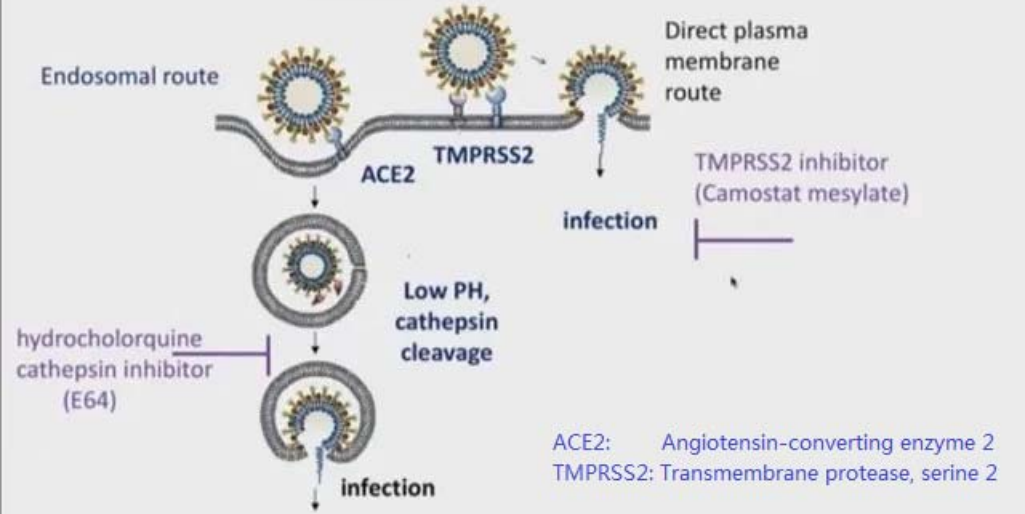
Coronaviruses use two routes of entry into the cell



ACE2: Angiotensin-converting enzyme 2
TMPRSS2: Transmembrane protease, serine 2

Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry

Graham Simmons*†, Dhaval N. Gosalia‡, Andrew J. Rennekamp*, Jacqueline D. Reeves*, Scott L. Diamond‡§, and Paul Bates*†

*Department of Microbiology, School of Medicine and Departments of ‡Bioengineering and §Chemical and Biomolecular Engineering, Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, PA 19104

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Severe acute respiratory syndrome (SARS) is caused by an emergent coronavirus (SARS-CoV), for which there is currently no effective treatment. SARS-CoV mediates receptor binding and entry by its spike (S) glycoprotein, and infection is sensitive to lysosomotropic agents that perturb endosomal pH. We demonstrate here that the lysosomotropic-agent-mediated block to SARS-CoV infection is overcome by protease treatment of target-cell-associated virus. In addition, SARS-CoV infection was blocked by specific inhibitors of the pH-sensitive endosomal protease cathepsin L. A cell-free membrane-fusion system demonstrates that engagement of receptor followed by proteolysis is required for SARS-CoV membrane fusion and indicates that cathepsin L is sufficient to activate membrane fusion by SARS-CoV S. These results suggest that SARS-CoV infection results from a unique, three-step process: receptor binding and induced conformational changes in S glycoprotein followed by cathepsin L proteolysis within endosomes. The requirement for cathepsin L proteolysis identifies a previously uncharacterized class of inhibitor for SARS-CoV infection.

SARS | viral entry | proteolysis | membrane fusion | viral envelope

Severe acute respiratory syndrome (SARS) is an acute respiratory illness caused by a newly described coronavirus (SARS-CoV) (1), the result of a zoonosis of a highly related animal coronavirus (2). There continues to be potential for further zoonotic transmission events, leading to the reintroduction of SARS-CoV into the human population. No effective antiviral treatments have been described for SARS, and, although several promising studies are ongoing, there is currently no licensed protective vaccine.

SARS-CoV entry into target cells is initiated by engagement of its cellular receptor, angiotensin-converting enzyme 2 (ACE2) by spike (S) glycoprotein (3). Subsequent infection is sensitive to inhibitors of endosomal acidification such as ammonium chloride (4–6), suggesting that SARS-CoV requires a low-pH milieu for infection. On the other hand, S protein can mediate cell–cell fusion at neutral pH (3, 4), indicating that S protein-mediated fusion does not include an absolute requirement for an acidic environment. Given these discordant findings, we hypothesized that cellular factors sensitive to ammonium chloride, such as pH-dependent endosomal proteins, may play a role in mediating SARS-CoV entry. In this study, the requirements for proteases in the activation of viral infectivity and the effect of protease inhibitors on SARS-CoV infection are examined. Our results are consistent with a model in which SARS-CoV employs a unique three-step method for membrane fusion, involving receptor-binding and induced conformational changes in S glycoprotein followed by cathepsin L (CTSL) proteolysis and activation of membrane fusion within endosomes.

Methods

Cell Lines and Plasmids. Human ACE2 was amplified by PCR from a cDNA library and cloned into pcDNA3.1. pCAGGS SARS-CoV S, as described in ref. 4. pCB6 vesicular stomatitis virus (VSV)-G, amphotropic murine leukemia virus (MLV-A) envelope, and avian sarcoma and leukosis virus (ASLV-A) envelope are described in refs. 4 and 7.

Cells were maintained in DMEM10 (DMEM supplemented with 10% FBS). A HeLa/Tva cell line was produced by using pcDNA6-Tva and selection with blasticidin. The 293T cells were transiently transfected with human ACE2 (293T/ACE2), by using standard calcium phosphate transfection techniques and challenged 48 h posttransfection.

Pseudotype Preparation. Pseudotypes were produced, essentially as described in ref. 4, by using 10 μg of luciferase of GFP vector (pNL-luc or pNL-gfp) (8) and 30 μg of plasmid-encoding viral envelope or ACE2. Dual-envelope-expressing virions were transfected with 10 μg of pNL-GFP, 15 μg of pCB6 ASLV-A envelope, and 20 μg of pCAGGS SARS-CoV S. If required, virions were concentrated by ultracentrifuge concentration at 40,000 rpm in a SW41 rotor (Beckman) through a 20% sucrose cushion for 1 h at 4°C. The pellets were resuspended in PBS overnight at 4°C.

Trypsin Pretreatment. Concentrated pseudovirions were exposed to L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) for 10 min at 25°C. DMEM10 supplemented with 75 μg/ml soybean trypsin inhibitor (STI) was then added. Samples were used to spin-infect 293T/ACE2 cells at 1,200 $\times$ *g* for 2 h at 4°C. After incubation for 5 h at 37°C, the medium was changed, and the cells were incubated for an additional 40 h. The cells were analyzed for luciferase activity by using a commercial assay (Promega).

Trypsin Bypass. Preincubation of 293T/ACE2 cells took place at 37°C for 45 min with DMEM10 in the presence or absence of ammonium chloride (20 mM). The medium was replaced with cold DMEM10 in the presence or absence of ammonium chloride (40 mM) and incubated for an additional 15 min at 4°C. An equal volume of diluted cold virus was added [a 1-in-10 dilution of HIV-luc(SARS S) or a 1-in-100 dilution of HIV-luc(VSV-G)], and the cells were spin-infected at 4°C to allow virus-binding to cells. The medium was replaced with warm serum-free DMEM in the presence or absence of ammonium chloride (20 mM) and incubated at 37°C for 15 min. The medium was removed, and fresh DMEM in the presence or absence of TPCK-trypsin (15 μg/ml) was added for 10 min at 25°C. The trypsin was removed, and DMEM10 supplemented with STI (75 μg/ml) in the presence or absence of ammonium chloride (20

Abbreviations: ACE2, angiotensin-converting enzyme 2; ASLV, avian sarcoma and leukosis virus; CTSB, cathepsin B; CTSL, cathepsin L; MLV, murine leukemia virus; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; RLU, relative light units; S, spike (glycoprotein); SARS, severe acute respiratory syndrome; SARS-CoV, SARS-associated coronavirus; STI, soybean trypsin inhibitor; VSV, vesicular stomatitis virus; Z-lll-FMK, Z-leu-leu-leu-fluoromethyl ketone.

†To whom correspondence may be addressed at: Department of Microbiology, University of Pennsylvania, 225 Johnson Pavilion, 3610 Hamilton Walk, Philadelphia, PA 19104. E-mail: pbates@mail.med.upenn.edu or gsimmons@mail.med.upenn.edu.

mM) was added. The medium was replaced with fresh DMEM10 12 h later. Cells were analyzed for luciferase activity 36 h later.

Replication-Competent SARS-CoV Assays. SARS-CoV (strain Tor2) was handled under biosafety-level 3 conditions and grown and titered on Vero E6 cells. For trypsin-bypass experiments, Vero E6 cells were incubated on ice for 1 h with DMEM2.5 (in the presence or absence of 25 mM ammonium chloride or 500 μg/ml leupeptin). SARS-CoV, at a multiplicity of infection of $\approx$0.5, was then added, and the cells were spin-infected at 4°C for 1 h at 1,200 $\times$ *g*. The virus was removed, and the cells were incubated for 10 min with serum-free DMEM at 37°C. The medium was then replaced with DMEM in the presence or absence of TPCK-trypsin (15 μg/ml), and the cells were incubated at room temperature for 10 min. The trypsin was removed and replaced with DMEM2.5 containing STI (75 μg/ml) in the presence or absence of ammonium chloride (25 mM) or leupeptin (500 μg/ml). The cells were incubated at 37°C for 4 h, the medium was replaced with DMEM2.5 without inhibitors, and the cells were incubated for an additional 40 h. The cells were fixed for 10 min in cold methanol/acetone, washed in PBS, and incubated for 2 h at 65°C. The cells were immunostained with anti-S protein antibodies IMG-557 and IMG-5010 (Imgenex, San Diego), at 0.5 μg/ml, followed by a mixture of anti-rabbit and anti-mouse FITC conjugates.

For leupeptin sensitivity assays, 293T/ACE2 cells were pretreated for 1 h with DMEM2.5 in the presence or absence of leupeptin and challenged with an equal volume of virus at a multiplicity of infection of $\approx$5. After 3 h, the cells were washed twice and incubated with DMEM2.5 in the presence or absence of leupeptin for an additional 4 h. The medium was then replaced with DMEM2.5, and the cells were incubated for 72 h. The supernatant was harvested, centrifuged to remove cell debris, and incubated at 65°C for 1 h in 1% Empigen (Calbiochem). Samples were analyzed for SARS-CoV nucleocapsid by using a commercial ELISA kit (Imgenex).

Intervirion Fusion. HIV-luc(ACE2) (500 ng of p24) was mixed with 1,000 ng of p24 of HIV-gfp particles incorporating ASLV-A envelope, SARS-CoV S protein, or both envelopes in PBS at 4°C for 30 min to allow binding. Samples were raised to 37°C for 15 min to allow for conformational rearrangements. Virions were adjusted to the desired pH with 0.1 M citric acid. PBS, TPCK-trypsin (final concentration 10 μg/ml), CTSL, cathepsin B (CTSB) (final concentrations 2 μg/ml) or CTSL buffer alone was then added. Recombinant CTSL (R & D Systems) was preactivated by incubation for 15 min at 10 μg/ml in 50 mM Mes, pH 6.0, on ice. Recombinant CTSB (R & D Systems) was preactivated in 25 mM Mes, 5 mM DTT, pH 5.0, for 30 min at 25°C. After a 10-min incubation at 25°C, proteolysis was halted by the addition of 300 μl of DMEM10 containing leupeptin (25 μg/ml) and STI (75 μg/ml). Virions were then incubated at 37°C for 30 min to allow membrane fusion. 100 μl of the virion mixture was added in quadruplicate to HeLa-Tva cells pretreated for 1 h with leupeptin (20 μg/ml). The cells were spin-infected and incubated at 37°C for 5 h. The medium was replaced with fresh DMEM10 and the cells were assayed for luciferase activity 40 h later.

Temperature-Sensitivity Intervirion-Fusion Assay. Intervirion-fusion assays were performed as above, except that binding was performed wholly at 4°C for 50 min for some samples, whereas others were allowed to bind at 4°C for 30 min, followed by 15 min at 37°C. The samples incubated at 37°C were returned to 4°C for 5 min, and cold TPCK-trypsin (final concentration of 10 μg/ml) was added. After a 15-min incubation at 4°C, proteolysis was halted by the addition of DMEM10 with STI (75 μg/ml) and leupeptin (25 μg/ml). Virions were then incubated at 37°C for 30 min to allow membrane fusion to occur, and the assay was completed as described above.

Protease Inhibitors. Vero E6 cells or 293T cells were pretreated for 1 h with leupeptin (Roche Molecular Biochemicals), CA-074, E64c, aprotinin, Z-leu-leu-leu-fluoromethyl ketone (Z-lll-FMK), or MDL28170 (Sigma). Inhibitors were removed and replaced with the same inhibitors at double the final concentration. An equal volume of pseudotypes was then added, and cells were spin-infected as described above. After spin-infection, the cells were incubated for 5 h, and the medium was replaced with fresh DMEM10 without drug. Cells were assayed for luciferase activity after 40 h.

Chemical-Library Screening for Cathepsin L. A library of 1,000 pharmacologically active compounds in DMSO was diluted to 100 μM in 50% glycerol and printed in triplicate on polysine-coated glass, as described in ref. 9. The library was screened for inhibitors of human CTSL at 1 μM in 400 mM NaCl, 20 mM malonate buffer, and 1 mM EDTA, pH 5.5, with fluorogenic substrate Z-Phe-Arg-AMC (Bachem) at 1 mM for detection. Leupeptin and blank spots with no compounds were used as controls. After the addition of enzyme and substrate, the reactions were incubated for 4 h before imaging the slide, as described in ref. 9.

IC_{50} Determination Protease Inhibitor MDL28170. IC_{50} determination was carried out by mixing 20 μl of 50 nM CTSL with 60 μl of buffer (400 mM NaOAc/4 mM EDTA, pH 5.5) containing MDL28170, at a final concentration ranging from 10 μM to 100 pM. The reaction was activated by the addition of 20 μl of 10 μM Z-Phe-Arg-7-amino-4-methylcoumarin (AMC). Fluorescence (Ex, 355 nM; Em, 460 nM) from cleaved AMC was detected in a kinetic mode by using an Ascent Fluoroskan FL plate reader (Thermo Electron LabSystems, San Jose, CA), with eight replicates on the same plate. The kinetic data were plotted, and the IC_{50} curve was determined by using software from GraphPad (San Diego).

Results

Proteolysis Activates SARS-CoV S Protein's Membrane-Fusion Potential. Fusion between Vero and 293T cells expressing SARS-CoV S protein occurs at neutral pH and is greatly enhanced by trypsin activation; yet, lysosomotropic agents block SARS-CoV infection (4). To reconcile the observed effects of pH and proteolysis on SARS-CoV membrane fusion, we posited that exogenous trypsin cleavage mimics the action of a pH-dependent endosomal protease (4). This hypothesis predicts that protease treatment of cell-associated virus should overcome the block to viral entry mediated by lysosomotropic agents like ammonium chloride. As demonstrated in ref. 4, pretreatment of cells with ammonium chloride dramatically reduced infection mediated by SARS-CoV S glycoprotein (Fig. 1*A*) and the pH-dependent viral glycoprotein VSV-G incorporated into HIV virions. However, when cell-bound HIV(SARS S) pseudovirions were exposed to trypsin, infection occurred in the presence or absence of ammonium chloride (Fig. 1*A*). In fact, the combination of trypsin proteolysis and ammonium chloride increased viral infectivity by 3-fold. Proteolysis of replication-competent SARS-CoV (Tor2 strain) bound to Vero E6 cells also overcame the block to viral infection otherwise mediated by ammonium chloride (Fig. 1*C*). Thus, proteolysis of SARS-CoV bypasses the requirement for acid pH during the viral entry process.

In marked contrast to the studies in which SARS-CoV or HIV(SARS S) virions were bound to cells before trypsin treatment, proteolysis of free HIV(SARS S) pseudovirions dramatically diminished infectivity (Fig. 1*B*). Trypsin concentrations 10-fold lower than those used to activate fusion of cell-associated HIV(SARS S) were able to effectively inhibit infection by free virus. Similarly, in cell–cell fusion assays, proteolysis after mixing SARS-CoV S-expressing cells with target cells also resulted in more robust membrane fusion, compared with pretreatment with trypsin (data not shown). In addition, trypsin was

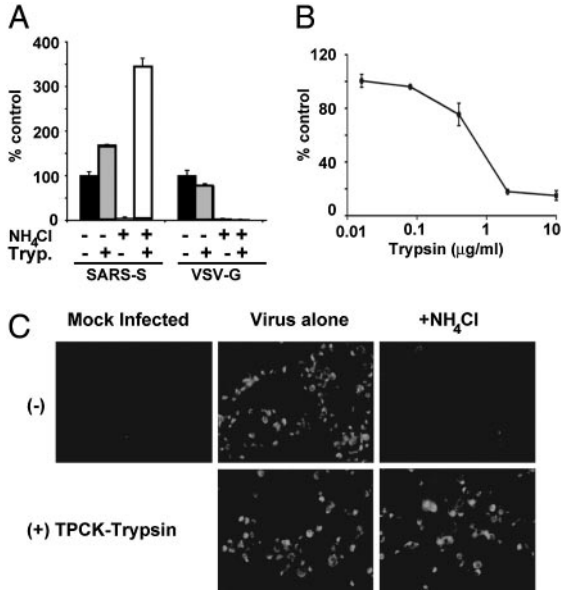

Fig. 1. Effect of trypsin on SARS-CoV infection. (*A*) Trypsin treatment bypasses ammonium chloride inhibition. HIV-luc(SARS S) or HIV-luc(VSV-G) were bound to mock (black and gray bars) or ammonium chloride-treated (third set of bars and white bars) 293T/ACE2 cells. The cells were incubated with either PBS (black bars and third set of bars) or TPCK-trypsin (gray and white bars). The results are presented as a percentage of no-ammonium-chloride (NH_4Cl), no-trypsin (Tryp.) controls (≈4,000 and 10,000 RLU for SARS S and VSV-G, respectively) and represent the means of samples run in triplicate (±SD). Similar results were seen in two subsequent assays. (*B*) Trypsin pretreatment of S protein inactivates infectivity. HIV-luc(SARS S) infection of 293T/ACE2 cells was assessed as luciferase activity, presented as a percentage of no-trypsin control (≈40,000 RLU). The results represent the means of samples run in triplicate (±SD). (*C*) Trypsin treatment bypasses ammonium chloride inhibition of SARS-CoV. Mock- (*Center*) or 25 mM ammonium chloride-pretreated (*Right*) Vero E6 cells were spin-infected with replication-competent SARS-CoV at a multiplicity of infection of 0.5 and incubated with either DMEM (*Upper*) or DMEM containing TPCK-trypsin (*Lower*). After 48 h, the cells were immunostained for S protein.

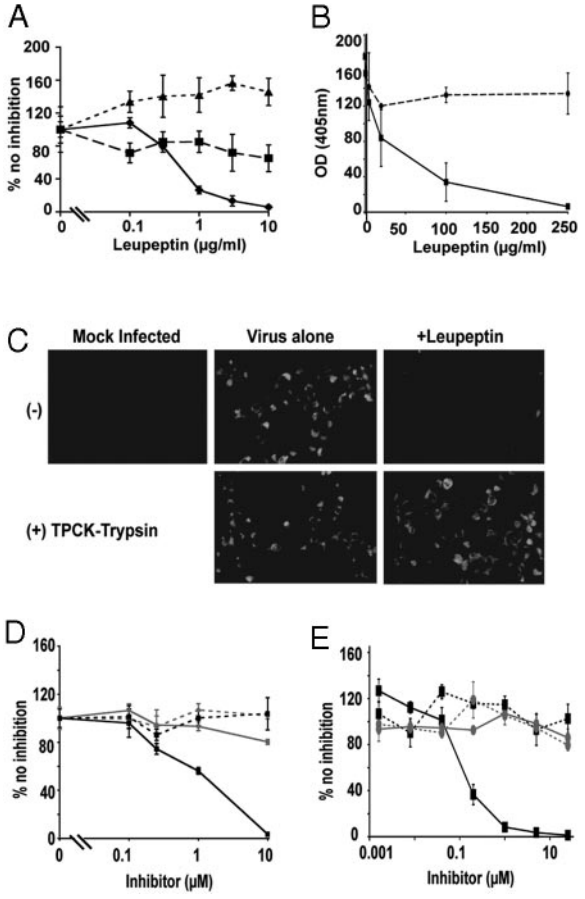

Fig. 2. Protease-inhibitor sensitivity. (*A*) Leupeptin inhibits S protein-mediated infection. The 293T cells were preincubated with leupeptin and challenged with HIV-luc SARS S (solid line, ◆), VSV-G (dashed line, ■), or MLV-Ampho (dotted line, ▲). The results are presented as a percentage of infection of untreated cells (≈3,000 RLU) for each envelope) and represent the means of samples run in triplicate (±SD). Similar results were seen in two subsequent assays. (*B*) Leupeptin inhibits replication-competent SARS-CoV infection. Cells were either preincubated with leupeptin for 1 h and then exposed to virus for 3 h in the continued presence of leupeptin (solid line) or exposed to virus for 3 h and incubated for an additional 4 h with leupeptin (dashed line). At 3 days postexposure, the supernatant was analyzed for nucleoprotein by ELISA. The results are expressed as OD and represent the means of samples run in triplicate (±SD). Similar results were seen in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium cytoxicity assay. (*C*) Trypsin treatment bypasses leupeptin inhibition of live SARS-CoV. Mock- (*Center*) or 500 μg/ml leupeptin-pretreated (*Right*) Vero E6 cells were spin-infected with replication-competent SARS-CoV at a multiplicity of infection of 0.5 and incubated with either DMEM (*Upper*) or DMEM containing TPCK-trypsin (*Lower*). After 48 h, the cells were immunostained for S protein. (*D*) E64c blocks SARS-CoV S protein-mediated entry. The 293T cells were preincubated with E64c (solid lines) or aprotinin (dashed lines) and challenged with HIV-luc SARS S (black lines) or VSV-G (gray lines). The results are presented as a percentage of infection of untreated cells (≈1,500 RLU for VSV-G and 6,000 RLU for SARS S) and represent the means of samples run in triplicate (±SD). Similar results were seen in two additional experiments. (*E*) Z-lll-FMK inhibits S protein-mediated infection. Vero E6 cells were preincubated with Z-lll-FMK (solid lines) or CA-074 (dashed lines) and then challenged with HIV-luc SARS S (black lines) or VSV-G (gray lines). The results are presented as a percentage of infection of untreated cells (≈15,000 RLU for VSV-G and 20,000 RLU for SARS S) and represent the means of samples run in triplicate (±SD). Similar results were seen on 293T and 293T/ACE2 cells.

unable to bypass the requirement for ACE2 on receptor-null cell lines, such as QT6 cells, even upon stable expression of the attachment factors DC-SIGN or DC-SIGNR (data not shown), suggesting a requirement for receptor engagement. The finding that, in solution, proteolysis leads to SARS-CoV S inactivation, whereas proteolysis leads to activation when the virus is bound to receptor-expressing membranes, demonstrates that the context in which proteolysis occurs is an important determinant of SARS-CoV infectivity.

Sensitivity of SARS-CoV S Protein-Mediated Entry to Protease Inhibitors. The ability of trypsin cleavage to overcome inhibition of endosomal acidification suggested a requirement for endosomal protease activity. To test this hypothesis, the infection of 293T cells with HIV(SARS S) was examined in the presence of leupeptin, an inhibitor of endosomal trypsin-like serine and cysteine proteases (Fig. 2*A*). Similar results were seen with 293T/ACE2 and Vero E6 cells (data not shown). Entry mediated by SARS-CoV S protein was efficiently blocked by leupeptin, with >95% inhibition observed at 10 μg/ml. Infection mediated by VSV-G, a pH-dependent viral membrane-fusion protein, and the pH-independent envelope from amphotropic MLV was not inhibited by leupeptin (Fig. 2*A*).

Infection by replication-competent SARS-CoV was also inhibited by leupeptin (Fig. 2*B*). Efficient inhibition was observed only if leupeptin was present 1 h before and during the 3-h exposure to the virus. When leupeptin was added to cells after exposure to SARS-CoV and then removed 4 h later, there was little or no effect on SARS-CoV replication, even at a concentration of 250 μg/ml. Thus, it is unlikely that the concentrations of leupeptin required to efficiently inhibit a spreading SARS-

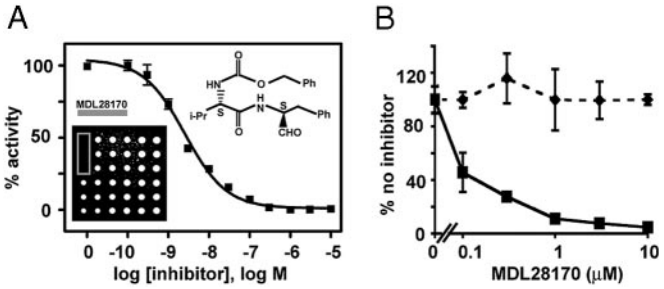

Fig. 3. Cathepsin-L-specific inhibitor blocks infection. (*A*) MDL28170 inhibits CTSL activity with an IC_{50} of 2.5 nM. A 1,000-compound library was screened for inhibitors of CTSL activity (*Inset*, bottom left). MDL28170 (*Inset*, top right) was found to be a potent inhibitor. The compound library was screened against several other cathepsins, including CTSB, with no hits. The activity of MDL28170 was confirmed in an *in vitro* CTSL-cleavage assay (inhibition curve). (*B*) MDL28170 inhibits S protein-mediated infection. The 293T cells were preincubated with MDL28170 and challenged with HIV-luc SARS S (solid line) or VSV-G (dashed line). The results are presented as a percentage of infection of untreated cells (≈100,000 RLU for VSV-G and 20,000 RLU for SARS S) and represent the means of samples run in triplicate (±SD). Similar results were seen on Vero E6 and 293T/ACE2 cells.

CoV infection are inhibiting postentry steps of replication or are merely toxic to the cells. Rather, leupeptin appears to inhibit an early step in viral entry. In a manner similar to inhibition by ammonium chloride (Fig. 1*C*), the leupeptin-mediated block to SARS-CoV infection of Vero E6 cells could be bypassed by proteolysis of virus bound to the cell surface (Fig. 2*C*). These findings are consistent with exogenous trypsin treatment compensating for cleavage normally mediated by leupeptin-sensitive endosomal proteases.

To more precisely define the protease(s) involved in SARS-CoV infection, a series of inhibitors were analyzed. E64c, an inhibitor of cysteine proteases, specifically inhibited infection by HIV(SARS S) pseudovirions, whereas aprotinin, an inhibitor of serine-type proteases, had no effect (Fig. 2*D*). Inhibitors of other classes of proteases, such as pepstatin, an aspartate protease inhibitor, also had no effect on either S protein- or VSV-G-mediated infection (data not shown). CA-074, a selective inhibitor of CTSB (10) did not dramatically affect infection by either HIV(SARS-CoV S) or HIV(VSV-G) (Fig. 2*E*; and see Table 1, which is published as supporting information on the PNAS web site). In contrast, Z-lll-FMK, an inhibitor of both CTSB and CTSL (11), efficiently inhibited infection by HIV(SARS S), but not by HIV(VSV-G) (Fig. 2*E*). In addition, a panel of four commercially available CTSL inhibitors specifically inhibited HIV(SARS S) infection (Table 1). Overall, these inhibitor results suggest that a pH-dependent cysteine protease, perhaps CTSL, is important for SARS-CoV infection.

Screen for Pharmacologically Active Inhibitors. To identify potential lead candidates for therapeutic inhibition of CTSL, a high-throughput screening of a library of pharmacologically active compounds was performed (see *Methods*). MDL28170 was identified as an efficient inhibitor of CTSL-mediated substrate cleavage, with an IC_{50} of 2.5 nM (Fig. 3*A*). MDL28170 (also known as calpain inhibitor III, or Z-Val-Phe-CHO) is an inhibitor of cytosolic calpains (12, 13). Inhibition of CTSB has also been noted (12). Interestingly, related calpain inhibitors have already been described as inhibitors of SARS-CoV replication (14), although it was assumed the action was through inhibition of viral proteases. Similarly, we found efficient inhibition of SARS-CoV replication using MDL28170 (data not shown). In addition, MDL28170 efficiently inhibited infection by HIV(SARS S), but not by HIV(VSV-G) pseudovirions (Fig. 3*B* and Table 1). Given that the pseudotype infection assay is a direct measure of S protein-mediated viral entry, these results suggest that MDL28170's action is due to inhibition of endosomal protease activity during viral entry. Thus, these experiments identify MDL28170 as a strong initial candidate for antiviral inhibitors of SARS-CoV viral entry.

Protease-Mediated Activation of Membrane Fusion. To further study the relative contributions of acid pH and specific proteases on SARS-CoV infection, we developed a cell-free, virus–virus membrane-fusion assay employing virions that carry either S glycoprotein or the SARS-CoV cellular receptor ACE2 (3). The HIV(ACE 2) pseudotypes encode luciferase, whereas S glycoprotein particles encode GFP and have on their surface not only SARS-CoV S but also the envelope glycoprotein from subgroup A ASLV-A envelope. Membrane fusion between the virions carrying SARS-CoV S and those with ACE2 is indicated by transfer of the genome encoding luciferase to HeLa/tva cells expressing the cellular receptor for ASLV-A but not SARS-CoV. A similar cell-free membrane-fusion assay has been used to analyze HIV and MLV-envelope-mediated membrane fusion and, in both instances, has been shown to accurately reflect normal virus infection requirements (15, 16).

Characterization of the peseudovirions demonstrated the efficient production of HIV particles containing ACE2 in their lipid coats, as determined by Western analysis of purified virions (data not shown). These HIV-luc(ACE2) particles were able to efficiently and specifically infect 293T cells expressing SARS-CoV S protein, as demonstrated by high levels of luciferase activity in the target cells (see Fig. 5, which is published as supporting information on the PNAS web site). HIV particles encoding GFP and incorporating both SARS-CoV S and ASLV-A envelope [referred to as HIV-gfp(SARS S/ASLV-A)] were also efficiently produced and infectious on cell lines expressing either ACE2 or the ASLV-A receptor Tva (data not shown).

The ability of SARS-CoV S and ACE2, on the surface of their respective virions, to mediate intervirion membrane fusion was assessed by coincubating the pseudotypes before infection of HeLa/Tva cells. In contrast to the results seen when individual pseudovirions were used, a mixture of HIV-luc(ACE2) and HIV-gfp(SARS S/ASLV-A) resulted in expression of the luciferase-encoding genome in HeLa/Tva cells (Fig. 4*A*). Luciferase activity was not observed when a pseudotype that did not carry ACE2 [termed HIV-luc(bald)] was mixed with HIV-gfp(SARS S/ASLV-A) or when HIV-gfp particles expressing ASLV-A env alone were mixed with HIV-luc(ACE2) (Fig. 4*A*). Thus, luciferase activity appears to be a measure of SARS-CoV S-mediated intervirion membrane fusion.

We used this virus–virus membrane-fusion assay to examine the effects of pH and proteolysis on SARS-CoV-mediated membrane fusion. Pretreatment of the HeLa/Tva cells with leupeptin before the addition of mixed virions abrogated S protein-mediated intervirion fusion, as demonstrated by the background levels of luciferase activity observed (Fig. 4*A*). As a control, leupeptin was found to have no effect on ASLV-A envelope-mediated infection of HeLa/Tva cells (data not shown). These results suggest that, for virus–virus membrane fusion to occur, the particles must be coendocytosed into endosomes, where proteases sensitive to leupeptin are able to alleviate a block to fusion between the virus particles. Thus, in all subsequent assays, target cells were pretreated with leupeptin to determine the effect of the addition of exogenous protease on virus–virus fusion before plating on target cells.

To more directly assess the requirement for proteolytic activation of S protein, we incubated the two pseudovirion populations to allow S protein and ACE2-mediated virus–virus binding. Trypsin proteolysis of the bound virus particles dramatically increased luciferase expression in target HeLa/Tva cells, despite endosomal proteolysis inhibition by leupeptin (Fig. 4*B*). In contrast to trypsin,

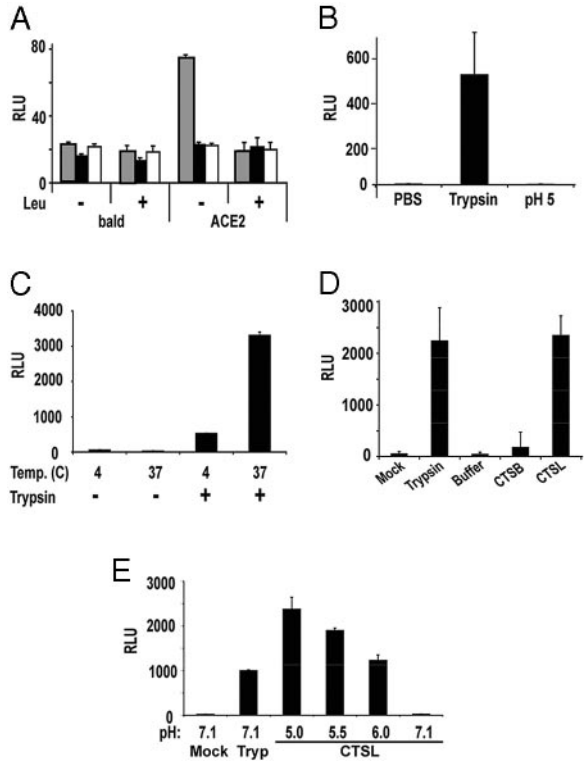

Fig. 4. S protein-mediated intervirion fusion. (*A*) Intervirion fusion requires ACE2 and S protein. Bald or ACE2 particles encoding luciferase (*x* axis) were incubated with particles encoding GFP (SARS S and ASLV-A envelope, gray bars; SARS S alone, black bars; or ASLV-A envelope alone, white bars). Virions were mixed and used to infect HeLa/Tva cells that had been pretreated with medium in the presence and absence of leupeptin (Leu) (20 μg/ml). Intervirion fusion was measured as luciferase activity 48 h postinfection. Results represent the means of samples run in triplicate (±SD). (*B*) Trypsin cleavage promotes fusion mediated by S protein. Intervirion fusion between HIV-luc(ACE2) and HIV-gfp(SARS S/ASLV-A) treated with TPCK-trypsin (10 μg/ml) for 10 min at 25°C or pulsed at pH 5.0 was quantified by luciferase activity 48 h postinfection of HeLa/Tva cells pretreated with leupeptin. The results represent the means of samples run in triplicate (±SD). Mixtures of HIV-gfp(SARS S), HIV-gfp(ASLV-A), and HIV-luc(ACE2) could not be activated by trypsin cleavage, suggesting that S and ASLV-A envelope are required to be incorporated into the same particle in order for transduction of target cells by fused particles. (*C*) Receptor interactions at elevated temperature are required before trypsin cleavage. HIV-luc(ACE2) and HIV-GFP(SARS S/ASLV-A) particles were mixed and incubated at 4°C to allow binding. Samples were then incubated at the noted temperatures. TPCK-trypsin digestion was carried out at 4°C for 15 min. The results represent the means of samples run in quadruplicate (±SD). Similar results were observed in two additional experiments. Temp., temperature. (*D*) CTSL enhances intervirion fusion. HIV-luc(ACE2) and HIV-GFP(SARS S/ASLV-A) particles were mixed and incubated for 10 min at 25°C with preactivated CTSB (at pH 5.0), CTSL (at pH 6.0), CTSL buffer alone (at pH 6.0), or TPCK-trypsin (at pH 7.0). The mixed virus was used to infect HeLa/Tva cells pretreated with leupeptin. The results represent the means of samples run in quadruplicate (±SD). Similar results were observed in two subsequent experiments. (*E*) Acidic conditions are required for CTSL-mediated S protein activation. HIV-luc(ACE2) and HIV-GFP(SARS S/ASLV-A) particles were mixed and adjusted to various pHs and CTSL was added. After neutralization of acid conditions, the mixed virus was used to infect HeLa/Tva cells pretreated with leupeptin. The results represent the means of samples run in quadruplicate (±SD). Tryp, trypsin. Similar results were observed in an additional experiment.

a brief low-pH pulse did not facilitate virus–virus membrane fusion, as assayed by luciferase gene transfer in the leupeptin-treated target cells (Fig. 4*B*). The higher levels of luciferase activity seen in these experiments compared with Fig. 4*A* may reflect a more efficient membrane fusion reaction, because this assay does not rely on traffic of the bound virions to the endosome for intervirion fusion. These results confirm that a low-pH environment does not appear to act as a direct trigger for SARS-CoV entry. In agreement with the studies above, showing proteolytic bypass of lysosomotropic-agent-mediated inhibition, these membrane-fusion data are most consistent with a model in which the low-pH environment of the endosome is needed for proteolytic activation of membrane-fusion activity.

Temperature-Dependence of Protease Activation. The fact that S protein needs to bind ACE2 in order for trypsin treatment to have an effect on membrane fusion (Fig. 1) suggested that conformational changes induced by SARS-CoV S protein–receptor interaction may be required before proteolysis. Conformational changes are generally slowed or arrested at low temperatures. Thus, we examined whether incubation of mixtures of HIV-luc(ACE2) and HIV-gfp(SARS S/ASLV-A) virions at 4°C, compared with 37°C before treatment with protease, affected subsequent membrane-fusion activity, possibly by preventing conformational changes in S protein induced by ACE2 binding. Only a small increase in intervirion fusion was seen with HIV-luc(ACE2) and HIV-gfp(SARS S/ASLV-A) virus particles maintained at 4°C, despite trypsin treatment (Fig. 4*C*). When the mixture of HIV-luc(ACE2) and HIV-gfp(SARS S/ASLV-A) particles was preincubated at 37°C for 15 min, however, before being returned to 4°C for trypsin treatment, efficient intervirion fusion was observed (Fig. 4*C*). These results indicate that a receptor and temperature-dependent step occurs before proteolysis of SARS-CoV S protein, possibly involving receptor-induced conformational changes within S to either expose a protease cleavage site or to undergo some of the steps leading up to membrane fusion.

Cathepsin L Activates SARS-CoV Membrane Fusion. The ability of specific inhibitors to block SARS-CoV entry and the requirement for proteolysis for S-mediated intervirion membrane fusion suggested that CTSL may play a role in directly modulating the fusion activity of SARS-CoV S. To test this hypothesis, recombinant cathepsins common to cellular endosomes, such as CTSB and CTSL, were used in the virus–virus membrane-fusion assay. Treatment of mixed HIV-luc(ACE2) and HIV-gfp(SARS S/ASLV-A) particles with CTSL at pH 6.0 mediated intervirion fusion as efficiently as did trypsin (Fig. 4*D*). In contrast, CTSB treatment did not produce a reproducible increase in intervirion fusion. Additionally, CTSL buffer alone at pH 6.0 had no effect. The sensitivity of SARS-CoV S protein-mediated entry to lysosomotropic agents is likely explained by the fact that endosomal proteases, such as CTSL, cleave more efficiently and are more stable at acidic pH. To address this question, CTSL-mediated activation of SARS-CoV S membrane fusion was performed at different pHs. With HIV-luc(ACE2) and HIV-gfp(SARS S/ASLV-A) particles and CTSL, a gradual reduction in levels of fusion was observed with increasing pH, and incubation at pH 7.1 resulted in no intervirion fusion (Fig. 4*E*).

Discussion

Distinct spikes of trimeric glycoproteins mediate the attachment, fusion, and entry of enveloped RNA viruses such as the orthomyxo-, paramyxo-, filo-, retro-, and coronaviruses. A hallmark of these class I viral membrane-fusion proteins is that they undergo a series of structural rearrangements that cause fusion between the viral and cellular membranes. The glycoproteins in the virion spikes are in an energetically unfavorable conformation, and an activating trigger is required to allow metastable protein complexes to refold into a more stable final form. For many viruses, binding to specific receptors can induce the conformational rearrangements within envelope proteins required for membrane fusion by binding to a single receptor, as is the case for Amphotropic MLV, or consecutive binding to a receptor and coreceptor, as is seen in HIV entry. Alternatively, viruses such as influenza require only an acidic milieu

to be triggered (17). More recently, a fourth means of achieving glycoprotein triggering has been described for the avian retrovirus ASLV-A, whereby both binding to a specific receptor and low pH are required in order for membrane fusion to be completed (18). We describe here a potential fifth model for glycoprotein triggering that requires the involvement of endosomal protease activity subsequent to receptor interactions.

A number of possibilities exist for the role of CTSL in SARS-CoV entry, including the cleavage of S protein, ACE2, or another cellular protein that aids in membrane fusion. One explanation is that, as in influenza, cleavage is required to expose the hydrophobic fusion peptide. Indeed, protease activation of influenza hemagglutinin can occur during entry in certain cell types (19). However, in the case of SARS-CoV, it appears that interaction with receptor is required before such cleavage. Although a fusion peptide has not been established for SARS-CoV S protein by mutagenesis mapping, prediction models place it immediately amino-terminal of the membrane-distal leucine/isoleucine heptad repeat (HR1) (20). Another likely scenario is that S protein is physically constrained from undergoing the necessary conformational changes required for fusion peptide insertion. Cleavage at sites exposed by receptor-binding then either relieves these constraints or even actively induces the conformational rearrangements leading to fusion peptide insertion. In this model, one can view proteolytic cleavage of S as the fusion-activating trigger comparable to pH for influenza HA or coreceptor-binding for HIV envelope. Analogous to the conformations of the influenza and HIV proteins induced by pH or coreceptor binding, it seems likely that the CTSL-cleaved SARS-CoV S may be a transient intermediate in the membrane-fusion process. It is, perhaps, this transient nature or the rather nonspecific character of cathepsin L that has made identification of the cleavage sites in S difficult. However, preliminary mutational analysis of the residues near the S1–S2 boundary of SARS S suggest that trypsin activation does not require cleavage at this location (G.S., A.J.R., and P.B., unpublished data).

An alternative model is that receptor-binding mediates the early conformational changes in the S protein, including fusion peptide insertion into the target membrane but that uncleaved S protein is constrained in such a way that the later steps in membrane fusion, such as stable six-helix bundle formation or fusion pore formation, cannot occur. The act of cleavage then releases this constraint. In support of this model, the ASLV envelope protein is thought to use receptor binding to activate the early steps of membrane fusion, including fusion peptide insertion and at least partial refolding into a six-helix bundle, but needs a low-pH step to complete membrane fusion (21–23). It may be that SARS-CoV S uses protease in an analogous manner to pH for ASLV as a second trigger acting late in the membrane-fusion process. The role, if any, that extracellular proteases commonly found in sites of SARS-CoV replication (such as the airways and the gut) may play in this model for viral entry is unclear. It is even possible that extracellular cleavage after receptor engagement would negate the requirement for endocytosis, as seen in the trypsin-bypass experiments.

Overall, these experiments suggest a previously undescribed paradigm for viral entry into target cells. Namely, that for SARS-CoV S protein, receptor-mediated conformational changes induce exposure of cryptic cleavage sites within viral envelope glycoprotein. Proteolysis by cellular proteases is then necessary to fully activate the viral glycoprotein's membrane-fusion potential. Further characterization of this phenomenon is likely to highlight steps in the activation of S protein that may yield targets for specific inhibitors of entry. Indeed, the finding that CTSL is an important activating protease for SARS-CoV infection suggests CTSL as a target for therapeutic intervention. MDL28170 represents an attractive starting point for specific inhibitors of CTSL as antiviral therapeutics targeting SARS-CoV entry.

The entry process described here for SARS-CoV S protein and the inhibitors of this process also raise the question whether other classically defined pH-dependent viruses display this dependence because of a requirement for acidic protease involvement and not pH-induced structural rearrangements, as is commonly assumed. Indeed, it has recently been suggested that Ebola glycoprotein undergoes similar processing by endosomal proteases (see ref. 24; G.S., A.J.R., and P.B., unpublished observations). Future investigation will reveal whether SARS-CoV and Ebola represent initial members of a previously uncharacterized category of viral fusion proteins.

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Early Outpatient Treatment of Symptomatic, High-Risk Covid-19 Patients that Should be Ramped-Up Immediately as Key to the Pandemic Crisis

Harvey A. Risch

Correspondence to Dr. Harvey A. Risch, Department of Chronic Disease Epidemiology, Yale School of Public Health, P.O. Box 208034, New Haven, CT 06520-8034 (e-mail: harvey.risch@yale.edu; phone: (203) 785-2848)

Author Affiliations: Department of Chronic Disease Epidemiology, Yale School of Public Health, New Haven, Connecticut (Harvey A. Risch).

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Running Head: Outpatient Treatment of High-Risk Covid-19

Abstract

More than 1.6 million Americans have been infected with SARS-CoV-2 and >10 times that number carry antibodies to it. High-risk patients presenting with progressing symptomatic disease have only hospitalization treatment with its high mortality. An outpatient treatment that prevents hospitalization is desperately needed. Two candidate medications have been widely discussed: remdesivir, and hydroxychloroquine+azithromycin. Remdesivir has shown mild effectiveness in hospitalized inpatients, but no trials have been registered in outpatients. Hydroxychloroquine+azithromycin has been widely misrepresented in both clinical reports and public media, and outpatient trials results are not expected until September. Early outpatient illness is very different than later hospitalized florid disease and the treatments differ. Evidence about use of hydroxychloroquine alone, or of hydroxychloroquine+azithromycin in inpatients, is irrelevant concerning efficacy of the pair in early high-risk outpatient disease. Five studies, including two controlled clinical trials, have demonstrated significant major outpatient treatment efficacy. Hydroxychloroquine+azithromycin has been used as standard-of-care in more than 300,000 older adults with multicomorbidities, with estimated proportion diagnosed with cardiac arrhythmias attributable to the medications 47/100,000 users, of which estimated mortality is <20%, 9/100,000 users, compared to the 10,000 Americans now dying each week. These medications need to be widely available and promoted immediately for physicians to prescribe.

Keywords: Azithromycin; Covid-19; Doxycycline; Hydroxychloroquine; Remdesivir; SARS-CoV-2; Zinc

Abbreviations: AZ, azithromycin; CDC, US Centers for Disease Control; FAERS, FDA Adverse Events Reporting System database; FDA, US Food and Drug Administration; HCQ, hydroxychloroquine; NIH, US National Institutes of Health; QTc, corrected electrocardiogram Q-T-wave duration; RCT, randomized controlled trial; RR, relative risk; R_t, epidemic reproduction number at time *t*.

Introduction

Aside from the now more than 1.6 million Americans found through testing and public-health reporting to be infected with SARS-CoV-2, seropositivity studies in California (1, 2), Colorado (3) and New York City and State (4) suggest that some 10-50-fold larger numbers of people carry antibodies to the virus. The workforce and effort required to carry out contact-tracing on these tens of millions of Americans is not practical. While these studies have generated some media criticism, recent similar studies of blood donor samples in the Netherlands found 3% with SARS-CoV-2 antibodies (5), and 5% among household volunteers in Spain (6). Even allowing for some degree of false-positivity of these antibody tests, they still indicate that appreciably larger fractions of the population have been infected than have been characterized by identified reported cases. "Flattening the curve," by social distancing, mask wearing and staying at home, serves to reduce hospital loads and spread them out over time, but to-date has pushed infection reproduction numbers R_t down only to about 1.0 (7), thus even if maintained, over time very large numbers of people in the US may eventually get the infection. The great majority of infected people are at low risk for progression or will manifest the infection asymptomatically. For the rest, outpatient treatment is required that prevents disease progression and hospitalization. Exposures will occur as isolation policies are lifted and people begin to mix, even with various degrees of public isolation such as mask usage and physical separation still in place. Thus, *the key to returning society toward normal functioning and to preventing huge loss of life*, especially among older individuals, people with comorbidities, African Americans and Hispanics and Latinos, *is a safe, effective and proactive outpatient treatment that prevents hospitalization in the first place*.

All treatments have costs and benefits. In an ideal world, randomized double-blinded controlled clinical trials establish evidence for the relative degree of benefit, and if large enough, for estimates of the frequencies of adverse events. These trials take time to conduct: to get formal approval, to get funding, to enroll enough eligible patients, to wait for the outcomes to occur, and to analyze the data. In the context of the Covid-19 pandemic, we are presently averaging about 10,000 deaths per week in the US, under moderately strong isolation policies that have put more than 36 million people out of work. Results of currently ongoing or planned randomized trials for use of a number of outpatient medications are many weeks or months off, and there are no guarantees that the results for these agents, even if statistically significant, will show sufficient magnitudes of effectiveness to be useful clinically. We are rapidly reaching a breaking point in the ability to maintain the status quo; states have begun the process of lifting their restrictions, and we thus need to evaluate what evidence we do have for promising outpatient treatments.

Review of Evidence

Based on laboratory and other preliminary evidence to-date, among many others, two candidate medication regimens have been widely discussed for outpatient treatment: remdesivir (Gilead Sciences, Inc., Foster City, California), and hydroxychloroquine (HCQ) plus azithromycin (AZ). Remdesivir has been studied extensively in laboratory work and in animals (8) and for other viral diseases and has good biological properties, suggesting utility for SARS-CoV-2 infection. In a study of remdesivir compassionate use in 53 hospitalized patients with severe disease (9), 13% died, which appears lower than what might have been expected without treatment, though greater than the deaths in the placebo arm of the Adaptive COVID-19

Treatment Trial (more below). In a randomized, controlled but relatively underpowered trial in severe non-ventilated hospitalized patients in China (10), benefit vs placebo was not able to be shown either in improvement or mortality. An appreciable fraction of the remdesivir patients left the trial early because of serious adverse events. The Adaptive COVID-19 Treatment Trial of hospitalized patients with advanced lung disease has released initial results (11) showing that patients on remdesivir had 31% faster recovery than patients on placebo, medians 11 vs 15 days, which difference was statistically significant, but these results involve patients who did indeed survive. Mortality of the two groups, 8.0% vs 11.6%, respectively, was better for remdesivir but not significantly so (*P*-value=.059). More specific for consideration here, remdesivir has not been studied in outpatient use. The Scientists to Stop Covid-19 "secret" Report (12, p. 7) recommends widespread use of remdesivir, and "as early in infection as possible," but no actual evidence as yet shows in humans that it would be helpful for routine outpatient circumstances and disease. The FDA recently approved use of remdesivir in the current public-health emergency circumstances (13), but only for patients with "severe disease defined as SpO2≤94% on room air, requiring supplemental oxygen, mechanical ventilation, or extracorporeal membrane oxygenation (ECMO)" and "administered in an in-patient hospital setting via intravenous (IV) infusion by a healthcare provider." This approval seems specifically not to allow outpatient use. Symptomatic outpatient infection is a pathologically and clinically different disease than the life-threatening inpatient acute respiratory distress syndrome caused by SARS-CoV-2, thus there is little reason to think that the same treatment would be useful for both (14). In any event, none of 20 currently registered trials is scheduled to provide data on outpatient use of remdesivir, thus we may not know whether it could be used effectively to prevent hospitalization of symptomatic outpatients unless or until it is actually tried that way.

The other suggestion is the combined regimen of HCQ+AZ (or its variant HCQ+doxycycline). The FDA has recently issued guidance (15) to physicians and the general public advising that the combination HCQ+AZ should not generally be used except by critically ill hospital inpatients or in the context of registered clinical trials. The NIH panel for Covid-19 treatment guidelines say essentially the same (16), and a similar statement has been released by the major cardiology societies (17). Numerous reviews of HCQ efficacy and adverse events have been and continue to be published. To my knowledge, *all of these reviews have omitted the two critical aspects of reasoning about these drugs: use of HCQ combined with AZ or with doxycycline, and use in the outpatient setting*. For example, the Veterans' Administration Medical Centers study (18) examined treated *hospitalized* patients and was fatally flawed (19). The same point about outpatient use of the combined medications has been raised by a panel of distinguished French physicians (20) in petitioning their national government to allow outpatient use of HCQ+AZ. It appears that the FDA, NIH and cardiology society positions have been based upon theoretical calculations about potential adverse events and from measured physiologic changes rather than from current real-world mortality experience with these medications and that their positions should be revised. In reviewing all available evidence, I will show that HCQ+AZ and HCQ+doxycycline are generally safe for short-term use in the early treatment of most symptomatic high-risk outpatients, where not contraindicated, and that they are effective in preventing hospitalization for the overwhelming majority of such patients. If these combined medications become standard-of-care, they are likely to save an enormous number of lives that would otherwise be lost to this endemic disease.

What is the evidence for these assertions? Similar to remdesivir, 16 clinical trials of HCQ+AZ are listed in the ClinicalTrials.gov database (21). Of these, only five involve treating

outpatients with the combined HCQ+AZ regimen (Web Table 1). For the earliest trial, between now and September, assuming a flat epidemic curve of 10,000 deaths per week, I estimate that approximately 180,000 more deaths will occur in the US before the trial results are known. The CDC has estimated substantially greater numbers of deaths (22).

In this context, we cannot afford the luxury of perfect knowledge and must evaluate, now and on an ongoing basis, the evidence for benefit and risk of these medications (23). Available evidence of efficacy of HCQ+AZ has been repeatedly described in the media as "anecdotal," but most certainly is not. The evidence is not perfect either. Each piece of evidence, contained in each study, must be carefully considered and not dismissed because in an ideal world such evidence would fall in a lower part of the evidence-quality triangle. Furthermore, and most critical to the correct understanding of what evidence is available, *evidence for single agents cannot be extrapolated to apply to combined agents, evidence for one biochemical form of a drug cannot be extrapolated to another form, and even more importantly, evidence for utility or lack thereof or toxicity in hospitalized patients cannot be extrapolated to apply to outpatient use*, outpatient use comprising the sole argument for application that I am making in this review.

Thus for example, studies of chloroquine or HCQ used alone do not bear upon evidence for efficacy of HCQ+AZ or HCQ+doxycycline. This point has been argued forcefully by the French doctors (20). The first study of HCQ+AZ (24) was controlled but not randomized or blinded, and involved 42 patients in Marseilles, France. This study showed a 50-fold benefit of HCQ+AZ vs standard-of-care, with *P*-value=.0007. In the study, six patients progressed, stopped medication use and left the trial before the day-6 planned outcome measure of swab-sampled nasopharyngeal viral clearance. Reanalysis of the raw study data elsewhere (25) and by myself shows that including these six patients does not much change the 50-fold benefit. What

does change the magnitude of benefit is presentation with asymptomatic or upper respiratory-tract infection, vs lower respiratory-tract infection, the latter cutting the efficacy in half, 25-fold vs standard-of-care. This shows that the sooner these medications are used, the better their effectiveness, as would be expected for viral early respiratory disease. The average start date of medication use in this study was day-4 of symptoms. This study has been criticized on various grounds that are not germane to the science, but the most salient criticism is the lack of randomization into the control and treatment groups. This is a valid general scientific criticism, but does not represent epidemiologic experience in this instance. If the study had shown a 2-fold or perhaps 3-fold benefit, that magnitude of result could be postulated to have occurred because of subject-group differences from lack of randomization. However, the 25-fold or 50-fold benefit found in this study is not amenable to lack of randomization as the sole reason for such a huge magnitude of benefit. Further, the study showed a significant, 7-fold benefit of taking HCQ+AZ over HCQ alone, *P*-value=.035, which cannot be explained by differential characteristics of the controls, since it compares one treatment group to the other, and the treated subjects who received AZ had more progressed pneumonia than the treated subjects receiving HCQ alone, which should otherwise have led to worse outcomes. The study has also been described as "small," but that criticism only applies to studies not finding statistical significance. Once a result has exceeded plausible chance finding, greater statistical significance does not contribute to evidence for causation (26). No different conclusion would have resulted had a study with 1000 patients found the same 50-fold benefit but with a *P*-value of 10^{-10}. Study size limitation only applies to studies having findings within the play of chance. That is not the case here.

A second study of the Marseilles group (27) involved 1061 patients tested positive for SARS-CoV-2 and treated with HCQ+AZ for at least 3 days and followed for at least 9 days. The authors state "No cardiac toxicity was observed." Good clinical outcome and virological cure were seen in 973 patients (92%). Five patients died, and the remainder were in various stages of recovery.

The third piece of evidence involves the cohort of 1450 patients treated by Dr. Vladimir Zelenko of Monsey, NY. Dr. Zelenko has released a two-page report (28) describing his clinical reasoning and procedures, dosing conditions and regimen, and patient results through April 28. Symptomatic patients presenting to Dr. Zelenko were treated with five days of HCQ+AZ+zinc sulfate if they were considered high-risk, as evidenced by one or more of: age 60 years or older; high-risk comorbidities; body-mass index>30; mild shortness of breath at presentation. Patients were considered to have Covid-19 based on clinical grounds and started treatment as soon as possible following symptom onset, rather than delaying for test results before starting treatment. Of the 1450 patients, 1045 were classified as low-risk and sent home to recuperate without active medications. No deaths or hospitalizations occurred among them. Of the remaining 405 treated with the combined regimen, 6 were ultimately hospitalized and 2 died. No cardiac arrhythmias were noted in these 405 patients.

The fourth relevant study was a controlled non-randomized trial of HCQ+AZ in 636 symptomatic high-risk outpatients in São Paulo, Brazil (29). All consecutive patients were informed about the utility and safety profile of the medications and offered the treatment, and those who declined (n=224) comprised the control group. Patients were monitored daily by telemedicine. The study outcome was need for hospitalization, defined as clinically worsening condition or significant shortness of breath (blood oxygen saturation <90%). Even though the

severities of all of the recorded flu-like signs and symptoms and of important comorbidities (diabetes, hypertension, asthma, stroke) were substantially greater in the treated patients than the controls, the need for hospitalization was significantly lower, 1.2% in patients starting treatment before day 7 of symptoms, 3.2% for patients starting treatment after day 7, and 5.4% for controls, *P*-value<.0001. No cardiac arrhythmias were reported in the 412 treated patients. The most common side effect of treatment was diarrhea (16.5%), but 12.9% of treated patients presented with diarrhea before treatment began.

Finally, a small study is ongoing in a long-term care facility in Long Island, NY. This study has been employing HCQ+doxycycline rather than HCQ+AZ for treatment of high-risk Covid-19 patients. Doxycycline itself has antiviral activity against SARS-CoV-2 at in vitro concentrations 5.6μM median (30). Among the first 54 residents treated in the Long Island study, 6 were hospitalized and 3 (5.6%) died (31). An unofficial update of these data indicates that of about 200 high-risk patients treated with HCQ+doxycycline, 9 (4.5%) have died.

The two non-randomized but controlled trials provide important evidence, if not "proof," for the major efficacy of early use of HCQ+AZ against SARS-CoV-2 infection in symptomatic high-risk outpatients. What can be said about the uncontrolled large case series of treated patients? Standard published case reports provide clinical evidence of the possibility of an exposure-outcome relationship, but not of the regularity, magnitude or representativeness of such a relationship. The same can be said of case series reports, meaning that subject entry into the series is not necessarily well-defined and no denominator information is provided from which to gauge what the series represents. However, a large series in the context of known risks of mortality or adverse events can allow for ballpark estimates of the denominator and thus provide a reasonable frame of reference for whether the outcomes likely represent beneficial or harmful

results. For example, among Connecticut cases 60 years of age or older, at present the mortality is 20% (32). Thus, it would be ballpark to estimate that some 20% of the 1466 treated high-risk patients in the Zelenko and Marseilles cohorts would have died without outpatient HCQ+AZ treatment, 293 patients, compared to the 7 who did die. An alternative is to use the 12-13% mortality of hospitalized patients in the placebo arms of the remdesivir trials (10, 11). This would give about 180 expected deaths.

Adverse Events

Both proposed drug regimens have shown side effects. Remdesivir, in its phase-3 trial of 10-day vs 5-day therapeutic courses in hospitalized patients, produced a range of adverse events in more than 70% of patients in both treatment arms (33). Adverse events requiring medication discontinuation were many fewer, 5% in the 5-day group and 10% in the 10-day group. In the Chinese trial, 12% of remdesivir patients stopped the medication before the end of the 10-day treatment because of drug-related adverse events (10).

For HCQ+AZ use, the argued issue concerns fatal cardiac arrhythmias: the warnings issued by the FDA, the NIH and the cardiology societies. Indeed, both HCQ and AZ produce QT prolongation, rare instances of fatal Torsades de Pointes and long QT-interval syndrome. A number of essays by cardiologists published in *JAMA* and other journals have anxiously warned about these risks, but have not examined mortality from them. The sole question is whether these fatal events, or even any fatal cardiac arrhythmia events, would occur with enough frequency that general treatment of non-contraindicated high-risk outpatients by HCQ+AZ would outweigh benefit in preventing hospitalization and mortality. A number of studies have examined hospital inpatient use, but these studies have had major flaws discussed at length in the

literature, not least of which is that patients hospitalized with multiple medical problems and more-advanced disease do not represent the mortality experience of outpatient use of these medications in patients otherwise well enough not to be hospitalized. One source of data on mortality associated with these medications is the FDA FAERS database (34). Examination of the database for adverse events reported from the beginning of the database in 1968 through 2019 and into the beginning of 2020, shows for hydroxychloroquine 1064 adverse event reports including 200 deaths for the total of cardiac causes that could be both specifically and broadly classified as rhythm-related. Of these, 57 events including 10 deaths were attributed to Torsades de Pointes and long QT-interval syndrome combined. This concerns the entirety of HCQ use over more than 50 years of data, likely millions of uses and of longer-term use than the 5 days recommended for Covid-19 treatment. For AZ use, the numbers of reported Torsades de Pointes and long QT-interval syndrome events total 37, of which 2 deaths. FAERS data are generated by patient, physician and pharmacist report initiation and likely underrepresent true event occurrences. However, even if the true numbers were 10-fold larger, they would still be minuscule compared to the amounts of medication usage. How much the risk of QT prolongation would be enhanced with HCQ and AZ taken together is unknown, but the Physicians' Desk Reference (35) says that coadministration of these medications risks "additive QT prolongation." Not multiplicative. "Pharmacokinetic drug interactions associated with the highest risk of TdP include antifungal agents, macrolide antibiotics (except azithromycin)" (36, p. 139). Nevertheless, even if the combined HCQ+AZ produced a 10-fold higher incidence of fatal Torsades de Pointes and long QT-interval syndrome than either agent alone, and even if both events were 10-fold underreported in FAERS, thus hypothetically giving 1200 fatal events, that would still be very small compared to the millions of uses of these medications that the

FAERS database represents. Therefore, while it is established that HCQ+AZ lengthens the QTc interval by 18-55ms on average (37-40), in 40, 84, 90 and 98 hospitalized severely ill patients in the four studies, respectively, treated with these medications and having this lengthening, a total of one case of Torsade de Pointes occurred and it was not fatal—there were no deaths. Substantial fractions of these hospitalized patients were taking diuretics, which may be contraindicated for HCQ+AZ use in the first place. This arrhythmia issue is a real, physiologically measurable effect of the use of these combined medications, but fatal arrhythmia outcomes are so rare that they are of much lesser clinical significance than the hospitalization and mortality that the drugs prevent. This fact is also clear from the lack of any cardiac arrhythmia events or arrhythmia mortality noted in the 405 Zelenko patients or the 1061 Marseilles patients or the 412 Brazil patients. Patients were not enrolled in these studies if they had known histories of QTc prolongation. History of cardiac arrhythmia or other possible contraindications for use of HCQ or AZ or doxycycline is a normal part of workup and clinical judgement in physician choice to use these medications and how to monitor the patients (see Web Appendix).

Further evidence of the real-world unimportance of arrhythmia and other cardiovascular adverse event endpoints of HCQ+AZ use is given in the large Oxford-based record-linkage study (41, 42). Fourteen large medical-records databases were examined for all-cause mortality and for 15 specified classes of adverse events among hundreds of thousands of patients with rheumatoid arthritis who had used these drugs. First, 323,122 users of HCQ+AZ were compared to 351,956 users of HCQ+amoxicillin. No significant difference in all-cause mortality was seen: as reported by the authors, relative risk (RR)=1.36, *P*-value=.10, and as I calculate from the data provided by the authors in their supplement to the paper (42), RR=1.18, *P*-value=.37; either way,

a null association within the range of chance. However, the authors selectively presented from among the 15 analyzed endpoints the three most significant associations: cardiovascular mortality RR=2.19, *P*-value=.0088; chest pain/angina RR=1.15, *P*-value=.0027; and heart failure RR=1.22, *P*-value=.027. What is misrepresented in the authors' presentation of these data in this way is that these three outcomes were not individually specified to be of more interest than any of the other 12 specific outcomes that they examined, and they did not correct their calculated levels of statistical significance for the 15 classes of outcomes. In lay terms, a fishing expedition. When accounting is done, by the standard Bonferroni correction of multiple comparisons, the respective *P*-values are .12, .040 and .35. The large amount of data in this study thus shows that there is no significant relationship of HCQ+AZ use vs HCQ+amoxicillin use for any of the 15 outcomes specified or for all-cause mortality, except a just-barely significant association with chest pain/angina, with a 15% higher risk which even if a true finding would still be of little clinical import for a relatively infrequent outcome in the context of the mortality to be saved by HCQ+AZ use in widespread symptomatic high-risk outpatient Covid-19 treatment.

Second, the stated concern of the FDA and NIH advisories and the cardiology society opinion restricting use of HCQ+AZ was for fatal Torsades de Pointes and long QT-interval syndrome, two rare types of cardiac arrhythmias, as well as for cardiac arrhythmias in general. The Oxford study (41, 42) examined cardiac arrhythmia outcomes and obtained for its random effects meta-analysis result, RR=1.08, *P*-value=.36 for HCQ+AZ use vs HCQ+amoxicillin use. The fixed-effects meta-analysis RR=1.04, *P*-value=.41. This study clearly demonstrates that cardiac arrhythmia adverse events are not appreciably increased by combining HCQ with AZ. The same study compared HCQ use to sulfasalazine use and again found no difference in cardiac

arrhythmia risk: for HCQ, a slightly lower RR=0.89, *P*-value=.13. The subjects analyzed in the Oxford study were largely older adults with multiple comorbidities in addition to rheumatoid arthritis.

Finally, the Oxford study allows for a direct estimate of the number of arrhythmia events attributable to HCQ+AZ use (41, 42). Among 306,106 people taking sulfasalazine (which is known not to produce QT prolongation), 877 with cardiac arrhythmias were identified, 0.287%. In 320,589 people taking HCQ+AZ, 1,068 had arrhythmias, 0.333%. The difference, 0.047% or 47/100,000 older multicomorbidity patients taking HCQ+AZ, is attributable to the HCQ+AZ use. These are events, not fatalities. As noted above, fatalities according to FAERS comprise <20% of HCQ-related arrhythmia events. The maintenance HCQ dose in the Oxford study patients, 200 mg/day, gives as large or larger plasma drug levels as five days of HCQ at 400 mg/day, the recommended dose for outpatient Covid-19. These very small numbers of arrhythmias, as well as the null results in this very large empirical study should therefore put to rest the anxieties about population excess mortality of HCQ+AZ outpatient use, either from cardiac arrhythmias, or as mortality from all causes.

This discussion thus shows that the FDA, NIH and cardiology society warnings about cardiac arrhythmia adverse events, while appropriate for theoretical and physiological considerations about use of these medications, are not borne out in mortality in real-world usage of them. Treatment-failure mortality will be much higher, but even that pales in comparison to the lives saved. It would therefore be incumbent upon all three organizations to reevaluate their positions as soon as possible. It is unclear why the FDA, NIH and cardiology societies made their recommendations about HCQ+AZ use now, when the Oxford study (41, 42) analyzed 323,122 users of HCQ+AZ compared to 351,956 users of HCQ+amoxicillin, i.e., that the

combination of HCQ+AZ has been in widespread standard-of-care use in the US and elsewhere for decades, use comparable to HCQ+amoxicillin as if it just involved an alternate antibiotic choice, this use predominantly in older adults with multiple comorbidities, with no such strident warnings about the use given during that time. I note that since doxycycline is believed to cause even fewer cardiac arrhythmias than AZ, in patients where that is a concern (43), the long-term care-facility evidence suggests that HCQ+doxycycline likely will work about as well.

Discussion

Given that a detailed and dispassionate review of all of the available relevant evidence leads to conclusions about outpatient HCQ+AZ use different than those of the FDA and NIH panels (which comprise wider expertise than the cardiology societies), I address how different underlying scientific worldviews might be involved. This is particularly reflected in the Scientists to Stop Covid-19 position about remdesivir use "as early as possible," i.e., early outpatient use implied (12, p. 5). All but one of the scientists on the Scientists to Stop Covid-19 panel are laboratory or clinical scientists; only one is an epidemiologist. Their recommendation for remdesivir use as early as possible was made without either FDA approval or RCT evidence of efficacy in the outpatient context. This recommendation therefore appears to be an extrapolation from animal and laboratory data and from use in severely ill hospitalized patients. However, a history of epidemiology shows numerous instances of failed extrapolation from animals to humans. "Animal research on almost any topic of epidemiologic interest is so heterogeneous and inadequately synthesized that it is possible to selectively assemble a body of evidence from the animal and in-vitro studies that support almost any epidemiologic result." (44, p. 221) For example, some carcinogens have been affirmed in animal studies but not shown in

human studies (acrylamide, alar, cyclamate, red dye #2, saccharin) (44). This is in part why the FDA has an approval system of phased RCTs leading to safety and efficacy of use in humans, *in the specific contexts* in which the drug is intended. It is not a question of off-label use, but of who are the patients for which to use the medication. For Covid-19, inpatient acute respiratory distress syndrome is typically a florid immune-system overreaction, whereas initial outpatient illness is a viral multiplication problem involving the beginnings of immune response. These are different diseases. Thus, how well remdesivir might perform in outpatients won't be known until it is tried in typical outpatient circumstances, whether in RCTs or in any other unbiased systematic study of such use. Further, to the degree that remdesivir is similar in temporal characteristics to an antiviral like Tamiflu, it would be used in general societal contexts where patients must first recognize that they might have symptoms of the disease and not something else and go to their physicians or clinics for care, and either be rapidly tested positive with an assay that has negligible false negatives, or be symptomatic enough for the disease to be clinically distinguished and diagnosed, but definably positive in this way not more than two days after symptoms start. This is a very narrow temporal window to be definitive and to obtain full antiviral effectiveness, and could be difficult to achieve in general in the mass-treatment circumstances that we are facing. So regardless of the strength of the *implied* evidence of outpatient efficacy when given shortly after the start of symptoms, remdesivir efficacy might be substantially less in the context of actual population outpatient usage. This is another reason why empirical studies of medication use in the full context of application are needed.

The extrapolation from laboratory theory to empirical use also seems to underlie resistance to the idea that combined HCQ regimens could work for early outpatient use. HCQ is known to interfere with toll-like receptor signaling, reducing dendritic cell activation and

immune response. This would seem to be counterproductive for suppressing SARS-CoV-2 multiplication in early treatment. Again, in extrapolation from physiologic theory to human data, the epidemiologic data are definitive. The fact that epidemiologic data to-date show strong evidence for efficacy of combined HCQ+AZ in early outpatient treatment, even if not "proof" yet at the level of several successful RCTs, is evidence that this medication regimen works in that context. The clash in scientific worldviews is that basic and clinical scientists seem to feel that biological and drug-development evidence for medication use in non-human and non-outpatient contexts can be extrapolated to recommendations for outpatient use without benefit of RCT evidence but don't accept epidemiologic evidence without RCTs, whereas epidemiologists have had career experience with laboratory and animal evidence that did not hold up under epidemiologic study, but do reason by including all types of epidemiologic study designs and derive causal conclusions in the standard way following Hill's Aspects (26) on the basis of strong totality of evidence, sometimes even without RCT evidence. There are contexts where each approach is valid. However, it is not my point to say that remdesivir has little evidence to support its potential outpatient utility, only efficacy considerations that have not been addressed and that could lead to lack of efficacy under general use, but that HCQ+AZ has been directly studied in actual early high-risk outpatient use with all of its temporal considerations and found empirically to have sufficient epidemiologic evidence for its effective and safe employment that way, and that requiring delay of such general use until availability of additional RCT evidence is untenable because of the ongoing and projected continuing mortality. No studies of Covid-19 outpatient HCQ+AZ use have shown higher mortality with such use than without, cardiac arrhythmias included, thus there is no empirical downside to this combined medication use.

Some of my medical colleagues still prefer to wait until more studies are done and stronger evidence such as from RCTs becomes available, and government and professional advisory panels do reevaluate the evidence. I strongly urge these panels to reconsider the data and arguments discussed above. Substantial fractions of physicians treating Covid-19 patients in Europe and elsewhere report use of HCQ+AZ: 72% in Spain, 49% in Italy, 41% in Brazil, 39% in Mexico, 28% in France, 23% in the US, 17% in Germany, 16% in Canada, 13% in the UK (45), much of the non-US use in outpatients. HCQ+AZ has been standard-of-care treatment at the four New York University hospitals, where a recent study showed that adding zinc sulfate to this regimen significantly cut both intubation and mortality risks by almost half (46). The French physicians are insistent that with careful clinical judgement and supervision, these medications are safe and should be used as early as possible for outpatients, and they provide a detailed clinical guide to their use (20). Until we have quantitative evidence for the utility and safety of other medications for preventing hospitalization and mortality in high-risk Covid-19 outpatients, the urgency of current mass mortality requires an immediate application of the best that we have available, even if knowledge is imperfect and even if yet unproven to the standards of double-blinded RCTs. This problem will get even worse as states and cities yield to the acute pressure at this moment to begin lifting stay-at-home restrictions and even more people become infected. Some people will have contraindications and will need other agents for treatment or to remain in isolation. But for the great majority, I conclude that HCQ+AZ and HCQ+doxycycline, preferably with zinc (47) can be this outpatient treatment, at least until we find or add something better, whether that could be remdesivir or something else. It is our obligation not to stand by, just "carefully watching," as the old and infirm and inner city of us are killed by this disease and our economy is destroyed by it and we have nothing to offer except high-mortality hospital

treatment. We have a solution, imperfect, to attempt to deal with the disease. We have to let physicians employing good clinical judgement use it and informed patients choose it. There is a small chance that it may not work. But the urgency demands that we at least start to take that risk and evaluate what happens, and if our situation does not improve we can stop it, but we will know that we did everything that we could instead of sitting by and letting hundreds of thousands die because we did not have the courage to act according to our rational calculations.

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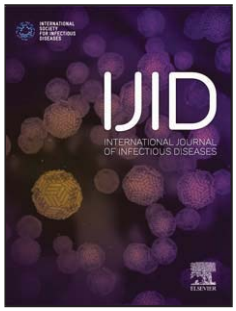

Treatment with Hydroxychloroquine, Azithromycin, and Combination in Patients Hospitalized with COVID-19

Samia Arshad, Paul Kilgore, Zohra S. Chaudhry, Gordon Jacobsen, Dee Dee Wang, Kylie Huitsing, Indira Brar, George J. Alangaden, Mayur S. Ramesh, John E. McKinnon, William O'Neill, Marcus Zervos, Henry Ford COVID-19 Task Force<ce:author-group id="aug0010">, Varidhi Nauriyal, Asif Abdul Hamed, Owais Nadeem, Jennifer Swiderek, Amanda Godfrey, Jeffrey Jennings, Jayna Gardner-Gray, Adam M Ackerman, Jonathan Lezotte, Joseph Ruhala, Raef Fadel, Amit Vahia, Smitha Gudipati, Tommy Parraga, Anita Shallal, Gina Maki, Zain Tariq, Geehan Suleyman, Nicholas Yared, Erica Herc, Johnathan Williams, Odaliz Abreu Lanfranco, Pallavi Bhargava, Katherine Reyes, Anne Chen

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Authors:

Samia Arshad, MPH[1*], Paul Kilgore, MD[2-3], Zohra S. Chaudhry, MD[1,*], Gordon Jacobsen, MS[10], Dee Dee Wang, MD[4], Kylie Huitsing, BS[1], Indira Brar, MD[1,*], George J. Alangaden, MD[1,3,*], Mayur S. Ramesh, MD[1,*], John E. McKinnon, MD[1], William O'Neill MD[4], Marcus Zervos, MD[1,3,*], Henry Ford COVID-19 Task Force[*]

[*]Henry Ford Covid-19 Task Force: Varidhi Nauriyal, MD[1,5], Asif Abdul Hamed MD[5], Owais Nadeem, MD[5], Jennifer Swiderek, MD[5], Amanda Godfrey, MD[5], Jeffrey Jennings, MD[5], Jayna Gardner-Gray, MD[6], Adam M Ackerman, MD[7], Jonathan Lezotte, DO[7], Joseph Ruhala, DO[7], Raef Fadel, DO[8], Amit Vahia, MD, MPH[1], Smitha Gudipati, MD[1], Tommy Parraga, MD[1], Anita Shallal, MD[1], Gina Maki, DO[1], Zain Tariq, MD[1], Geehan Suleyman, MD[1;] Nicholas Yared, MD[1], Erica Herc, MD[1;] Johnathan Williams, MD[1], Odaliz Abreu Lanfranco, MD[1], Pallavi Bhargava, MD[1], Katherine Reyes, MD, MPH[1], Anne Chen, MD[1]

[1]Infectious Diseases, Henry Ford Hospital, Detroit, MI

[2]Eugene Applebaum College of Pharmacy, Wayne State University, Detroit MI

[3]Wayne State University School of Medicine, Detroit, MI

[4]Division of Cardiovascular Disease & Structural Heart, Henry Ford Hospital, Detroit, MI

[5]Pulmonary Medicine, Henry Ford Hospital, Detroit, MI

[6]Emergency Medicine, Henry Ford Hospital, Detroit, MI

[7]Surgical Critical Care, Henry Ford Hospital, Detroit, MI

[8]Internal Medicine, Henry Ford Hospital, Detroit, MI

[9]Nephrology and Hypertension Division, Henry Ford Hospital, Detroit, MI

[10]Public Health Sciences, Henry Ford Hospital, Detroit, MI

Corresponding author

Marcus J. Zervos, M.D.

Division Head, Division of Infectious Diseases, Henry Ford Hospital

Professor of Medicine, and Assistant Dean of Global Affairs

Wayne State University School of Medicine

2799 West Grand Blvd, CFP 302

Detroit, MI 48202.

E-mail: MZervos1@hfhs.org

313 916 2573

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Highlights:

- As of May27, 2020 there are over 1,678,843 confirmed cases of COVID-19 claiming more than 100,000 lives in the Unites States. Currently there is no known effective therapy or vaccine.
- -According to a protocol-based treatment algorithm, among hospitalized patients, use of hydroxychloroquine alone and in combination with azithromycin was associated with a significant reduction in-hospital mortality compared to not receiving hydroxychloroquine.
- -Findings of this observational study provide crucial data on experience with hydroxychloroquine therapy, providing necessary interim guidance for COVID-19 therapeutic practice.

Abstract:

Significance: The United States is in an acceleration phase of the COVID-19 pandemic. Currently there is no known effective therapy or vaccine for treatment of SARS-CoV-2, highlighting urgency around identifying effective therapies.

Objective: The purpose of this study was to evaluate the role of hydroxychloroquine therapy alone and in combination with azithromycin in hospitalized patients positive for COVID-19.

Design: Multi-center retrospective observational study

Setting: The Henry Ford Health System (HFHS) in Southeast Michigan: large six hospital integrated health system; the largest of hospitals is an 802-bed quaternary academic teaching hospital in urban Detroit, Michigan.

Participants: Consecutive patients hospitalized with a COVID-related admission in the health system from March 10,2020 to May 2,2020 were included. Only the first admission was included for patients with multiple admissions. All patients evaluated were 18 years of age and older and were treated as inpatients for at least 48 hours unless expired within 24 hours.

Exposure: Receipt of hydroxychloroquine alone, hydroxychloroquine in combination with azithromycin, azithromycin alone, or neither.

Main Outcome: The primary outcome was in-hospital mortality.

Results: Of 2,541 patients, with a median total hospitalization time of 6 days (IQR: 4-10 days), median age was 64 years (IQR:53-76 years), 51% male, 56% African American, with median time to follow-up of 28.5 days (IQR:3-53). Overall in-hospital mortality was 18.1% (95% CI:16.6%-19.7%); by treatment: hydroxychloroquine+azithromycin, 157/783 (20.1% [95% CI: 17.3%-23.0%]), hydroxychloroquine alone, 162/1202 (13.5% [95% CI: 11.6%-15.5%]), azithromycin alone, 33/147 (22.4% [95% CI: 16.0%-30.1%]), and neither drug, 108/409 (26.4% [95% CI: 22.2%-31.0%]). Primary cause of mortality was respiratory failure (88%); no patient had documented torsades de pointes. From Cox regression modeling, predictors of mortality were age ≥65 years (HR:2.6 [95% CI:1.9-3.3]), white race (HR:1.7 [95% CI:1.4-2.1]), CKD (HR:1.7 [95%CI:1.4-2.1]), reduced O2 saturation level on admission (HR:1.5 [95%CI:1.1-2.1]), and ventilator use during admission (HR: 2.2 [95%CI:1.4-3.3]). Hydroxychloroquine provided a 66% hazard ratio reduction, and hydroxychloroquine+azithromycin 71% compared to neither treatment ($p<0.001$).

Conclusions and Relevance: In this multi-hospital assessment, when controlling for COVID-19 risk factors, treatment with hydroxychloroquine alone and in combination with azithromycin was associated with reduction in COVID-19 associated mortality. Prospective trials are needed to examine this impact.

Introduction

As of May 27, 2020, there were over 1,678,843 confirmed cases of COVID-19 claiming more than 100,000 lives in the Unites States.[1] Currently there is no known effective therapy or vaccine. The urgent need for therapeutic agents has resulted in repurposing and redeployment of experimental agents.[2,3]

Hydroxychloroquine, an antimalarial and immunomodulatory agent and a safer analogue of chloroquine, has demonstrated antiviral activity against SARS-CoV-2.[4-7] It is postulated to exert a direct antiviral activity by increasing intracellular pH resulting in decreased phago-lysosome fusion, impairing viral receptor glycosylation. In addition, it has immune-modulating effect by inhibiting toll-like receptor signaling, decreasing production of cytokines especially IL-1 and IL-6.[8] Prior data also suggests a potential anti-thrombotic effect.[9] Azithromycin, a macrolide antibiotic has *in vitro* antiviral properties such as decreased viral replication, blocking entrance into host cells, and a potential immunomodulating effect.[10] An *in vitro* study demonstrated synergistic activity of the combination of hydroxychloroquine and azithromycin against SARS-CoV-2 .[11] A small non-randomized, open-label trial from France reported higher frequency of SARS-CoV-2 clearance after six days of treatment with hydroxychloroquine alone or hydroxychloroquine in combination with azithromycin versus untreated control group (70% vs 12.5%; $P < 0.001$).[12] Other early studies of hydroxychloroquine have reported conflicting results.[13-22] The US FDA as of June 15, 2020 has revoked the prior emergency use authorization (EUA) to use hydroxychloroquine and chloroquine to treat COVID-19 in certain hospitalized patients when clinical trial data is unavailable or participation is not feasible.[23]

Currently, randomized trials of hydroxychloroquine for treatment and chemoprophylaxis are underway. [24-27] Based on these early reports, hydroxychloroquine alone and in combination with

azithromycin was incorporated into our institutional clinical guidelines for the treatment of hospitalized patients with COVID-19. We examined the association between hydroxychloroquine use and mortality in a large cohort of hospitalized COVID-19 patients.

Methods

SETTING

This is a comparative retrospective cohort study evaluating clinical outcomes of all consecutive patients hospitalized at the Henry Ford Health System (HFHS) in Southeast Michigan being treated for COVID-19. The organization is a large six hospital integrated health system; the largest of hospitals is an 802-bed quaternary academic teaching hospital in urban Detroit, Michigan. Approval for this study was granted by the Henry Ford Hospital IRB (#13897).

PATIENTS

Patients with a COVID-related admission in the health system from March 10, 2020 to May 2, 2020 were included. Only the first admission was included for patients with multiple admissions. All patients were hospitalized though our emergency department. A COVID-related admission was defined as hospitalization during which the patient had a positive SARS-CoV-2 test. Diagnosis with SARS-CoV-2 was confirmed by a positive reverse-transcriptase-polymerase-chain-reaction (RT-PCR) assay from a nasopharyngeal sample. All patients evaluated were 18 years of age and older and were treated as inpatients for at least 48 hours unless they expired within the time period. The primary objective was to assess treatment experience with hydroxychloroquine versus hydroxychloroquine+azithromycin, azithromycin alone, and other treatments for COVID-19. Treatments were protocol driven, uniform in all hospitals and established by a system-wide interdisciplinary COVID-19 Task Force.

Hydroxychloroquine was dosed as 400 mg twice daily for 2 doses on day 1, followed by 200 mg twice daily on days 2-5. Azithromycin was dosed as 500mg once daily on day 1 followed by 250mg once daily for the next 4 days. The combination of hydroxychloroquine+azithromycin was reserved for selected patients with severe COVID-19 and with minimal cardiac risk factors. An electrocardiogram (ECK) based algorithm was utilized for hydroxychloroquine use. QTc>500ms was considered an elevated cardiac risk and consequently hydroxychloroquine was reserved for patients with severe disease with telemetry monitoring and serial QTc checks. The clinical guidelines included adjunctive immunomodulatory therapy with corticosteroids and tocilizumab.

DATA SOURCES

The data source for analysis of patient information was derived from electronic medical records in the Electronic Information System. Study variables collected on each patient included the following; 1) patient demographics: age, gender, race, body mass index (BMI) on admission, stratified into four categories: <18.5; 18.5-24.9; 25.0-29.9 and ≥ 30; 2) clinical characteristics: admission date, discharge date, length of stay (LOS), comorbidities including: cardiovascular disease (CVD), chronic lung disease, chronic kidney disease (CKD), hypertension, asthma, chronic obstructive pulmonary disease (COPD), diabetes mellitus, immunodeficiency, and cancer (defined as active or past/resolved). Additionally, intensive care unit (ICU) status and ventilator use at any point during admission, minimum O2 saturation level collected on day of admission in the emergency department, and the maximal modified Sequential Organ Failure Assessment (mSOFA) score on admission were also collected. The mSOFA score is predictive of ICU mortality utilizing similar accuracy to the full SOFA score without substantial lab testing (ABG, LFTs) to complete.[28] The duration and dosages of all therapies for COVID-19 were

collected.

STUDY ENDPOINT

The primary endpoint was in-patient hospital mortality in each treatment group. All deaths were reviewed in detail by the study team.

STATISTICAL ANALYSIS

Demographic and clinical characteristics were descriptively summarized for all patients and subsets by treatment group, to test the null hypothesis that treatment course between hydroxychloroquine, hydroxychloroquine+azithromycin, azithromycin, and other (no hydroxychloroquine or azithromycin) were similar. Multivariable Cox regression models and Kaplan-Meier survival curves were used to compare survival among treatment groups while controlling for demographics (e.g., age, gender), preexisting medical conditions (e.g. CVD, lung disease) and clinical disease severity (mSOFA, O2 saturation). Bivariate comparisons of the 4 medication groups were made using analysis of variance or Kruskal-Wallis tests for continuous variables, and chi-square tests or Fisher exact tests for categorical variables. Additional analysis was performed using propensity score matching to compare outcomes in mortality across treatment groups. A propensity score was created for each patient based on the set of patient characteristics used in the Cox regression model. Subsequently, 1 to 1 matchups of patients given hydroxychloroquine (either hydroxychloroquine alone or in combination with azithromycin) and patients not given hydroxychloroquine based on the exact propensity score were observed. The resulting matched group status was placed into its own Cox regression model as a mortality predictor with a Kaplan-Meier plot summarizing the survival curves of the two matched groups. P values < 0.05 were considered statistically significant. Additionally, median survival times by treatment strata were calculated to approximate prognosis. No

imputations were made for missing data. All data were analyzed using SPSS software version 26 (IBM SPSS Statistics for Windows, version 26, IBM Corp., Armonk, N.Y., USA) and STATA (StataCorp. 2019. Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC), and SAS version 9.4.

Results

The first COVID-19 case confirmed at HFHS by RT-PCR was on March 10, 2020, any patients admitted before March 10th and subsequently tested positive were also included in the analyses. There was a total of 2,948 COVID-19 admissions, of these, 267 (9%) patients had not been discharged, 15 (0.5%) left against medical advice, and four (0.1%) were transferred to another healthcare facility; these patients were excluded from analysis as we could not ascertain their outcome. In addition, there were 121 (4.1%) readmissions, which were also excluded.

Overall, 2,541 consecutive patients were included in the analyses with a median age of 64 years (IQR: 53-76 years), 51% male, 56% African American, median inpatient LOS was 6 days (IQR: 4-10 days). The median time to follow-up was 28.5 days (IQR 3-53). Majority of patients (52%, n=1,250) had BMI ≥ 30. Additional underlying comorbidities are detailed in Table 1. On the day of admission, two variables predicting severity of disease and mortality: highest mSOFA score and lowest O2 saturation were recorded. However, 25% of the population did not have mSOFA scores available, as recording of this metric became institutional standard one month after the index admission. Other indicators of severity were ICU admission and mechanical ventilation status. All baseline characteristics were further stratified by the four treatment groups (hydroxychloroquine alone, hydroxychloroquine+azithromycin, azithromycin alone, and neither treatment). Median time (IQR) from admission to receipt of hydroxychloroquine was 1 day (1-

2). Overall crude mortality rates were 18.1% in the entire cohort, 13.5% in the hydroxychloroquine alone group, 20.1% among those receiving hydroxychloroquine+ azithromycin, 22.4% among the azithromycin alone group, and 26.4% for neither drug ($p < 0.001$). Adjunct therapy with corticosteroids (methylprednisolone and/or prednisone) and anti-IL-6 tocilizumab was provided in 68% and 4.5% of patients, respectively.

Primary cause of mortality in the 460 patients was: 88% respiratory failure, 4% cardiac arrest (with mean QTc interval from last ECG reading 471ms), 8% other cardiopulmonary arrest and multi-organ failure. No patient had documented torsades de pointes.

In the multivariable Cox regression model of mortality using the group receiving neither hydroxychloroquine or azithromycin as the reference, treatment with hydroxychloroquine alone decreased the mortality hazard ratio by 66% ($p<0.001$), and hydroxychloroquine+azithromycin decreased the mortality hazard ratio by 71% ($p<0.001$). We did not find statistical significance in the relative effect of adjunct therapy and mortality. Predictors of mortality were age ≥ 65 years (HR, 2.6 [95% CI: 1.9, 3.3]), white race (HR: 1.7 [95% CI: 1.4, 2.1]), CKD (HR, 1.7 [95%CI: 1.4, 2.1]), reduced O2 saturation level on admission (HR, 1.6 [95%CI: 1.1, 2.2]), and ventilator use during admission (HR, 2.2 [95%CI: 1.4, 3.0]), which were all significantly associated with mortality due to COVID-19 (Table 2).

Kaplan-Meier survival curves showed significantly improved survival among patients in the hydroxychloroquine alone and hydroxychloroquine+azithromycin group compared with groups not receiving hydroxychloroquine and those receiving azithromycin alone (Figure 1). The survival curves suggest that the enhanced survival in the hydroxychloroquine alone group persists all the way out to 28 days from admission.

Further, a total of 190 hydroxychloroquine patients exactly matched up with 190 corresponding non-hydroxychloroquine treated patients based on the exact underlying propensity score. Table 3 contains a descriptive summarization of these patients within both the unmatched and propensity matched settings, confirming that the propensity matched groups have identical underlying patient characteristics. The Cox regression result for the two propensity matched groups (table 4) indicates that treatment with hydroxychloroquine resulted in a mortality hazard ratio decrease of 51% (p=0.009). The resulting Kaplan-Meier survival curves within the propensity matched setting displayed significantly better survival in the hydroxychloroquine treated group, with the enhanced survival persisting all the way out to 28 days from admission (figure 2).

Discussion

The results of this study demonstrate that in a strictly monitored protocol-driven in-hospital setting, treatment with hydroxychloroquine alone and hydroxychloroquine + azithromycin was associated with a significant reduction in mortality among patients hospitalized with COVID-19. In this study, among one of the largest COVID-19 hospital patient cohorts (n=2,541) assembled in a single institution, overall in-hospital COVID-19 associated mortality was 18.1% reflecting a high prevalence of co-morbid conditions in COVID-19 patients admitted to our institution. The independent predictors of mortality in our study included age ≥ 65 years, CKD, and severe illness at initial presentation as measured by the oxygen saturation levels on admission, and ventilator use reflect findings similar to those reported in earlier studies.[29] These predictors also underscore the high-risk for COVID-19 experienced by residents in our hospital catchment population in Metropolitan Detroit, Michigan. Michigan is among the

states with the highest number of cases of COVID-19 and deaths. In Detroit, our residents suffer from substantial preexisting social and racial health disparities that place our patients at increased risk of severe disease and higher mortality.[1]

In the present study, multivariate analysis performed using Cox regression modeling and propensity score matching to control for potential confounders affirmed that treatment with hydroxychloroquine alone and hydroxychloroquine in combination with azithromycin was associated with higher survival among patients with COVID-19. Patients that received neither medication or azithromycin alone had the highest cumulative hazard. The benefits of hydroxychloroquine in our cohort as compared to previous studies maybe related to its use early in the disease course with standardized, and safe dosing, inclusion criteria, comorbidities, or larger cohort. The postulated pathophysiology of COVID-19 of the initial viral infection phase followed by the hyperimmune response suggest potential benefit of early administration of hydroxychloroquine for its antiviral and antithrombotic properties. Later therapy in patients that have already experienced hyperimmune response or critical illness is less likely to be of benefit. Others have shown that COVID-19 hospitalized patients are not diagnosed in the community and often rapidly deteriorate when hospitalized with fulminant illness.[30]

Limitations to our analysis include the retrospective, non-randomized, non-blinded study design. Also, information on duration of symptoms prior to hospitalization was not available for analysis. However, our study is notable for use of a cohort of consecutive patients from a multi-hospital institution, regularly updated and standardized institutional clinical treatment guidelines and a QTc interval-based algorithm specifically designed to ensure the safe use of hydroxychloroquine. To mitigate potential limitations associated with missing or inaccurate documentation in electronic medical records, we manually reviewed all deaths to confirm the

primary mortality outcome and ascertain the cause of death. A review of our COVID-19 mortality data demonstrated no major cardiac arrhythmias; specifically, no torsades de pointes that has been observed with hydroxychloroquine treatment. This finding may be explained in two ways. First, our patient population received aggressive early medical intervention, and were less prone to development of myocarditis, and cardiac inflammation commonly seen in later stages of COVID-19 disease. Second, and importantly, inpatient telemetry with established electrolyte protocols were stringently applied to our population and monitoring for cardiac dysrhythmias was effective in controlling for adverse events. Additional strengths were the inclusion of a multi-racial patient composition, confirmation of all patients for infection with PCR, and control for various confounding factors including patient characteristics such as severity of illness by propensity matching.

Recent observational retrospective studies and randomized trials of hydroxychloroquine have reported variable results.[12-22] In a randomized controlled study of 62 patients from China with COVID-19, hydroxychloroquine was associated with a shortened duration of fever and time to cough and pneumonia resolution.[17] In contrast, a study of 1376 consecutive hospitalized COVID-19 patients in New York that used respiratory failure as the primary endpoint found no significant reduction in the likelihood of death or intubation among those receiving hydroxychloroquine compared to those who did not.[19] In a separate multicenter cohort study of 1438 patients from 25 hospitals in New York, no reduction in hospitalized patient mortality was observed with hydroxychloroquine treatment.[20] Among a number of limitations, this study included patients who were initiated on hydroxychloroquine therapy at any time during their hospitalization. In contrast, in our patient population, 82% received hydroxychloroquine within the first 24 hours of admission, and 91% within 48 hours of admission. Because treatment

regimens likely varied substantially (including delayed initiation) across the 25 hospitals that contributed patients to the study, it is not surprising that the case-fatality rate among the New York patients was significantly higher than in our study.

Globally, the overall crude mortality from SARS-COV-2 is estimated to be approximately 6-7%.[1,31] Multiple descriptive studies report higher mortality in hospitalized COVID-19 patients from 10-30%.[32-40] Not surprisingly, mortality as high as 58% was observed among patients requiring ICU care and mechanical ventilation.[36,37] This high mortality associated with COVID-19 in many populations has led to a search for effective drug therapies. The randomized controlled trial of lopinavir–ritonavir in COVID-19 hospitalized patients showed a mortality of 19.2% on lopinavir–ritonavir and 25% for standard of care; therapy had to be terminated in 13.8% patients due to adverse events.[38] In the compassionate use remdesivir trial, 13% mortality was observed in the cohort of 61 patients.[39] The interim analysis randomized trial of remdesivir showed a mortality rate of 8.0% for the group receiving remdesivir versus 11.6% for the placebo group ($p = 0.059$).[40] In our study, overall mortality was 18.1% and in ICU patients 45%. Our cohort included patients with severe disease, with 24% and 18% requiring ICU care and mechanical ventilation at presentation, respectively.

Findings of this observational study provide crucial data on experience with hydroxychloroquine therapy, providing necessary interim guidance for COVID-19 therapeutic practice. These findings do support the recent NIH guidelines [24], indicating a potential role for hydroxychloroquine in treatment of hospitalized COVID-19 patients without co-administration of azithromycin. However, our results should be interpreted with some caution and should not be applied to patients treated outside of hospital settings. Our results also require further confirmation in prospective, randomized controlled trials that rigorously evaluate the safety, and

efficacy of hydroxychloroquine therapy for COVID-19 in hospitalized patients. Considered in the context of current studies on the use of hydroxychloroquine for COVID-19, our results suggest that hydroxychloroquine may have an important role to play in reducing COVID-19 mortality.

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Conflict of Interest: S.H. received speakers' bureau honoraria from Bayer. I.B. received speakers' bureau honoraria from Gilead, ViiV, and Jansssen, M.Z received consultation honoraria from contrafact. All others have no conflicts of interests.

Ethical Approval: Approval for this study was granted by the Henry Ford Hospital Institutional Review Board (#13897).

Declaration of interests

S.H. received speakers' bureau honoraria from Bayer. I.B. received speakers' bureau honoraria from Gilead, ViiV, and Jansssen, M.Z received consultation honoraria from contrafact. All others have no conflicts of interests.

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Table 1: Patient Characteristics by Treatment Group

Characteristics		Total (n=2541)	Neither Med (n=409)	HCQ Alone (n=1202)	AZM Alone (n=147)	HCQ+AZM (n=783)	P-value
Mortality, n (%)		460 (18.1)	108 (26.4)	162 (13.5)	33 (22.4)	157 (20.1)	<0.001 ***
Hospital LOS in Days, Mean ± SD, Median (IQR)		8.3 ± 6.5, 6 (4 – 10)	5.6 ± 4.8, (4 (3 – 7)	8.0 ± 5.8, 6 (4 – 10)	5.3 ± 4.5, 4 (2 – 6)	10.7 ± 7.5, 8 (5 – 14)	<0.001 ***
Age in Years, Mean ± SD, Median (IQR)		63.7 ± 16.5, 64 (53 – 76)	68.1 ± 18.9, 71 (56 – 83)	63.2 ± 15.6, 53 (64 – 74)	63.3 ± 17.3, 64 (52 – 76)	62.3 ± 15.9, 62 (51 – 74)	<0.001 ***
Age, n (%)	< 65 Years	1278 (50.3)	158 (38.6)	614 (51.1)	79 (53.7)	427 (54.5%)	<0.001 ***
	≥ 65 Years	1263 (49.7)	251 (64.1)	588 (48.9)	68 (46.3)	356 (45.5%)	
Gender, n (%)	Male	1298 (51.1)	199 (48.7)	634 (52.8)	62 (42.2)	403 (51.5%)	0.072
	Female	1243 (48.9)	210 (51.3)	568 (47.2)	85 (57.8)	380 (48.5%)	
Race, n (%)	Black	1411 (55.5)	187 (45.7)	724 (60.2)	76 (51.7)	424 (54.2%)	<0.001 ***
	White	852 (33.5)	186 (45.5)	332 (27.6)	63 (42.9)	271 (34.6%)	
	Asian/Pacific Islander	47 (1.8)	6 (1.5)	24 (2.0)	0 (0.0)	17 (2.2%)	
	Other	231 (9.1)	30 (7.3)	122 (10.1)	8 (5.4)	71 (9.1%)	
BMI, Mean ± SD, Median (IQR)		31.7 ± 8.5, 30 (26 – 36)	28.8 ± 7.6, 28 (23 – 33)	31.9 ± 8.6, 30 (26 – 36)	31.4 ± 8.7, 29 (25 – 36)	32.9 ± 8.4, 32 (27 – 37)	<0.001 ***
BMI, n (%)	<18.5	48 (2.0)	22 (5.7)	15 (1.4)	3 (2.1)	8 (1.1%)	<0.001 ***
	18.5-24.9	430 (18.0)	108 (28.2)	198 (17.9)	25 (17.5)	99 (13.1%)	
	25.0-29.9	662 (27.7)	104 (27.2)	314 (28.4)	49 (34.3)	195 (25.8%)	
	≥30.0	1250 (52.3)	149 (38.9)	580 (52.4	66 (46.2)	455 (60.1%)	
Chronic Lung Disease, n (%)		1619 (63.7)	195 (47.7)	806 (67.1)	93 (63.3)	525 (67.0)	<0.001 ***

Characteristics		Total (n=2541)	Neither Med (n=409)	HCQ Alone (n=1202)	AZM Alone (n=147)	HCQ+AZM (n=783)	P-value
Immunodeficiency, n (%)		30 (1.2)	2 (0.5)	15 (1.2)	2 (1.4)	11 (1.4)	0.502
Cardiovascular Disease, n (%)		222 (8.7)	45 (11.0)	100 (8.3)	10 (6.8)	67 (8.6)	0.306
Chronic Kidney Disease, n (%)		1099 (43.3)	196 (47.9)	528 (43.9)	62 (42.2)	313 (40.0)	0.062
COPD, n (%)		325 (12.8)	58 (14.2)	144 (12.0)	24 (16.3)	99 (12.6)	0.380
Hypertension, n (%)		1663 (65.4)	256 (62.6)	807 (67.1)	93 (63.3)	507 (64.8)	0.324
Asthma, n (%)		251 (9.9)	28 (6.8)	130 (10.8)	19 (12.9)	74 (9.5)	0.069
Cancer, n (%)		380 (15.0)	78 (19.1)	165 (13.7)	17 (11.6)	120 (15.3)	0.041 *
Diabetes Mellitus, n (%)		955 (37.6)	130 (31.8)	484 (40.3)	45 (30.6)	296 (37.8)	0.006 **
Max mSOFA Score on Admission, Mean ± SD, Median (IQR)		3.7 ± 3.0, 3 (1 – 5)	4.0 ± 3.6, 3 (1 – 6)	3.2 ± 2.7, 3 (1 – 5)	5.0 ± 3.9, 4 (2 – 6)	4.2 ± 3.1, 4 (2 – 6)	<0.001 ***
mSOFA Score, n (%)	≤1	497 (26.4)	92 (31.5)	295 (28.5)	12 (19.7)	98 (20.0%)	<0.001 ***
	2-4	799 (42.5)	95 (32.5)	481 (46.4)	19 (31.1)	204 (41.5%)	
	≥5	584 (31.1)	105 (36.0)	260 (25.1)	30 (49.2)	189 (38.5%)	
Max O2 Saturation on Admission, Mean ± SD, Median (IQR)		90.0 ± 8.1, (92 (89 – 94)	89.8 ± 10.9, 93 (89 – 95)	90.5 ± 6.7, 92 (89 – 94)	90.7 ± 8.7, 92 (90 – 94)	89.2 ± 8.1, 91 (88 – 93)	<0.001 ***
O2 Saturation, n (%)	Normal (≥95%)	504 (19.8)	126 (30.8)	233 (19.4)	34 (23.1)	111 (14.2%)	<0.001 ***
	Mild Hypoxemia (90-94%)	1275 (50.2)	180 (44.0)	619 (51.5)	84 (57.1)	392 (50.1%)	
	Mod Hypoxemia (86-89%)	408 (16.1)	38 (9.3)	202 (16.8)	13 (8.8)	155 (19.8%)	
	Severe Hypoxemia (≤85%)	354 (13.9)	65 (15.9)	148 (12.3)	16 (10.9)	125 (16.0%)	

Characteristics	Total (n=2541)	Neither Med (n=409)	HCQ Alone (n=1202)	AZM Alone (n=147)	HCQ+AZM (n=783)	P-value
Ever in ICU, n (%)	614 (24.2%)	62 (15.2)	243 (20.2)	19 (12.9)	290 (37.0)	<0.001 ***
Total ICU Days, Mean ± SD, Median (IQR)	2.3 ± 5.3, 0 (0 – 0)	0.8 ± 2.9, 0 (0 – 0)	1.9 ± 4.7, 0 (0 – 0)	0.7 ± 2.3, 0 (0 – 0)	4.0 ± 6.9, 0 (0 – 0)	<0.001 ***
Ever Mechanically Ventilated, n (%)	448 (17.6%)	34 (8.3)	166 (13.8)	14 (9.5)	234 (29.9)	<0.001 ***
Total Vent Days, Mean ± SD, Median (IQR)	1.6 ± 4.5, 0 (0 – 0)	0.5 ± 2.2, 0 (0 – 0)	1.2 ± 3.7, 0 (0 – 0)	0.5 ± 2.0, 0 (0 – 0)	3.1 ± 6.1, 0 (0 – 0)	<0.001 ***
Given Steroid, n (%)	1733 (68.2)	146 (35.7)	948 (78.9)	57 (38.8)	582 (74.3)	<0.001 ***
Given Tocilizumab, n (%)	114 (4.5)	5 (1.2)	32 (2.7)	5 (3.4)	72 (9.2)	<0.001 ***

* P-values between 0.01 and 0.05

** P-values between 0.001 and 0.01

*** P-values less than 0.001

Table 2. Multivariable Cox Regression Model for Mortality Prediction

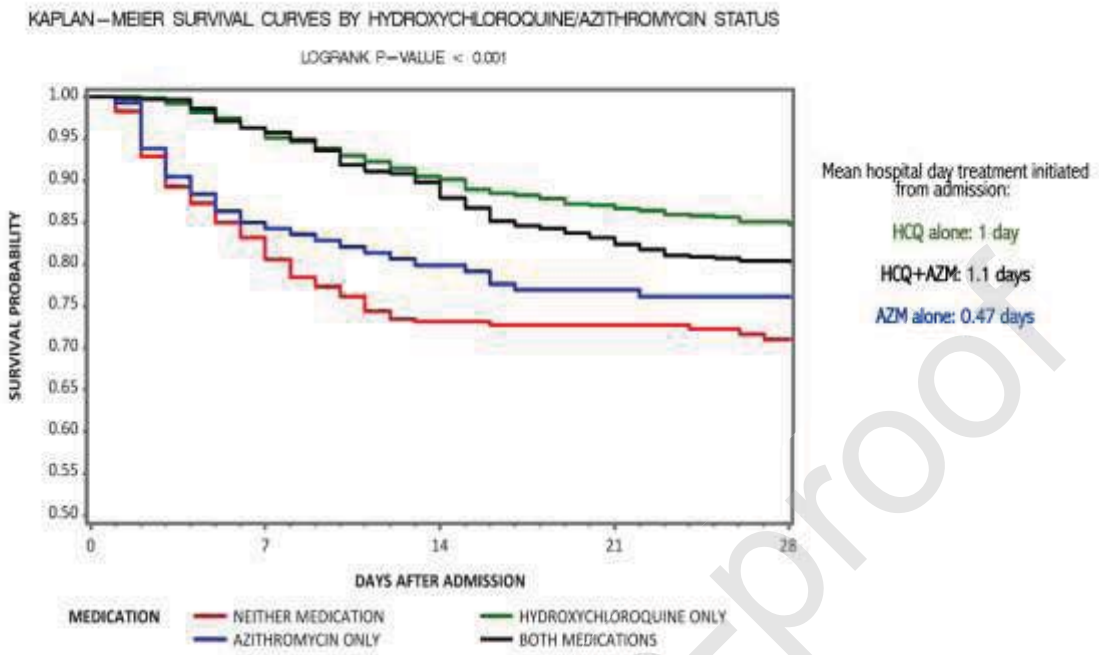
Parameter	P-value	Hazard Ratio	95% Hazard Ratio Confidence Limits	
HCQ Alone (vs. Neither Medication)	<0.001 ***	0.340	0.254	0.455
Azithromycin Alone (vs. Neither Medication)	0.825	1.050	0.682	1.616
HCQ+AZM (vs. Neither Medication)	<0.001 ***	0.294	0.218	0.396
Age ≥ 65 Years	<0.001 ***	2.579	1.989	3.345
M Gender	0.155	1.157	0.946	1.414
White Race	<0.001 ***	1.738	1.413	2.137
BMI ≥ 30	0.021 *	0.775	0.624	0.962
Lung Comorbidity	0.393	0.908	0.727	1.134
Immunodeficiency Comorbidity	0.429	1.398	0.609	3.206
Cardiovascular Comorbidity	0.678	1.062	0.800	1.410
Chronic Kidney Disease Comorbidity	<0.001 ***	1.699	1.370	2.108
COPD Comorbidity	0.170	1.202	0.924	1.563
Hypertension Comorbidity	0.064	0.798	0.628	1.014
Asthma Comorbidity	0.643	0.916	0.632	1.327
Cancer Comorbidity	0.577	0.933	0.731	1.190
Diabetes Comorbidity	0.822	0.975	0.786	1.211
Percent O2 Saturation < 95	0.021 *	1.488	1.063	2.084
Admitted to ICU	0.882	0.969	0.635	1.478
Ventilator	<0.001 ***	2.159	1.427	3.268
Given Steroid	0.085	0.802	0.625	1.031
Given Tocilizumab	0.490	0.894	0.651	1.228

* P-values between 0.01 and 0.05

** P-values between 0.001 and 0.01

*** P-values less than 0.001

Figure 1.



NUMBER AT RISK (IN HOSPITAL)

	ADMISSIONS	DAY 7	DAY 14	DAY 21	DAY 28
BOTH MEDICATIONS	783	751 (498)	675 (219)	593 (87)	490 (37)
HYDROXYCHLOROQUINE ONLY	1202	1144 (564)	999 (176)	786 (60)	463 (15)
AZITHROMYCIN ONLY	147	120 (31)	108 (8)	99 (2)	86 (1)
NEITHER MEDICATION	409	317 (117)	212 (16)	156 (6)	110 (3)

1

Table 3. Characteristics of Patients Given versus Not Given HCQ Before and after Propensity Score Matching

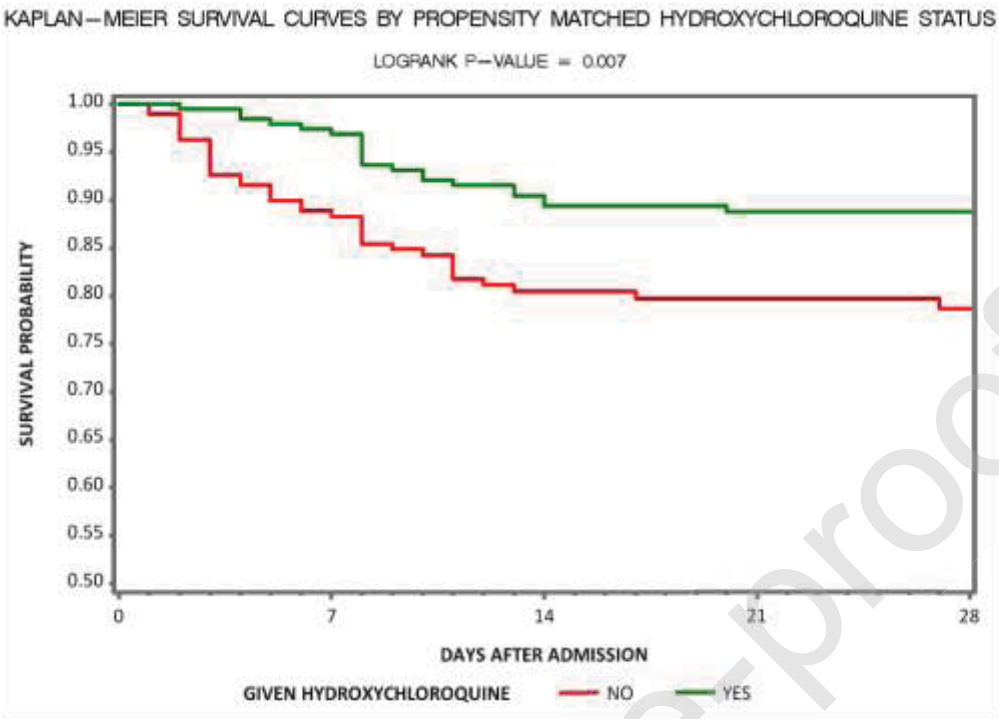
Characteristics	Unmatched Patients		Propensity-Matched Patients	
	Given HCQ (N=1985)	Not Given HCQ (N=556)	Given HCQ (N=190)	Not Given HCQ (N=190)
Age ≥ 65 Years	944 (47.6%)	319 (57.4%)	96 (50.5%)	96 (50.5%)
Male Gender	1037 (52.2%)	261 (46.9%)	88 (46.3%)	88 (46.3%)
White Race	603 (30.4%)	249 (44.8%)	67 (35.3%)	67 (35.3%)
BMI ≥ 30	1035 (55.5%)	215 (40.9%)	87 (45.8%)	87 (45.8%)
Lung Comorbidity	1331 (67.1%)	288 (51.8%)	103 (54.2%)	103 (54.2%)
Immunodeficiency Comorbidity	26 (1.3%)	4 (0.7%)	1 (0.5%)	1 (0.5%)
Cardiovascular Comorbidity	167 (8.4%)	55 (9.9%)	7 (3.7%)	7 (3.7%)
Chronic Kidney Disease Comorbidity	841 (42.4%)	258 (46.4%)	69 (36.3%)	69 (36.3%)
COPD Comorbidity	243 (12.2%)	82 (14.7%)	10 (5.3%)	10 (5.3%)
Hypertension Comorbidity	1314 (66.2%)	349 (62.8%)	118 (62.1%)	118 (62.1%)
Asthma Comorbidity	204 (10.3%)	47 (8.5%)	6 (3.2%)	6 (3.2%)
Cancer Comorbidity	285 (14.4%)	95 (17.1%)	8 (4.2%)	8 (4.2%)
Diabetes Comorbidity	780 (39.3%)	175 (31.5%)	51 (26.8%)	51 (26.8%)
Percent O2 Saturation ≤ 95	1641 (82.7%)	396 (71.2%)	141 (74.2%)	141 (74.2%)
Admitted to ICU	533 (26.9%)	81 (14.6%)	12 (6.3%)	12 (6.3%)
Ventilator	400 (20.2%)	48 (8.6%)	10 (5.3%)	10 (5.3%)
Given Steroid	1530 (77.1%)	203 (36.5%)	84 (44.2%)	84 (44.2%)
Given Tocilizumab	104 (5.2%)	10 (1.8%)	2 (1.1%)	2 (1.1%)

Table 4. Propensity Matched Cox Regression Result for Mortality Prediction

Parameter	P-value	Hazard Ratio	95% Hazard Ratio Confidence Limits	
Given HCQ	0.009 **	0.487	0.285	0.832

** P-value between 0.001 and 0.01

Figure 2.



NUMBER AT RISK (IN HOSPITAL)

	ADMISSIONS	DAY 7	DAY 14	DAY 21	DAY 28
GIVEN HYDROXYCHLOROQUINE	190	184 (84)	164 (16)	146 (8)	111 (2)
NOT GIVEN HYDROXYCHLOROQUINE	190	158 (49)	118 (7)	98 (2)	72 (1)

Preliminary evidence from a multicenter prospective observational study of the safety and efficacy of chloroquine for the treatment of COVID-19

Mingxing Huang[1*], Man Li[2,3*], Fei Xiao[1,2*], Pengfei Pang[2,3*], Jiabi Liang[4*], Tiantian Tang[5*], Shaoxuan Liu[6], Binghui Chen[7], Jingxian Shu[3], Yingying You[8], Yang Li[2,3], Meiwen Tang[9], Jianhui Zhou[10], Guanmin Jiang[10], Jingfen Xiang[11], Wenxin Hong[12], Songmei He[13], Zhaoqin Wang[14], Jianhua Feng[15], Changqing Lin[16], Yinong Ye[17], Zhilong Wu[18], Yaocai Li[19], Bei Zhong[20], Ruilin Sun[21], Zhongsi Hong[1], Jing Liu[22], Huili Chen[1], Xiaohua Wang [23], Zhonghe Li [24], Duanqing Pei[25,26†], Lin Tian[3†], Jinyu Xia[1†], Shanping Jiang[5†], Nanshan Zhong[27†], Hong Shan[2,3†]

1. Department of Infectious Diseases, The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai, Guangdong Province, China
2. Guangdong Provincial Key Laboratory of Biomedical Imaging and Guangdong Provincial Engineering Research Center of Molecular Imaging, The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai, Guangdong Province, China
3. Center for Interventional Medical, The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai, Guangdong Province, China
4. Department of Pharmacy, The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai, Guangdong Province, China
5. Department of Respiratory and Critical Care Medicine, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong Province, China
6. Clinical Research Center Office, The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai, Guangdong Province, China
7. Department of Radiology, The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai, Guangdong Province, China
8. Department of Stomatology, The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai, Guangdong Province, China

9. Department of Hematology, The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai, Guangdong Province, China
10. Department of Clinical Laboratory, The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai, Guangdong Province, China
11. Department of Emergency, Wuhan East West Lake Mobile Cabin Hospitals, Wuhan, Hubei Province, China
12. Department of Infectious Diseases, Guangzhou Eighth People's Hospital, Guangzhou Province, Guangdong Province, China
13. Department of Infectious Diseases, Dongguan Ninth People's Hospital, Dongguan, Guangdong Province, China
14. Department of Infectious Diseases, Shenzhen Third People's Hospital, Shenzhen, Guangdong Province, China
15. Department of Infectious Diseases, Zhongshan Second People's Hospital, Zhongshan, Guangdong Province, China
16. Department of Respiratory and Critical Care Medicine, Huizhou Central People's Hospital, Huizhou, Guangdong Province, China
17. Department of Infectious Diseases, Foshan First people's Hospital, Foshan, Guangdong Province, China
18. Department of Respiratory and Critical Care Medicine, The Fourth People's Hospital of Foshan City, Foshan, Guangdong Province, China
19. Department of Infectious Diseases, Maoming People's Hospital, Maoming, Guangdong Province, China
20. Department of Infectious Diseases, The Sixth Affiliated Hospital of Guangzhou Medical University, Qingyuan People's Hospital, Qingyuan, Guangdong Province, China
21. Pulmonary and Critical Care Medicine Department, Guangdong Second Provincial General Hospital, Guangzhou, Guangdong Province, China
22. Department of Respiratory and Critical Care Medicine, The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai, Guangdong Province, China
23. Intensive Care Unit, The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai, Guangdong Province, China
24. Department of Nephrology, The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai, Guangdong Province, China

25. Guangzhou Regenerative Medicine and Health Guangdong Laboratory, Guangzhou, Guangdong Province, China
26. Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, Guangdong Province, China
27. State Key Laboratory of Respiratory Diseases, Guangzhou Institute of Respiratory Health, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong Province, China.

* These authors contributed equally.

† These are the co-corresponding authors.

Abstract

Background Effective therapies are urgently needed for the SARS-CoV-2 pandemic. Chloroquine has been proved to have antiviral effect against coronavirus in vitro. In this study, we aimed to assess the efficacy and safety of chloroquine with different doses in COVID-19.

Method In this multicenter prospective observational study, we enrolled patients older than 18 years old with confirmed SARS-CoV-2 infection excluding critical cases from 12 hospitals in Guangdong and Hubei Provinces. Eligible patients received chloroquine phosphate 500mg, orally, once (half dose) or twice (full dose) daily. Patients treated with non-chloroquine therapy were included as historical controls. The primary endpoint is the time to undetectable viral RNA. Secondary outcomes include the proportion of patients with undetectable viral RNA by day 10 and 14, hospitalization time, duration of fever, and adverse events.

Results A total of 197 patients completed chloroquine treatment, and 176 patients were included as historical controls. The median time to achieve an undetectable viral RNA was shorter in chloroquine than in non-chloroquine (absolute difference in

medians -6.0 days; 95% CI -6.0 to -4.0). The duration of fever is shorter in chloroquine (geometric mean ratio 0.6; 95% CI 0.5 to 0.8). No serious adverse events were observed in the chloroquine group. Patients treated with half dose experienced lower rate of adverse events than with full dose.

Conclusions Although randomised trials are needed for further evaluation, this study provides evidence for safety and efficacy of chloroquine in COVID-19 and suggests that chloroquine can be a cost-effective therapy for combating the COVID-19 pandemic.

Introduction

The coronavirus disease 2019 (COVID-19) emerged in late 2019[1,2]. The responsible virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), belongs to a distinct clade from the human severe acute respiratory syndrome CoV (SARS-CoV) and Middle East respiratory syndrome CoV (MERS-CoV)[3]. It has become a global pandemic, affecting over 100 countries with more than 240,000 confirmed cases and over 10,000 deaths globally as of March 20, 2020, calling for an urgent demand of effective treatment.

Chloroquine has been proved effective in vitro to inhibit the replication of SARS-CoV[4], HCoV-229E[5], and the newly discovered SARS-CoV-2[6,7]. To evaluate the efficacy and safety of chloroquine for COVID-19, we previously conducted a single-arm pilot clinical study with 10 patients (Huang et al. Journal of Molecular Cell Biology, in press). Encouragingly, all patients achieved undetectable level of viral RNA within 14 days without serious adverse events. These results led us to conduct a multicenter prospective observational study in adult patients with COVID-19 to assess the efficacy and safety of chloroquine for COVID-19.

Result

Patients

Of the 233 enrolled patients for chloroquine, 197 (84.5%) completed treatment and were included in the final analysis (**Figure 1, study flowchart; Supplementary Table 1**). Of the 182 patients collected as historical controls, 176 (96.7%) were included in the final analysis. Their baseline demographic and clinical features are listed in **Table 1**. The median age of patients were 43 years (inter-quartile range [IQR], 33 to 55 years) in the chloroquine group and 47.5 years (IQR, 35.8 to 56 years) in the non-chloroquine group. Across the two treatment groups, the majority patients were classified as moderate cases (93.4% in chloroquine; 89.2% in non-chloroquine)[8]. Chloroquine was added into China's Diagnosis and Treatment Guidelines of COVID-19 later than the other therapies used in the non-chloroquine group. Therefore, we observed longer interval time between symptom onset and treatment initiation in chloroquine versus non-chloroquine (absolute difference 4 days; 95% CI 2 to 6 days; $P < 0.0001$). In addition, due to the rapid rise of patients in Wuhan and established mobile hospital in early February, the interval time between symptom onset and treatment initiation in Wuhan (median 17 days, IQR 10.5 to 21 days) is longer than that in Guangdong Province (median 5 days, IQR 3 to 10 days; **Table 1**). In the subgroup of patients from the Fifth Affiliated Hospital of Sun Yat-sen University (SYSU5), we obtained and evaluated the viral load at baseline between chloroquine (N=21) and non-chloroquine (N=8) group and did not observe statistically significant difference (absolute difference in medians = 2.93, 95% CI -0.8 to 6.6, p = 0.09).

Outcomes

In the analysis of the full study population, patients in the chloroquine group have an accelerated time to undetectable viral RNA from that of patients in the non-

chloroquine group (absolute difference in medians -5.4 days; 95% CI -6 to -4; $P < 0.0001$; **Figure 2**). Secondly, by day 10 and day 14 since treatment initiation, higher proportion of patients had undetectable viral RNA in the chloroquine group (91.4% and 95.9% respectively; **Table 2**) comparing to the non-chloroquine group (57.4% and 79.6% respectively; **Table 2**). In the aspect of clinical manifestations, we found that the duration of fevers is shorter in chloroquine versus non-chloroquine among patients experienced fever symptom (geometric mean ratio 0.6; 95% CI 0.5 to 0.8; $P = 0.0029$; **Supplementary Figure S1**). To note, the antipyretic effects of chloroquine may have also contributed to this result. We observed no difference in the length of hospital stay (**Supplementary Figure S2**). No patient died or admitted to ICU either in the chloroquine group or in the non-chloroquine group. Among patients who had moderate clinical symptoms at baseline, seven patients experienced aggravated symptoms from moderate to severe level, one in the chloroquine group and six in the non-chloroquine group. The proportion of patients having aggravated symptoms is lower in the chloroquine group but not statistically significant (absolute difference in proportions 3.28; 95% CI -6.96 to 1.43). All of the seven patients eventually were tested negative for the viral RNA within the study period.

Due to the significant difference observed in clinical classification between chloroquine and non-chloroquine group at baseline, we further analyzed the primary and secondary outcomes in patients with moderate symptoms only. The number of patients in mild or severe subgroup were too few to compare. The benefit of chloroquine in viral suppression is consistent with the full analysis, except for non-significant difference observed for the proportion of patients with undetectable viral RNA by day 14 (**Supplementary Table 2**).

In post hoc analysis, we examined the effect of chloroquine on the time to undetectable viral RNA stratified by different doses, types of clinical manifestation, the interaction between province and time from symptom onset to treatment initiation, and a representative center (**Figure 3**). Chloroquine showed beneficial effect in all stratum. However, the beneficial effect is not statistically significant in patients with severe COVID-19 symptoms, patients from Guangdong Province treated later than 14 days after symptom onset, or patients from SYSU5.

In order to assess the effect of chloroquine in more detailed clinical improvement outcomes in post hoc analysis, we collected detailed clinical data in patients from SYSU5, including the improvement of chest CT, the monitoring of serum chloroquine concentration, and the reappearance of positive viral RNA detection after hospital discharge. In this subgroup of patients, the interval time between symptom onset and treatment initiation were comparable. The medians are 7 days in chloroquine group (N=50) and 6 days in non-chloroquine group (N=21) (absolute difference in medians 1 day; 95% CI -3 to 4 days; P = 0.99; **Supplementary Table 3**). We did not find statistically significant difference in the time to undetectable viral RNA between the two groups (absolute difference in medians -3.5 days; 95% CI -6 to 1 days). The chloroquine group have higher percentage of patients with improved chest CT by day 10 (absolute difference in proportions 9.7; 95% CI -16.0 to 35.6) and day 14 (absolute difference in proportions 6.3; 95% CI -22.2 to 32.0) than the non-chloroquine group but the difference is not statistically significant (**Supplementary Table 3**). This could be due to the small sample size or the delayed chest CT absorption[9]. We did not observe beneficial effect of chloroquine in the length of hospital stay and the duration of oxygen support (**Supplementary Table 3**). Unprecedently, we observed 3 cases of so called “re-positive” patients in the

chloroquine group. They were identified with negative viral RNA test from respiratory tract samples but positive viral RNA test from fecal samples within 7 days following hospital discharge. No such observation in the non-chloroquine group. Investigation is underway to examine whether it is due to re-infection or other factors.

Among the 12 hospitals, one hospital explored different dosage of chloroquine, as 500 mg once daily, which is half of the protocol dosage. We compared the primary and secondary outcomes in patients from this subgroup (N=29) with the non-chloroquine group in Guangdong Province. The results mainly showed that chloroquine has benefit effect on the time to undetectable viral RNA (absolute difference in medians -5 days; 95% CI -6.0 to -4.0 days) and the proportion of patients with undetectable viral RNA by day 10 is higher in chloroquine group (absolute difference in proportions 32.7; 95% CI 23.9 to 42.1). The duration of fever was also shorter than those in the non-chloroquine group (geometric mean ratio 0.8; 95% CI 0.5 to 0.9) (**Supplementary Table 4**).

Safety

A total of 53 patients (26.9%) in the chloroquine group and 57 (32.4%) in the non-chloroquine group reported adverse events during study period (**Table 3**). Gastrointestinal events including vomiting, abdominal distension, nausea, decreased appetite, thirst were more common in chloroquine than in the non-chloroquine group. The percentage of patients with neurological adverse events, including dizziness and sleep order, were higher in the chloroquine than in the non-chloroquine group. In addition, anxiety was observed more frequently in chloroquine than in the non-chloroquine group. We observed fewer adverse events in patients with half dose of chloroquine than full dose (absolute difference in proportions -40; 95% CI -60 to -29).

Chloroquine phosphate has a long half-life (20-60 days)[10-12] and its mean residence time is approximately 20 days[10]. It may have cumulative effect[13]. In order to determine whether chloroquine has a cumulative effect in the short-term treatment with COVID-19, we measured the serum concentration of chloroquine in patients from SYSU5 during and off the treatment. The results showed that the mean of serum concentration of chloroquine gradually rising, with the highest reaching 1.80(±0.49) μmol/L during medication and reduced to 0.13(±0.08) μmol/L within 28±1 days off chloroquine (**Supplementary Figure 3**). We did not observe statistically significant difference in treatment effect of chloroquine when stratifying by tertiles of serum chloroquine concentrations (**Supplementary Figure 4**).

DISCUSSION

In this study, we found that patients in the chloroquine group experienced significantly faster and higher rate of viral suppression comparing to the non-chloroquine group in both the full analysis and the post hoc stratified analysis. Even when the dose reduced to half, the benefit of chloroquine still remained (**Figure 3**). These findings indicate that chloroquine could be effective in treating patients with COVID-19. To our knowledge, this is the first and largest clinical study on chloroquine phosphate for treating COVID-19 to date.

We recognize that our study has several limitations. This study was carried out under the COVID-19 public health emergency. Due to the limited medical capacity and urgent clinical situation, we were unable to conduct a standard randomised controlled study to formally evaluate efficacy and safety of chloroquine versus placebo. As an observational study, we have to note that several factors may influence the interpretation of the result. It is reasonable to suspect that the dramatic

improvement in the primary outcome in chloroquine could be due to the later treatment initiation since symptom onset. Firstly, gaining experience in treatment management and attenuation of the virus during the course of the epidemic could contribute to the improved outcomes. Secondly, we cannot rule out the possibility that among those with longer interval time between symptom onset and treatment, some may already have been on the course of recovery. Thirdly, although it is impossible to dissect the influence from other antiviral therapies used before chloroquine, it is a plausible assumption that chloroquine is the first antiviral therapy used in the group of patients treated within 3 days since symptom onset. The post hoc analysis dividing subgroups according to the interval time and the two provinces (**Figure 3**) indicating that the chloroquine group had a better outcome than the non-chloroquine group at early stage of the disease onset regardless of the locations. Lastly, due to the differences in personnel and technical equipment of among all hospitals, we could not fully collect clinical and laboratory data of all patients. However, detailed clinical data were obtained from the chloroquine patients enrolled from SYSU5, enabling advanced analysis of clinical outcomes and pharmacokinetics.

As of this time, there are more than 20 trials ongoing for evaluating the efficacy and safety of chloroquine or hydroxychloroquine in treating COVID-19. Magagnoli et al. recently published a retrospective study indicating that the use of hydroxychloroquine with or without azithromycin does not reduced the risk of mechanical ventilation in United States veterans hospitalized with COVID-19[14]. More recently, Geleris et al. presented an observational study of hydroxychloroquine indicating that no beneficial effect of hydroxychloroquine on the risk of intubation or death. Comparing with these studies, our study population was younger and fewer

patients with severe symptoms that requires ventilation[15]. Therefore, prospective randomised trials are needed to see if the results can be replicated.

Till now, the mechanism of chloroquine's effect against SARS-CoV-2 remained unelucidated. Clatherin-mediated endocytosis is required for entry of coronavirus into host cells and meanwhile autophagy involves in viral replication[16]. Chloroquine inhibits clatherin-mediated endocytosis by suppressing acidification of endosomes, and autophagy by raising its lysosomal PH and blocking fusion of autophagosome with lysosome and lysosomal protein degradation[17]. A recent study has shown that the development of COVID-19 disturbed metabolic patterns, which aligned with the progress and severity of COVID-19 (Wu et al. National Science Review 2020, in press). Chloroquine has a favorable effect on glucose and lipid metabolism[18]. Therefore, chloroquine may exert its antiviral effect against SARS-CoV-2 by inhibiting endocytosis and autophagy, and stabilizing glucose and lipid metabolism.

The adverse reactions of chloroquine drugs are of great concern to the community. Although it is an old anti-malarial drug, its safety in treating COVID-19 patients is still unknown. In the present study, we did not observe serious adverse events in patients with chloroquine. All adverse events observed during the study period are known side-effects for chloroquine (**Table 3**). The main adverse events were symptoms in gastrointestinal and neuropsychiatric systems. Chloroquine is known for its side effects in cardiovascular system. In the chloroquine group, we did not find significantly higher rate of adverse events in patients older than 65 or with pre-existing conditions (**Supplementary Table 5**). Adverse event appeared in 1 out of 29 patients (3.5%) with half dose while in 52 out 168 patients (31.0%) with full dose, indicating that the half dose group has lower adverse event rate (absolute rate

difference -27.5; 95% CI -45.0 to -19.2). Although previous studies suggested that chloroquine may have cumulative effect[11,19,20], we did not observe cumulative effects among 50 patients from SYSU5 by monitoring the serum concentration of chloroquine for up to 28 days after treatment completion. Chloroquine are thought to interfere with medications that influence the QT interval. Patients on chloroquine therapy concurrently taking drugs for the treatment of cardiac comorbidities should also be monitored for the potential risk of cardiac arrhythmia[21]. For patients in the non-chloroquine group, about half were treated with lopinavir/ritonavir alone or in combination with other medications and the other half were treated with Arbidol (**Supplementary Table 6**). There is no strong evidence that these antiviral treatments were safe and effective in COVID-19 patients[22]. In addition, a recent pharmacovigilance study reported that number of drugs used in hospital and underlying basic diseases are independent risk factor for adverse reactions in COVID-19 and majority of the adverse reactions can be explained by the use of lopinavir/ritonavir[23]. The different antiviral therapies used in the historical control group could potentially confound the risk of adverse events between chloroquine and non-chloroquine treatment. Future studies are needed to determine the optimal dosing for treating COVID-19 and the cumulative effect of chloroquine in tissues and organs. Severe cases are underrepresented in the present study, and thus should be focused in the future studies to evaluate the efficacy and safety profile in this population. In addition, it will be important to study the prophylaxical use of chloroquine in areas with high rate of COVID-19 or in health professionals working with COVID-19 patients.

In conclusion, our preliminary evidence showed that chloroquine has the potential to shorten the time to SARS-CoV-2 viral suppression and duration of fever

in patients with moderate symptoms at earlier stage of the disease, even with reduced dose. Further randomised studies are needed to determine the optimal dose, to assess its benefit for both severe cases and to assess its benefit in settings other than secondary care. Considering that there is no better option at present, chloroquine could be a viable option to combat the coronavirus pandemic under proper management.

METHODS

Study Design and participants

This study was a multicenter prospective observational study conducted from February 7 through March 8, 2020 at 11 hospitals in Guangdong Province and 1 mobile cabin hospital in Wuhan, Hubei Province, China. The study protocol was approved by the ethics committee of Fifth Affiliated Hospital of Sun Yat-sen University (SYSU5), located in Zhuhai, Guangdong Province, and registered at Chinese Clinical Trial Registry (ChiCTR2000029609). We did this study in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice. Written informed consent was obtained from all patients or their legal guardians. During the study period, each hospital had various choices of antiviral regimen, and the sample size of Lopinavir/Ritonavir (the historical control group in the original protocol) for single-use were underpowered. Thus, we updated the inclusion criteria of the historical control group as patients receiving non-chloroquine treatment.

Eligible patients were aged 18 years or older with confirmed SARS-CoV-2 infection, tested by the local Center for Disease Control (CDC) or by a designated diagnostic laboratory, using reverse-transcriptase-polymerase-chain-reaction (RT-PCR) assay (Shanghai ZJ Bio-Tech Co Ltd) for SARS-CoV-2 in a respiratory tract

sample. Patients were ineligible if he/she met any of the following criteria: pregnant women, with known allergies to 4-aminoquinoline compounds, blood system diseases, chronic liver or kidney diseases in end-stage, arrhythmia or second/third degree heart block, with known to have retinopathy, hypoacusis or hearing loss, mental disease, glucose-6-phosphate dehydrogenase (G6PD) deficiency, had received digitalis drugs within the 7 days preceding enrollment, or is classified as critical case according to China's Novel Coronavirus Pneumonia Diagnosis and Treatment Plan (4^{th} Edition). Enrolled patients received 500mg chloroquine Phosphate (equivalent of 300 mg chloroquine base, Shanghai Xinyi Pharmaceutical Co., Ltd) orally, once/twice-daily with no other antiviral therapies. The criteria of stopping chloroquine was defined as undetectable viral RNA for two consecutive respiratory tract samples. The duration of medication in chloroquine group is no more than 10 days. Patients in the historical control group were treated according to China's Novel Coronavirus Pneumonia Diagnosis and Treatment Plan (details described in **Supplementary Table 6**).

Outcome and measurements

The primary outcome is the time from treatment initiation to undetectable viral RNA for two consecutive respiratory tract samples. The secondary outcomes include the proportion of patients with undetectable viral RNA by day 10 and 14, duration of fevers, time in hospital, and adverse events. The detailed definition of outcomes is described in **Supplementary Methods**. Respiratory tract sample was collected from patients daily to conduct RT-PCR assay for SARS-CoV-2 infection. The epidemiological characteristics, clinical symptoms and signs, adverse reactions/events were collected with data collection forms. The outcomes, clinical

characteristics, laboratory findings, chest computed tomographic (CT) scans were recorded on case record forms and then double-entered into an electronic database and validated by trial staff. After hospital discharge, patients were followed up once weekly. Patients with “re-positive” viral RNA detection within one week after hospital discharge are defined as having either 2 consecutive RT-PCR positive result from either respiratory tract sample or fecal specimen. In the subgroup of patients in SYSU5, all CT images were reviewed by two fellowship-trained cardio-thoracic radiologists by using a viewing console. Images were reviewed independently, and final decisions were reached by consensus [9].

To fully assess the safety of chloroquine, we monitor the serum concentration of chloroquine at the day 1, 3, 5, 7, 10 during drug administration and day 1 to 7, and day 14, day 21 after treatment completion in a subgroup of samples enrolled from SYSU5 (N=50). Details about the measurement of serum concentration of chloroquine are described in **Supplementary Methods**.

Statistical Analysis

The original plan was to compare the efficacy between three groups, chloroquine only, Lopinavir/Ritonavir only, and chloroquine plus Lopinavor/Ritonavir. At the beginning of the outbreak, different therapies were proposed and tested for the treatment of COVID-19. Therefore, it is challenging to find sufficient patients with unified treatment across all centers. The epidemic in Guangdong had been brought under control rapidly during the study making it difficult to recruit patients as planned. The history of changes to the protocol is listed in **Supplementary Table 7**. Thus, a decision was made to focus on recruiting chloroquine only and compare the efficacy with historical controls. The current sample size was based on feasibility within the fixed trial recruitment window and was felt would provide sufficient precision for the

estimation of plausible effects. With right-censoring in time-to-event variables, generalized Wilcoxon test was used to compare the difference in medians and the 95% confidence intervals were calculated by bootstrapping[24]. For binary outcomes, Wilson test was implemented to calculate the difference in proportions and 95% confidence intervals. As this was an observational study, imbalance in the baseline characteristics of the two groups was expected. To adjust for this imbalance, we performed post hoc analyses within various subgroups by two dosage options, by clinical manifestation, by the interaction of province and the interval time between symptom onset and treatment initiation (≤ 3 days; 3~7 days; 7~14 days; > 14 days), and by center. For all comparative analyses, P <0.05 was considered statistically significant. No allowance for multiplicity. All P values are two tailed. All statistical analyses were performed in R, version 3.6.1 (R Foundation for Statistical Computing)[25].

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data and had final responsibility for the decision to submit for publication.

Contributors

S.H, N.Z, S.J. J.X. L.T and D.P. had the idea for and designed the study and had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. M.L, F.X, Y.L., M.H, J.L, P.P and T.T contributed to writing of the report. M.L, F.X, M.H, Y.L., J.L and P.P contributed to critical revision of the report. M.L contributed to the statistical analysis. All authors contributed to data acquisition, data analysis, or data interpretation, and reviewed and approved the final version.

Declaration of interest

All authors declare no competing interests.

Data sharing

The data that support the findings of this study are available from the corresponding author on reasonable request. Participant data without names and identifiers will be made available after approval from the corresponding author and Ministry of science and technology and Health Committee in Guangdong province. After publication of study findings, the data will be available for others to request. The research team will provide an email address for communication once the data are approved to be shared with others. The proposal with detailed description of study objectives and statistical analysis plan will be needed for evaluation of the reasonability to request for our data. The corresponding author and Ministry of science and technology and Health Committee in Guangdong province will make a decision based on these materials. Additional materials may also be required during the process.

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hospitals. We thank Jiaxing Taimei Medical Technology Co., Ltd for Electronic Data Capture service.

Table 1. Baseline characteristics in chloroquine and non-chloroquine among people with COVID-19.

	chloroquine (N=197)	Non-chloroquine (N=176)
Guangdong, N (%)	118 (60)	96 (54)
Hubei, N (%)	79 (40)	80 (46)
Age, mean (SD)	43.8 (13.1)	45.6 (13.5)
Age ≤ 65	190 (96)	171 (97)
Age > 65	7 (4)	5 (3)
Female sex, N (%)	101 (51)	97 (55)
Clinical manifestation†, N (%)		
Mild	9 (5)	5 (3)
Moderate	184 (93)	157 (89)
Severe	4 (2)	14 (8)
Comorbidities, N (%)*		
Hypertension	13 (17)	11 (17)
Type 2 diabetes	4 (5)	5 (8)
Interval time from symptom onset to treatment initiation, median (IQR)		
Guangdong	7 (3, 10.8)	4 (2, 7)
Hubei	19 (17, 24.5)	11 (7, 16)
Body temperature, median (IQR), ºC	36.7 (36.5, 37.0)	36.6 (36.4, 37.3)
Pneumonia from chest CT, N (%)§	173 (89)	137 (93)

* The number of patients with valid record of comorbidities are 78 in chloroquine group and 66 in non-chloroquine group

§ The number of patients with valid record of chest CT image are 194 in chloroquine group and 148 in non-chloroquine group.

† clinical manifestation type definitions: 1) Mild, mild clinical symptoms with no signs of pneumonia on chest radiological imaging; 2) Moderate, fever, respiratory symptoms, imaging with pneumonia changes; 3) Severe, meet any of the following criteria: shortness of breath, respiratory rate > 30 times per minute, resting stable

oxygen saturation in fingertip < 93%, oxygenation index < 300, pulmonary imaging showed that the lesion progressed significantly more than 50% within 24-48 hours.

Table 2. Outcomes in the overall population with confirmed SARS-CoV-2 infection[§].

	chloroquine (N=197)	Non-chloroquine (N=176)	Difference (95% CI) [†]	P value
Time to undetectable viral RNA, median no. of days (IQR)	3.0 (3.0, 5.0)	9.0 (6.0, 12.0)	-6.0 (-6.0, -4.0)	< 0.0001
Patients with undetectable viral RNA by, N (%)				
Day 10	180.0 (91.0)	101.0 (57.0)	34.0 (25.6, 42.9)	< 0.0001
Day 14	189.0 (96.0)	140.0 (80.0)	16.0 (9.2, 23.3)	< 0.0001
Duration of fever*, no. of days, geometric mean (CV)	1.2 (53.5)	1.9 (110.0)	0.6 (0.5, 0.8)	0.0029
Length of hospital stay, median no. of days (IQR)	19.0 (16.0, 23.0)	20.0 (15.8, 24.0)	-1.0 (-3.0, 0.0)	0.25

Abbreviations: CI, confidence interval; IQR, inter-quartile range; CV, coefficient of variation.
[§] Definitions of outcomes are listed in Supplementary Methods.
[†] 95% CI for continuous variables are calculated by bootstrapping. 95% CI for binary variables are calculated with Wilson method. The difference for duration of fever is geometric mean ratio of chloroquine group to non-chloroquine group. The differences for all other variables are the absolute difference between chloroquine group and non-chloroquine group.
* The number of patients had at least one day of fever is 42 and 51 in the chloroquine and non-chloroquine group respectively.

Table 3. Summary of adverse events[§].

Event, N (%)	chloroquine (N=197)	Non-chloroquine (N=176)
Any adverse event	53 (26.9)	57 (32.4)
Gastrointestinal		
Vomiting	9 (4.6)	2 (1.1)
Abdominal distension	2 (1.0)	1 (0.6)
Abdominal pain	2 (1.0)	2 (1.1)
Nausea	18 (9.1)	7 (4.0)
Diarrhea	6 (3.0)	11 (6.3)

Decreased appetite	7 (3.6)	0 (0)
Thisrt	4 (2.0)	0 (0)
Acid reflux	1 (0.5)	0 (0)
Belching	1 (0.5)	0 (0)
Neurological		
Dizziness	20 (10.2)	4 (2.3)
Headache	3 (1.5)	3 (1.7)
Sleep disorder	10 (5.1)	1 (0.6)
Psychological		
Anxiety	6 (3.0)	0 (0)
Depression	1 (0.5)	0 (0)
Delirious	1 (0.5)	1 (0.6)
Dysphoria	1 (0.5)	0 (0)
Emotional Unstable	1 (0.5)	0 (0)
Cardiovascular		
Pain under xiphoid	1 (0.5)	0 (0)
Chest tightness	2 (1.0)	6 (3.4)
Ventricular premature beat	0 (0)	1 (0.6)
Other		
Hand shaking/numbness	2 (1.0)	0 (0)
Muscle soreness	0 (0)	4 (2.3)
Blurred vision	3 (1.5)	0 (0)
Rash	1 (0.5)	0 (0)
Weight loss	1 (0.5)	0 (0)
Fatigue / Weakness	2 (1.0)	1 (0.6)
Shortness of breath	1 (0.5)	3 (1.7)
Unsteady gait	1 (0.5)	0 (0)

[§] Adverse events that occurred in more than 1 patient after treatment initiation during study period are shown. Some patients had more than one adverse event.

Figure 1. Study flowchart.

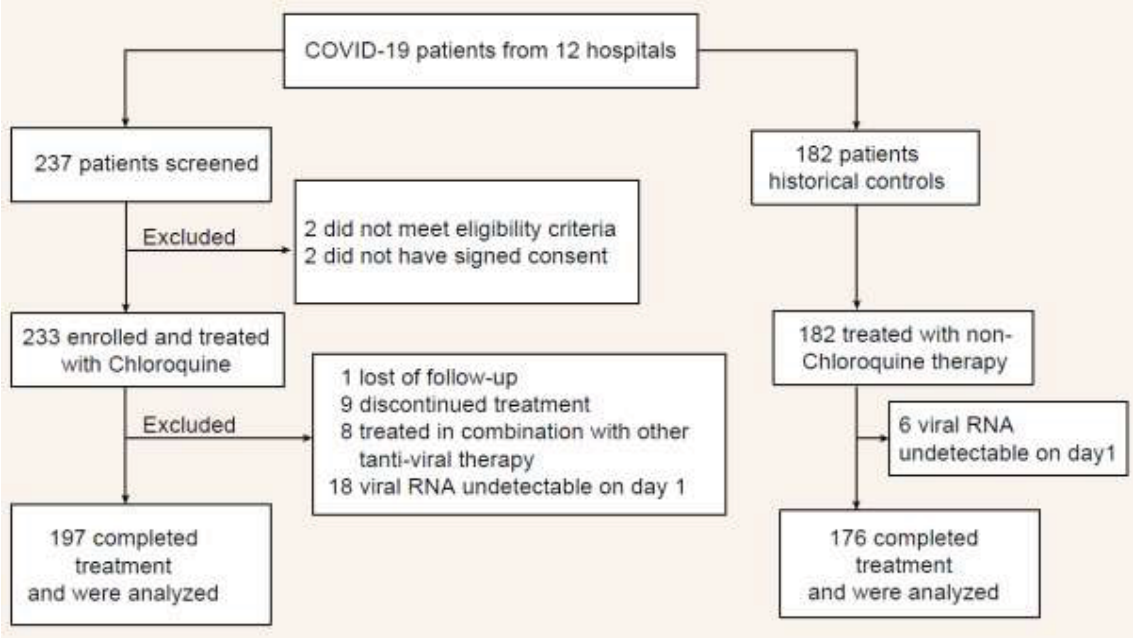


Figure 2. Kaplan-Meier curve for time to undetectable viral RNA comparing treatment groups.

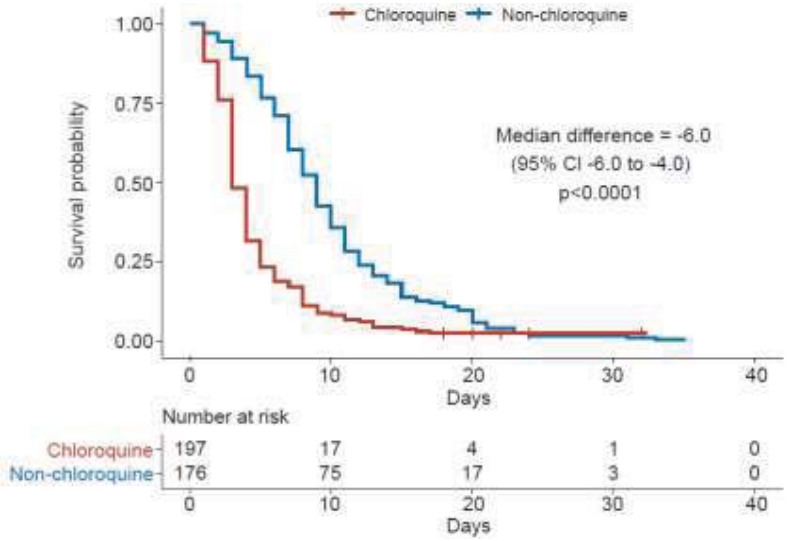
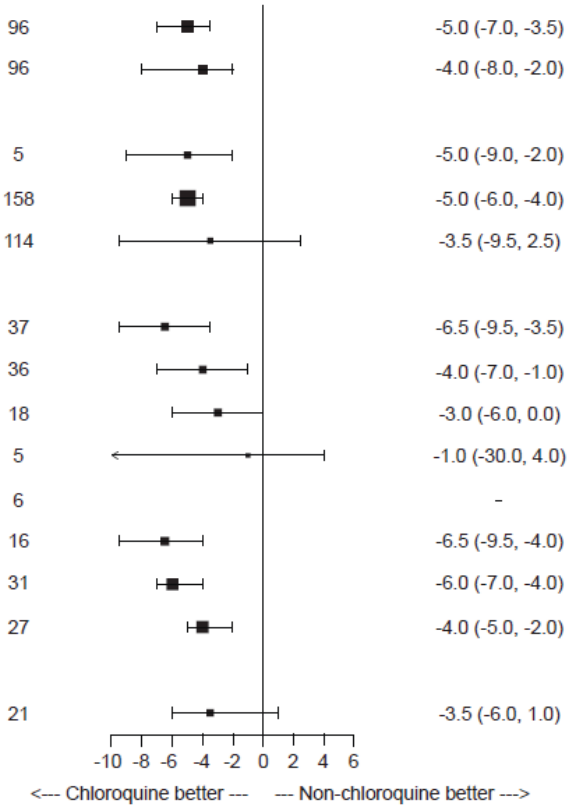


Figure 3. Post hoc analysis on the effect of chloroquine on time to undetectable viral RNA by stratification.

Stratum	Chloroquine median(IQR)	N	Non-chloroquine median(IQR)	N	Difference (95%CI)
Dosing					
Full dose	4.0 (2.0, 8.0)	89	9.0 (5.8, 14.0)	96	-5.0 (-7.0, -3.5)
Half dose	5.0 (2.0, 8.0)	29	9.0 (5.8, 14.0)	96	-4.0 (-8.0, -2.0)
Clinical manifestation					
Mild	4.0 (3.0, 5.0)	9	9.0 (7.0, 12.0)	5	-5.0 (-9.0, -2.0)
Moderate	3.0 (3.0, 5.0)	184	8.0 (6.0, 12.0)	158	-5.0 (-6.0, -4.0)
Severe	7.0 (4.5, 10.0)	4	10.5 (9.0, 14.0)	114	-3.5 (-9.5, 2.5)
Province * Time from onset to treatment initiation					
GD <=3	4.5 (2.0, 7.3)	32	11.0 (7.0, 15.0)	37	-6.5 (-9.5, -3.5)
GD 3-7	5.0 (2.0, 8.0)	32	9 (7.0, 12.0)	36	-4.0 (-7.0, -1.0)
GD 7-14	4.0 (2.0, 6.0)	45	7.0 (4.3, 11.0)	18	-3.0 (-6.0, 0.0)
GD >14	3.0 (1.0, 5.0)	9	4.0 (3.0, 31.0)	5	-1.0 (-30.0, 4.0)
HB <=3	-	0	11.0 (11.0, 17.8)	6	-
HB 3-7	2.0 (2.0, 3.0)	3	8.5 (6.8, 12.3)	16	-6.5 (-9.5, -4.0)
HB 7-14	3.0 (3.0, 3.3)	8	9.0 (7.0, 10.5)	31	-6.0 (-7.0, -4.0)
HB >14	3.0 (3.0, 4.0)	68	7.0 (4.0, 9.5)	27	-4.0 (-5.0, -2.0)
Center					
SYSU5	4.5 (2.0, 8.0)	50	8.0 (4.0, 11.0)	21	-3.5 (-6.0, 1.0)



Abbreviations: GD, Guangdong; HB, Hubei.
95% CI are calculated by bootstrapping. The differences for all other variables are the absolute difference between chloroquine group and non-chloroquine group.

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Hydroxychloroquine and azithromycin plus zinc vs hydroxychloroquine and azithromycin alone: outcomes in hospitalized COVID-19 patients

Philip M. Carlucci[1], Tania Ahuja[2], Christopher Petrilli[1,3], Harish Rajagopalan[3], Simon Jones[4.5], Joseph Rahimian[1]

[1]New York University Grossman School of Medicine, Department of Medicine, New York, NY

[2]New York University Langone Health, Department of Pharmacy, New York, NY

[3]NYU Langone Health, New York, NY

[4]Division of Healthcare Delivery Science, Department of Population Health, NYU Grossman School of Medicine, New York, NY

[5]Center for Healthcare Innovation and Delivery Science, NYU Langone Health, New York, NY

[6]Division of Infectious Diseases and Immunology, Department of Medicine, NYU Grossman School of Medicine, New York, NY

Corresponding author:
Joseph Rahimian, MD
NYU Grossman School of Medicine, Department of Medicine
31 Washington Square West, Floor number 4
New York, NY 10011
Joseph.Rahimian@nyulangone.org
(212) 465-8834

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Running head: Hydroxychloroquine and azithromycin plus Zinc

40-word summary: Zinc sulfate added to hydroxychloroquine and azithromycin may improve outcomes among hospitalized patients.

ABSTRACT

Background: COVID-19 has rapidly emerged as a pandemic infection that has caused significant mortality and economic losses. Potential therapies and means of prophylaxis against COVID-19 are urgently needed to combat this novel infection. As a result of *in vitro* evidence suggesting zinc sulfate may be efficacious against COVID-19, our hospitals began using zinc sulfate as add-on therapy to hydroxychloroquine and azithromycin. We performed a retrospective observational study to compare hospital outcomes among patients who received hydroxychloroquine and azithromycin plus zinc versus hydroxychloroquine and azithromycin alone.

Methods: Data was collected from electronic medical records for all patients being treated with admission dates ranging from March 2, 2020 through April 5, 2020. Initial clinical characteristics on presentation, medications given during the hospitalization, and hospital outcomes were recorded. Patients in the study were excluded if they were treated with other investigational medications.

Results: The addition of zinc sulfate did not impact the length of hospitalization, duration of ventilation, or ICU duration. In univariate analyses, zinc sulfate increased the frequency of patients being discharged home, and decreased the need for ventilation, admission to the ICU, and mortality or transfer to hospice for patients who were never admitted to the ICU. After adjusting for the time at which zinc sulfate was added to our protocol, an increased frequency of being discharged home (OR 1.53, 95% CI 1.12-2.09) reduction in mortality or transfer to hospice remained significant (OR 0.449, 95% CI 0.271-0.744).

Conclusion: This study provides the first *in vivo* evidence that zinc sulfate in combination with hydroxychloroquine may play a role in therapeutic management for COVID-19.

INTRODUCTION

The World Health Organization has declared a pandemic due to spread of the coronavirus disease of 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2)[1, 2]. SARS-CoV2 is a single-strand RNA coronavirus, which enters human cells mainly by binding the angiotensin converting enzyme 2 (ACE2)[3]. SARS-CoV2 is primarily transmitted after viral particles are inhaled and enter the respiratory tract and has the potential to cause a severe systemic inflammatory response, acute respiratory disease syndrome (ARDS), multi organ failure, and shock[2, 4]. Laboratory abnormalities found in patients with COVID-19 include lymphopenia, elevation in lactate dehydrogenase, C reactive protein, D-dimer, ferritin and interleukin-6 (IL-6)[5, 6].

Several medications are under investigation for the treatment of COVID-19. Despite limited and conflicting data, the U.S. Food and Drug Administration authorized the emergency use of hydroxychloroquine for the treatment of COVID-19 with or without azithromycin. Chloroquine analogues are weak bases that concentrate within acidic endosomes and lysosomes. Once intracellular, chloroquine analogues become protonated and increase pH resulting in prevention of endosomal trafficking, dysfunctional cellular enzymes, and impaired protein synthesis[7]. This inhibits viral replication through interference with endosome-mediated viral entry or late transport of the enveloped virus. Further, this results in interference with the terminal glycosylation of ACE2 receptor expression which prevents SARS-CoV-2 receptor binding and spread

of infection [8]. Hydroxychloroquine, a hydroxy-derivative of chloroquine, has also been proposed based on *in vitro* activity against SARS-CoV-2 with a three-fold higher cytotoxic potential compared to chloroquine [9]. However, clinical data in humans has yielded mixed results[10-12]. The anti-viral and anti-inflammatory effects of chloroquine have been suggested to account for its potential utility in preventing COVID-19-related pneumonia. Soon current studies will answer whether hydroxychloroquine is effective as monotherapy or in combination with azithromycin. In the case that hydroxychloroquine is found to be ineffective, it may still have a role to play when combined with zinc sulfate. Zinc inhibits RNA dependent RNA polymerase, and has been shown to do this *in vitro* against SARS-CoV[13]. However, it is difficult to generate substantial intracellular concentrations of zinc, therefore prophylactic administration of zinc alone may not play a role against SarCoV-2[14]. When combined with a zinc ionophore, such as chloroquine (hydroxychloroquine), cellular uptake is increased making it more likely to achieve suitably elevated intracellular concentrations[15]. This combination is already being tested as a prophylactic regimen in a randomized clinical trial.

As New York became the epicenter of the pandemic, hospitals in the area quickly adopted investigational therapies, including the use of hydroxychloroquine and azithromycin. Given this proposed synergistic effect of zinc with hydroxychloroquine, practices at NYULH changed and the addition of zinc sulfate 220 mg PO BID along with hydroxcychloroquine 400 mg once followed by 200 mg PO BID with azithromycin 500 mg once daily became part of the treatment approach for patients admitted to the hospital with COVID-19. This study sought to investigate outcomes among patients who

received hydroxychloroquine and azithromycin alone compared to those who received triple therapy with zinc sulfate.

METHODS

We performed a retrospective analysis of data from patients hospitalized with confirmed SARS-CoV-2 infection at NYU Langone Health. Data was collected from electronic medical records (Epic Systems, Verona, WI) for all patients being treated with admission dates ranging from March 2, 2020 through April 5, 2020. Patients were admitted to any of four acute care NYU Langone Health hospitals across New York City. COVID-19 positivity was determined by real-time reverse-transcriptase-polymerase-chain-reaction (RT-PCR) of nasopharyngeal or oropharyngeal swabs. Prior to March 16, tests were completed by the New York City Department of Health and Mental Hygiene. After that date, NYU Langone clinical laboratory conducted tests using the Roche SARS-CoV2 assay in the Cobas 6800 instruments. On March 31, testing was also conducted using the SARS-CoV2 Xpert Xpress assay in the Cepheid GeneXpert instruments. After March 16, only pharyngeal samples were tested.

Patients were included in the study if they were admitted to the hospital, had at least one positive test for COVID-19, received hydroxychloroquine and azithromycin, and had either been discharged from the hospital, transitioned to hospice, or expired. Patients were excluded from the study if they were never admitted to the hospital or if there was an order for other investigational therapies for COVID-19, including tocilizumab, nitazoxanide, rituximab, anakinra, remdesivir, or lopinavir/ritonavir during the course of

their hospitalization to avoid potential confounding effects of these medications. We collected demographics as reported by the patient and any past medical history of hypertension, hyperlipidemia, coronary artery disease, heart failure, chronic obstructive pulmonary disease, asthma, malignancy other than non-melanoma skin malignancy, and diabetes. We also recorded vital signs on admission, the first set of laboratory results as continuous variables, and relevant medications as categorical variables, including NSAIDs, anticoagulants, antihypertensive medications and corticosteroids ordered at any point during the course of the hospitalization.

Statistics

Patients were categorized based on their exposure to hydroxychloroquine (400 mg load followed by 200 mg twice daily for five days) and azithromycin (500 mg once daily) alone or with zinc sulfate (220 mg capsule containing 50 mg elemental zinc twice daily for five days) as treatment in addition to standard supportive care. Descriptive statistics are presented as mean and standard deviation or mean and interquartile range for continuous variables and frequencies for categorical variables. Normality of distribution for continuous variables was assessed by measures of skewness and kurtosis, deeming the dataset appropriate for parametric or nonparametric analysis. A 2-tailed Student's t test was used for parametric analysis, and a Mann Whitney U test was used for nonparametric data analysis. Pearson's chi-squared test was used to compare categorical characteristics between the two groups of patients. Linear regression for continuous variables or logistic regression for categorical variables was performed with the presence of zinc as the predictor variable and outcome measures (duration of

hospital stay, duration of mechanical ventilation, maximum oxygen flow rate, average oxygen flow rate, average FiO2, maximum FiO2, admission to the intensive care unit (ICU), duration of ICU stay, death/hospice, need for intubation, and discharge destination), as dependent variables. Data was log transformed where appropriate to render the distribution normal for linear regression analysis. Multivariate logistic regression was used to adjust for the timing that our protocol changed to include zinc therapy using admission before or after March 25th as a categorical variable. P-values less than 0.05 were considered to be significant. All analyses were performed using STATA/SE 16.0 software (STATA Corp.).

Study approval

The study was approved by the NYU Grossman School of Medicine Institutional Review Board. A waiver of informed consent and a waiver of the Health Information Portability Privacy act were granted. The protocol was conducted in accordance to Declaration of Helsinki.

RESULTS

Patients taking zinc sulfate in addition to hydroxychloroquine and azithromycin (n=411) and patients taking hydroxychloroquine and azithromycin alone (n=521) did not differ in age, race, sex, tobacco use or past medical history (Table 1). On hospital admission, vital signs differed by respiratory rate and baseline systolic blood pressure. The first laboratory measurements of inflammatory markers including white blood cell count, absolute neutrophil count, ferritin, D-dimer, creatine phosphokinase, creatinine, and C-

reactive protein did not differ between groups. Patients treated with zinc sulfate had higher baseline absolute lymphocyte counts [median (IQR), zinc: 1 (0.7-1.3) vs. no zinc: 0.9 (0.6-1.3), p-value: 0.0180] while patients who did not receive zinc had higher baseline troponin [0.01 (0.01-0.02) vs. 0.015 (0.01-0.02), p-value: 0.0111] and procalcitonin [0.12 (0.05-0.25) vs 0.12 (0.06-0.43), p-value: 0.0493) (Table 1).

In univariate analysis, the addition of zinc sulfate to hydroxychloroquine and azithromycin was not associated with a decrease in length of hospital stay, duration of mechanical ventilation, maximum oxygen flow rate, average oxygen flow rate, average fraction of inspired oxygen, or maximum fraction of inspired oxygen during hospitalization (Table 2). In bivariate logistic regression analysis, the addition of zinc sulfate was associated with decreased mortality or transition to hospice (OR 0.511, 95% CI 0.359-0.726), need for ICU (OR 0.545, 95% CI 0.362-0.821) and need for invasive ventilation (OR 0.562, 95% CI 0.354-0.891) (Table 3). However, after excluding all non-critically ill patients admitted to the intensive care unit, zinc sulfate no longer was found to be associated with a decrease in mortality (Table 3). Thus, this association was driven by patients who did not receive ICU care (OR 0.492, 95% CI 0.303-0.799). We also found that the addition of zinc sulfate was associated with likelihood of discharge to home in univariate analysis (OR 1.56, 95% CI 1.16-2.10) (Table 3). We performed a logistic regression model to account for the time-period when the addition of zinc sulfate to hydroxychloroquine plus azithromycin became utilized at NYULH. After adjusting for this date (March 25th), we still found an association for likelihood of discharge to home (OR 1.53, 95% CI 1.12-2.09) and decreased mortality or transition to hospice however

the other associations were no longer significant (Table 4). The decrease in mortality or transition to hospice was most striking when considering only patients who were not admitted to the ICU (OR: 0.449, p-value: 0.002) (Table 4).

DISCUSSION

While practicing at the epicenter of the pandemic in the United States, we were faced with unprecedented challenges of adopting investigational therapies quickly into clinical practice. Initially, antiviral options at our institution consisted of clinician preference for either ritonavir/lopinavir or hydroxychloroquine plus azithromycin. After the findings of ritonavir/lopinavir in NEJM, we noticed an increase in the use of hydroxychloroquine plus azithromycin[16]. Our providers within the infectious diseases division, clinical pharmacy, and hospitalists discussed the use of zinc sulfate as an addition to hydroxychloroquine, based on the potential synergistic mechanism, and low risk of harm associated with this therapy.

To our knowledge, we provide the first *in vivo* evidence on the efficacy of zinc in COVID-19 patients. After adjusting for the timing of zinc sulfate treatment, the associations between zinc and the need for ICU and invasive ventilation were no longer significant but we did still observe a trend. This observation may be because patients with COVID-19 were initially sent to the ICU quicker, but as time went on and resources became more limited, clinicians began treating COVID-19 patients on general medicine floors for longer periods of time before escalating to the ICU. Future studies are needed to confirm or refute the hypothesis that the addition of zinc sulfate to a zinc ionophore

such as hydroxychloroquine may reduce the need for ICU care in patients with COVID-19.

The main finding of this study is that after adjusting for the timing of zinc therapy, we found that the addition of zinc sulfate to hydroxychloroquine and azithromycin was found to associate with a decrease in mortality or transition to hospice among patients who did not require ICU level of care, but this association was not significant in patients who were treated in the ICU. This result may be reflective of the proposed mechanism of action of zinc sulfate in COVID-19. Zinc has been shown to reduce SARS-CoV RNA dependent RNA polymerase activity *in vitro* [13]. As such, zinc may have a role in preventing the virus from progressing to severe disease, but once the aberrant production of systemic immune mediators is initiated, known as the cytokine storm, the addition of zinc may no longer be effective [17]. Our findings suggest a potential therapeutic synergistic mechanism of zinc sulfate with hydroxychloroquine, if used early on in presentation with COVID-19. However, our findings do not suggest a prophylactic benefit of zinc sulfate in the absence of a zinc ionophore, despite interest in this therapy for prevention. A prophylactic strategy of zinc sulfate should be evaluated to help answer this question.

This study has several limitations. First, this was an observational retrospective analysis that could be impacted by confounding variables. This is well demonstrated by the analyses adjusting for the difference in timing between the patients who did not receive zinc and those who did. In addition, we only looked at patients taking

hydroxychloroquine and azithromycin. We do not know whether the observed added benefit of zinc sulfate to hydroxychloroquine and azithromycin on mortality would have been seen in patients who took zinc sulfate alone or in combination with just one of those medications. We also do not have data on the time at which the patients included in the study initiated therapy with hydroxychloroquine, azithromycin, and zinc. Those drugs would have been started at the same time as a combination therapy, but the point in clinical disease at which patients received those medications could have differed between our two groups. Finally, the cohorts were identified based on medications ordered rather than confirmed administration, which may bias findings towards favoring equipoise between the two groups. In light of these limitations, this study should not be used to guide clinical practice. Rather, our observations support the initiation of future randomized clinical trials investigating zinc sulfate against COVID-19.

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	Zinc N=411	**No Zinc** N=521	**P-value**
Demographics			
Age	63.19 ± 15.18	61.83 ± 15.97	0.0942
Female Sex	147 (35.7%)	201 (38.6%)	0.378
Race			0.428
African American	68 (16.5%)	81 (15.5%)	
White	189 (46.0%	244 (46.8%)	
Asian	30 (7.3%)	30 (5.8%)	
Other	97 (23.6%)	142 (27.2%)	
Multiracial/Unknown	27 (6.6%)	24 (4.6%)	
History			
Tobacco use			0.142
Never or Unknown	306 (74.5%)	382 (73.3%)	
Former	76 (18.5%)	115 (22.1%)	
Current	29 (7.1%)	24 (4.6%)	
Any cardiovascular condition	182 (44.3%)	248 (47.6%)	0.313
Hypertension	154 (37.5%)	208 (39.9%)	0.445
Hyperlipidemia	99 (24.1%)	148 (28.4%)	0.138
Coronary Artery Disease	36 (8.8%)	41 (7.9%)	0.624
Heart Failure	26 (6.3%)	22 (4.2%)	0.149

Asthma or COPD	50 (12.2%)	56 (10.7%)	0.499
Diabetes	105 (25.5%)	130 (25.0%)	0.835
Malignancy	23 (5.6%)	33 (6.3%)	0.638
Transplant	3 (0.7%)	2 (0.4%)	0.473
Chronic Kidney Disease	47 (11.4%)	44 (8.4%)	0.127
BMI kg/m^2	29.17 (25.8-33.42)	29.29 (25.77-33.2)	0.8611
Admission Characteristics			
Oxygen saturation at presentation	94 (91-96)*	94 (91-96)**	0.1729
Respiratory Rate, respirations per minute	20 (19-24)	20 (18-24)	**0.0460**
Pulse, beats per minute	97.66 ± 18.61	99.40 ± 19.82	0.0858
Baseline Systolic BP, mmHg	134.83 ± 20.84	132.41 ± 21.87	**0.0435**
Baseline Diastolic BP, mmHg	76.66 ± 12.62	76.59 ± 14.22	0.4670
Temperature, degrees Celsius	37.65 ± 0.82	37.72 ± 0.94	0.1354
White blood cell count 10^3/ul	6.9 (5.1-9.0) N=400	6.9 (5.1-9.3) N=500	0.5994
Absolute neutrophil count, 10^3/ul	5.15 (3.6-7.05) N=388	5.4 (3.8-7.5) N=488	0.0838
Absolute lymphocyte count, 10^3/ul	1 (0.7-1.3) N=388	0.9 (0.6-1.3) N=482	**0.0180**
Ferritin, ng/mL	739 (379-1528) N=397	658 (336.2-1279) N=473	0.1304
D-Dimer, ng/mL	341 (214-565) N=384	334 (215-587) N=435	0.7531
Troponin, ng/mL	0.01 (0.01-0.02) N=389	0.015 (0.01-0.02) N=467	**0.0111**
Creatine Phosphokinase, U/L	140 (68-330) N=343	151.5 (69.5-398.5) N=344	0.4371
Procalcitonin, ng/mL	0.12 (0.05-0.25) N=395	0.12 (0.06-0.43) N=478	**0.0493**

Creatinine, mg/dL	0.97 (0.8-1.34) N=400	0.99 (0.8-1.27) N=499	0.4140
C-Reactive Protein, mg/L	104.95 (51.1-158.69) N=398	108.13 (53-157.11) N=480	0.9586
Medications recorded during hospitalization			
NSAID	53 (12.9%)	74 (14.2%)	0.563
Anticoagulant	402 (97.8%)	511 (98.1%)	0.772
ACE inhibitor or ARB	138 (33.6%	175 (33.7%)	0.997
Beta Blocker	91 (22.1%)	132 (25.3%)	0.256
Calcium Channel Blocker	89 (21.7%)	104 (20.0%)	0.527
Corticosteroid	40 (9.7%)	47 (9.0%)	0.711

Table 1: Comparisons of baseline characteristics and hospital medications. Data are represented as median (IQR) or mean $\pm$ SD. Sample size is reported where it differed due to lab results not tested. P-values were calculated using 2-sided t-test for parametric variables and Mann Whitney U test for nonparametric continuous variables. Pearson χ^2 test was used for categorical comparisons. $P < .05$ was deemed significant. Laboratory results represent the first measured value while hospitalized.

*measured on supplemental oxygen for 86.4%

**measured on supplemental oxygen for 83.1%

	Zinc	No Zinc	β Coefficient	P-value
Length of Hospital stay (in days)*	6 (4-9) N=411	6 (3-9) N=521	0.015	0.646
Duration of mechanical* ventilation (in days)	5 (3-8) N=33	5 (3-9) N=86	0.040	0.667
ICU Duration (in days)*	4.85 (1.97-7.94) N=38	5.54 (2.65-9.32) N=82	-0.062	0.504
O2 Flow rate max*	6 (3-15) N=353	6 (3-15) N=426	-0.015	0.679
O2 Flow rate avg*	3.05 (2.1-6.3) N=353	3.5 (2.5-7.5) N=426	-0.062	0.082
FiO2 AVG	61.52 ± 32.03 N=107	65.26 ± 34.48 N=117	-.056	0.402
FIO2 MAX	74.94 ± 35.75 N=107	71.98 ± 35.85 N=117	0.041	0.538

Table 2: Comparisons of continuous hospital outcomes. Data are represented median (IQR) and as mean ± SD. Sample size is reported for each variable tested. β Coefficients and P-values were calculated using linear regression. N was specified for each comparison. *P* < .05 was deemed significant. *variables were log transformed for regression analysis

	Zinc N=411	No Zinc N=521	Odds Ratio	95% Confidence Interval	P-value
Discharged home	317 (77.1%)	356 (68.3%)	1.56	1.16-2.10	**0.003**
Needed ICU	38 (9.2%)	82 (15.7%)	0.545	0.362-0.821	**0.004**
Needed Invasive Ventilation	33 (8.0%)	86 (16.5%)	0.562	0.354-0.891	**0.014**
Expired/Hospice	54 (13.1%)	119 (22.8%)	0.511	0.359-0.726	**<0.0001**
Expired/Hospice**	28 (73.6%) N=38	61 (74.4%) N=82	0.964	0.401-2.31	0.934
Expired/Hospice***	26 (6.9%) N=373	58 (13.2%) N=439	0.492	0.303-0.799	**0.004**

Table 3: Comparison of categorical hospital outcomes. Data are represented as N(%). P-values were calculated using logistic regression. $P < .05$ was deemed significant. N was specified for subgroup analyses.

**After excluding all non ICU patients

***After excluding all ICU patients

	Zinc N=411	No Zinc N=521	Adjusted Odds Ratio	Adjusted 95% Confidence Interval	Adjusted P-value
Discharged home	317 (77.1%)	356 (68.3%)	1.53	1.12-2.09	**0.008**
Needed ICU	38 (9.2%)	82 (15.7%)	0.733	0.471-1.14	0.168
Needed Invasive Ventilation	33 (8.0%)	86 (16.5%)	0.804	0.487-1.33	0.396
Expired/Hospice	54 (13.1%)	119 (22.8%)	0.559	0.385-0.811	**0.002**
Expired/Hospice**	28 (73.6%) N=38	61 (74.4%) N=82	1.03	0.404-2.64	0.947
Expired/Hospice***	26 (6.9%) N=373	58 (13.2%) N=439	0.449	0.271-0.744	**0.002**

Table 4: Adjusted comparison of categorical hospital outcomes. Data are represented as N(%). P-values were calculated using multivariate logistic regression adjusting for patient admission after March 25th as a categorical variable. $P < .05$ was deemed significant. N was specified for subgroup analyses.

**After excluding all non ICU patients

***After excluding all ICU patients

Chloroquine or hydroxychloroquine for prophylaxis of COVID-19

In-vitro studies have shown that chloroquine is effective against several viruses, including severe acute respiratory syndrome coronavirus (SARS-CoV).[1] Multiple mechanisms of action have been identified for chloroquine that disrupt the early stage of coronavirus replication. Moreover, chloroquine affects immune system activity by mediating an anti-inflammatory response, which might reduce damage due to the exaggerated inflammatory response.[1] At the time of the SARS epidemic, chloroquine was suggested as a drug that could be used to treat this infection.[2] However, randomised, double-blind, controlled studies in humans to evaluate its efficacy for this use were not done, and the true clinical efficacy of chloroquine in treating coronavirus infections was not established.

Because coronavirus disease 2019 (COVID-19) is associated with substantial morbidity and mortality,[3] and no specific pharmacological treatment that is effective against it is available, chloroquine and chloroquine-related formulations have been tentatively included among drugs for use in limiting the total burden of COVID-19.[4,5] However, no studies have evaluated the use of chloroquine for prophylaxis.

Chloroquine is a cheap drug that has been used for decades—predominantly for malaria prophylaxis, for which it had excellent results and good safety and tolerability.[1] Severe adverse events, which mainly involve retinal and psychiatric symptoms, occur only when doses prescribed for malaria are substantially higher than required.[1] Inhibition of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) replication seems essential to reduce the risk of spread and development of COVID-19. SARS-CoV-2 is highly contagious.[5] Most people who live in areas with a high incidence of COVID-19 are apparently healthy, but they can be SARS-CoV-2 negative and healthy or healthy but with asymptomatic infection. In both cases, effective drugs such as chloroquine and its related formulations might prevent infection (ie, in those who are SARS-CoV-2 negative) or the development of severe symptomatic disease (ie, in those who are SARS-CoV-2 positive and asymptomatic or with minor symptoms), substantially reducing morbidity and mortality due to COVID-19. The dose used might be the same as that usually administered for malaria treatment given chloroquine inhibited SARS-CoV replication at a 50% effective concentration of 8·8 μmol/L. The half-maximal inhibitory concentration (IC_{50}) of chloroquine inhibition of SARS-CoV replication in Vero E6 cells, 8·8 μmol/L, is substantially lower than the plasma concentrations that are reached in humans when the drug is prescribed to treat malaria at a dose of 25 mg/kg over 3 days.[1] For long-term prophylaxis, even lower doses could be used. Doses of 3·6 mg/kg, similar to those generally prescribed to treat rheumatoid arthritis, lead to plasma concentrations of 1–3 μmol/L—ie, the same concentration range as the IC_{50} for SARS-CoV inhibition.[1] Alternatively, hydroxychloroquine could be used, for which even greater efficacy has been reported in in-vitro studies.[5] Prophylaxis could last for the whole duration of an outbreak, and in countries in which malaria is not endemic, there is no risk of negative events associated with the development of resistance to this drug. In countries where malaria is endemic, appropriate monitoring of resistance among *Plasmodium* spp is needed.

Future studies might better elucidate the most effective schedule of administration and potential adverse events. We advocate for studies to evaluate whether chloroquine or hydroxychloroquine prophylaxis should be considered in a country such as Italy, where there are thousands of cases and deaths as a result of COVID-19.

We declare no competing interests.

*Nicola Principi, *Susanna Esposito*
susanna.esposito@unimi.it

Università degli Studi di Milano, Milan Italy (NP); Paediatric Clinic, Pietro Barilla Children's Hospital, University of Parma, 43126 Parma, Italy (SE)

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Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial

Philippe Gautret[a,b$], Jean-Christophe Lagier[a,c$], Philippe Parola[a,b], Van Thuan Hoang[a,b,d], Line Meddeb[a], Morgane Mailhe[a], Barbara Doudier[a], Johan Courjon[e,f,g], Valérie Giordanengo[h], Vera Esteves Vieira[a], Hervé Tissot Dupont[a,c], Stéphane Honoré[i,j], Philippe Colson[a,c], Eric Chabrière[a,c], Bernard La Scola[a,c], Jean-Marc Rolain[a,c], Philippe Brouqui[a,c], Didier Raoult[a,c*].

[a]IHU-Méditerranée Infection, Marseille, France.

[b]Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille, France.

[c]Aix Marseille Univ, IRD, APHM, MEPHI, Marseille, France.

[d]Thai Binh University of Medicine and Pharmacy, Thai Binh, Viet Nam

[e]Infectiologie, Hôpital de l'Archet, Centre Hospitalier Universitaire de Nice, Nice, France

[f]Université Côte d'Azur, Nice, France

[g]U1065, Centre Méditerranéen de Médecine Moléculaire, C3M, Virulence Microbienne et Signalisation Inflammatoire, INSERM, Nice, France

[h]Department of Virology, Biological and Pathological Center, Centre Hospitalier Universitaire de Nice, 06200 Nice, France.

[i]Service Pharmacie, Hôpital Timone, AP-HM, Marseille, France

[j]Laboratoire de Pharmacie Clinique, Aix Marseille Université, Marseille, France.

[$]equal work

*Corresponding author:

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Didier Raoult

Didier.raoult@gmail.com

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Abstract

Background

Chloroquine and hydroxychloroquine have been found to be efficient on SARS-CoV-2, and reported to be efficient in Chinese COV-19 patients. We evaluate the role of hydroxychloroquine on respiratory viral loads.

Patients and methods

French Confirmed COVID-19 patients were included in a single arm protocol from early March to March 16th, to receive 600mg of hydroxychloroquine daily and their viral load in nasopharyngeal swabs was tested daily in a hospital setting. Depending on their clinical presentation, azithromycin was added to the treatment. Untreated patients from another center and cases refusing the protocol were included as negative controls. Presence and absence of virus at Day6-post inclusion was considered the end point.

Results

Six patients were asymptomatic, 22 had upper respiratory tract infection symptoms and eight had lower respiratory tract infection symptoms.

Twenty cases were treated in this study and showed a significant reduction of the viral carriage at D6-post inclusion compared to controls, and much lower average carrying duration than reported of untreated patients in the literature. Azithromycin added to hydroxychloroquine was significantly more efficient for virus elimination.

Conclusion

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Despite its small sample size our survey shows that hydroxychloroquine treatment is significantly associated with viral load reduction/disappearance in COVID-19 patients and its effect is reinforced by azithromycin.

Key words: 2019-nCoV; SARS-CoV-2; COVID-19; hydroxychloroquine; azithomycin; clinical trial

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1. Introduction

In late December 2019, an outbreak of an emerging disease (COVID-19) due to a novel coronavirus (named SARS-CoV-2 latter) started in Wuhan, China and rapidly spread in China and outside [1,2]. The WHO declared the epidemic of COVID-19 as a pandemic on March 12th 2020 [3]. According to a recent Chinese stud, about 80% of patients present with mild disease and the overall case-fatality rate is about 2.3% but reaches 8.0% in patients aged 70 to 79 years and 14.8% in those aged ≥80 years [4]. However, there is probably an important number of asymptomatic carriers in the population, and thus the mortality rate is probably overestimated. France is now facing the COVID-19 wave with more than 4500 cases, as of March 14th 2020 [5]. Thus, there is an urgent need for an effective treatment to treat symptomatic patients but also to decrease the duration of virus carriage in order to limit the transmission in the community. Among candidate drugs to treat COVID-19, repositioning of old drugs for use as antiviral treatment is an interesting strategy because knowledge on safety profile, side effects, posology and drug interactions are well known [6,7].

A recent paper reported an inhibitor effect of remdesivir (a new antiviral drug) and chloroquine (an old antimalarial drug) on the growth of SARS-CoV-2 *in vitro,* [8] and an early clinical trial conducted in COVID-19 Chinese patients, showed that chloroquine had a significant effect, both in terms of clinical outcome and viral clearance, when comparing to controls groups [9,10]. Chinese experts recommend that patients diagnosed as mild, moderate and severe cases of COVID-19 pneumonia and without contraindications to chloroquine, be treated with 500 mg chloroquine twice a day for ten days [11].

Hydroxychloroquine (an analogue of chloroquine) has been demonstrated to have an anti-SARS-CoV activity *in vitro* [12]. Hydroxychloroquine clinical safety profile is better than that of chloroquine (during long-term use) and allows higher daily dose [13] and has fewer

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concerns about drug-drug interactions [14]. Our team has a very comprehensive experience in successfully treating patients with chronic diseases due to intracellular bacteria (Q fever due to *Coxiella burnetii* and Whipple's disease due to *Tropheryma whipplei*) with long-term hydroxychloroquine treatment (600 mg/day for 12 to 18 months) since more than 20 years. [15,16] We therefore started to conduct a clinical trial aiming at assessing the effect of hydroxychloroquine on SARS-CoV-2-infected patients after approval by the French Ministry of Health. In this report we describe our early results, focusing on virological data in patients receiving hydroxychloroquine as compared to a control group.

2. Study population and Methods

Setting

This ongoing study is coordinated by The Méditerranée Infection University Hospital Institute in Marseille. Patients who were proposed a treatment with hydroxychloroquine were recruited and managed in Marseille centre. Controls without hydroxychloroquine treatment were recruited in Marseille, Nice, Avignon and Briançon centers, all located in South France.

Patients

Hospitalized patients with confirmed COVID-19 were included in this study if they fulfilled two primary criteria: i) age >12 years; ii) PCR documented SARS-CoV-2 carriage in nasopharyngeal sample at admission whatever their clinical status.

Patients were excluded if they had a known allergy to hydroxychloroquine or chloroquine or had another known contraindication to treatment with the study drug, including retinopathy, G6PD deficiency and QT prolongation. Breastfeeding and pregnant patients were excluded based on their declaration and pregnancy test results when required.

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Informed consent

Before being included in the study, patients meeting inclusion criteria had to give their consent to participate to the study. Written informed signed consent was obtained from adult participants (≥ 18 years) or from parents or legal guardians for minors (<18 years). An information document that clearly indicates the risks and the benefits associated with the participation to the study was given to each patient. Patients received information about their clinical status during care regardless of whether they participate in the study or not. Regarding patient identification, a study number was assigned sequentially to included participants, according to the range of patient numbers allocated to each study centre. The study was conducted in accordance with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines of good clinical practice, the Helsinki Declaration, and applicable standard operating procedures.

The protocol, appendices and any other relevant documentation were submitted to the French National Agency for Drug Safety (ANSM) (2020-000890-25) and to the French Ethic Committee (CPP Ile de France) (20.02.28.99113) for reviewing and approved on 5th and 6th March, 2020, respectively. This trial is registered with EU Clinical Trials Register, number 2020-000890-25.

Procedure

Patients were seen at baseline for enrolment, initial data collection and treatment at day-0, and again for daily follow-up during 14 days. Each day, patients received a standardized clinical examination and when possible, a nasopharyngeal sample was collected. All clinical data were collected using standardized questionnaires. All patients in Marseille center were proposed oral hydroxychloroquine sulfate 200 mg, three times per day during ten days (in this preliminary phase ,we did not enrolled children in the treatment group based in data indicating that children

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develop mild symptoms of COVID-19 [4]). Patients who refused the treatment or had an exclusion criteria, served as controls in Marseille centre. Patients in other centers did not receive hydroxychloroquine and served as controls. Symptomatic treatment and antibiotics as a measure to prevent bacterial super-infection was provided by investigators based on clinical judgment. Hydroxychloroquine was provided by the National Pharmacy of France on nominative demand.

Clinical classification

Patients were grouped into three categories: asymptomatic, upper respiratory tract infection (URTI) when presenting with rhinitis, pharyngitis, or isolated low-grade fever and myalgia, and lower respiratory tract infections (LRTI) when presenting with symptoms of pneumonia or bronchitis.

PCR assay

SARS-CoV-2 RNA was assessed by real-time reverse transcription-PCR [17].

Hydroxychloroquine dosage

Native hydroxychloroquine has been dosed from patients' serum samples by UHPLC-UV using a previously described protocol [18]. The peak of the chromatogram at 1.05 min of retention corresponds to hydroxychloroquine metabolite. The serum concentration of this metabolite is deduced from UV absorption, as for hydroxychloroquine concentration. Considering both concentrations provides an estimation of initial serum hydroxychloroquine concentration.

Culture

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For all patients, 500 µL of the liquid collected from the nasopharyngeal swab were passed through 0.22-µm pore sized centrifugal filter (Merck millipore, Darmstadt, Germany), then were inoculated in wells of 96-well culture microplates, of which 4 wells contained Vero E6 cells (ATCC CRL-1586) in Minimum Essential Medium culture medium with 4% fetal calf serum and 1% glutamine. After centrifigation at 4,000 g, microplates were incubated at 37°C. Plates were observed daily for evidence of cytopathogenic effect. Presumptive detection of virus in supernatant was done using SU5000 SEM (Hitachi) then confirmed by specific RT-PCR.

Outcome

The primary endpoint was virological clearance at day-6 post-inclusion. Secondary outcomes were virological clearance overtime during the study period, clinical follow-up (body temperature, respiratory rate, long of stay at hospital and mortality), and occurrence of side-effects.

Statistics

Assuming a 50% efficacy of hydroxychloroquine in reducing the viral load at day 7, a 85% power, a type I error rate of 5% and 10% loss to follow-up, we calculated that a total of 48 COVID-19 patients (ie, 24 cases in the hydroxychloroquine group and 24 in the control group) would be required for the analysis (Fleiss with CC). Statistical differences were evaluated by Pearson's chi-square or Fisher's exact tests as categorical variables, as appropriate. Means of quantitative data were compared using Student's t-test. Analyses were performed in Stata version 14.2.

3. **Results** (detailed results are available in supplementary Table 1)

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Demographics and clinical presentation

We enrolled 36 out of 42 patients meeting the inclusion criteria in this study that had at least six days of follow-up at the time of the present analysis. A total of 26 patients received hydroxychloroquine and 16 were control patients. Six hydroxychloroquine-treated patients were lost in follow-up during the survey because of early cessation of treatment. Reasons are as follows: three patients were transferred to intensive care unit, including one transferred on day2 post-inclusion who was PCR-positive on day1, one transferred on day3 post-inclusion who was PCR-positive on days1-2 and one transferred on day4 post-inclusion who was PCR-positive on day1 and day3; one patient died on day3 post inclusion and was PCR-negative on day2; one patient decided to leave the hospital on day3 post-inclusion and was PCR-negative on days1-2; finally, one patient stopped the treatment on day3 post-inclusion because of nausea and was PCR-positive on days1-2-3. The results presented here are therefore those of 36 patients (20 hydroxychloroquine-treated patients and 16 control patients). None of the control patients was lost in follow-up. Basic demographics and clinical status are presented in Table 1. Overall, 15 patients were male (41.7%), with a mean age of 45.1 years. The proportion of asymptomatic patients was 16.7%, that of patients with URTI symptoms was 61.1% and that of patients with LRTI symptoms was 22.2%). All patients with LRTI symptoms, had confirmed pneumonia by CTScan. Hydroxychloroquine-treated patients were older than control patients (51.2 years vs. 37.3 years). No significant difference was observed between hydroxychloroquine-treated patients and control patients with regard to gender, clinical status and duration of symptoms prior to inclusion (Table 1). Among hydroxychloroquine-treated patients six patients received azithromycin (500mg on day1 followed by 250mg per day, the next four days) to prevent bacterial super-infection under daily electrocardiogram control. Clinical follow-up and occurrence of side-effects will be described in a further paper at the end of the trial.

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Hydroxychloroquine dosage

Mean hydroxychloroquine serum concentration was 0.46 µg/ml±0.2 (N=20).

Effect of hydroxychloroquine on viral load

The proportion of patients that had negative PCR results in nasopharyngeal samples significantly differed between treated patients and controls at days 3-4-5 and 6 post-inclusion (Table 2). At day6 post-inclusion, 70% of hydroxychloroquine-treated patients were virologicaly cured comparing with 12.5% in the control group (p= 0.001).

When comparing the effect of hydroxychloroquine treatment as a single drug and the effect of hydroxychloroquine and azithromyc in combination, the proportion of patients that had negative PCR results in nasopharyngeal samples was significantly different between the two groups at days 3-4-5 and 6 post-inclusion (Table 3). At day6 post-inclusion, 100% of patients treated with hydroxychloroquine and azithromycin combination were virologicaly cured comparing with 57.1% in patients treated with hydroxychloroquine only, and 12.5% in the control group ($p<0.001$). These results are summarized in Figures 1 and 2. Drug effect was significantly higher in patients with symptoms of URTI and LRTI, as compared to asymptomatic patients with $p<0.05$ (data not show).

Of note, one patient who was still PCR-positive at day6-post inclusion under hydroxychloroquine treatment only, received azithromycin in addition to hydroxychloroquine at day8-post inclusion and cured her infection at day-9 post infection. In contrast, one of the patients under hydroxychloroquine and azithromycin combination who tested negative at day6 post-inclusion was tested positive at low titer at day8 post-inclusion.

Cultures

We could isolate SARS-CoV-2 in 19 out of 25 clinical samples from patients.

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4. Discussion

For ethical reasons and because our first results are so significant and evident we decide to share our findings with the medical community, given the urgent need for an effective drug against SARS-CoV-2 in the current pandemic context.

We show here that hydroxychloroquine is efficient in clearing viral nasopharyngeal carriage of SARS-CoV-2 in COVID-19 patients in only three to six days, in most patients. A significant difference was observed between hydroxychloroquine-treated patients and controls starting even on day3 post-inclusion. These results are of great importance because a recent paper has shown that the mean duration of viral shedding in patients suffering from COVID-19 in China was 20 days (even 37 days for the longest duration) [19]

Very recently, a Chinese team published results of a study demonstrating that chloroquine and hydroxychloroquine inhibit SARS-CoV-2 *in vitro* with hydroxychloroquine (EC50=0.72%μM) found to be more potent than chloroquine (EC50=5.47%μM) [14]. These *in vitro* results corroborate our clinical results. The target values indicated in this paper [14] were reached in our experiments. The safer dose-dependent toxicity profile of hydroxychloroquine in humans, compared to that of chloroquine [13] allows using clinical doses of hydroxychloroquine that will be over its EC50 observed *in vitro* [14].

Our preliminary results also suggest a synergistic effect of the combination of hydroxychloroquine and azithromycin. Azithromycin has been shown to be active *in vitro* against Zika and Ebola viruses [20-22] and to prevent severe respiratory tract infections when administrated to patients suffering viral infection [23]. This finding should be further explored to know whether a combination is more effective especially in severe cases. Speculated

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potential risk of severe QT prolongation induced by the association of the two drugs has not been established yet but should be considered. As for each treatment, the cost benefits of the risk should be evaluated individually. Further studies on this combination are needed, since such combination may both act as an antiviral therapy against SARS-CoV-2 and prevent bacterial super-infections.

The cause of failure for hydroxychloroquine treatment should be investigated by testing the isolated SARS-CoV-2 strains of the non-respondents and analyzing their genome, and by analyzing the host factors that may be associated with the metabolism of hydroxychloroquine. The existence of hydroxychloroquine failure in two patients (mother and son) is more suggestive of the last mechanism of resistance.

Such results are promising and open the possibility of an international strategy to decision-makers to fight this emerging viral infection in real-time even if other strategies and research including vaccine development could be also effective, but only in the future. We therefore recommend that COVID-19 patients be treated with hydroxychloroquine and azithromycin to cure their infection and to limit the transmission of the virus to other people in order to curb the spread of COVID-19 in the world. Further works are also warranted to determine if these compounds could be useful as chemoprophylaxis to prevent the transmission of the virus, especially for healthcare workers. Our study has some limitations including a small sample size, limited long-term outcome follow-up, and dropout of six patients from the study, however in the current context, we believe that our results should be shared with the scientific community.

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Titles for figures

Figure 1. Percentage of patients with PCR-positive nasopharyngeal samples from inclusion to day6 post-inclusion in COVID-19 patients treated with hydroxychloroquine and in COVID-19 control patients.

Figure 2. Percentage of patients with PCR-positive nasopharyngeal samples from inclusion to day6 post-inclusion in COVID-19 patients treated with hydroxychloroquine only, in COVID-19 patients treated with hydroxychloroquine and azithomycin combination, and in COVID-19 control patients.

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Table 1 Characteristics of the study population.

	Age (years)			Male gender		Clinical status				Time between onset of symptoms and inclusion (days)		
	Mean ± SD	t	p-value	n (%)	p-value	Asymptomatic	URTI	LRTI	p-value	Mean ± SD	t	p-value
Hydroxychloroquine treated patients (N=20)	51.2 ± 18.7	-1.95	0.06	9 (45.0)	0.65	2 (10.0)	12 (60.0)	6 (30.0)	0.30	4.1 ± 2.6	-0.15	0.88
Control patients (N=16)	37.3 ± 24.0			6 (37.5)		4 (25.0)	10 (62.5)	2 (12.5)		3.9 ± 2.8		
All patients (36)	45.1 ± 22.0			15 (41.7)		6 (16.7)	22 (61.1)	8 (22.2)		4.0 ± 2.6		

URTI: upper tract respiratory infection, LRTI: lower tract respiratory infection

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Table 2. Proportion of patients with virological cure (negative nasopharyngeal PCR) by day, in COVID-19 patients treated with hydroxychloroquine and in COVID-19 control patients.

	Day3 post inclusion			Day4 post inclusion			Day5 post inclusion			Day6 post inclusion		
	Number of negative patients/total number of patients	%	p-value	Number of negative patients/total number of patients	%	p-value	Number of negative patients/total number of patients	%	p-value	Number of negative patients/total number of patients	%	p-value
Hydroxychloroquine treated patients (N=20)	10/20	50.0	0.005	12/20	60.0	0.04	13/20	65.0	0.006	14/20	70.0	0.001
Control patients (N=16)	1/16	6.3		4/16	25.0		3/16	18.8		2/16	12.5	

[a]control patients from centers other than Marseille did not underwent daily sampling, but were sampled every other day in most cases, they were considered positive for PCR when actually positive the day(s) before and the day(s) after the day(s) with missing data.

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Table 3. Proportion of patients with virological cure (negative nasopharyngeal PCR) by day, in COVID-19 patients treated with hydroxychloroquine only, in COVID-19 patients treated with hydroxychloroquine and azithomycin combination, and in COVID-19 control patients.

	Day3 post inclusion			Day4 post inclusion			Day5 post inclusion			Day6 post inclusion		
	Number of negative patients/total number of patients	%	p-value	Number of negative patients/total number of patients	%	p-value	Number of negative patients/total number of patients	%	p-value	Number of negative patients/total number of patients	%	p-value
Control patients	1/16	6.3		4/16	25.0		3/16	18.8		2/16	12.5	
Hydroxychloroquine treatment only	5/14	35.7	0.002	7/14	50.0	0.05	7/14	50.0	0.002	8/14	57.1	<0.001
Hydroxychloroquine and azithromycin combined treatment	5/6	83.3		5/6	83.3		6/6	100		6/6	100	

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Supplementary Table 1.

Patient	Age (years)	Sex	Clinical status	Time between onset of symptoms and inclusion (days)	Hydroxychloroquine treatment	Hydroxychloroquine serum concentration µg/ml (day of dosage)	Azithromycin treatment	D0	D1	D2	D3	D4	D5	D6
1	10	M	Asymptomatic	-	No	-	No	31	NEG	NEG	NEG	NEG	NEG	NEG
2	12	F	Asymptomatic	-	No	-	No	26	ND	33	34	NEG	34	NEG
3	14	F	Asymptomatic	-	No	-	No	26	31	23	22	27	NEG	26
4	10	M	Asymptomatic	-	No	-	No	24	NEG	33	33	NEG	NEG	32
5	20	M	URTI	4	No	-	No	24	24	24	27	NEG	31	29
6	65	F	URTI	2	No	-	No	POS	ND	POS	ND	POS	ND	POS
7	46	M	URTI	Unknown	No	-	No	28	ND	ND	ND	26	ND	30
8	69	M	LRTI	2	No	-	No	POS	ND	POS	ND	POS	POS	POS
9	62	F	LRTI	10	No	-	No	POS	ND	POS	ND	POS	ND	POS
10	66	F	URTI	0	No	-	No	POS	ND	POS	ND	ND	ND	POS
11	75	F	URTI	3	No	-	No	POS	ND	POS	ND	POS	ND	ND
12	23	F	URTI	5	No	-	No	ND	ND	POS	ND	POS	ND	ND
13	45	F	URTI	Unknown	No	-	No	POS	ND	POS	ND	POS	ND	POS
14	16	M	URTI	2	No	-	No	POS	ND	POS	ND	ND	POS	ND
15	42	F	URTI	5	No	-	No	ND	ND	ND	POS	ND	POS	ND
16	23	F	URTI	6	No	-	No	POS	ND	ND	ND	ND	POS	ND
17	44	F	URTI	6	Yes	0.519 (D6)	No	30	ND	29	26	32	26	31
18	54	M	Asymptomatic	-	Yes	0.462 (D6)	No	29	NEG	NEG	NEG	NEG	NEG	NEG
19	25	M	URTI	3	Yes	0.419 (D6)	No	23	25	28	25	NEG	NEG	NEG
20	59	F	Asymptomatic	-	Yes	0.288 (D4)	No	30	NEG	NEG	NEG	NEG	NEG	NEG
21	49	F	URTI	1	Yes	0.621 (D6)	No	34	27	19	16	34	24	22
22	24	F	URTI	10	Yes	0.723 (D6)	No	28	NEG	32	34	NEG	NEG	NEG
23	81	F	LRTI	2	Yes	0.591 (D6)	No	22	21	30	NEG	32	28	NEG
24	85	F	LRTI	1	Yes	0.619 (D6)	No	17	21	23	21	26	24	24
25	40	M	URTI	3	Yes	0.418 (D6)	No	22	ND	28	21	15	20	17
26	53	M	URTI	5	Yes	0.515 (D6)	No	27	28	32	31	NEG	NEG	NEG
27	63	F	URTI	8	Yes	0.319 (D4)	No	34	NEG	30	NEG	NEG	NEG	NEG
28	42	F	URTI	1	Yes	0.453 (D6)	No	19	16	17	17	19	20	31
29	87	F	URTI	5	Yes	0.557 (D6)	No	25	30	NEG	NEG	NEG	ND	ND
30	33	M	URTI	2	Yes	0.194 (D2)	No	15	23	26	26	NF	32	32
31	53	F	LTRI	7	Yes	1.076 (D6)	Yes	28	31	34	NEG	34	NEG	NEG
32	48	M	URTI	2	Yes	0.57 (D6)	Yes	23	29	29	NEG	NEG	NEG	NEG
33	50	F	LRTI	5	Yes	0.827 (D6)	Yes	30	27	NEG	NEG	NEG	NEG	NEG
34	20	M	URTI	2	Yes	0.381 (D6)	Yes	27	31	29	NEG	NEG	NEG	NEG
35	54	M	LRTI	6	Yes	0.366 (D4)	Yes	24	ND	ND	29	NEG	NEG	NEG
36	60	M	LRTI	4	Yes	0.319 (D4)	Yes	29	31	31	NEG	NEG	NEG	NEG

URTI: upper tract respiratory infection, LRTI: lower tract respiratory infection, POS: positive PCR, NEG: negative PCR (CT value $\geq$35), ND: PCR not done

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Figure 1. Percentage of patients with PCR-positive nasopharyngeal samples from inclusion to day6 post-inclusion in COVID-19 patients treated with hydroxychloroquine and in COVID-19 control patients.

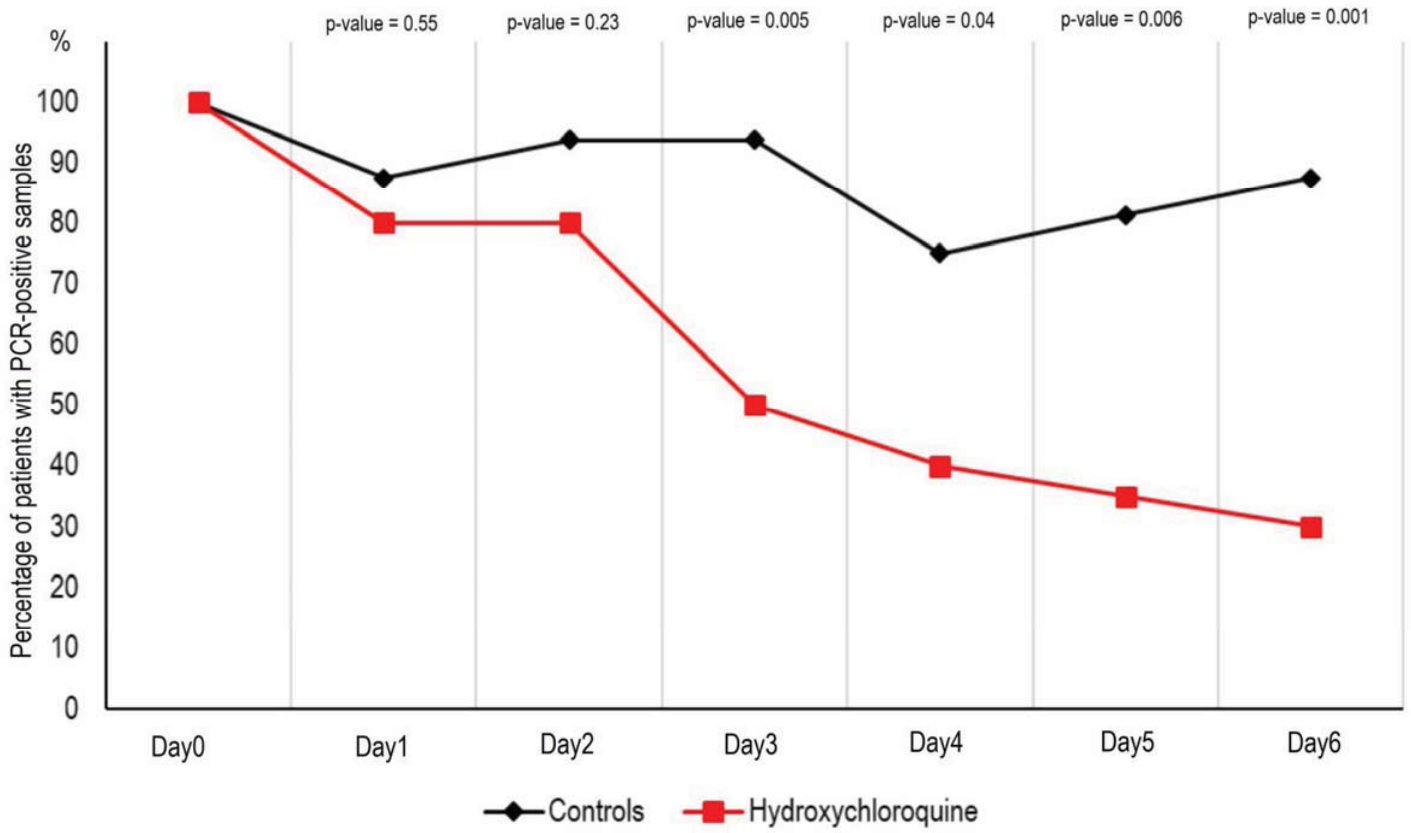

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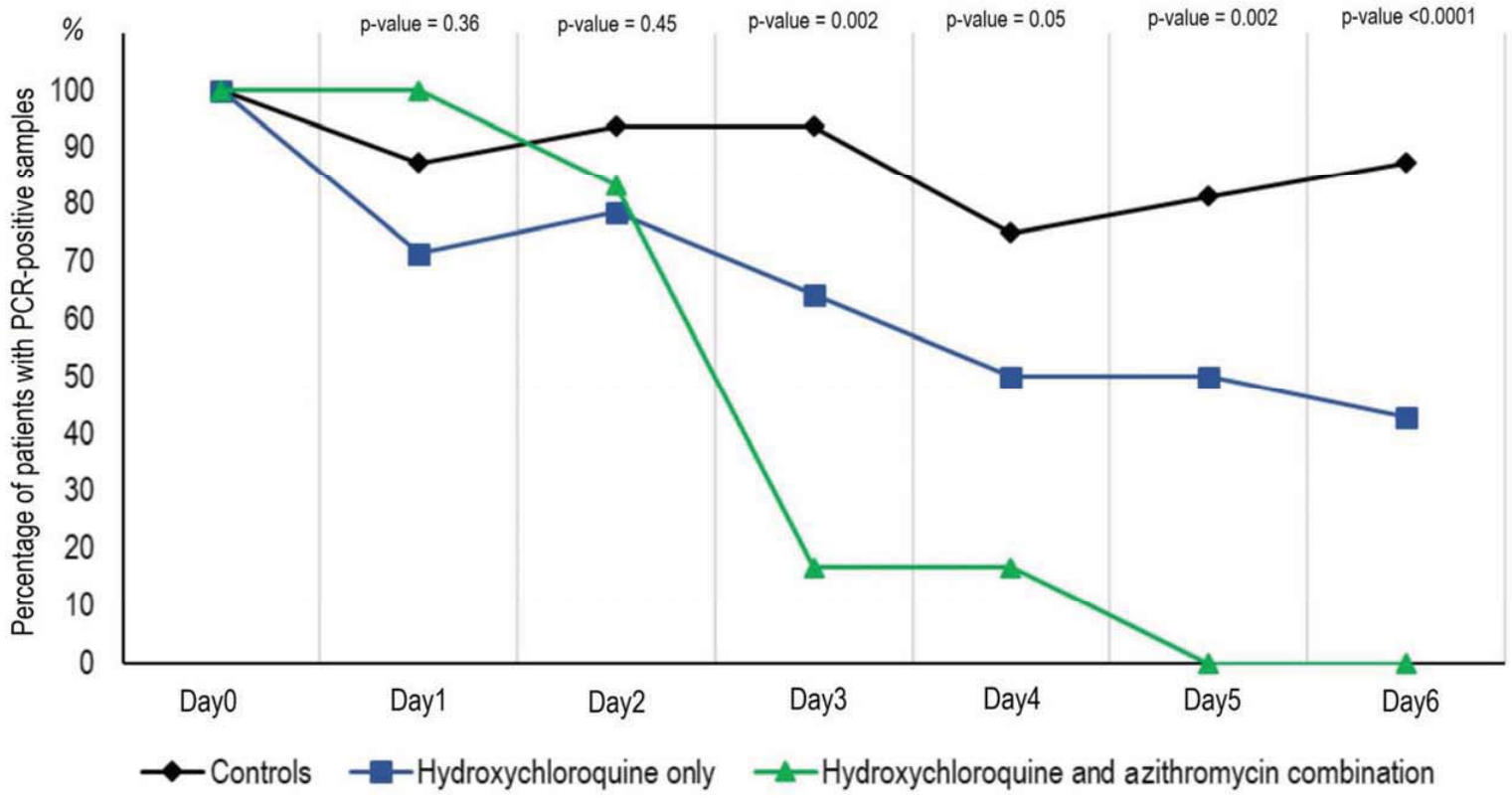
Figure 2. Percentage of patients with PCR-positive nasopharyngeal samples from inclusion to day6 post-inclusion in COVID-19 patients treated with hydroxychloroquine only, in COVID-19 patients treated with hydroxychloroquine and azithomycin combination, and in COVID-19 control patients.



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ABSTRACT

Background

In a recent survey, most physicians worldwide considered that hydroxychloroquine (HCQ) and azithromycin (AZ) are the two most effective drugs among available molecules against COVID-19. Nevertheless, to date, one preliminary clinical trial only has demonstrated its efficacy on the viral load. Additionally, a clinical study including 80 patients was published, and *in vitro* efficiency of this association was demonstrated.

Methods

The study was performed at IHU *Méditerranée Infection*, Marseille, France. A cohort of 1061 COVID-19 patients, treated for at least 3 days with the HCQ-AZ combination and a follow-up of at least 9 days was investigated. Endpoints were death, worsening and viral shedding persistence.

Findings

From March 3rd to April 9th, 2020, 59,655 specimens from 38,617 patients were tested for COVID-19 by PCR. Of the 3,165 positive patients placed in the care of our institute, 1061 previously unpublished patients met our inclusion criteria. Their mean age was 43.6 years old and 492 were male (46.4%). No cardiac toxicity was observed. A good clinical outcome and virological cure was obtained in 973 patients within 10 days (91.7%). Prolonged viral carriage at completion of treatment was observed in 47 patients (4.4%) and was associated to a higher viral load at diagnosis ($p < 10^{-2}$) but viral culture was negative at day 10 and all but one were PCR-cleared at day 15. A poor outcome was observed for 46 patients (4.3%); 10 were transferred to intensive care units, 5 patients died (0.47%) (74-95 years old) and 31 required 10 days of hospitalization or more. Among this group, 25 patients are now cured and 16 are still hospitalized (98% of patients cured so far). Poor clinical outcome was significantly associated to older age (OR 1.11), initial higher severity (OR 10.05) and low

hydroxychloroquine serum concentration. In addition, both poor clinical and virological outcomes were associated to the use of selective beta-blocking agents and angiotensin II receptor blockers ($P<0.05$). Mortality was significantly lower in patients who had received $\geq$ 3 days of HCQ-AZ than in patients treated with other regimens both at IHU and in all Marseille public hospitals ($p< 10^{-2}$).

Interpretation

The HCQ-AZ combination, when started immediately after diagnosis, is a safe and efficient treatment for COVID-19, with a mortality rate of 0.5%, in elderly patients. It avoids worsening and clears virus persistence and contagiosity in most cases.