a positive molecular test detects viral RNA but not necessarily a viable virus. Confirming infectivity requires isolating and culturing the virus. Although Ct values are often used as proxies for viral load or infectiousness, they are imperfect indicators of these measures. However, it remains unclear how many SARS-CoV-2 virions are required to cause infection or whether Ct values correlate reliably with the number of infectious virus particles. Some studies have even cultured viable SARS-CoV-2 from samples with fewer than the previously suggested 10⁶ genome copies.⁶²

Factors such as specimen quality, sample type, and laboratory conditions all influence Ct values and viral viability. These findings reinforce that we cannot entirely rely on Ct-values as a direct measure of infectiousness.⁶³ Moreover, Ct values vary significantly between variants. For example, Omicron tends to show higher Ct values compared to earlier strains despite active infection, reinforcing the idea that Ct values alone cannot reliably indicate infectiousness.⁶⁴

While the presence of SARS-CoV-2 RNA could theoretically indicate non-functional or 'inert' RNA, this is unlikely because such RNA is quickly broken down in the human body.^{65,66} In our study, all RT-qPCR-positive semen samples were cultured in Vero CCL-81 cells to confirm viral replication, followed by quantification of the viral load. Immunofluorescence confirmed the presence of the virus's S protein—not just RNA fragments—in all positive cultures. Three highly fluorescent samples (≤30 days, 3 months, and 6 months) were further analyzed by TEM, revealing intact, potentially infectious viruses, confirming that we detected viable, fully assembled viral particles. To date, only three other studies have attempted semen culture in Vero cells.^{8,36,38} However, despite detecting positive RT-qPCR results in one patient in one study,⁸ all of their cultures returned negative results.

We investigated whether SARS-CoV-2 detected in semen reflected viral persistence or reinfection. Due to low viral loads, genotyping was inconclusive for most samples. Only one sample (Sample 66), collected 21 days post-symptom onset, yielded sufficient coverage (65%) and was classified as lineage 19A—consistent with the variant circulating at the time of infection. Although genotyping could not definitively distinguish between persistence and reinfection, several findings favor persistence. All patients were asymptomatic at the time of semen collection, and the likelihood of coincidental, asymptomatic reinfections in nearly all moderate cases between 4 and 9 months post-infection is low. While not conclusive, these observations support the hypothesis that SARS-CoV-2 persists in semen following infection.

This study has several important strengths. It is the first longitudinal investigation to demonstrate long-term SARS-CoV-2 infectivity in semen, offering direct evidence of viral viability and addressing a key clinical question. Integrating laboratory and clinical data provides a translational perspective that informs the development of highly sensitive diagnostic tools and enhances understanding of COVID-19's impact on male reproductive health.

However, some limitations must be acknowledged—the sample size, while in line with previous studies, was relatively small. In the moderate group, only one semen sample was collected at 6 months due to clinical constraints, limiting earlier comparisons. Additionally, the absence of a control group—such as pre-infection samples from the same patients—precludes direct before-and-after comparisons of semen quality.

In conclusion, we detected viable SARS-CoV-2 in the semen of patients recovered from mild to moderate COVID-19, indicating the long-term presence/persistence of the virus in the male genital tract. This supports the possibility of testicular viral replication and offers new insight into SARS-CoV-2 biology. Semen, as a non-invasive sample, may serve as a valuable tool for detecting viral persistence in long COVID. Its presence could serve as a biomarker for diagnosis, risk stratification, treatment monitoring, and assessment of disease severity and prognosis.

Contributors

MHF: conceptualisation, data curation, literature search, investigation, methodology, project administration, figures, study design, data collection, data analysis, data interpretation, writing – original draft, and writing – review & editing; LMA: investigation, methodology, figures, study design, data collection, data analysis, data interpretation; RSA: study design, data analysis, data interpretation, and writing – review & editing; FRS: investigation, methodology, data collection, data analysis, data interpretation; JCG: writing – review & editing; BPM: data curation, investigation, methodology, project administration; TPF: literature search, study design, data collection, writing – review & editing; APC: data curation, investigation, methodology; SMSNL: conceptualisation, data interpretation, and writing – review & editing; GMJC: conceptualisation, data curation, literature search, investigation, methodology, project administration, figures, study design, data collection, data analysis, data interpretation, writing – original draft, and writing – review & editing.

Declaration of interests

We declare no competing interests.

Data sharing

All data will be made available upon request to the corresponding author, subject to approval of a proposal and a signed data access agreement.

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Tables

Table 1 - Baseline characteristics and study design

		All	Mild	Moderate
N		38	27	11
Age (years, mean, $\pm$ SD; range)		38.3 (6.4; 28-52)	38.5 (6.8; 28-52)	37.9 (5.7; 31-48)
Testicular symptoms (n(%))		1/38 (2.6%)	1/27 (3.7%)	0/11 (0%)
Smoke		1/38 (2.6%)	1/27 (3.7%)	0/11 (0%)
Infertility factor		3/38 (7.9%)	2/27 (7.4%)*	1/11 (9.1%)**
Vaccination***		20/38 (52.6%)	11/27 (40.7%)	9/11 (81.8%)
Semen collection	1 month	n (%) .. (days, median (IQR)) ..	27/27 (100%)\$ 27 (21, 30)	
	3 months	n (%) .. (days, median (IQR)) ..	11/27 (40.7%)& 129 (122, 142)	
	6 months	n (%) .. (days, median (IQR)) ..	8/27 (29.6%)@ 221 (218, 221)	11/11 (100%) 200 (184, 239)
\$ 14 only 1st collection		* varicocelelectomy and criptorchidism		
& 5 only 1st and 2nd collection		** anabolic steroids use		
@ 1 collected only the 1st and 3rd		*** at least 1 dose of vaccine		

Table 2 - Semen parameters

	Collection (months)	All*	Mild	Moderate*
Volume (ml, median (IQR))	1	-	3.0 (2.3, 4.0)	-
	3	-	3.4 (1.8, 3.8)	-
	6	3.3 (2.3, 4.0)	2.9 (2.3, 5.1)	3.4 (2.3, 4.0)
Count (millions, median (IQR))	1	-	210.6 (64.0, 317.3)	-
	3	-	139.7 (89.3, 220.0)	-
	6	216.9 (101.2, 363.0)	185.9 (91.0, 285.7)	255.6 (123.2, 533.5)
Concentration (millions/ml, median (IQR))	1	-	69.3 (18.2, 96.8)	-
	3	-	51.2 (38.2, 57.9)	-
	6	63.9 (44.0, 97.3)	58.4 (38.1, 87.1)	68.8 (50.0, 136.8)
Total Motility (% , median (IQR))	1	-	62.0 (39.0, 73.0)	-
	3	-	60.0 (53.0, 79.0)	-
	6	76.5 (73.0, 81.0)	74.5 (60.5, 79.0)	80.0 (74.0, 87.0)
Progressive Motility (% , median (IQR))	1	-	51.0 (33.0, 63.0)	-
	3	-	51.5 (43.0, 70.0)	-
	6	71.0 (64.0, 77.0)	68.5 (52.5, 71.5)	75.5 (68.0, 82.0)
Morphology (% , median (IQR))	1	-	3 (2, 5)	-
	3	-	4 (4, 6)	-
	6	4 (4, 5)	4 (4, 5)	4 (3, 6)
Round Cells (millions/ml, median (IQR))	1	-	3.0 (1.9, 7.6)	-
	3	-	3.0 (1.8, 4.5)	-
	6	2.2 (1.2, 5.5)	1.7 (1.1, 6.7)	2.6 (1.4, 5.1)
Leukocytes (millions/ml, median (IQR))	1	-	0.3 (0.1, 1.3)	-
	3	-	0.3 (0.04, 0.8)	-
	6	0.1 (0.05, 0.2)	0.15 (0.06, 0.2)	0.1 (0.04, 0.2)
Any seminal abnormality** (n, %)	1	-	20/27 (74.1)	-
	3	-	5/12 (41.7)	-
	6	5/18 (27.8)	2/8 (25.0)	3/10 (30.0)

* excluded one patient on anabolic steroids use

**volume, count, concentration, total motility, progressive motility, morphology, leukocytes

Table 3 - Lymphocytic profile

	Collection (months)	All*	Mild	Moderate*
CD3 (% , median (IQR))	1	-	1.36 (0.96, 3.15)	-
	3	-	2.15 (1.06, 3,25)	-
	6	0.36 (0.22, 0.80)	0.36 (0.23, 2.97)	0.32 (0.11, 0.61)
CD4 (% , median (IQR))	1	-	0.60 (0.34, 1.66)	-
	3	-	1.21 (0.73, 2.89)	-
	6	0.20 (0.13, 0.44)	0.21 (0.13, 2.08)	0.18 (0.08, 0.32)
CD8 (% , median (IQR))	1	-	0.84 (0.40, 2.18)	-
	3	-	1.19 (0.63, 1.84)	-
	6	0.02 (0.01, 0.03)	0.02 (0.01, 2,15)	0.02 (0.01, 0.03)
CD45 (% , median (IQR))	1	-	4.58 (2.61, 8.13)	-
	3	-	5.06 (1.73, 10,74)	-
	6	1.72 (1.15, 5.07)	1.58 (1.15, 5.07)	2,21 (1.08, 6,54)

* excluded one patient on anabolic steroids use

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Table 4 - Fluorescence intensity according to the Vero cells immunofluorescence

The samples with the highest intensity for each time period (1, 3, or 6 months) are highlighted in blue.
Those samples were taken to the TEM.

Sample	#7	#10	#11	#16	#17	#19
Value 1	1680,00	1893,00	1618,00	1736,00	1894,00	1481,00
Value 2	1675,00	1791,00	1605,00	1713,00	1894,00	1513,00
Value 3	1676,00	1891,00	1609,00	1682,00	1895,00	1469,00
Sample	#20	#21	#22	#26	#29	#30
Value 1	1845,00	1726,00	1700,00	925,00	1716,00	1606,00
Value 2	1799,00	1709,00	1668,00	953,00	1773,00	1581,00
Value 3	1873,00	1707,00	1689,00	892,00	1707,00	1566,00
Sample	#33	#35	#36	#38	#39	#40
Value 1	2044,00	1701,00	1409,00	1843,00	2077,00	1418,00
Value 2	2069,00	1668,00	1357,00	1798,00	2170,00	1393,00
Value 3	2079,00	1699,00	1415,00	1793,00	2128,00	1367,00
Sample	#52	#60	#64	#66	#67	#69
Value 1	1876,00	2119,00	2217,00	2536,00	2205,00	2355,00
Value 2	1839,00	2084,00	2193,00	2431,00	2259,00	2281,00
Value 3	1828,00	2070,00	2235,00	2480,00	2200,00	2348,00

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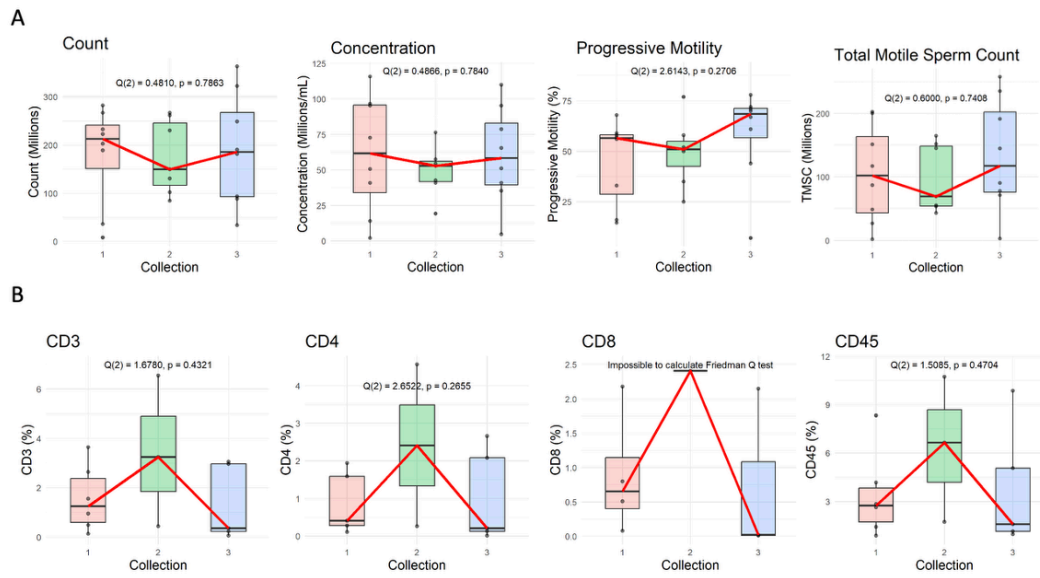


Figure 1 - Comparison of semen parameters and seminal lymphocytic profile.

Figure 1A shows the tendency of semen parameters to decrease from the 1-month-sample to the 3-month-sample and recovery at baseline at 6 months. Figure 1B shows the inverse tendency with an increase of lymphocytes between the 1-month-sample to the 3-month-sample and returning below baseline values at the 6-month-sample.

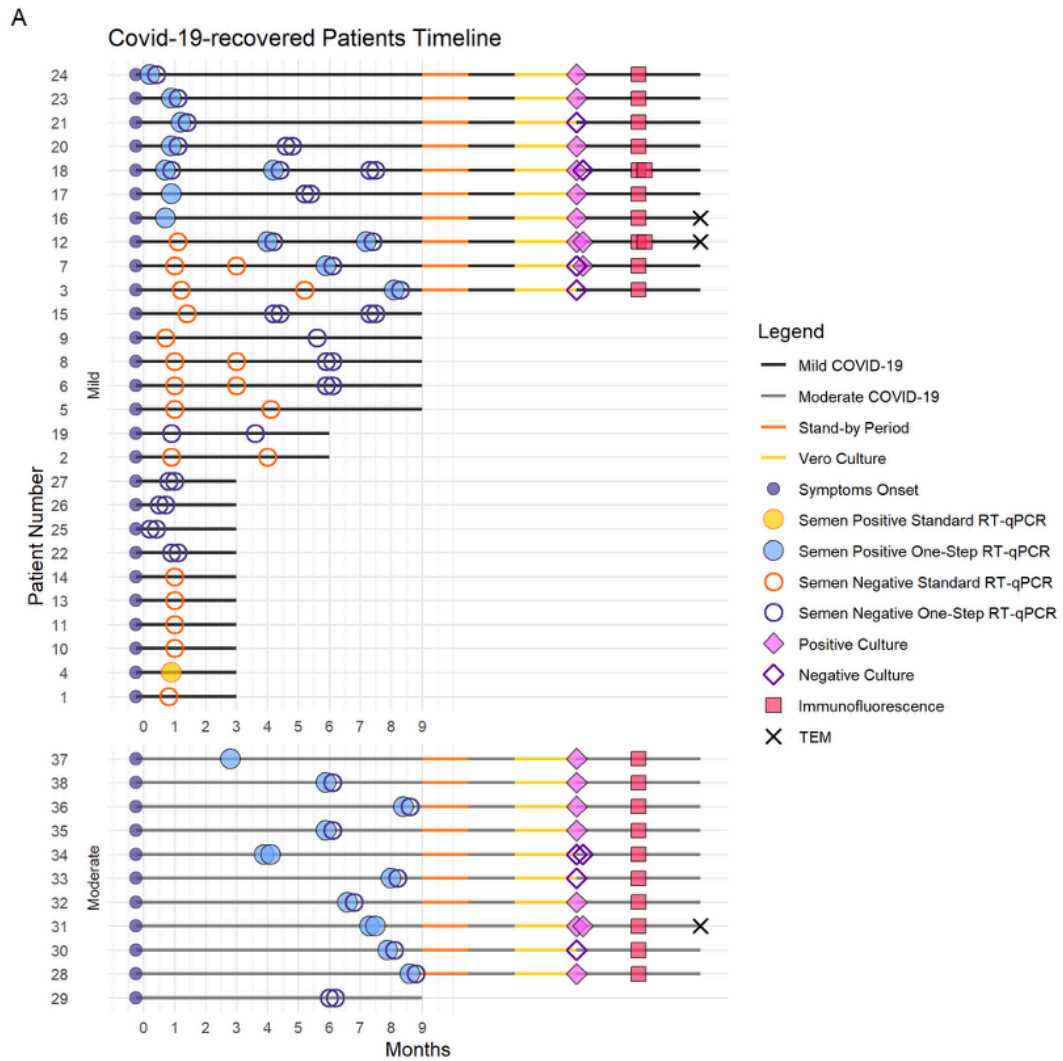
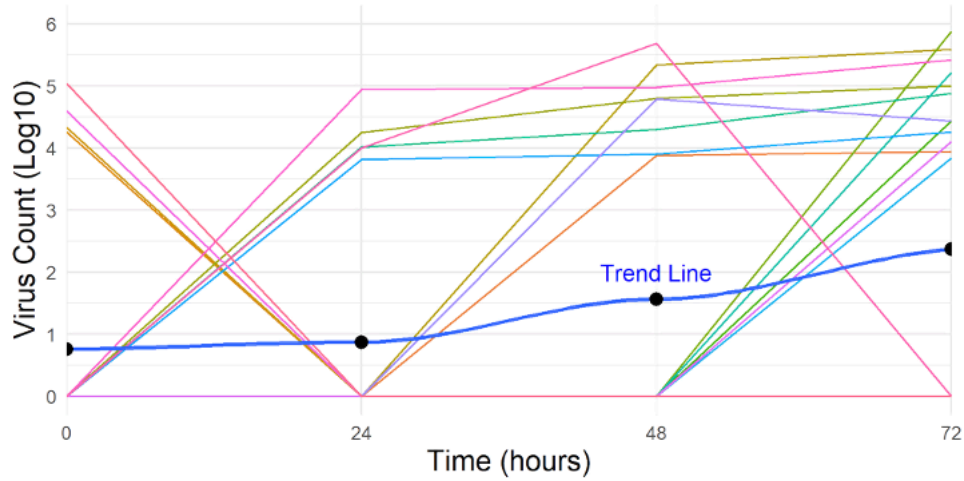


Figure 2 – Timeline of the studied patients

A

Vero Cell Virus Isolation



B

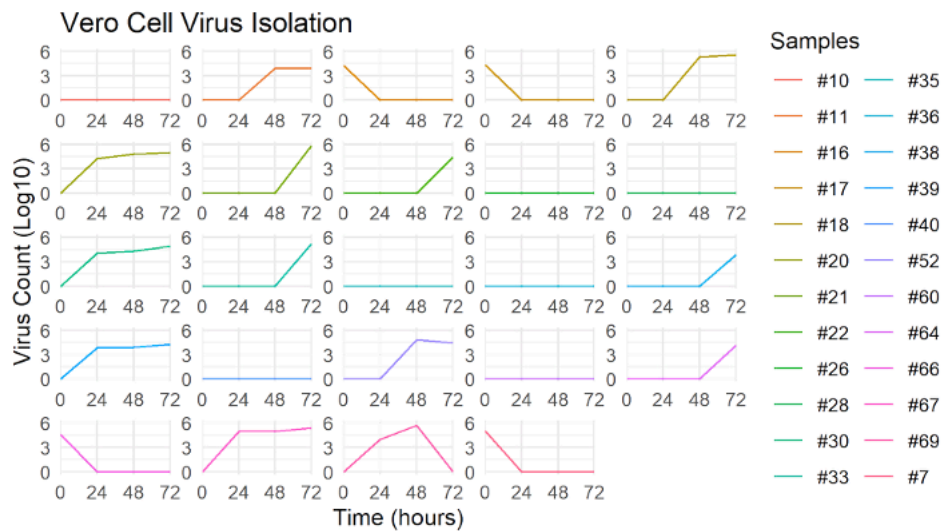
**Figure 3 – Vero cells virus isolation.**

Figure A shows the tendency of the number of viruses to increase over time in the Vero cell culture of all RT-qPCR-positive samples. Figure B depicts the behavior of each sample separately over time, showing that 13 of the 24 samples increased the viral load. Samples #20, #30, #38, #39, #40, #52, #64, #66, #67 and #69 are from mild patients. All the other samples belongs to moderate group.

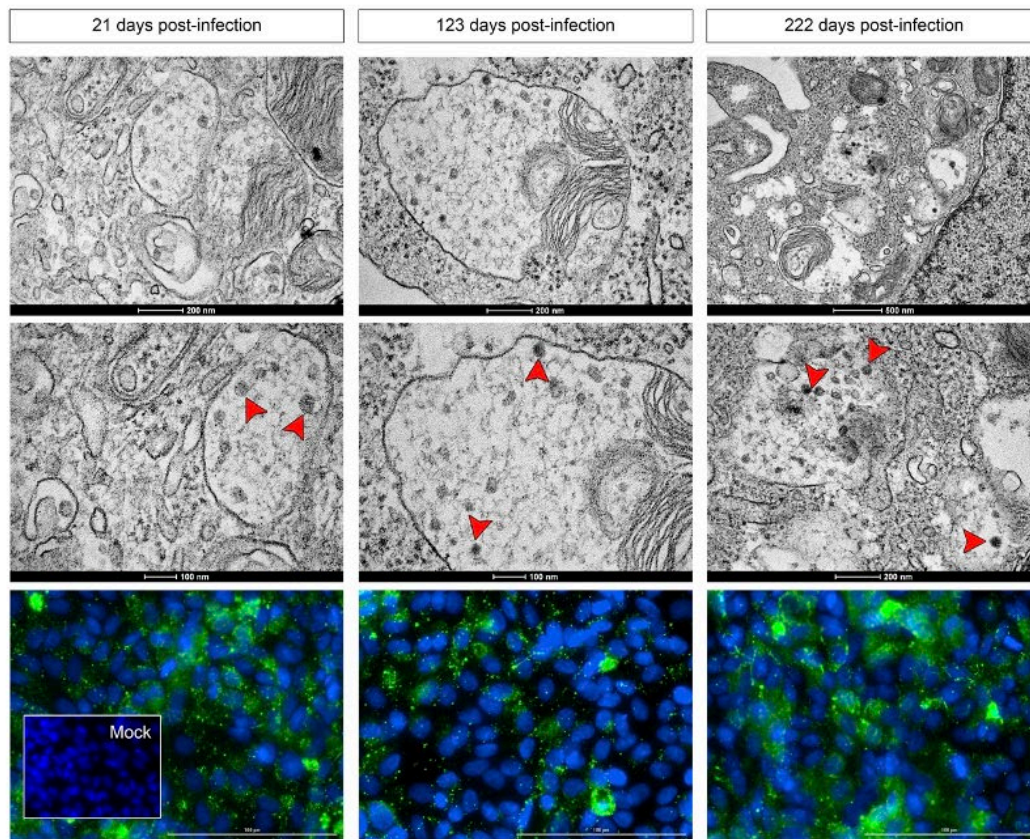


Figure 4 – TEM and Immunofluorescence of the Vero cells. This figure shows the TEM of 3 different patients chosen with the highest fluorescence intensity by fluoroscopy, representative of the 3 time points of collection—1, 2, and 3 months. Line B depicts a higher augmentation of the line A images, and the red arrowheads show the SRAS-CoV-2 virus inside vesicles in the Vero Cells. The images in line C show the immunofluorescence for spike protein of the same samples studied by TEM

3.3 Artigo 3

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Belo Horizonte, October 30th, 2025.

To:

Professor Ralf Henkel, Ph.D.,

Editor-in-Chief

Dear Editor,

We would like to submit our manuscript, "**AI-Driven Evaluation of Post-COVID-19 Effects on Male Reproductive Health: A Comprehensive Analysis Beyond WHO Semen Parameters**", by Vinícius A. C. G. Baliza, Talita de O. Farias, Marcelo H. Furtado, Paulo H. R. Amaral, João C. B. Sepulveda, Victor. H. S. De Paiva, Maria Ivonete N. da Silva, Lídia M. de Andrade, Samyra M. S. N. Lacerda, Guilherme M. J. Costa, Juan C. González for consideration for publication in *Andrologia* – Wiley Online Library.

The COVID-19 pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a member of the Coronaviridae family, has had a profound impact on global health, with over 775 million cases and more than 7 million deaths reported as of August 2024. SARS-CoV-2 has been shown to have detrimental effects on the male reproductive system, with most recovered patients exhibiting no significant differences in mean sperm parameters three months post-infection. However, despite the recovery of sperm parameters, SARS-CoV-2 infection has been associated with negative impacts on assisted reproduction techniques for up to six months. Further research is required to determine whether other seminal alterations, not detected by routine sperm analyses, could explain these adverse reproductive outcomes.

This study presents for the first time the use of two different artificial intelligence approaches to distinguish between semen from individuals who have never been diagnosed with COVID-19 and semen from COVID-19-recovered patients. Firstly, machine learning was used to analyze sperm parameters to uncover underlying information not revealed by the standard statistical protocols recommended by the World Health Organization and used in clinical practice. This tool showed a good accuracy of 77 % in detecting semen samples from COVID-19-recovered patients. Next, a machine learning spectroscopy (MLS) approach applied to optical ellipsometry measurements of semen samples was demonstrated with excellent accuracy of 90%.

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This study paves the way for a new research field, utilizing innovative methods to analyze traditional exams and incorporating optical data that has been little explored in semen to understand the reproductive outcomes following SARS-CoV-2 infection. From a technological standpoint, the implementation of a simple optical device with embedded AI processing to record reflectivity spectra from sperm samples could potentially revolutionize precision reproductive medicine in clinics and hospitals, offering new hope and excitement for the future of reproductive health research.

Given its relevance and significance, we are confident that our work aligns with the scope of *Andrologia – Wiley Online Library* and meets its rigorous publication standards. We believe it will attract a wide readership, and thus, we are submitting it for your consideration.

The manuscript has not been published and is not under consideration for publication elsewhere. All authors approve its submission, and we declare no conflicts of interest.

Yours Sincerely,

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AI-Driven Evaluation of Post-COVID-19 Effects on Male Reproductive Health: A Comprehensive Analysis Beyond WHO Semen Parameters

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Abstract

The male reproductive system can be affected by viral infections, which may impact reproductive function and fertility. Although it is believed that seminal parameters generally return to normal after recovery from COVID-19, subtle changes may still affect reproductive outcomes. These changes may not be detectable using standard protocols for semen analysis outlined by the World Health Organization, requiring the use of advanced, but facile, artificial intelligence tools. This study presents the use of two different artificial intelligence approaches to distinguish semen samples from COVID-19-recovered patients. The first approach focuses on analyzing sperm parameters from COVID-19-recovered men and a control group with no history of infection, though the second is based on optical ellipsometry measurements of those semen samples. Although seminal parameters of both groups fell within the normal World Health Organization reference ranges, several machine-learning classification algorithms demonstrated significant differences between them. The Support Vector Machine model showed the best classification performance (accuracy of 77%). At the same time, SHAP interpretability analysis identified progressive motility and immotile sperm as the most important parameters to separate the two groups of samples. The machine learning spectroscopy approach showed an even better performance, with an accuracy of 90% for the Support Vector Machine model. These results demonstrated that artificial intelligence approaches can detect important differences in the semen of COVID-19-recovered patients that traditional seminal analysis methods fail to reveal, underscoring the potential of using artificial intelligence tools to improve reproductive health assessments.

Keywords: Covid-19, Seminal parameters, Artificial Intelligence, Optical Spectroscopy, Support Vector Machine.

1. Introduction

The male reproductive tract is vulnerable to infections by various viruses, which can significantly impair reproductive function and male fertility [1,2]. Notably, male genital organs can act as reservoirs for these viruses [2,3]. Viral infections in the male reproductive system can result in numerous adverse outcomes, including testicular inflammation, germ cell apoptosis, fibrosis, disrupted spermatogenesis, decreased testosterone synthesis, Leydig cell apoptosis, and impairment of the Sertoli cell blood-testis barrier. These infections can lead to infertility, germline infection, cancers of the male genital organs, sexual transmission of diseases, and transmission to embryos, potentially causing congenital abnormalities, which are long-term implications that cannot be overlooked [1-4]. Furthermore, over thirty viruses have been detected in semen, altering sperm parameters such as count, vitality, motility, and morphology [1, 2, 5].

The COVID-19 pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a member of the Coronaviridae family, has had a profound impact on global health, with over 775 million cases and more than 7 million deaths reported as of August 2024 [6]. The severity of the impact is also strongly related to the infected individuals' regional development level, socio-economic background [7], and SARS-CoV-2 variants [8]. Histopathological changes due to SARS-CoV-2 have been documented in multiple organs and tissues [9, 10]. Specifically, SARS-CoV-2 has been shown to have detrimental effects on the testis, epididymis, and sperm parameters [3,11-14].

In COVID-19 patients previously examined for varicocele and infertility, significant decreases were observed in sperm concentration, total sperm number, and normal sperm morphology percentage following SARS-CoV-2 infection [15]. However, three months post-infection, recovered patients exhibited no significant differences in mean sperm vitality, concentration, total number, and abnormal sperm percentage [16]. Despite the recovery of sperm parameters, SARS-CoV-2 infection has been associated with negative impacts on assisted reproduction techniques for up to six months, with higher miscarriage rates reported [17]. Further research is required to determine whether other seminal alterations, not detected by routine sperm analyses, could explain these adverse reproductive outcomes.

Artificial intelligence (AI) has recently been used for COVID-19 medical diagnosis [18] and forecasting [19], as well as is increasingly being integrated into reproductive medicine [20]. Multiple research groups have been exploring AI applications for sperm selection to improve reproductive outcomes [20]. Using a machine learning spectroscopy approach, our group has achieved high accuracy in distinguishing sperm samples, by using murine models, from healthy and Zika virus-infected mice [4].

This study presents the use of two different artificial intelligence approaches to distinguish between semen from individuals who have never been diagnosed with COVID-19 and semen from COVID-19-recovered patients. Firstly, machine learning (ML) will be used to analyze sperm parameters to uncover underlying information not revealed by the standard statistical protocols, recommended by the World Health Organization, and used in clinical practice. Next, a machine learning spectroscopy (MLS) approach applied to optical ellipsometry measurements of semen samples will be demonstrated. These new, rapid, and cost-effective methods could aid urologists and reproductive centers in screening for or even diagnosing fertility issues, as well as understanding the reproductive outcomes following SARS-CoV-2 infection.

2. Materials and methods

2.1 Covid-19 recovered patients and control patients

This cross-sectional study recruited adult men aged 18 to 50 who attended Hospital Mater Dei between October 2020 to October 2021. These individuals were diagnosed with COVID-19, confirmed via detecting the SARS-CoV-2 virus in an oropharyngeal swab using RT-qPCR, and subsequently fully recovered. Following RT-qPCR analysis for the SARS-CoV-2 virus in all semen samples, we randomly selected 29 negative samples to include in this study. The control group consisted of 13 male patients aged 18 to 50 without a history of COVID-19 infection. These individuals attended the MF Male Fertility Clinic for fertility evaluations between May 2020 and October 2020.

Semen samples were collected from all participants after the subject informed consent for research participation was obtained. This study was authorized by the National Research Ethics Committee (CONEP) and by the Research Ethics Committee of Hospital Mater Dei

under the protocol's numbers CAAE: 36122420.5.0000.5128 and CAAE: 18456819.1.0000.5128, respectively. All procedures were conducted in accordance with the ethical standards outlined in the Declaration of Helsinki.

2.2 Semen specimens

For the semen examination, the ejaculate was collected in a sterile container via masturbation after 2 to 3 days of sexual abstinence, and its volume was measured. Patients were instructed on proper hygiene procedures, including hand and genital washing with soap or disinfectant, to prevent viral contamination. The samples were then liquefied at 37°C for up to 60 minutes in an incubator before analysis. The same andrologist estimated routine sperm parameters. After semen analysis, the remaining raw semen was immediately frozen and stored in liquid nitrogen at -196°C in a bio-repository established for this project, located at the MF Male Fertility Clinic under the supervision of one of the authors (MHF).

2.3 Semen Analysis

Semen samples were analyzed following World Health Organization (WHO) guidelines [21]. Different semen parameters were assessed in both groups of patients, which included sperm concentration and motility, all measured within 1 hour of ejaculation. The semen samples were homogenized for analysis in replicate.

The sperm concentration was quantified using 10 µL of the semen sample in a Neubauer chamber (4×4; Lo-Laboroptik, United Kingdom). At least 200 spermatozoa were counted. The total number of sperm was obtained by multiplying the sperm concentration by the ejaculate volume. Sperm motility was evaluated using 10 µL of semen sample on a Makler counting chamber (Sefi-Medical Instrument, Haifa, Israel). In this analysis, at least 200 spermatozoa were examined under light microscopy at 400x magnification and categorized as progressive (PM), non-progressive (NPM), and immotile (IM). The concentration of round cells was determined using a Makler counting chamber (Sefi-Medical Instrument, Haifa, Israel).

For sperm morphology analysis, a sperm smear was prepared by spreading 10 µL of the semen sample along the surface of a microscope slide. The slide was air-dried, then fixed with 95% ethanol for 15 minutes, and stained using the Papanicolaou staining method. We used

Kruger's strict criteria to assess morphology. In this analysis, 200 spermatozoa were classified as normal or abnormal based on their head, midpiece, and tail morphology.

2.4 Spectroscopy measurements

The spectroscopy measurements followed protocols already implemented and tested for the diagnosis of COVID-19 and sperm alterations in Zika virus-infected mice [4,18]. Semen from control and COVID-19-recovered patients was collected, with a concentration of $\sim 10^7$ cells per mL, and fixed in 70% ethanol. The samples were meticulously prepared by drop casting Corning No. 1½ coverslips with aliquots of 20 μ L, and the resulting liquid biofilm was carefully covered with a second Corning No.1½ coverslip.

The samples were measured in standard configuration mounted in a J.A. Woollam M-2000 variable angle spectroscopy ellipsometer. In this configuration, the spectra of the ellipsometric angles Ψ and Δ angles, as well as the depolarization degree (dPol) and the unpolarized reflection (uR), were acquired from 240 nm to 1700 nm, in steps of 5 deg from the angle of incidence (AOI) of 45 deg to 70 deg. Ellipsometry measurements were carried out in 9 different locations, covering approximately the whole surface of the glass coverslips. The acquired spectra were considered for MLS modeling.

2.5 ML of sperm parameters

To classify the patients based on sperm parameters, an ML classification solution was built in the Python computer language [18, 22]. Six different classification algorithms were trained to predict patient classification: Decision Tree Classifier (DT) [23], eXtreme Gradient Boosting (XGBoost) [24], K-Nearest Neighbors (KNN) [25], Logistic Regression (LR) [26], Multilayer Perceptron (MLP) [27], and Support Vector Machine (SVM) [28].

The classification algorithms were optimized by adopting the Optuna [29] library, using 100 trials to search for optimal parameters and hyperparameter values to enhance model performance. The optimization was carried out by maximizing the Area Under the Receiver Operating Characteristic Curve (AUROC), or simply AUC (Area Under the Curve), following a 5-fold stratified cross-validation. The classification probability of each sample in the 5 test folds was determined during the cross-validation process. At the end of the cycles, AUROC

was calculated considering the classification probabilities of all samples. The search space for the hyperparameters/parameters of all algorithms is summarized in Table S1 of the supplemental material.

After the optimization stage, the algorithms were trained and validated. In the validation stage, 30 cross-validation (CV) experiments with five folds each were conducted, generating scores for six different performance metrics: AUC, Accuracy (Acc), F1 score (F1), Matthews Correlation Coefficient (MCC), Specificity (Spec), and Sensitivity (Sens). In each of the 30 cross-validations, the samples were randomly distributed (with different random seeds) along the five folds. A mobile classification threshold was implemented from the ROC curves of the training database, using the closest to (0,1) criteria [30], to segregate both classes better. The average classification threshold was used to calculate F1, MCC, Acc, Spec, and Sen metrics. An outer loop in the validation stage was implemented to evaluate the variance of the performance metrics. This implementation of the K-fold CV process was adapted from [31] and is illustrated in Figure 1. The optimized values of hyperparameters and parameters of all algorithms are summarized in Table S2 and S3 of the supplemental material, respectively.

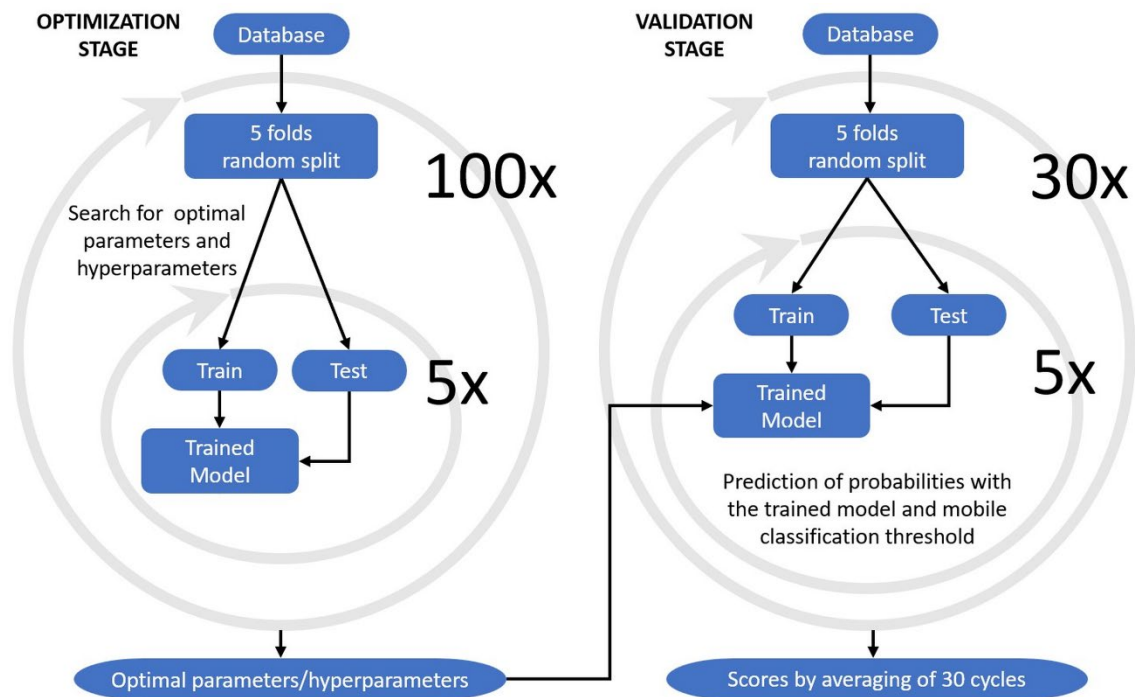


Figure 1. Scheme of the optimization and validation process in the 5-fold cross validation process.

To better understand the importance of different features in the classification process of the best ML model, the SHapley Additive exPlanations (SHAP) method was used [32]. SHAP values are a way to explain the output of any ML model. It uses a game-theoretic approach that measures each player's contribution to the outcome. In ML, each feature is assigned an importance value representing its contribution to the model's output. In this sense, the sum of the SHAP values of all features (sperm parameters) of a specific instance (a patient) plus the base SHAP value of the model (considering all patients) gives the probability of that particular patient being classified as part of the COVID-19-recovered group. SHAP values are also model-agnostic and have several important properties that make them effective for interpreting models: additivity, local accuracy, missingness, and consistency. Overall, SHAP values provide a consistent and objective way to gain insights into how a machine learning model makes predictions and which features have the most significant influence.

2.6 ML of spectroscopy data

A similar 5-fold CV methodology was used to train, optimize, and validate the classification algorithms for the spectroscopy data. However, in this case, many features are considered since 674 different wavelengths for each AOI and measured properties (Ψ , Δ , dPol, and uR) were considered. After the best models were selected from the k-fold CV step, a nested CV process (Figure S1) was applied to the best models to evaluate their performance without biases.

2.7 Statistical analysis

Data is presented using descriptive statistics. All quantitative data were tested for normality by the Shapiro–Wilk tests. The seminal data with normal distribution was submitted to the Student's t-test. In contrast, the non-parametric seminal data was submitted to the Mann-Whitney test (U-test) to evaluate possible variations in the seminal characteristics. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc.). The level of significance considered was $P < 0.05$.

3. Results

3.1 Standard semen analysis

The seminal parameters of both the control group and COVID-19-recovered patients were found to be similar and above the fifth percentile of World Health Organization (WHO) reference values. It is noteworthy that there were no significant differences in seminal parameters such as volume, concentration, total sperm number (total count), normal morphology, motility, and the presence of round cells in semen between the two groups (Figure S2). These findings demonstrate that the parameters were within the normal range and consistent with WHO reference values, as indicated in Table 1 and Figure 2.

Table 1. Median (IQR) values of seminal parameters of control patients and the COVID-19-recovered patients, and WHO standards (6th, fifth percentile, 2021).

Seminal characteristics	Control patients	Covid-19 patients	WHO 2021
Semen volume (mL)	3.0 (2.7 - 3.8)	2.6 (2 - 4.3)	> 1.4
Sperm concentration (10 ⁶ /mL)	24.5 (14.1 - 54.1)	40.8 (19.3 - 72)	> 16
Total sperm number (10 ⁶ /ejaculate)	83 (48.2 - 135.4)	123.2 (71.2 - 210.6)	> 39
Normal morphology (%)	4 (3.5 - 4.5)	4 (3 - 5.75)	> 4
Progressive motility (%)	47 (27.5 - 51.5)	50 (42.4 - 64)	> 30
Non-progressive motility (%)	9 (8 - 13)	6.4 (5 - 11)	> 1
Immotile sperm (%)	43 (37 - 60)	41 (26 - 50)	> 20
Round cells (10 ⁶ /mL)	0.9 (0.8 - 1.4)	2.4 (1.4 - 3.7)	-

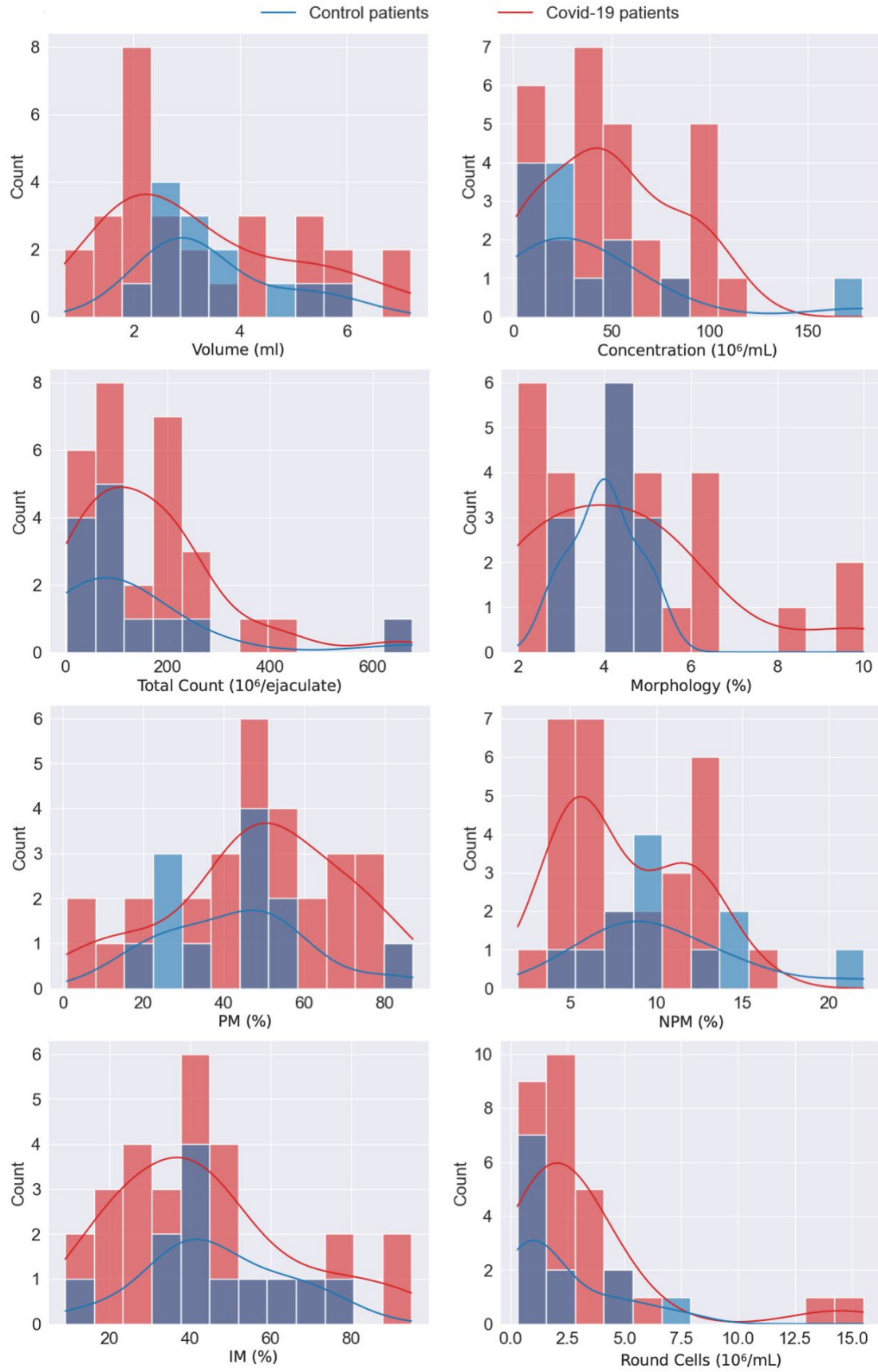


Figure 2. Comparison between the distribution of seminal parameters of control patients (blue) and patients recovered from COVID-19 (red). The distributions are superimposed, hence the dark blue effect, indicating the red distribution behind the blue one. PM: Progressive Motility; NPM: Non-Progressive Motility; IM: Immotile sperm.

3.2 ML semen analysis

Figure 3 shows the correlation matrix of the sperm parameters. Total count and concentration are strongly correlated since $\text{Total count} = \text{Concentration} \times \text{Volume}$. A strong correlation is also observed between PM and IM since $\text{PM} + \text{IM} + \text{NPM} = 100\%$, and NPM presents a lower and less variable value for all patients in this study. Therefore, a manual feature selection process was implemented to eliminate correlated features while increasing the performance of the ML models. Removing concentration from the pool of sperm parameters increases the performance of the ML models. However, removing any of the other parameters reduces the performance of the ML models. Therefore, the decision to train the classification algorithms using only seven features, Volume, Total Count, Morphology, PM, NPM, IM, and Round Cells, was critical in ensuring the robustness and reliability of the models.

Table 2 summarizes the performance of the 5 tested classification algorithms. AUC train corresponds to the AUC value obtained from the train dataset, while AUC test corresponds to the value obtained from the test dataset. Th corresponds to the optimum mobile threshold value.

Several ML models present good ($0.7 < \text{AUC} < 0.8$) or very good ($0.8 < \text{AUC} < 0.9$) performance. However, SVM was the best model overall, with a classification performance of $\text{AUC} = (0.81 \pm 0.05)$, $\text{F1} = (0.82 \pm 0.05)$, and $\text{MCC} = (0.53 \pm 0.08)$, and a strong equilibrium between Accuracy, Sensitivity, and Specificity.

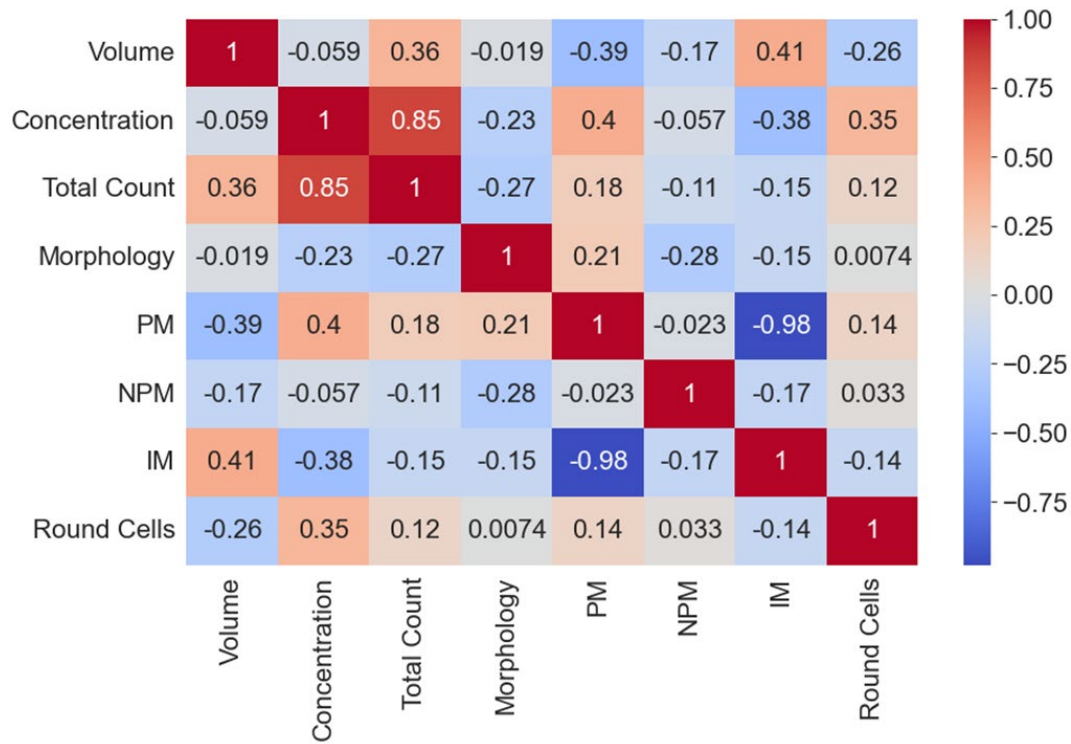


Figure 3. Correlation matrix of all sperm parameters.

Table 2. Performance metrics and optimum threshold value for classification algorithms.

Models	AUC train	AUC test	F1	MCC	Acc			Th
					(%)	Spec (%)	Sens (%)	(%)
KNN	1.00± 0.00	0.72 ± 0.05	0.74 ± 0.09	0.44 ± 0.09	70 ± 8	80 ± 12	66 ± 14	47 ± 18
LR	0.75± 0.01	0.56 ± 0.04	0.65 ± 0.08	0.22 ± 0.05	60 ± 6	67 ± 11	56 ± 12	98 ± 6
DT	1.00± 0.00	0.71 ± 0.05	0.81 ± 0.04	0.42 ± 0.11	75 ± 5	61 ± 8	81 ± 7	100 ± 5
SVM	0.99± 0.01	0.81 ± 0.05	0.82 ± 0.05	0.53 ± 0.08	77 ± 5	77 ± 12	78 ± 11	57 ± 9
XGBoost	0.95± 0.01	0.75 ± 0.04	0.86 ± 0.04	0.56 ± 0.07	81 ± 4	65 ± 11	88 ± 9	52 ± 14
MLP	0.99± 0.01	0.76 ± 0.04	0.81 ± 0.06	0.52 ± 0.08	77 ± 5	81 ± 6	75 ± 8	96 ± 7

Figure 4 showcases a SHAP summary plot stratified by class for the SVM mode. This plot assigns a different symbol to each class and plots the data from all patients. Open circles represent patients from the control group (class 0), while close triangles represent patients from the COVID-19-recovered group (class 1). The vertical axis ranks the sperm parameters by importance, with positive SHAP values contributing to classifying a patient in the Covid-19-

recovered class, and negative SHAP values contributing to classifying a patient in the control class.

PM and IM are the two most important sperm parameters, with SHAP values increasing with feature values. Volume, morphology, and total count were the next group of sperm parameters necessary for the classification process. Volume and morphology follow an opposite trend to PM and IM, where lower feature values result in higher SHAP values, while the SHAP values for Total Count increase with the feature value. Although some features from the COVID-19-recovered class patients show negative SHAP values, and features from the control class patients show positive SHAP values, the positive SHAP values part of the graph is dominated by the IM, PM, volume, morphology, total count, and NPM features of the COVID-19-recovered class patients. Therefore, the SVM model tends to classify as Covid-19-recovered patients when the semen has high PM, high IM, high NPM, high total count, and low volume.

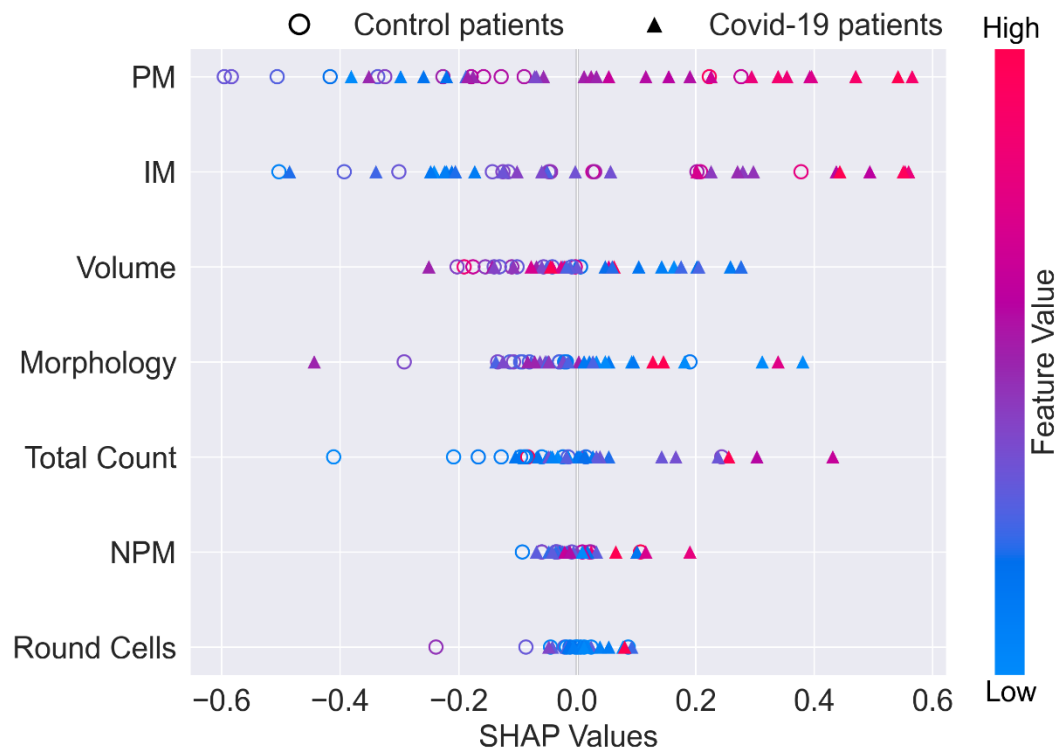


Figure 4. Class stratified SHAP summary plot to explain the importance of different features in the model for control patients (circles) and COVID-19 patients (triangles). The vertical axis lists the features and the horizontal axis shows the SHAP values, indicating the contribution of each feature to the model prediction. The color bar on the right side ranges from blue (low feature value) to red (high feature value), reflecting the magnitude of the feature values. Similar

or divergent contribution patterns between the two groups can be observed through the position and color of the points.

3.3 MLS

Figure 5 shows the average spectra of each sample class for the four measured properties at $\text{AOI} = 55^\circ$. The spectra are affected by the strong optical absorption of the top glass slide of the sample for wavelengths below ~ 350 nm. Above that wavelength value, the Δ spectra are relatively flat and close to 180° , indicating that the sample is almost transparent, with minimal optical absorption in that range. The sharp intensity increase in uR at about 1000 nm is not due to the sample itself, but to the change of the spectrometer used to measure the NIR part of the spectra. In this spectral region, there are thickness oscillations in the uR spectra, due to the layered nature of the samples. The differences between the average spectra (Ψ , Δ , and dPol) of both classes of samples are minimized in the NIR region. From these average spectra, the largest differences between the two classes of samples are observed in the visible region.

Due to the slight differences between the spectra of the four ellipsometric properties measured, the above-described ML learning approach will be used to classify each sample correctly. Table 3 presents the performance of the trained ML models obtained by k-fold cross-validation. All considered ML models can correctly classify the patients from their measured spectra with good, very good, or excellent ($0.9 < \text{AUC} < 1.0$) performance, regardless of the AOI and the measured property. However, SVM, XGBoost, and MLP (combined with the uR data) perform best, reaching AUC values of approximately 0.95.

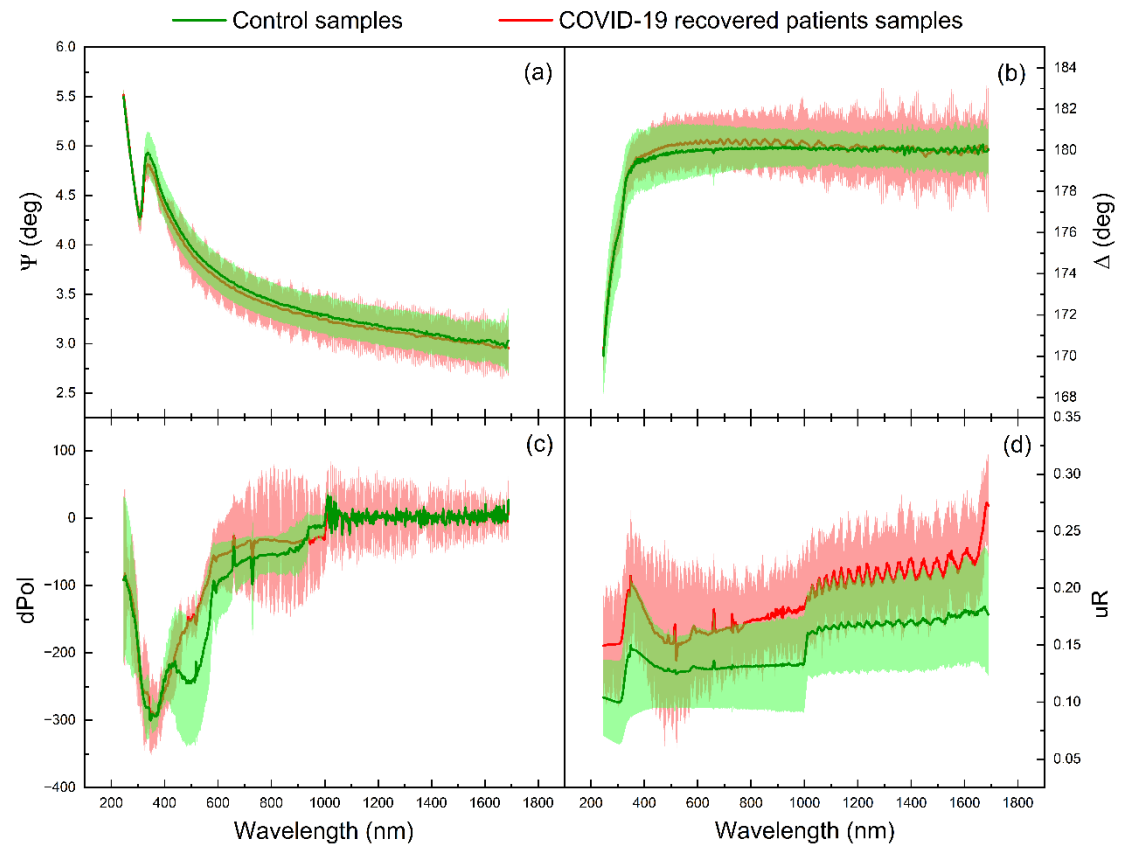


Figure 5. Averaged spectra of Ψ , Δ , dPol, and uR for both classes of sample in the study.

Table 3. k-fold cross-validation results for the MLS models. The best-performing models are highlighted in blue

Model	Prop	Angle	AUC train	AUC test	F1	MCC	Acc (%)	Spec (%)	Sens (%)	Th (%)
KNN	Ψ	45	0.90 ± 0.01	0.75 ± 0.06	0.20 ± 0.10	0.10 ± 0.10	68 ± 3	90 ± 4	18 ± 8	50 ± 1
	Δ	65	0.83 ± 0.01	0.74 ± 0.05	0.30 ± 0.10	0.20 ± 0.10	69 ± 4	90 ± 5	20 ± 10	55 ± 2
	dPol	60	0.89 ± 0.01	0.85 ± 0.05	0.62 ± 0.09	0.50 ± 0.10	77 ± 5	83 ± 4	60 ± 10	56 ± 2
	uR	70	0.92 ± 0.01	0.91 ± 0.03	0.81 ± 0.04	0.73 ± 0.06	89 ± 2	94 ± 3	78 ± 5	56 ± 2
LR	Ψ	45	0.81 ± 0.01	0.80 ± 0.04	0.61 ± 0.05	0.40 ± 0.09	70 ± 5	67 ± 7	76 ± 8	21 ± 2
	Δ	45	0.89 ± 0.01	0.73 ± 0.07	0.02 ± 0.05	0.04 ± 0.09	69 ± 1	100 ± 1	1 ± 3	71 ± 2
	dPol	50	0.92 ± 0.01	0.86 ± 0.05	0.66 ± 0.05	0.50 ± 0.08	78 ± 4	81 ± 5	70 ± 7	41 ± 3
	uR	60	0.93 ± 0.01	0.92 ± 0.06	0.85 ± 0.03	0.79 ± 0.05	91 ± 2	94 ± 2	84 ± 5	67 ± 2
SVM	Ψ	55	0.75 ± 0.02	0.64 ± 0.07	0.71 ± 0.05	0.20 ± 0.10	63 ± 6	50 ± 10	67 ± 7	51 ± 2
	Δ	65	0.82 ± 0.01	0.67 ± 0.08	0.82 ± 0.03	0.30 ± 0.20	73 ± 5	30 ± 10	91 ± 6	52 ± 2
	dPol	50	0.91 ± 0.01	0.93 ± 0.04	0.86 ± 0.03	0.67 ± 0.08	82 ± 4	95 ± 8	77 ± 3	59 ± 2
	uR	50	0.95 ± 0.01	0.95 ± 0.04	0.94 ± 0.02	0.80 ± 0.07	92 ± 3	81 ± 9	96 ± 1	27 ± 4
XGBoost	Ψ	60	0.91 ± 0.01	0.80 ± 0.04	0.81 ± 0.01	0.02 ± 0.10	69 ± 2	2 ± 3	99 ± 2	32 ± 4
	Δ	60	0.82 ± 0.01	0.74 ± 0.05	0.80 ± 0.01	-0.04 ± 0.10	67 ± 2	3 ± 5	96 ± 2	21 ± 3
	dPol	50	0.93 ± 0.01	0.91 ± 0.03	0.88 ± 0.03	0.60 ± 0.10	82 ± 4	60 ± 20	91 ± 5	34 ± 6
	uR	45	0.95 ± 0.01	0.94 ± 0.04	0.90 ± 0.03	0.60 ± 0.10	85 ± 5	60 ± 20	96 ± 1	30 ± 6
MLP	Ψ	45	0.88 ± 0.01	0.84 ± 0.04	0.86 ± 0.03	0.50 ± 0.10	79 ± 5	40 ± 20	96 ± 3	47 ± 4
	Δ	65	0.90 ± 0.01	0.72 ± 0.07	0.82 ± 0.01	0.01 ± 0.04	69 ± 1	0.3 ± 1	100 ± 1	11 ± 4
	dPol	50	0.93 ± 0.01	0.93 ± 0.03	0.87 ± 0.04	0.67 ± 0.08	84 ± 5	90 ± 10	81 ± 8	52 ± 9
	uR	50	0.96 ± 0.01	0.95 ± 0.04	0.93 ± 0.02	0.80 ± 0.07	91 ± 3	89 ± 9	92 ± 3	42 ± 7
DT	Ψ	45	0.77 ± 0.01	0.77 ± 0.06	0.52 ± 0.08	0.40 ± 0.10	73 ± 5	85 ± 6	47 ± 9	50 ± 1
	Δ	55	0.71 ± 0.01	0.63 ± 0.08	0.30 ± 0.20	0.06 ± 0.20	63 ± 6	80 ± 8	30 ± 10	55 ± 2
	dPol	50	0.85 ± 0.01	0.84 ± 0.04	0.61 ± 0.09	0.40 ± 0.10	75 ± 5	81 ± 5	60 ± 10	50 ± 1
	uR	55	0.83 ± 0.01	0.88 ± 0.06	0.74 ± 0.08	0.60 ± 0.10	83 ± 5	85 ± 5	78 ± 9	50 ± 1

Figure 6 presents the normalized histogram of the samples' classification probability using the SVM + uR and AOI = 50° model. The vertical dotted line represents the optimal classification threshold, or cut-off point, between the two classes. The solid lines, representing the probability density functions, are obtained using Kernel Density Estimation (KDE).

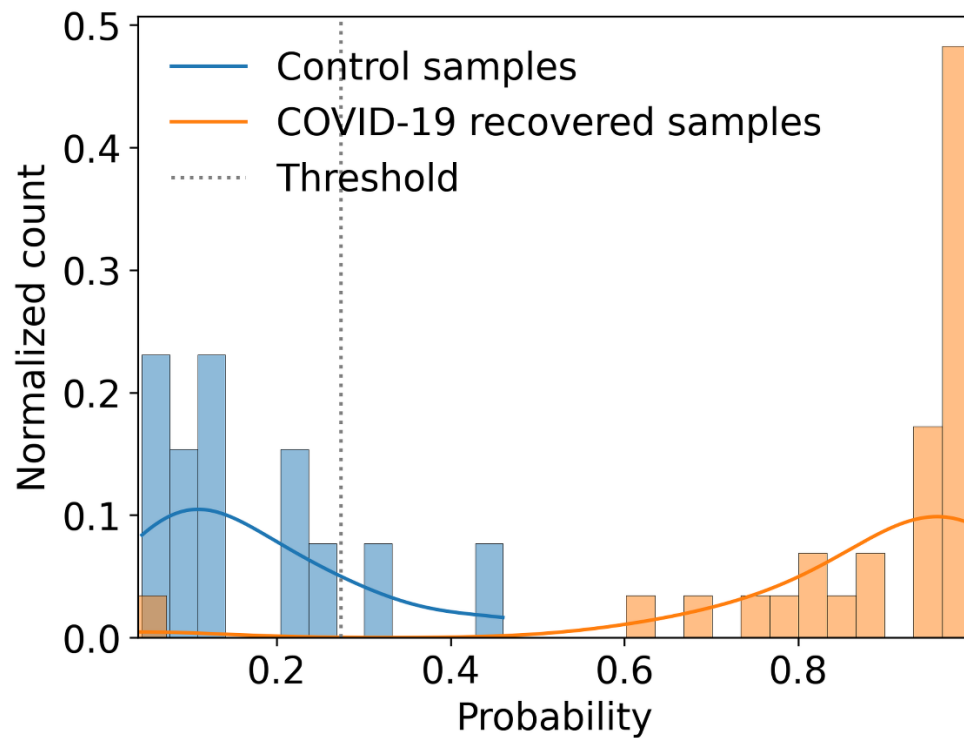


Figure 6. Normalized histogram of the classification probability of the control (blue bars) and COVID-19 recovered (orange bars) samples obtained with SVM, uR, and AOI = 50°. The blue and orange solid lines represent the probability density functions obtained from the histogram for the control and COVID-19 recovered groups, respectively. The vertical dotted line represents the optimum cut-off point between both classes.

To realistically evaluate the performance of the best models, avoiding all possible biases, a nested CV methodology (see Figure S1 of supplementary material) was used for the best MLS models (MLP, SVM, and XGBoost). The performance of these models is summarized in Table 4. All models present the same values of AUC for the test data set. However, considering the remaining metrics, the SVM model exhibits the best and most equilibrated performance.

Table 4. Scores for different validation metrics obtained from nested cross-validation for the best-performing models on k-fold cross-validation.

Model	Property	AUC_train	AUC_test	F1	MCC	Acc (%)	Spec (%)	Sens (%)
MLP	uR	0.98 ± 0.03	0.9 ± 0.2	0.9 ± 0.2	0.8 ± 0.4	90 ± 20	80 ± 30	90 ± 20
XGBoost	uR	0.98 ± 0.03	0.9 ± 0.1	0.85 ± 0.09	0.8 ± 0.3	90 ± 10	80 ± 40	90 ± 10
SVM	uR	0.96 ± 0.03	0.9 ± 0.1	0.9 ± 0.2	0.6 ± 0.2	90 ± 10	90 ± 20	90 ± 20

4. Discussion

The seminal characteristics observed in both control and COVID-19-recovered patient groups were similar and aligned with WHO standards for semen quality in fertile men [21]. Previous studies [16,33-35] have reported no significant differences in semen characteristics or transient changes in seminal parameters after COVID-19 infection. On the other hand, despite the recovery of sperm parameters, SARS-CoV-2 infection has been associated with negative impacts on assisted reproduction techniques for up to six months, with higher miscarriage rates reported [17]. Consequently, traditional semen analysis alone proves to be insufficient to distinguish between individuals with no history of COVID-19 and those who have recovered from the infection. Modern AI techniques can be valuable in this context. Both AI approaches in this study effectively separate these samples with very good or excellent performance.

The SVM ML model has presented the best overall performance in analyzing sperm parameters and optical spectroscopy data. It is particularly effective in distinguishing between semen specimens of different patient groups, regardless of the WHO-established guidelines for evaluating each parameter. This is evident in the comparison between the AUC scores obtained with the training and test databases, which shows no overfitting or underfitting. The quality of the training and optimization process of the ML algorithms is also apparent in the equilibrium between different metrics and their elevated values. The SHAP analysis brings interesting insight into the ranking of importance for the sperm parameters to distinguish the samples collected from patients who have never been diagnosed with COVID-19 or semen samples from COVID-19-recovered patients.

Interestingly, PM and IM were the two most important factors in classifying the sample as control or COVID-19-recovered patients, as depicted by the SHAP summary plot for the

SVM model. The traditional statistical analyses did not show any difference when comparing these parameters. According to the more robust AI evaluation, higher PM or IM is more related to the samples classified as belonging to the COVID-19-recovered group. Due to the deleterious effect of SARS-CoV-2 on the reproductive system, high IM data in COVID-19 patients would be somehow expected [36, 37]. However, this SHAP data also underscores the need for further investigations to explain the underlying reasons for the elevated PM values observed in this group. This research is crucial for our understanding of critical seminal characteristics after COVID-19 recovery.

Research on semen quality following SARS-CoV-2 infection has revealed a complex and individualized picture. In COVID-19-recovered patients, azoospermia was observed in 14.3% to 18.6% of cases, with a strong correlation to the severity of the illness [36,38]. Oligozoospermia was noted in 7% of patients [36]. Significant reductions in sperm concentration and total sperm count were reported in several studies [15, 33, 35, 38, 39], while others found no significant changes pre- and post-infection [37, 40]. Progressive motility also showed a significant decrease in some studies [33,35,41], although others reported no significant difference [40, 42]. Additionally, a decrease in normal sperm morphology was noted in both COVID-19 patients and recovered individuals [15, 42], while some studies found no significant differences [33]. This variability underscores the complexity and individuality of the impact of COVID-19 on semen quality.

While the current research provides valuable insights, it also underscores the need for further investigation. Semen volume was generally not significantly different in some studies [33], although a decrease was reported in others [40, 41, 43]. An increase in round cells was observed in COVID-19-recovered patients [15], yet no significant differences in leukocytes were noted among various patient groups [36]. Interestingly, some studies found better outcomes for sperm concentration and total sperm number in recovered patients compared to infertile controls, with greater progressive motility [16]. Furthermore, seminal parameters in recovered patients were consistent with the WHO 2010 fifth percentile [16], indicating that while COVID-19 impacts semen quality, the effects may vary widely among individuals. This research highlights the urgency and importance of further studies in this area.

With the wide variation in the seminal parameters results of COVID-19 patients, the MLS approach presents a promising addition to the reproductive health arsenal. This label-free,

cost-effective, and fast diagnosis solution can be further engineered to a completely automatic and compact version. The MLS approach outperforms the analysis of sperm parameters via traditional statistical methods or even modern AI models. It relies on the morphological and chemical alterations of the semen [4,18,22] induced by the response of the human organism to COVID-19. These alterations modify the dielectric function of the semen and consequently the interaction of light with these samples. Importantly, these alterations could not be attributed to direct infection of the sperm cells by the SARS-CoV-2 virus, as the semen samples tested negative for viral presence via RT-PCR.

This study paves the way for a new research field, utilizing innovative methods to analyze traditional exams and incorporating optical data that has been little explored in semen to understand the reproductive outcomes following SARS-CoV-2 infection. Although this approach has the limitation of not revealing the biological nature of the alterations in the semen samples, this methodology effectively classified the semen samples with a very good accuracy. From a technological standpoint, the implementation of a simple optical device with embedded AI processing to record uR spectra from sperm samples could potentially revolutionize precision medicine in reproduction clinics and hospitals, offering new hope and excitement for the future of reproductive health research.

5. Conclusion

Our study showed that we could accurately classify semen from patients who have never been diagnosed with COVID-19 and semen from COVID-19-recovered patients using AI methods that could assist urologists and reproductive centers in screening or even diagnosing fertility issues.

Conflict of Interest

The authors declare that they have no competing interests, either financial or non-financial.

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Supplementary material

Additional figures and tables are provided in the Supplementary Material file (PDF).

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Supplementary Material

Table S1. Hyperparameters along with their chosen spaces for the machine learning models

Model	Hyperparameters	Space
KNN	n neighbors	2 and 3
	weights	uniform, distance
	p	1, 2 and 3
LR	C	0.01 - 10.00
	penalty	l1, l2
	class weight [†]	0.05 - 0.95
	solver	saga
	max iter	100000
SVM	C	0.01 - 100000
	gamma	0.0001 - 0.0100
	class weight [†]	0.1 - 0.9
XGBoost	n estimators	1 - 1000
	learning rate	0.001 - 0.900
	max depth	1 - 10
	subsample	0.1 - 1.0
	colsample bytree	0.1 - 1.0
	colsample bylevel	0.1 - 1.0
	colsample bynode	0.1 - 1.0
	scale pos weight	0.1 - 2.0
MLP	hln1*	1 to the 2x features number
	hln2**	1 to the 2x features number
	solver	sgd, adam
	activation	logistic, tanh, relu
	momentum	0.01 - 1.00
	max iter	100000
DT	criterion	entropy, log loss, gini
	max depth	1 - 50
	max features	auto, sqrt, log2, None

[†] The value here refers to class 0. For class 1, the value is the complement: 1 - class_weight.

* Number of neurons for the first hidden layer.

** Number of neurons for the second hidden layer.

Table S2. Hyperparameters optimized for each of the models

Model	Hyperparameters	Ψ	Δ	dPol	uR
KNN	n neighbors	2	2	2	2
	weights	uniform	distance	distance	distance
	p	3	2	1	2
LR	C	2.27	6.05	8.98	7.31
	penalty	l2	l1	l1	l1
	class weight	0: 0.85	0: 0.57	0: 0.60	0: 0.37
		1: 0.15	1: 0.43	1: 0.40	1: 0.63
	solver	saga	saga	saga	saga
	max iter	100000	10000	10000	10000
SVM	C	3561	92885	1567	64787
	gamma	0.00063	0.0081	0.0092	0.0095
	weight0	0.49	0.37	0.87	0.17
XGBoost	n estimators	999	545	683	477
	learning rate	0.079	0.34	0.89	0.53
	max depth	9	9	3	2
	subsample	0.94	0.53	1.00	0.58
	colsample bytree	0.66	0.73	0.56	0.88
	colsample bylevel	0.97	0.98	0.37	0.96
	colsample bynode	0.63	0.35	0.55	0.36
	scale pos weight	1.13	0.11	0.34	1.01
	hln1	31	136	35	27
	hln2	37	112	35	14
MLP	solver	sgd	sgd	adam	adam
	activation	tanh	tanh	tanh	relu
	momentum	0.87	0.99	0.57	0.58
	max iter	100000	100000	100000	100000
	criterion	log loss	gini	gini	log loss
DT	max depth	36	8	16	48
	max features	None	sqrt	None	auto

Table S3. Parameters optimized for each of the models

Model	Parameters	Ψ	Δ	dPol	uR
KNN	angle	45	65	60	70
	spectral range (nm)	270 - 654	338 - 414	1438 - 1525	1255 - 1587
	feature number	164	27	13	27
LR	angle	45	45	50	60
	spectral range (nm)	265 - 361	701 - 1408	1166 - 1577	567 - 1660
	feature number	55	281	98	368
SVM	angle	55	65	50	50
	spectral range (nm)	506 - 1138	1131 - 1277	1026 - 1360	957 - 1666
	feature number	9	24	8	168
XGBoost	angle	60	60	50	45
	spectral range (nm)	300 - 455	334 - 408	1059 - 1321	834 - 1674
	feature number	77	23	25	222
MLP	angle	45	65	50	50
	spectral range (nm)	268 - 344	936 - 1321	1161 - 1388	1510 - 1692
	feature number	44	92	29	15
DT	angle	45	55	50	55
	spectral range (nm)	328 - 429	257 - 580	1307 - 1466	867 - 1683
	feature number	26	90	25	267

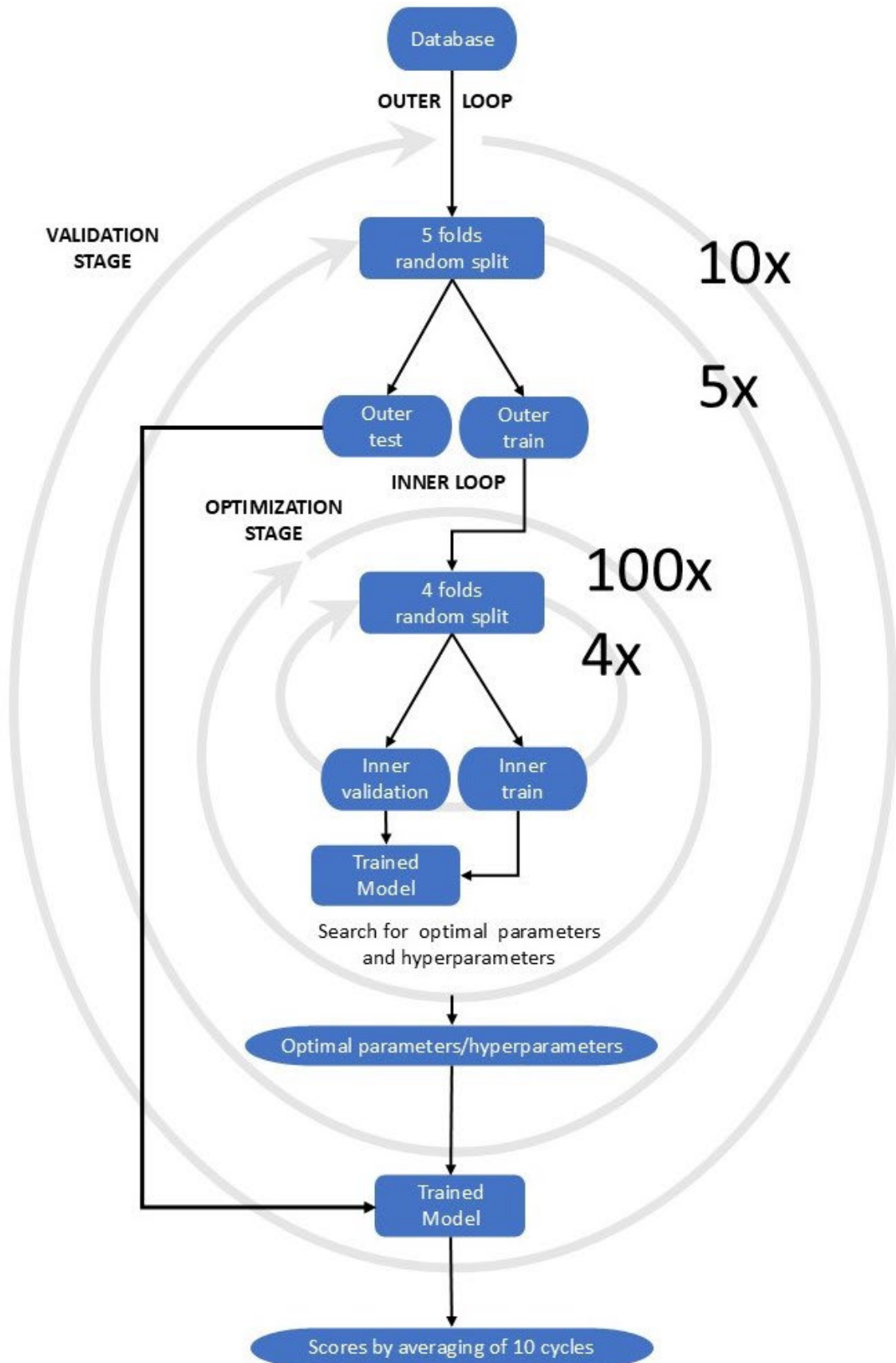


Figure S1. Scheme of the optimization and validation process in the Nested cross-validation method for the best performing ML models.

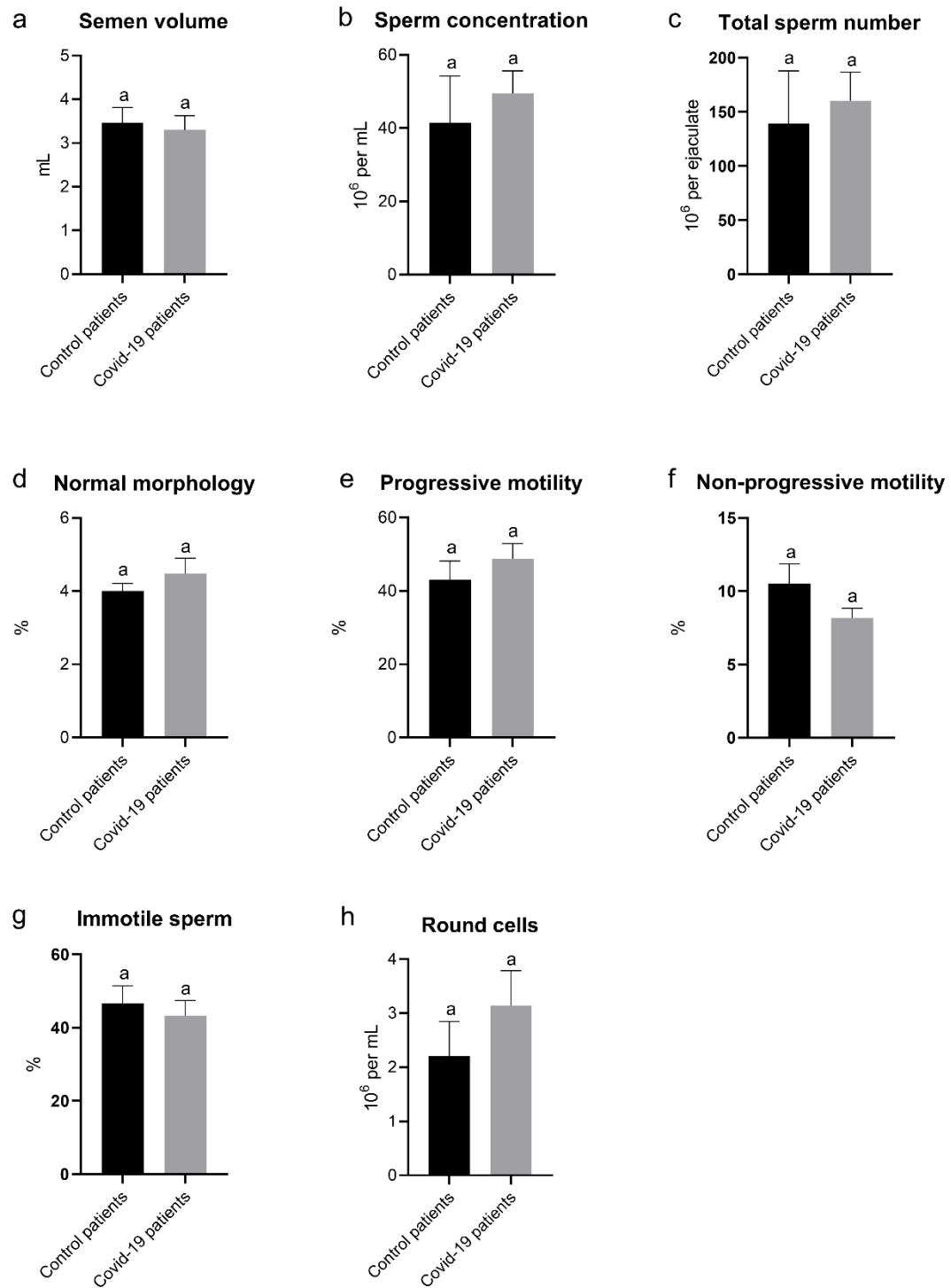


Figure S2. Mean ($\pm$ SEM) values of seminal parameters of control patients and the COVID-19-recovered patients. No statistical differences were observed between the groups ($p > 0.05$).

4. DISCUSSÃO

O impacto do SARS-CoV-2 sobre o sistema reprodutor masculino tem recebido atenção crescente desde os primeiros momentos da pandemia de COVID-19. A compreensão abrangente dessa relação é essencial, não apenas para abordar possíveis preocupações relacionadas à fertilidade, mas também para explorar mecanismos de persistência viral e COVID longa. Os achados de três estudos complementares — abrangendo desde a histopatologia testicular pós-morte até a virologia seminal longitudinal e análises funcionais — revelam, coletivamente, uma influência multifacetada do SARS-CoV-2 sobre a saúde reprodutiva masculina.

O estudo de Costa et al. (2023) fornece evidências diretas da infecção e da replicação do SARS-CoV-2 nos testículos de pacientes falecidos não vacinados. Utilizando metodologias diagnósticas sensíveis — incluindo RT-qPCR com primers específicos para genes virais, imunofluorescência, nanosensores e microscopia eletrônica de transmissão (TEM) — os autores demonstraram que o vírus infecta macrófagos, células espermátogoniais e, em menor grau, células de Sertoli e de Leydig. A presença de fábricas de replicação viral nas células testiculares, a confirmação de infectividade em culturas de células VERO e a correlação entre alterações histopatológicas e o tempo de permanência na UTI reforçam a ideia de que o SARS-CoV-2 pode replicar-se ativamente e induzir danos progressivos aos testículos em casos graves. Essas alterações — incluindo apoptose de células germinativas, ruptura da barreira de células de Sertoli, disfunção das células de Leydig, fibrose e inflamação — ocorreram apesar da ausência de sintomas testiculares evidentes, o que destaca o caráter subclínico do comprometimento testicular.

Aprofundando essas observações, o estudo longitudinal submetido à *Journal of Medical Virology* por Furtado et al. avaliou a persistência e a infectividade do SARS-CoV-2 no sêmen por até seis meses após a recuperação, em pacientes com doença leve a moderada. O RNA viral foi detectado no sêmen de mais da metade dos participantes, e a infectividade viral foi confirmada em uma proporção substancial dessas amostras por meio de cultura em células VERO, imunofluorescência e TEM. Esses achados indicam que o SARS-CoV-2 pode persistir no sêmen em forma viável por longos períodos após a recuperação clínica, inclusive em pacientes sem doença grave. Curiosamente, apesar da persistência viral, os parâmetros seminais

permaneceram dentro dos limites de referência da OMS, e apenas foram observados aumentos transitórios e modestos nos linfócitos T seminiais (CD3+, CD4+, CD8+), especialmente em torno do terceiro mês. Esses dados ressaltam o potencial do trato genital masculino como reservatório viral e evidenciam as limitações da análise seminal convencional para detectar alterações biologicamente relevantes após a infecção.

Para investigar se a infecção pelo SARS-CoV-2 deixa assinaturas funcionais sutis no sêmen, não detectáveis por análises convencionais, nosso grupo empregou aprendizado de máquina (machine learning, ML) e espectroscopia. Apesar de todas as amostras de sêmen estarem dentro dos parâmetros normais segundo a OMS, os algoritmos de ML conseguiram distinguir com precisão amostras de pacientes recuperados das amostras de controle. Com base em parâmetros seminiais convencionais, o classificador de máquinas de vetores de suporte (SVM) atingiu 77% de acurácia na distinção entre os grupos infectado e controle; entre as variáveis avaliadas, a motilidade progressiva e a proporção de espermatozoides imóveis foram as de maior influência na discriminação entre os grupos. Os modelos baseados em espectroscopia apresentaram desempenho ainda melhor, atingindo até 90% de acurácia. Esses resultados indicam que alterações bioquímicas ou físicas sutis nos espermatozoides — possivelmente reflexo residual da infecção viral ou da inflamação — persistem mesmo após a recuperação e só são detectáveis por meio de ferramentas computacionais e ópticas avançadas.

Em conjunto, esses três estudos constroem uma narrativa coerente ao longo de diferentes estágios da doença e de diferentes níveis de complexidade biológica. O SARS-CoV-2 demonstra um tropismo marcante pelos testículos e pode replicar-se ativamente nas células testiculares em casos graves. O vírus pode persistir em forma viável no sêmen por até seis meses após o início dos sintomas, mesmo na ausência de sintomas reprodutivos ou alterações detectáveis nos parâmetros seminiais. Por fim, o uso de aprendizado de máquina e espectroscopia revela alterações funcionais relevantes que persistem após a infecção, mesmo quando os exames clínicos tradicionais são normais.

Do ponto de vista clínico, esses achados têm diversas implicações. Em primeiro lugar, pacientes recuperados da COVID-19 — independentemente da gravidade da doença — podem se beneficiar de acompanhamento reprodutivo prolongado, especialmente aqueles com desejo reprodutivo ou candidatos a técnicas de reprodução assistida. Em segundo lugar, a dependência exclusiva da análise seminal convencional pode subestimar o real impacto da infecção; métodos

avançados, como classificação baseada em IA e espectroscopia, podem oferecer maior sensibilidade diagnóstica. Em terceiro lugar, a detecção de vírus viável no sêmen suscita a possibilidade de transmissão sexual durante a fase de convalescença, o que merece investigação adicional, particularmente em termos de diretrizes de saúde pública.

Por fim, o status imunológico privilegiado dos testículos, combinado com a demonstração da persistência do SARS-CoV-2 no compartimento testicular e seminal, posiciona o trato genital masculino como um modelo valioso para o estudo de santuários virais do SARS-CoV-2 e das sequelas pós-agudas. Assim, pode servir como um local acessível e clinicamente relevante para o avanço da compreensão da COVID longa e da persistência viral de forma mais ampla.

5. CONCLUSÃO

Após a investigação de pacientes que apresentaram COVID-19 clinicamente severa, moderada e leve, conclui-se que:

- **O SARS-CoV-2 apresenta alto tropismo pelos testículos**, especialmente em casos graves de COVID-19, com infecção ativa documentada em macrófagos, células germinativas, células de Sertoli e de Leydig.
- **Alterações histopatológicas testiculares graves** foram observadas, incluindo apoptose de células germinativas, ruptura da barreira hematotesticular, inibição das células de Leydig, fibrose e inflamação, mesmo na ausência de sintomas clínicos testiculares.
- **O vírus pode persistir no sêmen por até seis meses** após a infecção, inclusive em pacientes com quadros leves e moderados de COVID-19, com confirmação de infectividade em culturas de células VERO.
- **Embora existam alterações nos parâmetros seminais no contexto pós-infecção por SARS-CoV-2, a avaliação seminal convencional frequentemente não as evidencia, dado que a maior parte dos pacientes apresenta resultados dentro das faixas de referência da OMS, apesar da persistência viral e de inflamação seminal transitória. Modelos de IA e espectroscopia óptica** conseguem identificar alterações sutis e persistentes no sêmen de pacientes recuperados, com alta acurácia na

classificação entre o grupo controle e o pós-COVID-19, mesmo quando os exames tradicionais são normais.

- **A presença viral no sêmen observada neste estudo sustenta o potencial do trato genital masculino como reservatório do SARS-CoV-2, com implicações para transmissão sexual e para o entendimento da COVID longa. É recomendável o monitoramento reprodutivo de homens que se recuperaram da COVID-19, especialmente os com desejo reprodutivo ou em avaliação para reprodução assistida, com atenção para limitações dos métodos diagnósticos convencionais.**
- **O uso de ferramentas avançadas como IA e espectroscopia pode ampliar significativamente a sensibilidade diagnóstica, permitindo detectar disfunções subclínicas que afetam a fertilidade masculina mesmo após a recuperação clínica da infecção.**

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