

# Understanding Severe Acute Respiratory Syndrome Coronavirus 2 Replication to Design Efficient Drug Combination Therapies

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## Keywords

Coronavirus · Virus-host interactions · SARS-CoV-2 · Treatment · Combination · Protease · Entry

## Abstract

**Background:** The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and its disease COVID-19 has strongly encouraged the search for antiviral compounds. Most of the evaluated drugs against SARS-CoV-2 derive from drug repurposing of Food and Drug Administration-approved molecules. These drugs have as target three major processes: (1) early stages of virus-cell interaction, (2) viral proteases, and (3) the viral RNA-dependent RNA polymerase. **Summary:** This review focused on the basic principles of virology and pharmacology to understand the importance of early stages of virus-cell interaction as therapeutic targets and other main processes vital for SARS-CoV-2 replication. Furthermore, we focused on describing the main targets associated with SARS-CoV-2 antiviral therapy and the rationale of drug combinations for efficiently suppressing viral replication. **Key Messages:** We hypothesized that blocking of both entry mechanisms could allow a more effective antiviral effect compared to the partial results obtained with

chloroquine or its derivatives alone. This approach, already used to achieve an antiviral effect higher than that offered by every single drug administered separately, has been successfully applied in several viral infections such as HIV and HCV. This review will contribute to expanding the perception of the possible therapeutic targets in SARS-CoV-2 infection and highlight the benefits of using combination therapies.

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## Introduction

In the last trimester of 2019 a new coronavirus, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and responsible for coronavirus disease 2019 (COVID-19), emerged in Wuhan, China. There is no yet an approved therapy to treat the infection by SARS-CoV-2 and its associated disease, COVID-19, and the therapeutic approaches used have been changing from the beginning of the outbreak [1–3]. The standard care of patients with COVID-19 is mainly focused on symptomatic and respiratory support according to the diagnosis and treatment of pneumonia and coagulopathies caused

by COVID-19 [4]. Several research groups have been working to determine the possible targets to control the viral infection [5–7]. Three major targets have been identified and can be summarized in the following: early stages of virus-cell interaction, viral proteases, and the viral RNA-dependent RNA polymerase (RdRp) [7–9]. The replication cycle of SARS-CoV-2 and the main targets for the antiviral therapy described in this review are shown in Figure 1.

### Early Infection Stages: Blocking Viral Entry

After interaction with its receptor ACE2, SARS-CoV-2 enters the cell through the fusion of its viral membrane with the cellular one (Fig. 1) [10]. The viral fusion protein is exposed by priming with a proteolytic enzyme. Three different types of proteases have been proposed for this cleavage: (1) Priming by the cellular transmembrane serine protease 2 (TMPRSS2) and also TMPRSS4 in enterocytes. This priming allows viral entry by the early endosomal pathway, although the exact mechanism of entry is unknown [8, 11, 12]. (2) Priming by cathepsin B in the late endosomes [13]. (3) Activation during virus egress from the cell by furin [14]. Furin-dependent pre-cleavage of the spike has been shown to be necessary for activation through the TMPRSS2 pathway [15].

In the early stages of infection, different therapeutic agents have been used, including hydroxychloroquine (HCQ) [16, 17], benzoic acid derivatives such as nafamostat and camostat [18], and neutralizing antibodies [19]. The antiviral effect of HCQ is related to an elevation of the pH in endosomes/lysosomes, which is essential for the fusion between the viral and vesicle membranes. In addition, HCQ could inhibit SARS-CoV cellular entry by changing the glycosylation pattern of ACE2, the key receptor for SARS-CoV-2 entry [16, 17]. Furthermore, HCQ exerts immunomodulatory effects through attenuation of cytokine production [20]. The effects of HCQ on cellular targets are considered nonspecific antiviral effects.

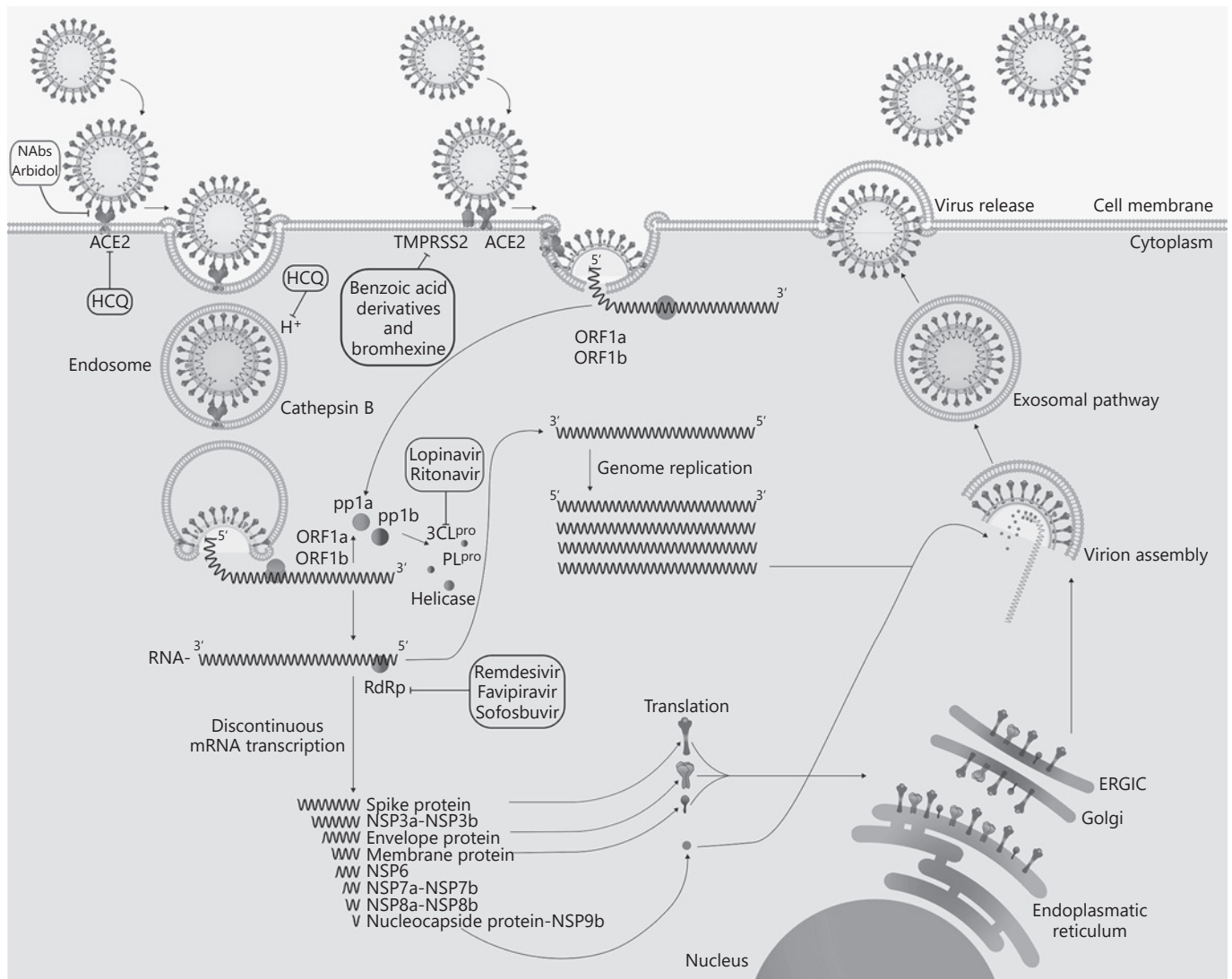
Another early target evaluated against SARS-CoV-2 is a cellular protease related to the priming of the spike protein (S), which exposes the fusion motive and allows the release of viral RNA into the cytosol. The proteolytic processing of the viral spike protein is a key step during the infection process of SARS-CoV-2. This reaction is driven by TMPRSS2 and could be inhibited by camostat, nafamostat, and bromhexine [18, 21]. Interestingly, some reports showed that SARS-CoV-2 could gain entry into the

host cell without using the late endocytic pathway. The processing of TMPRSS2 in the extracellular lumen could likely be sufficient to activate the fusion between the viral and host membrane [22, 23]. This alternative entry pathway would explain the low success rates in the use of HCQ/chloroquine alone in COVID-19 therapy. In addition, chloroquine does not seem to prevent SARS-CoV-2 entry into human lung cells, suggesting that the TMPRSS2-primed pathway predominates in the lung [24].

Arbidol (umifenovir) is an antiviral agent that targets S/ACE2 interaction, inhibiting membrane fusion of the viral envelope [2]. This compound could be considered as an early-stage inhibitor of SARS-CoV-2. Another therapy for COVID-19 is the use of convalescent plasma or hyperimmune immunoglobulins (NAbs) [25]. Antibodies from recovered patients or neutralizing monoclonal antibodies may neutralize the free virus and induce the immune clearance of infected cells. On March 24, 2020 the Food and Drug Administration (FDA) released an emergency approval for the use of COVID-19 convalescent plasma. The development of neutralizing monoclonal antibodies against SARS-CoV-2 infection is ongoing [19, 26].

### Targeting Viral Enzymes

Other promising therapeutic targets against SARS-CoV-2 infection are viral proteases (Fig. 1) due to their low homology with host protease and the structural knowledge available for SARS-CoV [7, 27]. SARS-CoV-2 has two viral proteases related to the proteolytic processing of the viral polyprotein into mature and functional proteins. These enzymes are the main protease (3CL<sup>Pro</sup>) and the papain-like protease (PL<sup>Pro</sup>), both belonging to the family of cysteine proteases. They exhibit a high structural and functional homology with SARS-CoV proteases [27–29]. The main protease is responsible for the proteolysis of pre-proteins associated with the viral replication machinery such as the RdRp, the helicase, and the exoribonuclease, among others [29]. Several groups have shown by using *in silico* and *in vitro* approaches that this protease may be blocked by protease inhibitors effective in HIV therapy [7, 27, 29, 30]. Furthermore, lopinavir/ritonavir, an FDA-approved oral drug combination for treating HIV, exhibited *in vitro* activity against other coronaviruses. However, in the case of SARS-CoV-2 infection, the timing of the administration of these drugs during the early peak of the viral replication phase (at initial 7–10 days) appears to be critical. Delayed initiation of



**Fig. 1.** SARS-CoV-2 replication. The viral cycle begins with the interaction between the viral spike and the cellular receptor. Several membrane proteins have been proposed as possible receptors for SARS-CoV-2; however, ACE2 is likely to be the most important one [10]. After the viral spike has interacted with the receptor, the virus gains entry into the host cytosol by two mechanisms: (1) by late endocytosis, releasing the viral RNA after the fusion with the lysosome (this entry is blocked by HCO), or (2) by early endocytosis by fusion of the viral and host membrane without the participation of the lysosome. In these early stages, the priming processing by cellular proteases is the key for the exposure of the viral fusion motive. TMPRSS2 could act in both early and late endosome entry processes and could be inhibited by benzoic acid derivatives such as nafamostat, camostat, and bromhexine [18, 21]. Furthermore, other proteases such as cathepsin B could mediate the entry in the late lysosomal pathway, while only TMPRSS2 has been related to the early entry endosome [11, 13, 15, 18]. After fusion has occurred, the viral RNA is released into the cytoplasm and open reading frame 1 (ORF1) is translated to produce the RdRp. Subgenomic mRNAs are produced by discontinuous transcrip-

tion, a process characteristic of this RdRp, which favors recombination. Compounds such as remdesivir, favipiravir, and sofosbuvir block this enzyme [39–41, 45]. The subgenomic mRNAs are then translated into protein. The genome has eight ORFs. The gene segments that encode nonstructural polyproteins are processed first and translated into ORF1a and ORF1b producing pp1a and pp1ab proteins, respectively. Protein pp1a and pp1ab are cleaved by the viral proteases (3CL<sup>pro</sup> and PL<sup>pro</sup>). The main protease is also the target of protease inhibitors such as lopinavir/ritonavir [7]. The structural proteins – spike, envelope, and membrane proteins – enter into the endoplasmic reticulum/Golgi complexes. Then, the nucleoprotein combines with the (+) strand genomic RNA (nucleoprotein complex) and merges with the other structural proteins in the endoplasmic reticulum-Golgi apparatus compartment [64]. Finally, the virion is excreted to the extracellular region through the exosomal pathway [64]. HCO, hydroxychloroquine; RdRp, RNA-dependent RNA polymerase; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TMPRSS2, transmembrane serine protease 2; ORF, open reading frame.

therapy with lopinavir/ritonavir did not result in positive clinical outcomes mainly due to the high viral load [31]. Another important concern with lopinavir/ritonavir therapy against SARS-CoV-2 is related to the administration route. This drug combination is administered to patients with COVID-19 by the oral pathway, applicable for drugs with good gastrointestinal absorption and low volume of distribution (the volume of distribution for lopinavir/ritonavir is around 17 L) [32, 33]. However, these compounds bind tightly to plasma proteins (in the plasma, >98% of the drug is in bound form), which decreases the ability to reach tissues such as the lung [32]. This important pharmacological issue related to the pharmacokinetic parameters of lopinavir/ritonavir needs to be addressed in order to improve the therapeutic outcome of treatment with this drug combination. The second protease related to the SARS-CoV-2 replication cycle, PL<sup>Pro</sup>, is responsible for the cleavages of the N-terminus of the polyprotein to release Nsp1, Nsp2, and Nsp3, nonstructural proteins associated with viral replication [34–36]. In addition, PL<sup>Pro</sup> also possesses de-ubiquitination and deISGylation activities that could antagonize the host's innate immunity [37].

The SARS-CoV-2 genome replication is carried out by an RdRp and could be one of the best targets in the SARS-CoV-2 replication (Fig. 1) [38]. This enzymatic activity has no parallel process in the eukaryotic cells. Thus, attacking this target might reduce side effects that might occur by affecting any similar protein in the host. The sequences of RdRp in SARS-CoV and SARS-CoV-2 have a high homology and encode structurally similar proteins. Remdesivir is a nucleotide analog inhibitor of RdRp and has shown a broad spectrum of antiviral activity against several RNA viruses [38, 39]. Remdesivir similarly to sofosbuvir, a direct-acting antiviral used in HCV therapy, is a chain-terminating nucleotide analog. These drugs produce their therapeutic effect by directly interacting with the RdRp and incorporating the active form of the inhibitor into the growing RNA strand, preventing the replication to continue [40, 41]. Several clinical trials have been conducted evaluating the activity of remdesivir and other direct-acting antivirals used in HCV against SARS-CoV-2 [42]. Only remdesivir reached the FDA emergency approval status for its use in patients with severe COVID-19. However, the clinical improvements by using remdesivir alone in SARS-CoV-2 patients are modest. Favipiravir, another antiviral agent with broad activity against other RNA viruses by inhibiting the RdRp, halting viral replication, was evaluated against SARS-CoV-2, showing effects *in vitro* and *in vivo* [43–45].

## Combined Therapy

Drug combinations that have been widely used in antiviral therapy have become the leading choice for treating HIV and HCV, among other viral pathologies [46–50]. The use of multiple drugs with different mechanisms of action increases the efficacy of the therapeutic effect. It also allows decreasing the dose of a single drug, thus preventing host toxicity [51–54]. Moreover, one important advantage of combination therapies besides enhancing the selective effect over the target is that this approach minimizes or slows down the development of drug resistance, which is the main issue in antiviral therapy of RNA viruses. Experimental conditions for drug combinations *in vitro* can be easily defined. Information for most individual drugs used against SARS-CoV-2 is available, thus conducting *in vitro* assays for the effect of their combinations could take a very short time (approximately <2 weeks) by using methods such as the combination index-isobologram method of Chou [55]. However, there are some issues associated with the evaluation of drug combinations in clinical trials related to dose, timing, route of administration, efficacy, and toxicity, among others, that make the extrapolation of *in vitro* data difficult. These factors are associated with, but not limited to, variables in the patient population such as sex, age, race, and disease stage. Furthermore, drug combination trials need to fulfill all the ethical concerns to treat patients with placebo or suboptimal therapeutic doses as required for a drug combination study design according to the guidelines of regulatory agencies, such as the FDA.

As explained above, HCQ could produce a non-direct-acting specific antiviral effect against SARS-CoV-2 by several mechanisms. However, the results obtained in the clinical trial by using HCQ alone were less satisfactory than expected [56]. Although HCQ affects the early stages of the viral infection, *i.e.*, the endosome-mediated entry, some reports showed that SARS-CoV-2 could enter the host cells bypassing the pathways blocked by HCQ [15]. Thus, we hypothesize that this could be avoided by combining HCQ and camostat or nafamostat [18] or bromhexine [21, 57]. These TMPRSS2 inhibitors could block the other mechanism associated with SARS-CoV-2 entry. In addition to interfering with the different proteases involved in the activation of the fusion peptide of S, a combined scheme might include an inhibitor of this domain [8]. Furthermore, we support the fact that other TMPRSS2 inhibitors such as bromhexine, a drug used as mucolytic, could be an important pharmacological tool to design prophylactic therapies. Bromhexine has a well-

**Table 1.** Clinical trials registered in the United States National Library of Medicine that included drug combinations against SARS-CoV-2 targeting early steps of viral replication or viral enzymes

Drug combination	Clinical trial number (NIH)	Country	Registration date
<b>Camostat mesilate, HCQ</b>	NCT04338906	Germany	April 8, 2020
	NCT04355052	Israel	April 21, 2020
Baricitinib, <b>HCQ, lopinavir/ritonavir, remdesivir</b>	NCT04373044	US	May 4, 2020
<b>HCQ, azithromycin, ivermectin, camostat mesilate</b>	NCT04374019	US	May 5, 2020
<b>Favipiravir, HCQ</b>	NCT04411433	Turkey	June 2, 2020
<b>Favipiravir, lopinavir</b>	NCT04499677	UK	August 5, 2020

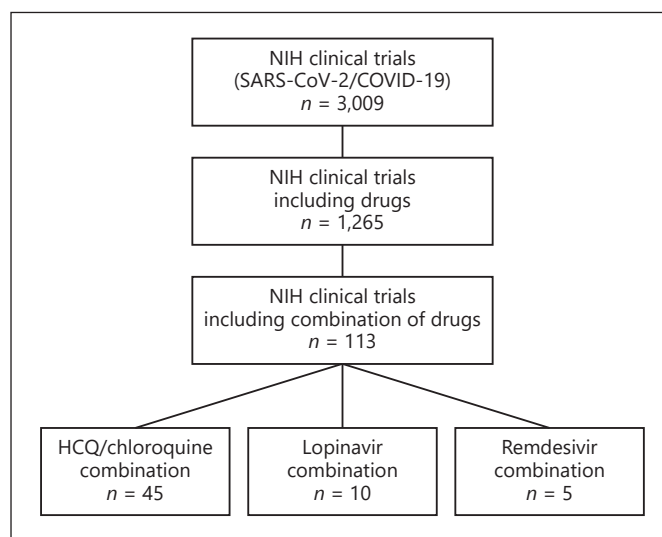
The studies shown were retrieved from the website ClinicalTrials.gov (see also Fig. 2). SARS-CoV-2/COVID-19 as condition or disease and the drug names and their combinations were used as keywords for the search. Drugs related to blocking early stages of viral replication or viral enzymes are shown in bold. COVID-19, coronavirus disease 2019; NIH, National Institutes of Health; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

known drug safety profile and it is marketed as an over-the-counter medication. Thus, the design of clinical trials and extrapolation into effective therapy should be less complicated in comparison with drugs as camostat or nafamostat. Furthermore, some reports showed that bromhexine could overcome the main pharmacokinetic problem in SARS-CoV-2 therapy, reaching the pulmonary and bronchial epithelial cells in concentrations higher than those found in the plasma [58].

The above combination targets viral entry into the cell. Similarly, neutralizing antibodies and fusion inhibitors could be combined in a triple combination format. Most of the targets associated with the early stages of the viral infection are host targets that significantly decrease the probability of developing resistance in comparison with viral targets [51].

The early study published by Cao et al. [59] showed that lopinavir/ritonavir could produce a modest positive effect in comparison to the standard care in patients with SARS-CoV-2. However, several researchers have criticized the structural design of the study of Cao et al. [59], suggesting that the antiviral effect of lopinavir/ritonavir against SARS-CoV-2 could be higher; thus, more studies are needed [60–62]. Nevertheless, the side effects associated with these protease inhibitors are important. Thus, antiviral therapy based on combining these protease inhibitors with another antiviral compound such as remdesivir could decrease the effective dose required to produce the antiviral effect and then decreasing the possibility of side effects. The possibility of the occurrence of viral resistance with this strategy should be kept in mind.

Of the 1,265 clinical trials based on drugs to treat SARS-CoV-2 registered in the National Institutes of Health Library, only 113 (<10%) are based on drug com-



**Fig. 2.** Clinical trials using a combination of drugs targeting early replication steps or viral enzymes. An exhaustive revision of the ongoing clinical trials related to SARS-CoV-2 was performed using the database of the National Institutes of Health (NIH) of the United States. The data were accessed from the NIH webserver ClinicalTrials.gov. The search terms were “COVID” or “SARS-CoV-2,” resulting in 3,009 trials. Then, the search parameter drug was applied as a further term, retrieving 1,616 clinical trials. In each search, the trials included those not yet recruiting, recruiting, active, or completed. A visual inspection of each of the 1,616 trials was conducted and only 1,265 included drugs as the main intervention. Moreover, the drug combination trials were also reviewed, and only 113 included a combination of pharmacological therapy for the treatment of COVID-19 patients. The database search was conducted on August 14, 2020. COVID-19, coronavirus disease 2019; HCO, hydroxychloroquine; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

binations. One-third of them include HCQ under combination therapy (Fig. 2). Table 1 summarizes the ongoing clinical trials combining drugs targeting early viral replication events or viral enzymes.

## Conclusions

Altogether, the virology and pharmacological principles discussed in this review emphasize the importance of early stages of the infection as therapeutic targets and the advantage of simultaneously blocking alternative processes critical for viral replication. The development of effective therapies against this new viral pathogen has proven to be a challenge. Useful in vitro and in vivo models are needed to address the effectiveness of drugs or their combination to be used as preclinical data, followed by randomized clinical trials. Prior studies with SARS-CoV might help to develop and validate models for this new infection. More studies will help to fully understand the possible advantages of drug combinations against SARS-CoV-2 infection. Based on the experience obtained with the treatment of other viral infections, the combination of two or more antiviral drugs could produce a better effect than the one reached with a single drug. These antiviral effects could be additive or synergistic, depending on the degree of inhibition of viral load. Furthermore, besides the improvement in the antiviral effect achieved,

an eventual reduction of the dosage used could represent fewer side effects associated with the therapy. Thus, enhancing the antiviral effect and reducing side effects could represent a main change in SARS-CoV-2 therapy. There are at present many concerns because of the toxicity of chloroquine or its derivative [24]. Drug combination might help to reduce the dose or the time of application of this mixed therapy. Nasal administration is also a promising alternative that might overcome both toxicity effects and effective concentration challenges [63].

## Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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## Author Contributions

F.H. Pujol, J.T. Ortega, and H.R. Rangel visualized and designed the manuscript. F.H. Pujol and J.T. Ortega wrote the manuscript. J.L. Zambrano performed imaging and art creation. F. Liprandi and B. Jastrzebska were responsible for data curation and revision. All authors read and contributed to the final version of the manuscript.

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