**Frontiers Medicine**

**February 3, 2021**

**Title**: **Cyclosporin A: a repurposable drug in the treatment of COVID-19 ?**

**Running title**: Cyclosporin A and COVID-19

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Abstract length: 190 words; Manuscript length:,7817 words

Figures: 7

Table 4

**Keywords:** SARS-CoV-2; COVID-19; Cyclosporin A; Cyclophilin; ACE2

**Summary:**

COVID-19 is now at the forefront of major health challenge faced globally, creating an urgent need for safe and efficient therapeutic strategies. Given the high attrition rates, high costs and quite slow development of drug discovery, repurposing of known FDA-approved molecules is increasingly becoming an attractive issue in order to quickly find molecules capable of preventing and/or curing COVID-19 patients. Cyclosporin A (CsA), a common anti-rejection drug widely used in transplantation, has recently been shown to exhibit substantial anti-SARS-CoV-2 antiviral activity and anti-COVID-19 effect. Here we review the molecular mechanisms of action of CsA in order to highlight why this molecule seems to be an interesting candidate for the therapeutic management of COVID-19 patients. We conclude that CsA could have at least three major targets in COVID-19 patients: i) an anti-inflammatory effect reducing the production of pro-inflammatory cytokines; ii) an antiviral effect preventing the formation of the viral RNA synthesis complex; and, iii) an effect on tissue damage and thrombosis by acting against the deleterious action of angiotensin II. Several preliminary CsA clinical trials performed on COVID-19 patients report encouraging data and suggest that this strategy should be investigated further.

## Introduction

The first outbreak of Coronavirus disease 2019 (COVID-19) was reported by China at the end of 2019 (Zhu et al., 2020; Huang et al., 2020; Frutos et al., 2020). Evidence was rapidly reported that the patients were infected by a novel Betacoronavirus lineage 2b/Sarbecovirus tentatively named 2019 novel coronavirus (2019-nCoV) before being known as SARS-CoV-2 with respect to its phylogenetic relationship (80% nucleotide identity) with the SARS-CoV (Zhou et al., 2020). To date, it is the seventh characterized coronavirus described as capable of causing a respiratory infection in human. From the start of 2020, COVID-19 has become a global pandemic and has been declared a global health emergency by the World Health Organization (WHO). In one year, more than 75 million people were infected worldwide and this virus has caused more than 1.6 million deaths (https://coronavirus.jhu.edu/map.html, 18 December, 2020). Depending on the health status, age and comorbidities (hypertension, coronay heart diseases, cerebrovascular diseases, diabetes, chronic kidney diseases) of the infected persons, SARS-CoV-2, may either be asymptomatic, give a picture of influenza infection, or induce severe forms of COVID-19 with acute respiratory distress syndrome and multiple organ failure syndrome which can lead to death in about 2,27% of infected individuals (Huang et al., 2020; Ksiazek t al., 2020, Qin et al., 2020).

The SARS-CoV-2 is an enveloped $RNA^{+}$ virus surrounded by spike (S) glycoproteins. The genomic length of SARS-CoV-2 is about 30 kb and encodes as many as 14 open-reading frames (ORFs) leading to the synthesis of 29 proteins (Wu et al., 2020; Chang et al., 2020). CoV have the largest viral RNA genomes known to date (e.g., human immunodeficiency virus genome is only 10 kb) and it was hypothesized that their expansion and selection was likely enabled by acquiring enzyme functions that counter the high error frequency of RNA polymerases (Snijder et al., 2016). During the early infection process, the trimeric SARS-CoV-2 S1 spike first binds to the N-terminal portion of the angiotensin I converting enzyme 2

(ACE2) which acts as viral receptor at the surface of susceptible cells (Yan et al., 2020). The cellular transmembrane protease serine 2 (TMPRSS2) contributes to enhance the S-protein-driven viral entry (Hoffmann et al., 2020). After cleavage at the S1/S2 junction, the S2 take the conformation required for insertion of the fusion peptide into the cellular lipid bilayers. The viral nucleocapsid is thus delivered into the cytoplasm through the endocytic vesicle. After acidification of the late endosome, the action of cathepsin enables the uncoating of the genomic RNA. SARS-CoV-2 like other pathogenic CoVs, possesses a linear plus-sense strand RNA genome (gRNA) that has a 5' methylated cap and 3' poly-A tail, allowing its anchorage to ribosomes for the synthesis of polyprotein precursor. The two-thirds of this gRNA (about 20Kb) is occupied by the ORF1a (expressed by genome translation) and ORF1ab (expressed by genome translation and ribosomal frameshift) and encodes the polyproteins precursors pp1a and pp1ab, respectively, giving rise to the production of 16 non-structural proteins (Nsps) by auto-proteolytic processing (Baruah et al., 2020). Among these Nsps, Nsp12 is an RNA-dependent RNA polymerase, Nsp3 and Nsp5 are proteinases, Nsp13 is a helicase, Nsp14 and Nsp15 are ribonucleases, and Nsp14 is a methyltransferase (involved in RNA cap formation). Regarding the other proteins, Nsp1 triggers host mRNA degradation and inhibits interferon signaling, Nsp2 modulates host survival signaling, Nsp3 acts as an interferon antagonist, Nsp4 participates to the assembly of virally-induced cytoplasmic double membrane vesicle formation, Nsp6 inhibits STAT1 nuclear translocation, among other functions while Nsp12, Nsp8, Nsp7 and Nsp13 forms a complex known as replicative machinery (Hillen et al., 2020; Wang et al., 2020) that bind the gRNA to neosynthesize different viral RNA molecules. The 3'-proximal third sequence of the gRNA serves as template for several sub-genomic mRNAs having common 3′ UTRs (Hussain et al., 2005) that encode the viral structural (the spike/S, the envelope/E, the membrane/M, and the nucleocapsid/N) and accessory proteins. The S, E, and M proteins are synthesised and

anchored on the endoplasmic reticulum (ER) with the N protein translated in the cytosol. Post-translational modifications of viral proteins occur within the endoplasmic reticulum and trans-Golgi network vesicles. After assembly in the ER-Golgi intermediate compartment (ERGIC), where the E protein plays an essential role in virus assembly and the mature M protein shapes the virus. Mature virions are released from smooth-walled vesicles by exocytosis. The accumulation of knowledge relating to the intracellular cycle of replication of the virus as well as the nature of the interactions between the viral and cellular proteins is essential to choose in the large panel of FDA-approved therapeutic compound the molecules capable of blocking the deleterious effects of this virus in infected persons or to design new antiviral drugs.

Because of the urgent need for safe and efficient therapeutic drugs able to lower morbidity and mortality of COVID-19, multiple clinical trials have been conducted including repurposing of antiviral drugs, anti-inflammatory molecules and also all kinds of low cost 'old' drugs known for their *in vitro* antiviral properties. Several independent studies reported in the literature had revealed the *in vitro* antiviral properties of cyclosporin A (CsA), a well characterized immunosuppressant largely used in the prevention of graft rejection. *In vitro,* this drug was shown to be active against different viruses and to inhibit coronaviruses replication, including that of HCoV-229E and SARS-CoV-1 (De wilde et al., 2011; Tanaka et al., 2013). (**Table I**). Unsurprisingly, when tested *in vitro* on SARS-CoV-2, CsA was also found to inhibit the replication of this new virus (Pizzorno et al., 2020). Moreover, the CsA-analog alisporivir (called Debio-025) was also shown to block SARS-CoV-2 replication *in vitro* (Ogando et al., 2020; Softic et al., 2020). The question of CsA or CsA analogs use in the treatment of COVID-19 is now more pressing.

## Discovery of cyclosporin A, a cyclophins inhibitor, and FK506, an FKBPs inhibitor

The cyclosporin story started in the 1969-70 at the Sandoz laboratories in Basel (Switzerland) The 11-amino-acid lipophilic cyclic peptide cyclosporin (CsA, also known as ciclosporin) of 1.2 kDa molecular weight produced from the fungus *Tolypocladium inflatum*, and other microorganism such as *Fusarium solani*, *Neocosmospora varinfecta* and *Aspergillus terreus* (Borel et al., 1976), was found to exhibit immunosuppressive properties offering new hope to transplant surgeons to avoid patients' transplant rejection. The CsA cyclic peptide is insoluble in water and soluble in ethanol or in olive or sesame oil at 60°C and next can be kept in solution at room temperature. The olive oil soluble form of the peptide supplemented with 12.5% ethanol was the first form of manufactured CsA for oral administration, which must be dispersed in juice or milk for ingestion (Nussenblatt and Palestine, 1986). CsA was introduced in clinical practice in 1978 (Calne et al., 1978). The bioavailability of the original corn-oil based preparation of cylosporine (Sandimmune®, Novartis Pharma) largely varied in cyclosporine blood levels among patients leading to the development of microemulsion formulation (Neoral®, Novartis Pharma) (Dun et al., 2001; Schiff et al., 2007). Usually, dose of 20 mg CsA/kg daily are recommended after solid organ transplant with progressive decrease every week down to 5 mg/kg daily while dose of 1 mg/kg daily is recommended after hematopoietic stem cell transplantation (Flores et al., 2019). Upon administration, CsA is absorbed at the intestinal level by the epithelial cells and the efficiency of this process is influenced by different factors such as dietary composition or bile flow. In the plasma, CsA is found bound to lipoproteins and spreads in the extravascular space (Kahan, 1989). CsA is metabolized by liver cells through the P450 3A4 (CYP3A4) leading to the generation of a number of metabolites (Wang et al., 2018). After a single dose of CsA, there is a peak of drug blood concentrations (Cmax) during the first 2 hours followed by elimination (C0) and the drug bioavailability should be carefully monitored in clinical settings using the Cmax and a

154 measure of concentration each 2-hours (C0, C2, C4, C6, C8) to determine when an additional
155 dose should be administered (Pedroso and Citteri, 2015).

156 The mechanism of action of CsA was elucidated in 1984 with the isolation from thymocytes
157 of cyclophilin (CyP), a 18 kDa highly basic charged cytosolic protein that binds CsA with
158 high affinity (Handschumacher et al., 1984). Next, a structurally different
159 immunosuppressant, a macrolide named FK506 isolated from *Streptomyces tsukubaensis*,
160 emerged and was found to interfere with T cell activation through a similar mode of action
161 than CsA leading to suppression of mixed lymphocyte reaction (MLR), IL-2 and IL-2
162 receptor, IL-3, and γ-interferon (Kino et al., 1987). Like CsA, FK506 binds to a member of
163 peptidylproline cis-trans isomerase acitivity (PPIase), but instead of binding cyclophilin (also
164 called rotamase) it binds the FK506-binding protein (FKBP) (Harding et al., 1989). Similarly,
165 rapamycin, another immunosuppressant synthesized by *Streptomyces hygroscopicus* (a
166 macrolid originally described in 1975 as an antifungal agent), also bind FKBP and more likely
167 the FKBP12 and FKBP52 isoforms (Liu, 1993; Kang et al., 2008). The immunosuppressive
168 effects of FK506 as well as rapamycin are considered independent of the chaperone function
169 of FKBP. When complexed with ligands, FKBP adopts a conformation allowing its binding to
170 calcineurin and the mammalian target of rapamycin (mTOR). FKBP can also bind the inositol
171 1,4,5-triphosphate receptor (IP3R) $Ca^{2+}$ channel, which is activated through phosphorylation
172 by the protein kinase A (PKA), while its inactivation is induced through dephosphorylation by
173 calcineurin (Cameron et al., 1995; Cameron et al, 1997). FKBP also binds to the ryanodine
174 receptor (RyR) chanel, and the type 1 transforming growth factor beta (TGFβ) receptor
175 (Wang et al., 1994). Both CsA, FK506 (also known as fujimycin or tacrolimus) and
176 rapamycin (or sirolimus) inhibit the phosphatase activity of calcineurin thereby preventing the
177 dephosphorylation of the nuclear factor of activated T-cells (NF-AT) that is usually induced
178 after $Ca^{2+}$ binds to calmodulin, leading to the binding of calmodulin to calcineurin, a calcium-

calmodulin-activated serine/threonine-specific posphatase, which in turn is activated (Kang et al., 2008). In a model of liver fibrosis in rats, rapamycin was reported to inhibit mTOR, to demonstrate potent antifibrotic activity and to improve portal pressure (Patsenker et al., 2011).

## Cyclophilin function

The main function of peptidylproline cis-trans isomerase, PPIases, is that of chaperone proteins involved in folding, assembly and trafficking of other proteins (Galat et al., 1993, Galat and Bouet, 1994). The human genome encodes seventeen cyclophilins, the peptidyl-prolyl isomerase A (PPIA or CyPA also called Cyp-18a a cytosolic protein of molecular mass 18 kDa) encoded by a gene located on chromosome 7, PPIB (CypB also called Cyp-22/p, an endoplasmic reticulum and golgi protein of molecular mass 22 kDa) encoded by a gene on chromosome 15, PPIC (CypC an endoplasmic reticulum and golgi protein of molecular mass 33 kDa), PPID (CypD a mitochondrial protein of molecular mass 20 kDa; the cytosolic CyPD and CyPF are named CyP40), PPIE (CypE, a component of the spliceosomal apparatus), PPIF (CypF is a component of the mitochondrial permeability transition pore involved in apoptosis regulation), PPIG (CypG or SR-cyclophin or matrix-cyclophilin is a nuclear matrix protein which interacts with RNA polymerase II is a component of the spliceosomal apparatus), PPIH (CypH), NKTR (Cypp), PPIL1 encoded by the X-chromosome, PPIL2, PPIL3, PPIL4, PPIL6, PPWD1, RANBP2, and SDCCAG-10, respectively (Wang and Heitman 2005; Davis et al., 2010). The CyPA exhibits multiple functions including folding of the procollagen I and transferrin, nuclear translocation of ERK1/2 kinases, transport of molecules to the plasma membrane through interaction with the Ig-like CD147 receptor, Cholesterol transport, nuclear export of zinc-finger protein-1, and stimulation of apoptosis (Uittenbogaard et al., 1998; Nigro et al., 2013). Although CyPA is mainly a cytosolic protein, there is also a secreted form

of this molecule, which is produced in response to different inflammatory stimuli, particularly infection (Sherry et al., 1992). The secretion of CyPA is mediated via a vesicular transport pathway that depends on the Rho kinase activation (Bukrinsky, 2015). The secreted form of CyPA acts as a chemoattractant for monocytes, and leukocytes (Sherry et al., 1992; Xu et al., 1992; Jin et al., 2004). To date, although several functions of most cyclophilin isoforms remain unknown, the different isoforms of cyclophilins exhibit domain-specific properties apart from their function as chaperones. For example, PPIA was found to bind the non-receptor tyrosine kinase Itk playing a role in the maturation of thymocytes, PPIH and PPIL1 respectively interacts with the hPRP4 and SKIP protein in the spliceosome, PPIE shows a RNA-specific isomerase activity. Beside encoding nineteen cyclophilins, the human genome encodes eighteen FK506-binding proteins (FKBPs) and a three parvulins, the smallest PPIases (Gray et al., 2015).

It was reported that CsA can bind PPIA, PPIB, PPIC, PPID, PPIE, PPIF, PPIG, PPIH, PPIL1, NKTR, PPWD1, while PPIL2, PPIL6, RANBP2, SDCCAG-10 are incompetent to ligate CsA (Davis et al., 2010). ). (**Figure 1**). Special attention was reported to the CsA/CypA interaction and quantitative transcriptomics analysis (RNA-Seq) to classify the tissue-specific expression of the CypA gene indicated that this molecules is ubiquitously expressed (Fagerberg et al., 2014).

**Recollection of CsA repurposing in AIDS' therapy**

Although the ability of CsA to block SARS-CoV-2 replication *in vitro* draw attention of clinicians for a possible repurposing of CsA in COVID-19, there is a precedent in the case of treatment of viral diseases with CsA which moderates the enthusiasm for a rapid experimentation of this drug in COVID-19. However, there is currently evidence that CsA can

be beneficial in HIV treatment when CsA is given post-primo-infection in association with HAART (**Table II**), suggesting it may also be suitable in COVID-19.

Based on the hypothesis according to which the multiplication of the human immunodeficiency virus (HIV) in the organism is all the more important as the CD4 cells are activated, 25-years ago CsA was considered as a possible drug to treat AIDS. During a press conference, the results of a preliminary CsA clinical trial carried out on AIDS patients by a medical doctors' team from the Laënnec hospital (Paris, France) in October 1985 were reported (Andrieu et al., 1986). Unfortunately, after the death of two HIV patients under CsA therapy, a campaign fueled by media tended to discredite this work (Nau and Nouchi, 1985; Dodier and Barbot, 2008). Among the critics it was emphasized that it was surprising to suggest using an immunosuppressant to treat a disease characterized by an immunosuppression (e.g., virus-induced progressive depleted of $CD4^+$ lymphocytes being at the origin of AIDS). Despite the media attacks the pilot phase was continued by the Andrieu's team who reported on the CsA treatment of eight patients who were given 7.5 mg CsA/kg daily and concluded based on their observation that clinical trials with CsA would be worth pursuing (Andrieu et al., 1988). However, adverse effects of this experimental treatment were reported by another team, which published the results of a CsA pilot study on nine patients with AIDS (six presented with *P. carinii* pneumonia and three had Kaposi's sarcoma) who experienced severe toxic symptoms, one developed massive intravascular hemolysis and was withdrawn from the study after 13 days of treatment, the other also experienced severe symptoms which necessitated discontinuation of CsA therapy in six of them and the condition of all patients improves after therapy was stopped (Phillips et al., 1989). Although the results from this last clinical studies were disappointing, another study that enrolled 53 patients with renal transplantation the HIV-infection of whom was caused by an infected transplant or by blood transfusion indicated that after 5-years, the cumulative incidence of AIDS was lower in

40 patients who received CsA than in 13 transplant patients receiving immunosuppressive treatment without CsA (Schwarz et al., 1993). Coming back to animal model to explore pathophysiology without putting patients at risk, it was shown by the Fauci's team that administration of CsA to monkeys inoculated with the simian immunodeficiency virus (SIV), was beneficial relatively to the kinetics of CD4 cells depletion (Martin et al., 1997). This result revived scientific debate on the use of CsA in the treatment of AIDS, but rather than using it as monotherapy on patients with declared AIDS (low $CD4^+$ cell count), the choice fell on use of CsA in combination with highly active antiretroviral therapy (HAART) during primary infection based on the hypothesis that rapid shutdown of T cell activation in the early phase of primary infection could have long-term beneficial effect on the outcome of the disease. Pantaleo's team reported that during a 64 weeks follow-up, patients receiving CsA in combination with HAART consistently maintained significantly higher levels of $CD4^+$ T cells than those taking HAART alone (Rizzardi et al., 2002). This promising result relaunched investigation on the use of CsA in AIDS (Vogel et al., 2004; Argyropoulos and Athanasia, 2006; Markowitz et al., 2010; Sokolskaja et al., 2010; Hawley et al., 2013). More recently, Nicolas and colleagues reported the results of a clinical investigation, which concluded that unintegrated DNA forms of viral genome increased in the CsA treated group compared to controls, suggesting an anti-integration effect of the drug (Nicolas et al., 2017) (**Figure 2**). This is consistent with earlier data demonstrating that cell activation is dispensable for viral entry but is required for the HIV-1 provirus integration (Zack et al., 1990; Bukrinsky et al., 1991; Benkirane et al., 1993). It will therefore have taken more than 30 years of research to begin to understand in which specific therapeutic conditions CsA can be beneficial in the treatment of AIDS. Altogether these results suggest that the treatment with CsA can be beneficial in the prevention of AIDS but that the window of action of this treatment is narrow,

limited to primary infection to prevent the integration of the viral genome while it is no longer efficient on the chronic infection once the provirus is integrated.

**Is there a perspective for the CsA repurposing in COVID-19?**

Immunocompromised patients, include patients with HIV, those receiving immunomodulatory therapy for autoimmune disease, patients with cancer, solid organ transplant recipients who are immunosuppressed to prevent complication associated to alloimmune responses are generally considered at risk for more severe viral infection because of their poor immune response. In transplant recipients, CsA and tacrolimus calcineurin inhibitors are the most prescribed drugs for prevention of alloimmune responses (Calne et al., 1978; Starzl et al., 1989). Therefore the question of using CsA in COVID-19 recently come into debate since it remains unclear if immunosuppression in transplanted patients alters the predisposition to acquiring COVID-19 and/or modifies the disease outcome for better or worse (Rudnicka et al., 2020). Today, solid organ transplant recipients are listed as high-risk individuals for the development of severe forms of COVID-19 (Azzi et al., 2020) and there is a specific follow up of transplanted patients to evaluate their outcome when they become infected with SARS-CoV-2. It is generally admitted that immunosuppressive therapy in transplanted patients modulates humoral and cell-mediated immunity increasing the risk of severe infection when exposed to viruses (Kaltsas and Sepkowitz, 2012). In regard to this idea, some authors suggested pausing immunosuppressants drugs as a precaution in transplanted patients found positive for SARS-CoV-2 (Romanelli et al., 2020). Yet, it was also reported that transplanted patients have not been found more susceptible to viral infections and severe forms of COVID-19 than the general population (Colombo et al., 2014; Poulsen et al., 2020; Cour et al., 2020), which begs questions about the relationship between CsA treatment and COVID-19. An observational clinical study from Spain which followed 29

kidney transplant recipients with COVID-19 reported a mortality of 12.5% in the group of patients under CsA therapy (n=23) compared to 50% mortality in the control group reduced in CsA (n=6), supporting the hypothesis that CsA therapy is safe and might be beneficial to transplanted patients with COVID-19 (Rodriguez-Cubillo et al. 2020). However, this study should be interpreted with caution due to other drugs used in these patients with differences according to the subgroups: Mycophenolate and/or mammalian target of rapamycin inhibitors (mTORi) were discontinued in all patients, hydroxychloroquine was used in all patients, two third of the patients were given high-dose steroid, one third received intraveinous immunoglobulin, one third were given an interleukin-6 (IL-6) inhibitor. Observational studies have shown that patients receiving CsA for the prevention of graft versus host (GVH) disease have a lower risk of developing a COVID-19 infection than patients receiving basic treatment with tacrolimus or corticosteroids (**Table III**). Interestingly, in a recent study including 40 kidney-transplanted patients, Demir and colleagues identified by using a multivariable analysis that the use of CsA was associated with a lower incidence of death (0.077 [95% CI, 0.018-0.324; $P \leq .001$]) (Demir et al., 2020). The question currently being raised is whether the background immunosuppressive therapy in transplanted patients should be modified, when possible, by CsA to prevent the occurrence of COVID-19 (Poulsen et al., 2020).

At least eight FDA-approved clinical trials of CsA are currently underway in patients with severe COVID-19 (**Table IV**). Recently, an open-label, non-randomized pilot clinical study on 209 adult patients confirmed positive for SARS-CoV-2 receiving enoxaparin, methylprednisolone or prednisone compared the clinical outcome of 105 patients who received CsA (oral CsA at a dose of 1-2 mg/kg daily) plus steroids to that of 104 patients treated with steroids alone and concluded that CsA used as adjuvant to steroid treatment improves outcomes of patients with moderate to severe forms of COVID-19 and reduces mortality (Galvez-Romero et al., 2020).

Altogether, these results suggest that CsA could have a beneficial effect in the treatment of COVID-19 patients and that such repurposing strategy should be further investigated while being aware of possible side effects. In addition, these data also raise questions about the mechanisms by which CsA might influence the outcome of COVID-19.

## CsA and Cyclophilin in proinflammation processes: implication forCOVID-19

Upon entering the cell, the immunosuppressants CsA and FK506 bind with high affinity to CyPs (also named immunophilins) and inhibit their peptidyl prolyl cis-trans isomerase activities. The CyP-CsA (or FKP-FK506) complex bind to calcineurin and inhibit its phosphatase activity. Many of the suppressive actions of CsA on T cells appear to be due to an inhibition of T cell receptor (TCR)-induced activation signals with minimal effects on already activated $CD8^+$ cytotoxic T cells (Shevach, 1985). Although CSA affects T cell differentiation, proliferation and cytokines production, these cells still express the interleukin-2 receptor (IL-2R) and proliferate under IL-2 stimulation (Herold et al., 1986, Granelli-Piperno, 1988). However, CsA can apparently also trigger a status on T cell-mediated autoimmunity (Prud'homme et al., 1991). CsA inhibits the development of both $CD4^+CD8^{neg}$ T-cells and $CD4^{neg}CD8^+$ T cells lineages (Jenkins et al., 1988). CsA inhibits a T cell receptor dependant calcium-dependent signal-transduction pathway and blocks T cell proliferation by inhibition of the IL-2 synthesis and this is achieved after forming a complex with CyPA. In absence of CsA, TCR-induced activation signal trigger $Ca^{2+}$ binding to calmodulin, that leads calmodulin to form a complex with calcineurin, a calcium/calmodulin-dependent serine threonine phosphatase. The activation of calcineurin triggers dephosphorylation of the cytoplasmic nuclear factor of activated T-cells (NF-ATcP). Once dephosphorylated, NF-ATc translocates from the cell cytoplasm into the cell nucleus and activates the transcription of the

IL-2 gene (Chow et al., 1999). Under CsA treatment, the CsA/CyPA complex specifically binds to calcineurin and inhibits its phosphatase function (Liu et al., 1991; Kang et al., 2007). Due to a lack of phosphatase activity, the nuclear factor of activated T cells (NFAT) remain under its inactive cytoplasmic phosphorylated form (NF-ATcP). *In vivo* studies have highlighted that CsA promote the expansion of $Foxp3^+$ T regulator cells (Treg) (Ruppert et al., 2015). Indeed, the result of CsA treatment is a change in the balance between T helper cells and Treg that favor the Treg population. The CypA is regulated by inflammatory stimuli, and several cell-types secrete CypA in response to oxidative stress. Zhang and colleagues also reported that serum CypA correlated with serum interleukin-6 (IL-6), matrix metalloproteinase-9 (MMP-9) and C-reactive protein expression (Zhang et al., 2018). It was recently reported that the secreted CypA can be used as a potential inflammatory biomarker of chronic obstructive pulmonary disease (COPD), as its expression levels are elevated in serum of COPD' patients and reflects the severity of inflammation (Zhang et al., 2018).

## Pathological similarities between transplanted patients and COVID-19 patients: tissues injuried with picture of chronic vascular rejection

In our experience, significant parallels are observed between SARS-CoV-2 tissue injury and allograft rejection and especially with chronic vascular rejection (Stewart et al., 2007; Roden and Tazelaar, 2018). In tissues of patients died from COVID-19 (**Figure 3**), similar lesions to those observed in chronic vascular rejection grade D were observed (Stewart et al., 2007). Vascular rejection is characterized by concentric thickened arteries and/or veins, due to fibrointimal connective tissue. These lesions usually starts with intimal proliferation, then fragmented and discontinuous internal elastic lamina. Concurrent endovasculitis has also been observed (Roden and Tazelaar, 2018). In patients suffering from GVH disease, lung

histological lesions are characterized by alveolar changes (intra-alveolar fibrin, organizing pneumonia, and chronic interstitial pneumonia), atypical pneumocytes, intra-epithelial bronchiolar T cells and perivenular cuffing (Yousem, 1995; Xu et al., 2013; Goker et al., 2001; Murphy, 2020).

Lung analysis of patients died from COVID-19 showed an inflammatory perivascular lymphocytes infiltration that presents some similarities to those observed in GVH (**Figure 4**), although non-specific (Deshmukh et al., 2020). Perivascular inflammation was reported to be patchy and scattered, composed mainly of lymphocytes, with thrombi in the branches of the pulmonary artery and focal areas of congestion in the alveolar septal capillaries, as well as septal capillary lesions with wall and luminal fibrin deposition (Deshmukh et al., 2020).

In these diseases, critical epithelial stem cell populations are preferentially targeted, in one instance by cytotoxic immune pathways, in the other by a viral protein-receptor interaction. Moreover, in both diseases again, severe injuries are mediated by cytokine deregulation named the « cytokine storm syndrome » which lead to cells apoptosis. Cytokine dysregulation has historically been reported in the early phase of acute GVH disease described by Ferrara as a "cytokine storm" (Ferrara et al., 1993) and subsequently used to describe the exacerbated immune response observed in severe COVID-19 infection (Mehta et al., 2020, Melenotte et al., 2020). Thus, it could explain some of the histological similarities observed, even chronic, since physiological mechanisms involved in these lesions are, in part, common. Stem cells death by apoptosis is associated with activation of the p53-p73 ‘suicide pathway’ observed in GVH disease and perivascular lymphocyte infiltrates were identified in case of GVH disease (Sostak et al., 2009, 2010; Al-Hashmi et al., 2011; Zhan et al., 2012).

## COVID-19 infection in transplanted patients

Recipients of allogeneic hematopoietic stem cell transplant (HSCT) are generally considered at particular risk of developing severe forms of COVID-19 when infected with SARS-CoV-2 due to the profound immunosuppression relates to this procedure expected to reduce the immune defense of the host thereby favoring *in vivo* viral replication. It was reported that treatment with the selective JAK1/2 inhibitor ruxolitinib has shown promising results in the context of COVID-19 patients with GVH disease (Saraceni et al., 2020). In COVID-19 the tissues injury observed in patients with severe forms of the disease appears to be related to a massive increase of inflammatory cytokines level and increase of $CD15^{+}CD16^{+}$ neutrophils known for being involved in proinflammatory processes (Li et al., 2019; Vitte et al., 2020). It is currently admitted that the severe forms of COVID-19 are associated with a release of cytokines and chemokines such as IL-2, IL-6, IL-7, IL-10, tumor necrosis factor (TNF), and granulocyte colony-stimulating factor (GCSF) (Huang et al., 2020; Tay et al., 2020).

Among these cytokines therapeutic approaches targeting excessive inflammation caused by IL-6 interaction with its cellular receptor IL-6R have been under investigation using IL-6 antagonists such as tocilizumab and sarilumab used in the treatment of autoimmunity (Hojyo et al., 2020; de Caceres et al., 2020; Tsai et al. 2020; Gremese et al., 2020). It was recently shown that the total number of $CD4^{+}$ T cells, CD8+ T cells, B cells, and NK cells in patients was markedly decreased in the most severe forms of COVID-19 and that there is an increase of IL-2, IL-6, IL-10 and IFN-γ (Zheng et al., 2020; Luo et al., 2020; Liu et al., 2020)(Melenotte,OncoImmunology,2020). There is likely space for investigating the possible beneficial effect of immunosuppressant CsA therapy in COVID-19, since this molecule is known to reduce the IL-2 production that contribute to the cytokine storm reported in the severe forms of COVID-19 (**Figure 5**). It is also worth noting that the Nsp1 protein found to have multiple functions (e.g., binds to 40S ribosomal subunit and inhibit translation; triggers

host mRNA degradation by endonucleolytic cleavage; induces cell cycle arrest; inhibits IFN signaling) was reported in SARS-CoV to enhance IL-2 production when overexpressed and that SARS-CoV infection increase signaling through the Calcineurin/ NFAT (Pfefferle et al., 2011). Such Nsp1 induction of IL-2 production is probably also occurring with SARS-CoV-2.

**CsA and Cyclophilin in viral infectious processes: implication for COVID-19**

Different isoforms of cyclophilins CyPA and CypB were reported to specifically bind a proline-containing sequence in the polyprotein $Pr55^{gag}$ and the $p24^{gag}$ capsid protein of the human immunodeficiency virus type 1 (HIV-1) and CsA disrupts the interaction of these proteins with CyPA and also with CyPB although with less efficiency (Luban et al., 1993). *In vitro*, CsA was reported to inhibit the replication of HIV-1 (Briggs et al., 1999). The nonimmunosuppressant analogue of CsA, SDZ NIM 811 (Sandoz), was also found to inhibit HIV-1 *in vitro* (Steinkasserer et al., 1995)

Beside HIV-1, CsA was reported to inhibit the vesicular stomatitis virus (Bose et al., 2003), the hepatitis C virus (HCV) (Watashi et al., 2003; Nakagawa et al., 2004), the human papillomavirus (HPV)-16 (Bienkowska-Haba et al., 2009), the influenza A virus (Liu et al., 2009), the Rift valley fever virus (Ianevski et al., 2018). Regarding the HCV, the RNA-dependent RNA polymerase NS5B from the virus binds the human CypA and CypB proteins (Watashi et al., 2005; Chatterji et al., 2009) and CypA was also found to interact with the NS2 protein of HCV (Ciesek et al., 2009) while CypB appeared to regulate with the HCV polymerase and CyP40 seems to also be involved in HCV replication (Goto et al., 2009). First a 3.5 log reduction of HCV load was demonstrated with the CsA analog DEBIO-025 (Flisiak et al., 2008). In light of these results, clinical trials of Cyp inhibitors (DEBIO-025, SCY635, and NIM811) have started against HCV and a very elegant *in vitro* work evidenced that

NIM811 reduces HCV replication by inhibiting CyPs, including CyPA, CypH and CyPE and identified many cellular compounds interacting with these CyPs (Gaither et al., 2010).

Similarly, in flaviviruses, it was reported that CsA blocks West Nile virus, Dengue -2 virus and Yellow Fever virus replication. CsA was found to inhibit the interaction between CypA and the NS5 protein (and also CyPA and viral RNA) of the West Nile Virus (Qing et al., 2009), while CyPB was found to interact with the NS4A protein of the Japanese encephalitis virus (Kambara et al., 2011) suggesting that CyP isoforms are essential to the replication complex of flaviviruses.

Regarding coronaviruses, it was reported that CsA inhibits the human coronavirus HCoV-NL63, HCoV-229, and SARS-CoV-1 as well as animal coronaviruses such as feline CoV and porcine CoV, suggesting that CyPs are required for successful replication of most coronaviruses (Pfefferle et al., 2011). Once inside cell, the genomic RNA (positive) from each coronavirus is released from the viral particle present in late endosomes. Covered with a cap allowing its anchorage to the ribosome level, this genomic RNA serves as template for the translation of two large open reading frames (ORF1a and ORF1b). This yields to the synthesis of the polyprotein 1a (pp1a) and following a -1 ribosomal frameshift it leads to the extended pp1ab polyprotein. After proteolysis, several non structural proteins (Nsp) are produced including a RNA-dependent RNA polymerase which interacts with other Nsp compounds to form, together with host protein including CyP proteins, the endoplasmic-reticulum-derived double- membrane-associated replication transcription complex required for the synthesis of all viral molecules which enter in the composition of de novo viral particles (Pedersen et al., 1999; Hagemeijer et al., 2012; Van Hemert et al., 2008). The antiviral properties of CsA against HCoV-229E and SARS-CoV-1 were confirmed in an independent *in vitro* work which conclude that CsA strongly affect replication of coronavirus HCoV-229E and SARS-CoV-1 rendering RNA and protein synthesis almost undetectable (de Wilde et al., 2011). It was also

reported that CyPA interacts with the SARS-CoV-1 nucleocapsid (N) protein (Luo et al., 2004; Chen et al., 2005). A genome-wide SARS-CoV-1 screening of viral proteins interacting with cellular compounds (human cDNA libraries) performed using the yeast two hybrid strategy revealed that the Nsp1 protein of SARS-CoV-1 binds FKBPs (Pfefferle et al., 2011). It was also reported that FK506 inhibits the replication HCoV-NL63, HCoV-229, and SARS-CoV-1 and that the inhibition of HCoV-NL63 replication by FK506 occurs through inhibition of the FKBP1A/B, suggesting that both FKBPs and CyPs families of PPIases are involved in coronaviruses replication (Carbajo-Lozoya et al., 2012). It is worth noting that both siRNA-mediated CyPA depletion and shRNA-mediated CyPA depletion so far failed to trigger reduction of SARS-CoV-1 replication, suggesting either that SARS-CoV-1 transcription mainly involves FKBPs and/or CyP other than CyPA or that the residual CyPA present in cells after treatment was sufficient to achieve the building of the replication complex (de Wilde et al., 2011; de Wilde et al., 2018). CsA was also reported to inhibit the replication of MERS-CoV, a result which was more drastic when CsA was combined with interferon (IFN)-α (Li et al., 2018). It was reported that CsA upregulates the interferon regulatory factor 1 (IRF1) signaling pathway and that inhibition of IRF1 allows viral replication despite the presence of CsA. The SARS-CoV-1 virulence factor Nsp1 antagonize the IFN immune response (Wathelet et al., 2007; Zust et al, 2007).

During the replication cycle of SARS-CoV-2, the RNA-dependent RNA polymerase (RdRp) required for the replication of the virus is active within a complex that assemble several non-structural protein of the virus including Nsp12, Nsp8, and Nsp7 as well as cellular proteins likely including members of the CyP protein family. Within this replicative machinery (that is a target for the FDA-approved triphosphate metabolite Remdesivir), the active site cleft of nsp12 (RdRp) binds to the first turn of gRNA template , while nsp8 is involved in the formation of sliding poles regulating the processivity of the RdRp (Hillen et al., 2020; Wang

et al., 2020). The Nsp12 needs to associate with Nsp8 and Nsp7 to activate is capability to replicate long RNA. The nsp13 helicase is also present in the SARS-CoV-2 replication complex and facilitate the RdRp function (Yan et al., 2020). Recently, the antiviral activity of CsA was evaluated *in vitro* on Vero E6 cells infected by SARS-CoV-2 and treated 1 hour post infection with serial drug dilutions and it was reported an anti-SARS-CoV-2 at 50% effective concentration ($EC_{50}$) of 3.5 μM to be compared to 1.5 μM for chloroquine and 5.2 μM for lopinavir (Pizzorno et al., 2020). Interestingly, the non-immunosuppressive CsA-derivatives Alisporivir (Debio025) previously reported to inhibit the in vitro replication of the human coronavirus HCoV-NL63 (Carbajo-Lozoya et al., 2014), was assayed for SARS-CoV-2 inhibition on Vero E6 cells infected for 3 hours at a MOI of 0.05 and was found to reduce SARS-CoV-2 production in a dose-dependent manner, with an EC50 of 0.46 μM (Softic et al., 2020). These results suggest that CsA inhibits the viral replicative machinery likely though interaction with a member of the CyP family. Although CyPA depletion so far failed to trigger reduction of SARS-CoV-1 replication (see above) a function for CyPA in SARS-CoV-2 replication cannot be excluded. It was also previously reported that the transmembrane glycoprotein CD147 (also known as extracellular matrix metalloproteinase inducer EMMPRIN) is facilitating viral replication by interacting with the N protein of SARS-CoV-1 through CyPA (Liu et al., 2020). CD147 was also reported to bind extracellular CyPB and to stimulates T-lymphocytes (Allain et al., 2002). In COVID-19 patients the anti-CD147 antibody Meplazumab was claimed to improve patients' recovery, suggesting a role for the CyPA/CD147 complex in SARS-CoV-2 replication similar to that previously described for SARS-CoV-1 (Bian et al., 2020). Finally, in their very elegant work, Gordon and colleagues set up a SARS-CoV-2 protein interactome map which identified 332 high-confidence protein interactions between SARS-CoV-2 proteins and human cellular compounds. This study revealed that the nsp2 protein of SARS-CoV-2 interacts with FKBP15, and that the ORF8 of

SARS-CoV-2 interacts with FKBP7 and FKBP10 (Gordon et al., 2020). Altogether, these results suggest that CsA acts at different levels in infected cells to prevent the SARS-CoV-2 replication cycle (**Figure 6**).

## CsA and Cyclophilin in the renin angiotensin system (RAS) pathway: implication for COVID-19

More than two decade ago, it was shown that the formation of abdominal aortic aneurysm in the rat model of elastase infusion was attenuated by CsA treatment (Dobrin et al., 1996). CyPA is known to promote atherosclerosis through stimulation of low-density lipoproteins uptake, decrease of endothelial nitric oxide synthase (eNOS) expression, increase of vascular cell adhesion molecule 1 (VCAM-1), and induction of tumor necrosis factor alpha (TNFα) (Nigro et al., 2011). It was reported that deletion of CyPA in mice prevents the formation of abdominal aortic aneurysm in response to infusion of angiotensin II (Ang II) (Satoh et al., 2009).

Although CyPA is an intracellular molecule, it can be secreted from macrophages in response to inflammatory stimuli acting as a chemoattractant of monocytes (Sherry et al., 1992) and it is also secreted by endothelial cells and vascular smooth muscle (VSM) cells, stimulates proinflammatory signals thereby contributing to cardiovascular diseases (Jin et al., 2000; Suzuki et al., 2006). Extracellular CyPA triggers IκBα phosphorylation that activates the nuclear translocation of NF-κB into the cell nucleus stimulating the transcription of vascular cell adhesion molecule 1 (VCAM-1) and E-selectin (Jin et al., 2004). Indeed, CypA secretion is regulated by Rho-kinase and behave as a secreted oxidative-stress molecule contributing to the pathogenesis of arteriosclerosis, hypertension and heart failure and inhibition of Rho-kinase by fasudil reduces the angiotensin II-induced aortic aneurysm formation (Wang et al.,

2005; Satoh, 2015). Reactive oxygen species (ROS) were found to contribute to the pathogeneis of artheriosclerosis through induction of extracellular signal regulated kinases ERK1/2 and p38 MAP kianse signaling which stimulated VSM cells growth (Rao et al. 1992; Baas et al., 1995; Taniyama et al., 2004). ROS-induced VSM cells growth and proinflammatory signal have been implicated in the revascularization of obstructive coronary artery disease and the pathogenesis of neointima following vascular injury (Satoh et al., 2010). Serum levels of CyPA were found elevated in coronary artery disease (Ramachandran et al., 2014; McClements et al., 2016; Alfonso et al., 2019). CypA secreted from blood vessels and heart cells regulates signal pathways and causes a decline of diastolic and systolic function leading to proliferation of cardiac fibroblasts, the occurrence of cardiac hypertrophy and remodeling (Cao et al., 2019).

Taniyama and colleagues reported that Ang II activates p38 MAPK inducing an Akt signaling pathway that results in VSM cells activation and suggested that the ROS-sensitive 3-phosphoinositide-dependent proteine kinase 1 (PDK1) phosphorylates Akt and that a parallel pathway that requires NADPH oxidase (NOX)-dependent production of ROS (including superoxide anions $O_2^-$, hydrogen peroxide $H_2O_2$ and hydroxyl radical OH) triggers p38 MAPK activation that in turn activates Akt (Taniyama et al., 2004). CyPA was also found to be involved in the translocation of NOX enzymes and the two molecules synergizes to increase ROS production (Soe et al., 2013). Finally, it was also reported that Ang II trigger the release of CyPA and the activation of metalloproeinase 2 (MMP-2) in VSM cells derived from human abdominal aortic aneurysm (Nigro et al., 2013). AngII type 1 receptor (AT1R) blockers have been shown to prevent cardiovascular diseases (Cassis et al., 2007). During treatment with simvastatin (a member of the statin family which inhibits the hydroxymethylglutaryl CoA reductase), patients with abdominal aortic aneurysm were found to have reduced CypA mRNA expression as well as reduced CyPA intracellular protein levels

571 (Piechota-Polanczyk et al., 2013). Interestingly, in a mice model, the deletion of CyPA gene
572 prevented the formation of abdominal aortic aneurysm usually observed in response to
573 infusion of Ang II (Satoh et al., 2009).

574 In SARS-CoV-2 infected individuals, the host angiotensin-converting enzyme A (ACE2)
575 monocarboxypeptidase serves as cell-surface receptor for the virus which interacts with ACE2
576 by the receptor binding domain present in its spike (S) protein (reviewed in Devaux et al.,
577 2020b). We have recently found evidence that SARS-CoV-2 infected cells have a down
578 regulation of ACE2 mRNA expression and a reduced cell surface expression of ACE2, and
579 that COVID-19 patients have decreased soluble ACE2 and increased levels of AngII in their
580 plasma (Submitted for publication). Beside a vasoconstrictor and thrombotic effects of AngII,
581 the dysregulation of the renin-angiotensin pathway with the massive AngII accumulation is
582 likely to promote the production of proinflammatory cytokine via AT1R interaction, by
583 activating the metalloprotease 17 (ADAM-17) which can process the membrane anchored
584 TNFα to a soluble TNFα which acts as an activator of NF-KB and, IL-6Rα to a soluble forms
585 sIL6Rα which can form complex with IL-6 and activates a STAT3 signaling pathway
586 (Eguchi et al, 2018, Hirano and Murakami, 2020). Since Ang II triggers the release of
587 extracellular CyPA through regulation of Rho-kinase and that extracellular CyPA behave as a
588 secreted oxidative-stress molecule triggering the activation of the NF-κB that stimulate the
589 transcription of vascular cell adhesion molecule 1 (VCAM-1), E-selectin and overexpression
590 of TNFα, the inhibition of CyPA with CsA in COVID-19 patients could reduce
591 atherosclerosis, hypertension and heart failure. Interestingly, the treatment of COVID-19
592 patients with a recombinant soluble human ACE2 (hrsACE2 from Apeiron Biologics) which
593 can interfere with virus binding but also with AngII reduced SARS-CoV-2 load, and induced
594 a massive decrease of AngII levels, IL-6 and TNF in patients and showed strong benefit for
595 the outcome of the patients (Zoufaly et al., 2020) (**Figure 7**).

## Conclusion

The emergence of the COVID-19 pandemic about one year ago has stressed healthcare systems worlwide and beside improving patients' care as knowledge of disease improves, there was a global race to identify as fast as possible effective drugs to treat SARS-CoV-2 infected patients while waiting to be able to protect individuals with an effective vaccine (Gautret el al., 2020). Since no antiviral was specifically developed against this new coronavirus, the number of clinical trials of molecules expects to interfere with the viral replication cycle or to modulate the immune response has been greater than ever. In this emergency context, the fastest strategy that has been followed by the majority of healthcare teams has been the repositioning of molecules already approved by the US Food and Drugs Administration. Among other molecules, there is ample evidence that CsA may represent a molecule to be tested further in its repurposing therapeutic strategy to treat patients with severe forms of COVID-19. This molecule is widely available, it is FDA-approved, it is affordable, it prevents pro-inflammatory processes, it blocks SARS-CoV-2 replication, and it interferes with angiotensin II harmful effects.

Therapeutic doses of CsA are usually in the range of 10 to 20 mg/kg daily when given orally. A wide variability in CsA pharmacokinetics has been observed after the oral or intraveinous administration of this drug to patients and varies with respect to organ grafted, age of patient and patient health status. CsA is absorbed in the gastrointestinal tract and almost completely metabolized in both the liver and small intestine by cytochrome P450 family 3 (CYP3A). CsA is also given as intravenous infusion using 2.5 to 5 mg/kg daily. CsA bioavailability in patients range from 5% to 90%. The CsA concentration required to inhibit virus replication exceeds the serum concentration of the drug that are usually well below 200ng/mL (Ptachcinski et al., 1986). A major challenge is to obtain appropriate concentration of CsA in

infected tissues, which will likely require 3-6 fold higher doses than those usually given to the patients, which will strongly increase the risks of toxic effects (Poulsen et al., 2020). Given the variety of side effects of CsA, a careful evaluation of cost/benefit should be done before considering this molecule in COVID-19 treatment. Nephrotoxicity is the most common adverse effect of CsA treatment and is frequently associated with arterial hypertension (Palestine et al., 1984; Olivari et al., 1989; Meyer-Lehnert et al., 1993). This could be a problem as many patients with mild or severe forms of COVID-19 have high blood pressure. In addition, several animal studies have highlighted a vasoconstrictor effect of CsA (Lamb et al., 1987; Zimmerhackl et al., 1990; Perico et al., 1990). Moreover, many drug including amphotericin B, aminoglycoside antibiotics and co-trimoxazole are at risk to potentiate the nephrotoxicity of CsA (Ptachcinski et al., 1986). Indeed there is a long list of drugs that have proven or suspected to clinically interact with CsA (Aronson, 2016) such as anticonvulsants (carbamazepine, phenobarbital, phenytoin, primidone) that reduce CsA blood concentration, antidepressants (fluvoxamine, Nefazodone), antimicrobial and antifungal drugs (ketoconazole, fluconazole, itraconazole, metronidazole, fluoroquinolones, macrolides, clarithromycin, erythromycin), antiviral drugs (ritonavir, saquinavir), cardiovascular drugs (amiodarone, calcium channel blockers, amlodipine, nicardipine, verapamil, carvedilol), hypoglycemic drugs (glibenclamide, glipizide) among others. This list also includes chloroquine, and glucocorticoids, which are sometime used in COVID-19 therapy. The adverse effects of CsA treatment include nephrotoxicity (risk increased by ACE inhibitors among many other drugs), hypertension, hyperkaliemia (risk increased by potassium salts), hyperlipidemia, hypomagnesemia, neurotoxicity (risk increased by imipenem), hepatotoxicity (risk increased by androgens) post transplant diabetes, gingival hyperplasia (risk increased by nifedipine), hirsutism.

The data in the literature are clear regarding the effects of CsA on *in vitro* SARS-CoV-2 replication, but these are not the only possible beneficial effects one would expect from CsA experimental use in treatment of COVID-19 since it can modulate both pro-inflammatory responses and the RAS pathaway. Moreover, as summarized in Table III, several preliminary CsA clinical trials performed on COVID-19 patients are encouraging and suggest that this strategy should be pursued further. In this review we describe at least three possible mechanisms for which it can be postulated that they are likely to produce a favorable effect on the outcome of COVID-19 patients: i) an anti-inflammatory effect reducing the production of pro-inflammatory cytokines; ii) an antiviral effect preventing the formation of the viral RNA synthesis complex; and, iii) an effect on tissue damage and thrombosis by acting against the deleterious action of angiotensin II. Even if CsA has many effects that are likely to improve the outcome of patients infected with SARS-CoV-2, one can of course wonder about the consequence of using a therapeutic drug that exhibits immunosuppressive effects in severe forms of COVID-19 because this could reduce the innate and adaptive immune responses of the patients against the virus. However, there is an increasing panel of available cyclophilin inhibitors such as Alisporivir/ Debio-025 (Novartis), Debio-064 (Novartis), SDZ NIM811 (Sandoz, Novartis), SCY-635 (Scynexis Inc), STG-175 (S &T Global), CRV431 (Hepion Pharmaceuticals) or CPI-431-32 (Ciclofilin Pharmaceuticals Inc.), and it is still possible to replace CsA by one of these compounds or compare these molecules in clinical trials. Finally, it will be very important to decide when CsA should be admistratred to SARS-CoV-2 infected patients to obtain the the most beneficial effects.

## Acknowledgment

The figures were designed using predefined graphic elements from the Servier Medical Art supply of images available under a Creative Commons CC BY 3.0 license. We thank the Cookie Trad company for English editing. This article is dedicated to Prof. Philippe Even.

## Funding

This work was supported by the French Government under the « Investissements d'avenir » (Investments for the Future) programme managed by the Agence Nationale de la Recherche (French ANR: National Agency for Research), (reference: Méditerranée Infection 10-IAHU-03), the Région Provence Alpes Côte d'Azur and European funding FEDER PRIMI

## Authorship

CAD, CM, MDP and DR contributed to conceived the manuscript. CM designed the tables and CAD designed the figures. CD provided the histological data. CAD wrote the paper. DR obtained the funding for this study. All authors reviewed and approved the final version of the manuscript

## Competing Interests

CAD declares a link of interest with the Sanofi and Merck pharmaceutical companies. The other authors declare that they have no competing interests.

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**Table 1. In vitro activity of cyclosporine A against viruses**

| Virus | Cyclophilin inhibitor | Read out | Dose of action | Effect | Reference |
|---|---|---|---|---|---|
| SARS-CoV-2 | Cyclosporine A | Vero E6 cells model of SARS-CoV-2 infection | $IC_{50}$ : 3 µM | Reduce viral production | Pizzorno et al., 2020 |
| SARS-CoV-2 | Debio-025 | Vero E6 cells | 0.46 ± 0.04 µM | Reduced SARS-CoV-2 RNA production in a dose-dependent manner | Softic et al., 2020 |
| SARS-CoV-2 | Debio-025 | Vero E6 cells | 4.3 µM | Reduced SARS-CoV-2 progeny virions production | Ogando et al., 2020 |
| SARS-CoV-1 | Cyclosporine A | Vero E6 cells and 293/ACE2 cells. | 16 µM | Reduced viral replication and reporter gene expression of SARS-CoV–GFP: inhibition of SARS-CoV RNA synthesis; the protein synthesis was almost undetectable | De Wilde et al., 2011 |
| SARS-CoV-1 | Debio-025 | Vero E6 cells | 4.3 µM | Reduced SARS-CoV progeny virions production | Ogando et al., 2020 |
| SARS-CoV-1 | FK506 | VeroFM cells | $EC_{50}$ : 6.9 µM | Decreased viral infection and inhibition of SARS-CoV-1 replication | Carbajo-Lozoya et al., 2012 |
| HCoV- 229E | Cyclosporine A | Huh7 cells | 32 µM | Reduced reporter gene expression and the production of infectious progeny were also significantly decreased | De Wilde et al., 2011 |
| HCoV-229E | FK506 | HuH7 cells | $EC_{50}$ : 5.4 µM | Decreased viral infection and inhibition of HCoV-229E replication | Carbajo-Lozoya et al., 2012 |
| HCoV-NL63 | FK506 | CaCo2 cells | $EC_{50}$ of about 13.4 M | Decrease viral infection and inhibition of HcoV-NL63replication | Carbajo-Lozoya et al., 2012 |
| Human immunodeficiency virus type 1 (HIV-1) | Cyclosporine A | human $CD4^+$ T cells<br>Jurkat target cells | 2.5 µM<br>2.5 µM | Reduced viral infectivity | Sokolskaja et al., 2004 |
| HIV-1 | Cyclosporine A | Jurkat T cells | 10 µM | Decreases $gp120^{env}$ and $gp41^{env}$ incorporation into HIV-1 virions and impaired fusion of these virions with susceptible target cells | Sokolskaja et al., 2010 |
| HIV-1 ($HIV\text{-}1_{NL4\text{-}3}$) | Cyclosporine A | HIV Rev-dependent indicator cell line and Peripheral blood mononuclear cells (PBMCs) | All dosage s from 100 to 600 nM | Inhibits HIV-1 replication (including subtherapeutic concentrations) | Hawley et al., 2013 |
| HIV-1 | SDZ NIM 811 | MT4 cell line (human T-cell leukemia virus-transformed T4 cell line) | $IC_{50}$ : 0.084 g/ml | Inhibits HIV-1 replication | Mlynar et al., 1997 |
| HIV-1 | STG-175 | peripheral blood mononuclear cells (PBMCs) | 0.5 and 5 µM | Inhibits HIV-1 replication | Gallay et al., 2016 |
| HIV-1 ($HIV\text{-}1_{LAI}$) | FK506-modified HIV-protease inhibitor | T cells | $IC_{50}$ of 4.2 nM | The FK506-modified HIV-protease inhibitor retains anti-HIV-1 protease Activity *in vitro* and is partitioned into the cellular component of whole blood via binding to FKBP | Marinec et al., 2009 |
| HIV-1 | Cyclophilin Inhibitor CPI-431-32 | blood-derived $CD4^+$ T-lymphocytes | 2 µM | Inhibits HIV-1 replication | Gallay et al., 2015 |
| Hepatitis B virus (HBV) | Cyclosporine A | HepaRG; HepAD38; primary human hepatocytes | 4 µM | Inhibits HBV entry into cultured hepatocytes (Inhibits the transporter activity of sodium taurocholate cotransporting polypeptide, NTCP) | Watashi et al., 2014 |
| HBV | STG-175 | Human hepatoma Huh7.5.1 cells | 0.5 and 5 µM | Decreased HBV replication | Gallay et al., 2016 |
| Hepatitis C virus (HCV) | Cyclosporine A | Huh 5-2 cells | $EC_{50}$ : 2.8 ± 0.4 µg/mL | Inhibition of HCV subgenomic replicons | Paeshuyse et al., 2006 |
| HCV | Debio-025 in combination with other antiviral | hepatoma cells | 0.1 or 0.5 µM | Antiviral activity in short-term antiviral assays | Ogando et al., 2020 |

**Table II: In vitro effect of CsA on HIV replication and on disease progression in HIV-infected patients**

| Date | Type of study | Results | Reference |
|---|---|---|---|
| In vitro | | | |
| 1988 | HIV in vitro infection and replication<br>H-9 T-cell leukemic line<br>human peripheral blood-derived lymphocytes | Pretreatment of cells and human lymphocytes with CsA over 24 hours prevented viral infection over a 21-day period, whereas the addition of drug at two hours postinfection with HIV-1 had a significant inhibitory effect on viral replication and expression of the virus-specific antigens p17 and $p24^{gag}$ | Wainberg et al., 1988 |
| 1992 | HIV and CD4 T cells | CsA induced a 100 fold reduction in the yield of HIV infection<br>CsA inhibited the growth of HIV infected cells | Karpas et al., 1992 |
| 1994 | HIV T4 lymphoid cell lines, in a monocytic cell line, and in HeLa T4 cells | SDZ NIM 811 selectively inhibited HIV-1 replication in CD4+ lymphoid cell lines, in a monocytic cell line, and in HeLa T4 cells | Rosenwirth et al., 1994 |
| 2010 | HIV and Human CD4+-T cells | CsA inhibited HIV infectivity | Sokolskaja et al., 2010 |
| 2013 | HIV and T cell line or peripheral blood mononuclear cells | CsA inhibited HIV-1 replication in a GFP indicator T cell line and peripheral blood mononuclear cells | Hawley et al., 2013 |
| In patients | | | |
| 1978 | Transplanted patients (n=7) | CsA was effective in inhibiting rejection (adverse effect : nephrotoxicity and hepatotoxicity.) | Calne et al., 1978 |
| 1988 | AIDS patients (n=8) | CsA (7.5 mg/kg daily)<br>Sustained and increased> 600 CD4+ cells/mm3, decreased CD8+ cell count. Lymphadenopathy disappeared.<br>Reversibility once CsA was stopped | Andrieu et al., 1988 |
| 1989 | AIDS patients (n=8) | Severe toxic syndrome requiring discontinuation of CsA | Phillips et al., 1989 |
| | | Decreased lymphocyte count, CD4+ and CD8+ T- cells, and no resolution of symptoms | |
| 1993 | Transplanted kidney patients & HIV-1(n=53) | 5-year cumulative risk of AIDS: 31% in CsA group versus 90% in non CsA group, P = 0.001 | Schwarz et al., 1993 |
| 2002 | 9 early HIV patients treated HAART + CsA | Significantly higher CD4+ T cells in patients treated with CsA | Rizzardi et al., 2002 |
| 2004 | 3 HIV patients treated HAART + CsA | Pharmacological adjustment of CsA in association with HAART | Vogel et al., 2004 |
| 2010 | 54 early HIV (ART + CsA vs ART) | No apparent immunological and virological benefit | Markowitz et al., 2010 |
| 2017 | 20 early HIV (ART+CsA vs ART) | increased non-integrated DNA in the CsA arm between weeks 0 and 36 weeks<br>CsA has unintegrated effect | Nicolas et al., 2017 |

Table III. Cyclosporin A based treatment in transplanted patients

| N° of transplanted patients | Cyclosporine A | Corticoids | Intensive Care Unit (ICU) | Death | reference |
|---|---|---|---|---|---|
| **HEART** | | | | | |
| 6 transplanted patients | 6/6 patients received cyclosporine A (70-200 mg/d) | NA | 2/6 patients admitted inICU (2 days and 16 days) | 2 died : 1 with acute respiratory distress syndrome. 1 with sepsis. Their cyclosporine A therapy was reduced in both cases (100% and 40%, respectively) | Caraffa et al., 2020 |
| **KIDNEY TRANSPLANTATION** | | | | | |
| 2 patients | 1 patients | NA | 1 patient not treated with cyclosporine A | 1 patient not treated with cyclosporine A | Wei et al., 2020 |
| 40 patients | 5 patients (12%) | 40 (100%) | SEVERITY<br>Cyclosporine A associated reduction risk of mortality multivariate analysis<br>OR: 0,077 (IC0,018-0,32) p<0,001 | | Demir et al., 2020 |
| 19/2493 kidney transplant recipient | 9/19 patients (47,4%) | NA | NA | 2 patients (22%) died in the cyclosporin A treated group vs 7 patients alive (70%) p=0,03 | Rahbar et al., 2020 |
| 23 patients | 6 patients already treated with cyclosporine A<br>19 patients switched to cyclosporine A therapy | NA | NA | Mortality was higher in the immunosuppression minimization strategy group, 3/6 patients (50%), as compared to the cyclosporine A strategy group 3/23 patients (13%) | Rodriguez-Cubillo et al., 2020 |
| **LIVER TRANSPLANTATION** | | | | | |
| 151 reports SARS CoV 2 with liver transplantation | 8 patients | 67 (44%) | NA | 4/28 died patients received cyclosporine A *versus* 4/123 alive patients<br>(non significative) | Webb et al., 2020 |

**Table IV. FDA approved clinical trial proposing cyclosporine A to treat SARS-CoV-2 infection.**

| | Clinical trial | Study title | Intervention | countries |
|---|---|---|---|---|
| 1 | NCT04412785 | Cyclosporine in Patients With Moderate COVID-19 | Phase 1 safety study to determine the tolerability, clinical effects, and changes in laboratory parameters of short course oral or IV **cyclosporine** (CSA) administration in patients with COVID-19 disease requiring oxygen supplementation but not requiring ventilator support. | University of Pennsylvania Philadelphia, Pennsylvania, United States |
| 2 | NCT04392531 | Clinical Trial to Assess Efficacy of cYclosporine Plus Standard of Care in Hospitalized Patients With COVID19 | Open, Controlled, Randomized Clinical Trial to Evaluate the Efficacy and Safety of **Cyclosporine** Plus Standard Treatment vs Standard Treatment Only in Hospitalized Patients With **COVID**-19 Infection | Complejo Hospitalario Universitario La Coruña La Coruña, Galicia, Spain<br>Hospital Quiron La Coruña La Coruña, Galicia, Spain<br>Hospital Rey Juan Carlos Mostoles, Madrid, Spain |
| 3 | NCT04540926 | Cyclosporine A Plus Low-steroid Treatment in COVID-19 Pneumonia | Consecutive patients with suspected or confirmed diagnosis of COVID-19 were assigned, in an unblinded and non-randomized fashion, to receive either steroids plus CsA (intervention group) or steroids only (standard of treatment in this hospital, control group), as per individual clinical judgment | Jose Luis Jl Galvez-Romero Puebla, Mexico |
| 4 | NCT04492891 | Cyclosporine For The Treatment Of COVID-19(+) | Phase IIa clinical trial in which 75 non-ICU hospital inpatients will be randomized 2:1 to 7 days of Neoral (2.5mg/kg PO BID) + standard of care (SOC) or no CSA + SOC. | Baylor College of Medicine Houston, Texas, United States |
| 5 | NCT04451239 | Topical Steroids and Cyclosporin-A for COVID-19 Keratoconjunctivitis | Single Group Assignment<br>All patient will be treated with Topical 1% prednisolone acetate for 7 days as initial treatment +non-preserved artificial tears and **cyclosporin** A 0.5% four times daily . | Farawaniya hospital Kuwait, Farawaniya, Kuwait |
| 6 | NCT04341038 | Clinical Trial to Evaluate Methylprednisolone Pulses and Tacrolimus in Patients With COVID-19 Lung Injury | Open Randomized Single Centre Clinical Trial to Evaluate Methylprednisolone Pulses and Tacrolimus in Patients With Severe Lung Injury Secondary to **COVID**-19 | Hospital Universitari de Bellvitge L'Hospitalet de Llobregat, Barcelona, Spain |
| 7 | NCT04420364 | Maintenance Versus Reduction of Immunosuppression for Renal Transplant Patients Hospitalized With COVID-19 Disease | Maintenance or reduction of immunosuppression, phase II-III Single-blind, parallel-group, randomized, active-controlled trial | Birgham and Women's Hospital, Boston, Massachusetts |
| 8 | NCT04569851 | Clinical Characteristics and Prognostic Factors of Patients With **COVID-19** (Coronavirus Disease 2019) | Retrospective, observationnal<br>Clinical Characteristics and Prognostic Factors of Patients With **COVID**-19 Using Big Data and Artificial Intelligence Techniques (BigCoviData) | Hospital Universitario de Guadalajara Guadalajara, Spain<br>Hospital Universitario La Princesa Madrid, Spain |

**Figures**:

**Figure 1**: Schematic representation of the subcellular localization of cyclophilins and FKBP proteins. The red arrow indicates interaction between Cyclosporin A and Cyclophilins. The blue arrow indicates interaction between FK506 and FKBP. CsA: Cyclosporin A; CyPA, CyPB, CyPC, CyPD, CyP40 : Cyclophilins A, B, C, D, 40; FKBP: FK506-binding protein; Caln: Calcineurin; MPTP: Mitochondrial permeability transition pore ; Ca2+: Calcium.

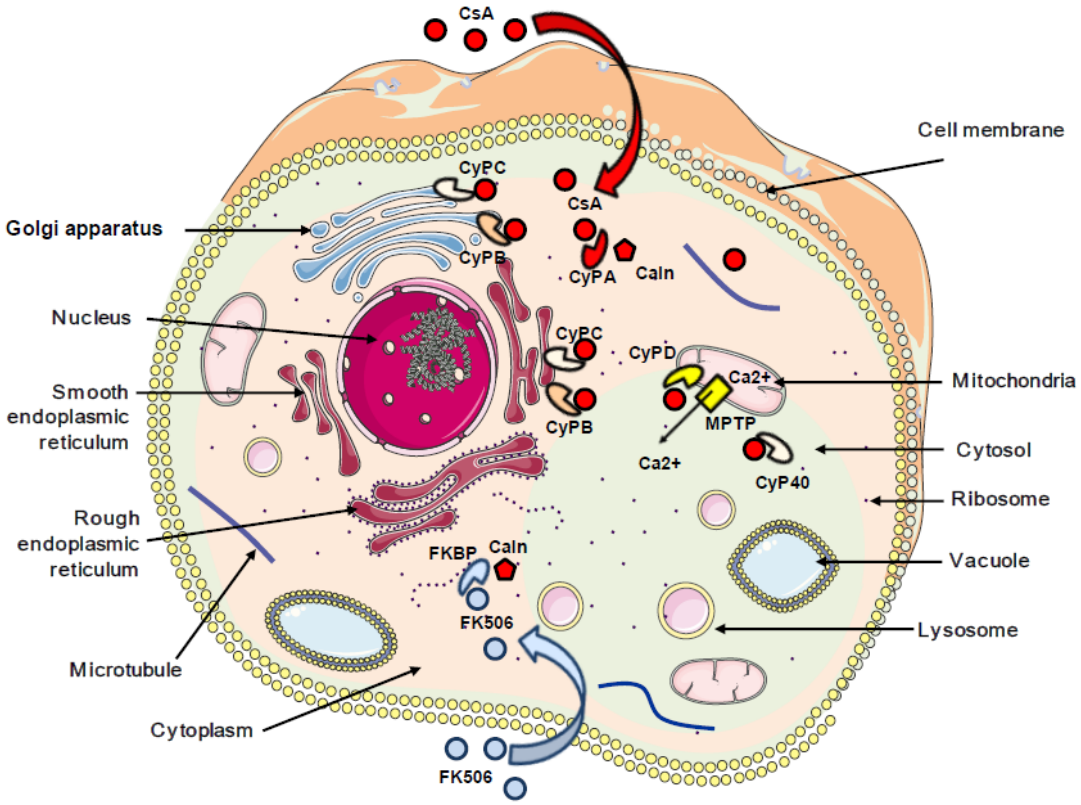

**Figure 2:**

Schematic representation of the antiviral effect of CsA treatment on the HIV-1 disease progression regarding the clinical trials reported in the literature. The effectiveness and beneficial effects of CsA depend on the stage of the disease at which the treatment is given. Unintegrated DNA forms of viral genome increased in the CsA treated group compared to controls when CsA is given post-primo-infection in association with HAART. AIDS: Acquired ImmunoDeficiency Syndrome; HAART: Highly Active Antiretroviral Therapy. CsA : Cyclosporine A

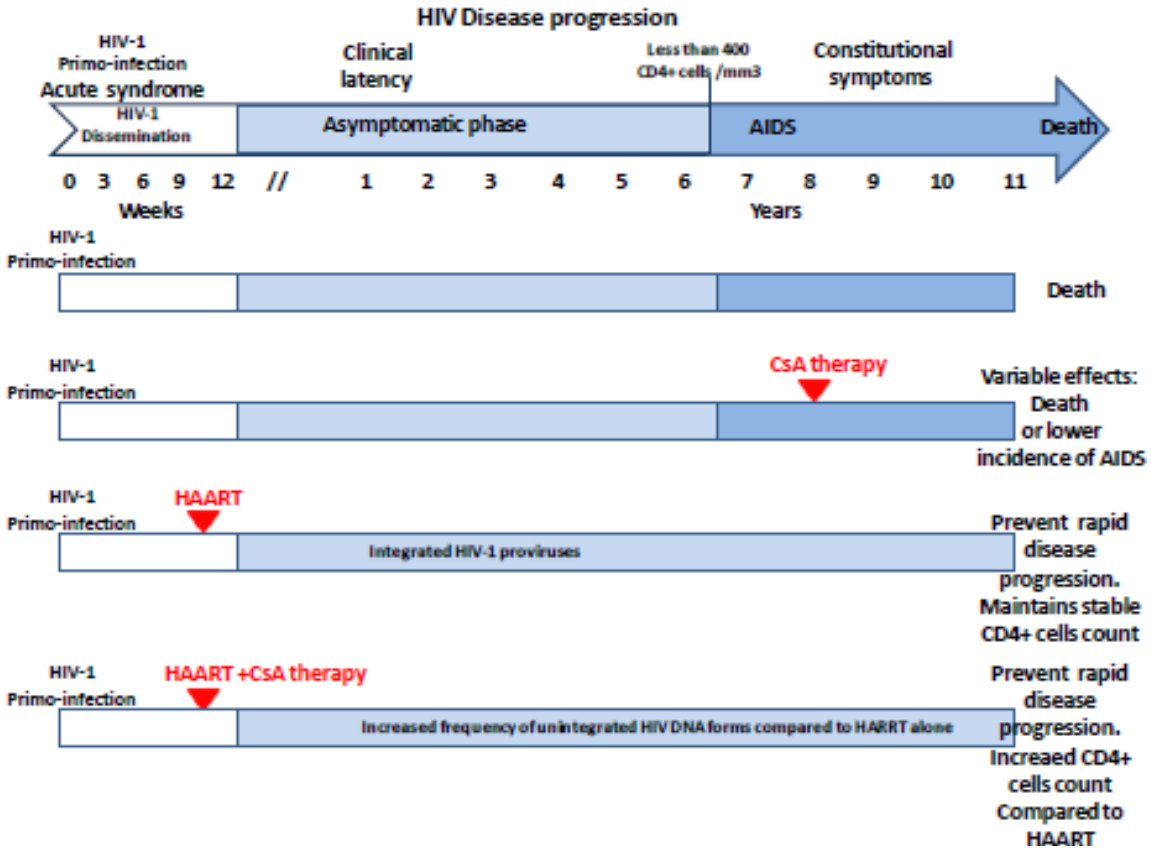

**Figure 3:** Microscopic examination of histological section of tissues from patients died of COVID-19 after hematoxylin, eosin and saffron staining (the hematoxylin stains cell nuclei blue, eosin stains the extracellular matrix and cytoplasm pink, the saffron stain in orange conjonctive matrix). A) Vascular rejection is characterized by concentric thikened arterie secondary to intimal proliferation and endovasculitis. Original magnification x 150. B) concentric thikened arterie secondary to fibro-intinal proliferation. Original magnification x 200 µm.

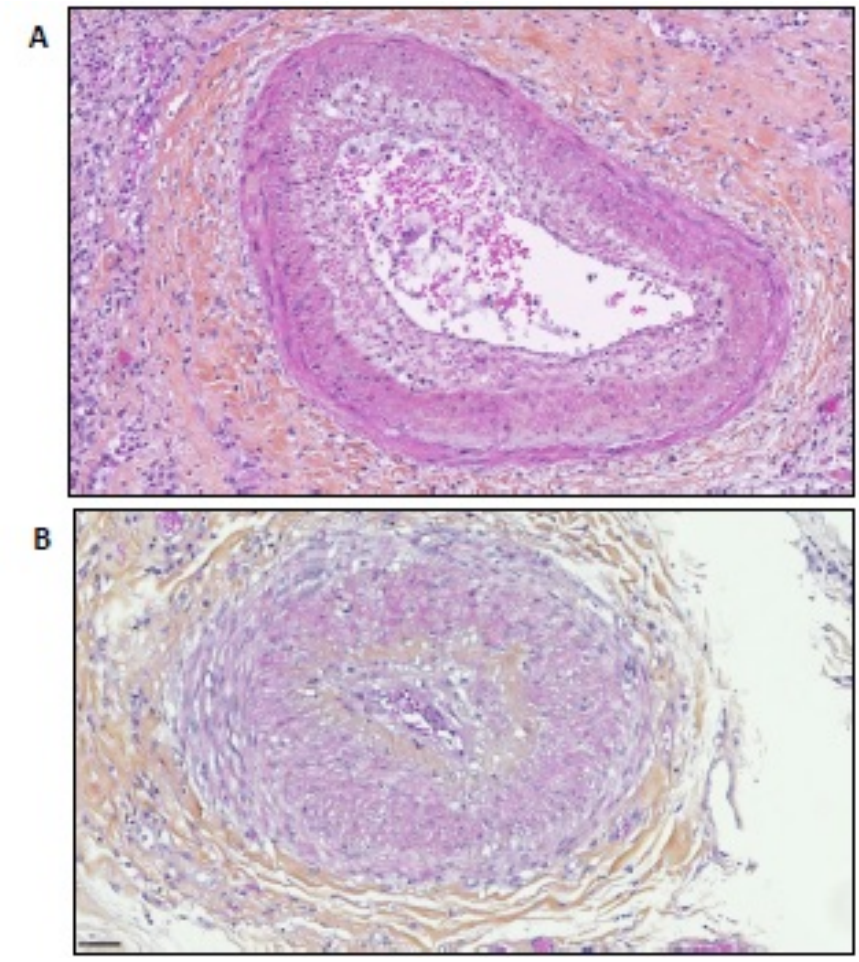

**Figure 4:** Microscopic examination of tissues from patients died of COVID-19. A) hematoxylin, eosin and saffron staining showing intra-alveolar fibrin. Original magnification x 70. B) Inflammatory perivascular lymphocytes T infiltration evidenced by anti-CD3 monoclonal antibody immunostaining. Original magnification x 170.

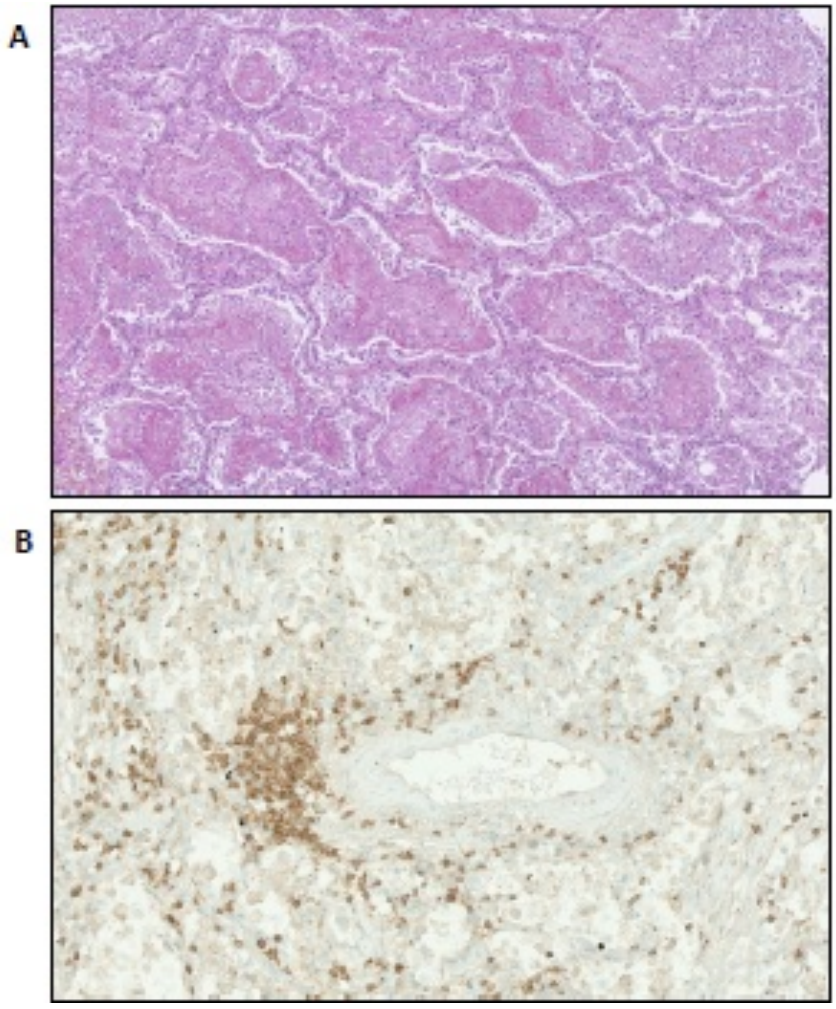

**Figure 5:** Schematic representation of the classical of TcR/CD3 induced activation of IL-2 production. During infection with SARS-CoV-2, the virally-induced cell dysregulation lead to the aberrant opening of MPTP inducing mitochondrial release of $Ca^{2+}$ that triggers an abnormal Ca2+/Calmodulin activation of calcineurin and dephosphorylation of the cytoplasmic NFAT leading to NFAT nuclear translocation and the synthesis of IL-2 and other inflammatory cytokines. Under CsA treatment, the CsA/CyPA complex specifically binds to calcineurin and inhibits its phosphatase function. Consequently, the nuclear factor of activated T cells (NFAT) remain under its inactive cytoplasmic phosphorylated form. Moereover by interacting with CyPD, CsA prevents the opening of MPTP and release of Ca2+ that usually lead to cell death. In addition, through binding to CyPA, CsA is expected to upregulate interferon that block the virus replication. HLA class II: Human leukocyte antigen class II; TcR-CD3 complex: T cell receptor-CD3 complex; PLC: Phospholipase C; IP3: Inositol 1,4,5-triphosphate; Calm: Calmodulin; Caln: Calcineurin; NFATc-P: Nuclear factor of activated T-cell cytoplasmic phosporylated form; NFATc: NFAT cytoplasmic dephoryled; PKC: Protein kinase C; CsA: Cyclosporin A.

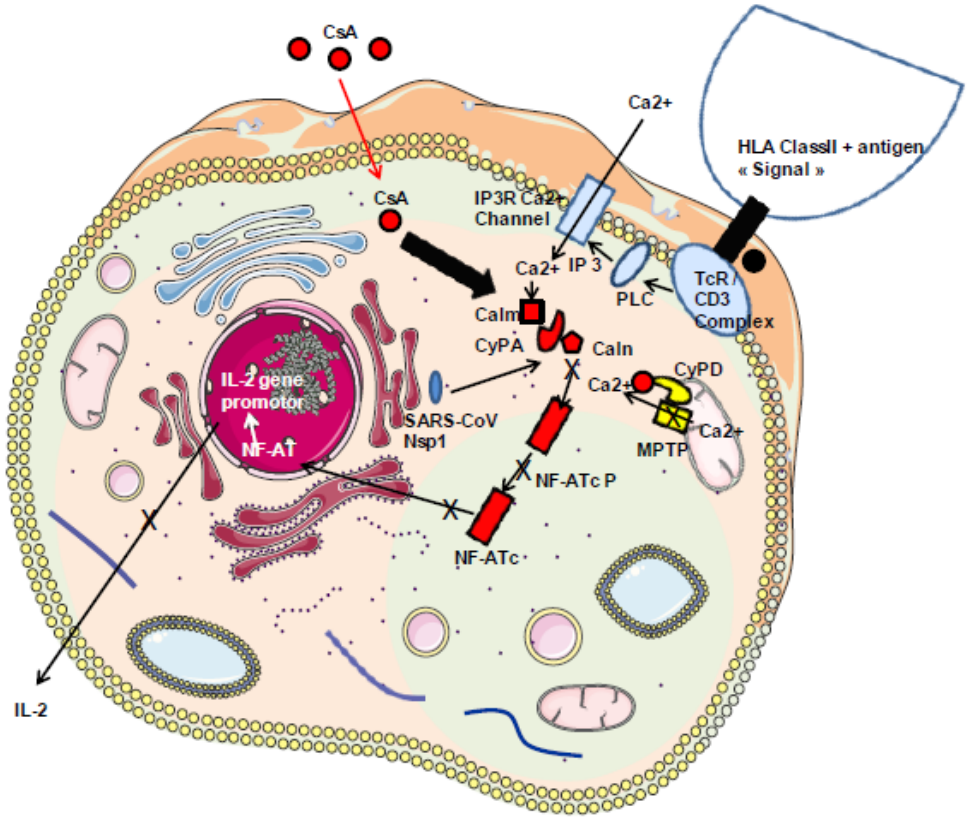

**Figure 6:** Schematic representation of the antiviral properties of CsA. Once the SARS-CoV-2 genome starts to be transcribed into pp1a et pp1ab, the RNA dependent RNA polymerase (Nsp12) should interact with several other viral (Nsp8, Nsp7, Nsp13) and cellular (CypA) proteins to construct a replication complex require for the viral replication cycle to be completed with the synthesis of the structural proteins S, E, M, and N. This step can be inhibited through the interaction between CsA and CypA (see the text for details regarding the different steps of the SARS-CoV-2 cycle which can be inhibited by CsA). ACE2: angiotensin-converting enzyme 2; CsA: Cyclosporin A; CyPA, CyPB, CyPC, CyP : Cyclophilins A, B, C, D; gRNA: genomic RNA; Nsps: nonstructural proteins, ERGIC: Endoplasmic reticulum Golgi intermediate compartment.

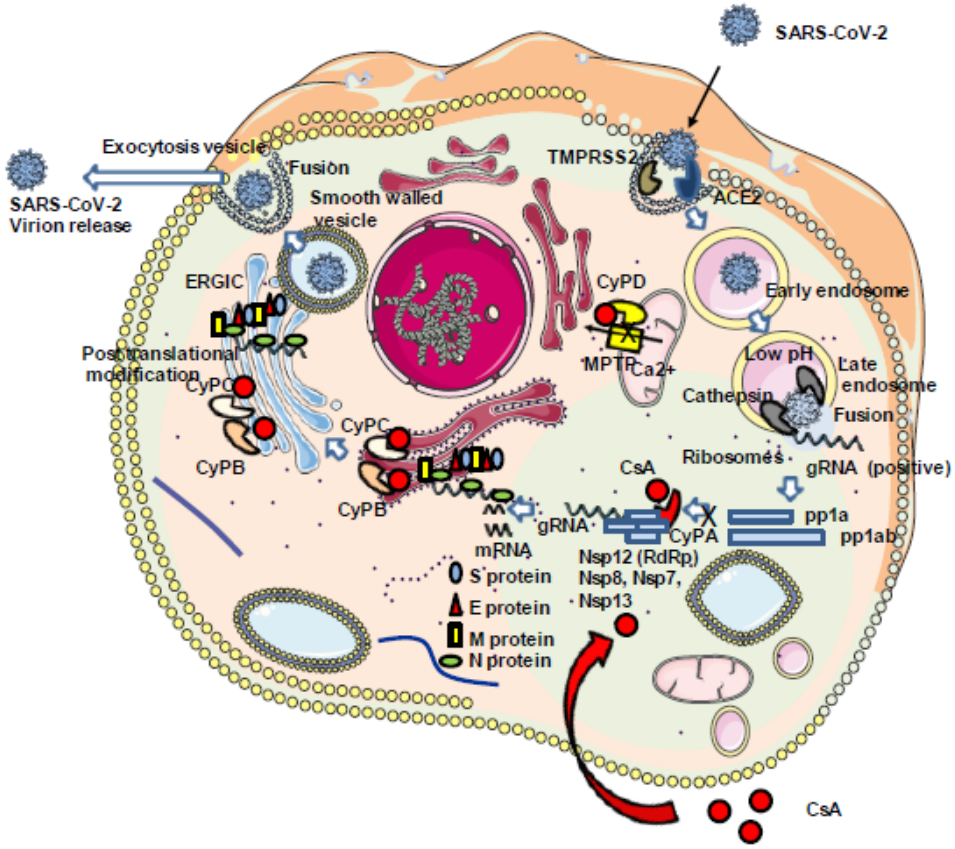

**Figure 7:** Schematic representation of Ang II/AT1R induced inflammatory pathway with cytokines release. During infection with SARS-CoV-2 , the virus binds ACE2 reducing the ACE2 transcription and inhibiting the capacity of ACE2 to mediate the cleavage of Angiotensin II (Ang II) into Angiotensin 1-7. The accumulation of AngII triggers signals through its receptor AT1R inducing ROS production. ROS triggers secretion of CyPA that act as a stress factor activating the ERK1/2 kinase and overproduction of ROS through a positive feeback loop. ROS-sensitive 3-phosphoinositide-dependent protein kinase (PDK1) activation that contributes to phosphorylation and activation of Akt. A parallel pathway involves the NOX-dependent generation of ROS that activates the p38 MAP kinase (p38MAPK) which recruits MAPKAPK2 leading to AkT phosphorylation on a second amino acid position leading to full activation of the p38 MAPK-Akt-complex, the activation of IKKαβ inducing the release of IkB from the IκB-NF-κB complexes, nuclear translocation of NF-κB and the production of cytokines including TNF-α and soluble IL-6 receptor (sIL-6R) via disintegrin and metalloprotease 17 (ADAM 17) followed by the activation of the IL-6 amplifier (IL-6 AMP) which, by feedbach regulation, activates both the NF-κB and STAT3 transcription factors and the production of IL-6. SARS-CoV-2 itself activates NF-κB via the TLR3 receptor. AngII: Angiotensin II; AT1R: Angiotensin II type 1 receptor ; ROS: Reactive oxygen species; NOX: NADPH oxidase ; IKK: IkB kinase; CyPA: cyclophilin A; TLR3: Toll-like receptor 3; NF-κB: nuclear factor κB.