The Top 10 Papers Related to COVID-19 of June 27, 2020

Compiled by

College of Health Sciences

June 27, 2020

Addis Ababa

Epidemiology

1. Horton R. **Offline: The second wave**. Lancet 2020, June 27, 395, ISSUE 10242, P1960. Full-text: https://doi.org/10.1016/S0140-6736(20)31451-3

Not in a good mood today? Then don't read this important comment on what will likely happen during the next months. The first wave of the 1918 influenza pandemic took place between March and July. It proved relatively mild. The second wave arrived in August. It was much worse. Most of the 50–100 million deaths caused by influenza took place during 13 weeks between September and December, 1918. In many countries, the test, trace, and isolate system is still not fully functional and we have angry debates about whether physical distancing should be 1 m or 2 m. Scientists predict that a second wave will arrive in September, peaking by the end of 2020. Just sayin'.

Transmission

2. Ortega R, Gonzalez M, Nozari A, et al. **Personal Protective Equipment and Covid-19**. N Engl J Med 2020; June 25. Full-text: https://doi.org/10.1056/NEJMvcm2014809

Helpful video, demonstrating the complex procedure for putting on and removing PPE that has been recommended by the CDC to minimize the risk of exposure to infectious material during the care of patients with COVID-19.

3. Prather KA, Wang CC, Schooley RT. **Reducing transmission of SARS-CoV-2**. Science 26 Jun 2020: Vol. 368, Issue 6498, pp. 1422-1424. Full-text: https://doi.org/10.1126/science.abc6197

Aerosol transmission of viruses must be acknowledged as a key factor leading to the spread of infectious respiratory diseases. This viewpoint summarizes current research that is already leading to a better understanding of the importance of airborne transmission.

Diagnostics

4. Mak GC, Cheng PK, Lau SS, et al. **Evaluation of rapid antigen test for detection of SARS-CoV-2 virus**. J Clin Virol. 2020 Jun 8;129:104500. PubMed: https://pubmed.gov/32585619 . Full-text: https://doi.org/10.1016/j.jcv.2020.104500

Bad performance of the commercially available rapid BIOCREDIT COVID-19 antigen test. This test was 10,000 fold less sensitive than RT-PCR and detected between 11.1 % and 45.7 % of RT-PCR-positive samples from COVID-19 patients. It serves only as adjunct to RT-PCR test because of the potential for false-negative results.

5. Ben-Ami R, Klochendler A, Seidel M, et al. **Large-scale implementation of pooled RNA extraction and RT-PCR for SARS-CoV-2 detection**. Clin Microbiol Infect. 2020 Jun 22:S1198-743X(20)30349-9. PubMed: https://pubmed.gov/32585353 . Full-text: https://doi.org/10.1016/j.cmi.2020.06.009

Due to the overwhelming use of SARS-CoV-2 RT-PCR tests worldwide, availability of test kits has become a major bottleneck. The authors show how to overcome these challenges by pooling samples, performing RNA extraction and RT-PCR in pools. A comparison of 184 samples tested individually and in pools of 8 samples, showed that test results were not significantly affected.

6. Amanat F, White KM, Miorin L, et al. **An In Vitro Microneutralization Assay for SARS-CoV-2 Serology and Drug Screening**. Curr Protoc Microbiol. 2020 Sep;58(1):e108. PubMed: https://pubmed.gov/32585083 . Full-text: https://doi.org/10.1002/cpmc.108

A new microneutralization assay is described in detail. This assay can be used to assess in a quantitative manner if antibodies or drugs can block entry and/or replication of SARS-CoV-2 *in vitro*. Compared to the most common neutralization assay, the plaque reduction neutralization test (PRNT), more samples can be analyzed. Compared to RBD-ACE2 inhibition assays, the test will also detect neutralizing antibodies binding to epitopes outside of the RBD. Different virus isolates can be used, and the assay can likely be adapted for staining antibodies other than mAbs (e.g., polyclonal sera, antibodies targeting S or M, etc.).

7. Deeks JJ, Dinnes J, Takwoingi Y, et al. **Antibody tests for identification of current and past infection with SARS-CoV-2**. Cochrane Database Syst Rev. 2020 Jun 25;6:CD013652. PubMed: https://pubmed.gov/32584464 . Full-text: https://doi.org/10.1002/14651858.CD013652

This Cochrane analysis on 57 publications with 15,976 samples says that the sensitivity of antibody tests is too low in the first week from symptom onset to have a primary role in the diagnosis of COVID-19. However, these tests may still have a role complementing other testing in individuals presenting later, when RT-PCR tests are negative, or are not done. Antibody tests are likely to have a useful role for detecting previous SARS-CoV-2 infection if used 15 or more days after the onset of symptoms. Data beyond 35 days post-symptom onset is scarce. According to the authors, studies of the accuracy of COVID-19 tests require considerable improvement. Studies must report data on sensitivity disaggregated by time from onset of symptoms. Updates of this living systematic review are planned.

Clinical

8. Lockhart SM, O'Rahilly S. **When two pandemics meet: Why is obesity associated with increased COVID-19 mortality**? Med 2020,June 25. Full-text: https://www.cell.com/med/fulltext/S2666-6340(20)30010-6

What a nice understatement. The authors describe "some hypotheses regarding the deleterious impact of obesity on the course of COVID-19". This brilliant overview summarizes current knowledge on the underlying mechanisms. These are: 1. Increased inflammatory cytokines (potentiate the inflammatory response), 2. reduction in adiponectin secretion (abundant in the pulmonary endothelium), 3. increases in circulating complement components, 4. systemic insulin resistance (associated with endothelial dysfunction and with increased plasminogen activator inhibitor-1), and 5. ectopic lipid deposited in type 2 pneumocytes (pre-disposing to lung injury).

Comorbidities

9. Louapre C, Collongues N, Stankoff B, et al. **Clinical Characteristics and Outcomes in Patients With Coronavirus Disease 2019 and Multiple Sclerosis**. JAMA Neurol 2020, June 26. Full-text: https://doi.org/10.1001/jamaneurol.2020.2581

This registry-based cohort study from France has included 347 patients with MS with a confirmed or highly suspected diagnosis of COVID-19. In total, 73 patients (21.0%) had a COVID-19 severity score of 3 or more, and 12 patients (3.5%) died. Age, Expanded Disability Severity Scale score (EDSS; ranging from 0 to 10, with cutoffs at 3 and 6), and obesity were independent risk factors for severe COVID-19; there was no association found between exposure to disease-modifying therapies and severity.

Treatment

10. Li S, Hu Z, Song X. **High-dose but not low-dose corticosteroids potentially delay viral shedding of patients with COVID-19**. Clinical Infectious Diseases 2020, June 26. Full-text: https://doi.org/10.1093/cid/ciaa829

This study with 206 patients suggests that the effect of corticosteroids on viral shedding may be in a dose-response manner. High-dose (80 mg/d) but not low-dose corticosteroids (40 mg/d) delayed viral shedding of patients with COVID-19.

Offline: The second wave

Fabrice Coffrini/AFP/Getty Images

The spectre of a second wave of COVID-19 hangs over us. Some infectious disease specialists believe that SARS-CoV-2 might be losing virulence. Most are less sanguine. Dr Tedros Adhanom Ghebreyesus, WHO's Director-General, reported last week that "the pandemic is accelerating"—across the Americas, south Asia, and the Middle East. "The world is in a new and dangerous phase", he said. "The virus is still spreading fast, it is still deadly, and most people are still susceptible." The shadow of the 1918 influenza pandemic darkens our perspective. The first wave of that outbreak took place between March and July. It proved relatively mild. The second wave arrived in August. It was much worse. Most of the 50–100 million deaths caused by influenza took place during 13 weeks between September and December, 1918. The past century has incubated the idea that a second wave should justify mortal fear. Whether true or not, it is right to ask: what should we do to prepare?

*

SolStock/Getty Images

In the UK, new infections are still taking place across the whole country. This week, Tim Spector, Professor of Genetic Epidemiology at King's College London, wrote to Prime Minister Boris Johnson. His COVID Symptom Study app has 3·9 million contributors across the UK. Those who have signed up to Spector's survey self-report their symptoms. These data are the most reliable information we have about the spread of coronavirus. The government's testing regime is missing two-thirds of people with COVID-19. If we could diagnose new infections more rapidly, we could exit lockdown faster and more safely. Spector argues that what is needed is a campaign to educate the public to suspect infection not when they have later stage symptoms of fever and cough, but when they have earlier signs of muscle pain, fatigue, headache, diarrhoea, and rashes. Self-isolation at this earlier stage would reduce the risk of others becoming infected. If all new infections could be identified within 48 h, there is every possibility that a second wave could be avoided. And once one gets to fewer than 1000 new infections per day—the current number is estimated to be 3612 per day—the embryonic test, trace, and isolate system would have the capacity to detect and follow up every new case.

Tim Spector app

*

Prolonged lockdowns are certainly not the answer to future waves of COVID-19. School closures are not sustainable. The economy cannot be refrigerated again. Risks to mental health are real. Work at the Institute for Health Metrics and Evaluation (IHME) in Seattle suggests that SARS-CoV-2 displays strong seasonality. In the Northern Hemisphere, IHME scientists predict that a second wave will arrive in September, peaking by the end of 2020. They expect the public will have less tolerance for future government mandates to shut down societies. So what if local outbreaks do take off? Modelling suggests that brief lockdowns (eg, for 2 weeks) followed by relaxations for between 2 and 6 weeks might be enough to cut lines of virus transmission. But one casualty of COVID-19 has been public and political trust in models attempting to forecast the course of the pandemic. Gabriel Leung's team at the University of Hong Kong has described one solution to managing a second wave—real-time tracking of transmissibility by closely monitoring the instantaneous effective reproduction number (Rt). Measurement of Rt should be supplemented by early diagnosis (Spector's data are important here), contact tracing, isolation, and continual efforts to keep public awareness high. In the UK, the test, trace, and isolate system is still not fully functional. There have been angry debates about whether physical distancing should be 1 m or 2 m. The lesson from the HIV pandemic is that no single preventive measure is adequate to control virus transmission. What matters is combination prevention—in the case of coronavirus, a mix of measures that include handwashing, respiratory hygiene, mask wearing, physical distancing (as much as is reasonably possible), and avoiding mass gatherings. So far, politicians and public health officials have not advocated the idea of combination prevention—they should. Another lesson from HIV is the importance of protecting key populations. COVID-19 is not socially neutral. SARS-CoV-2 exploits and accentuates inequalities. And on the dangers of a second wave to the most socially vulnerable, there has been not a word.

Richard Horton
richard.horton@lancet.com

VIDEOS IN CLINICAL MEDICINE
SUMMARY POINTS

Julie R. Ingelfinger, M.D., *Editor*

Personal Protective Equipment and Covid-19

Rafael Ortega, M.D., Mauricio Gonzalez, M.D., Ala Nozari, M.D., Ph.D., and Robert Canelli, M.D.

The following text summarizes information provided in the video.

OVERVIEW

Given the threat of coronavirus disease 2019, or Covid-19, it is important to emphasize the use of proper precautions for infection control in health care settings. Severe acute respiratory syndrome coronavirus 2, or SARS-CoV-2, is the virus that causes Covid-19.[1] The routes of SARS-CoV-2 transmission include direct contact — that is, contact with the respiratory droplets and aerosols from an affected person — and indirect contact, such as contact with contaminated surfaces or supplies.[2]

The best way for health care workers to prevent infection with SARS-CoV-2 is through training and demonstrated competency in putting on and removing, also known as donning and doffing, personal protective equipment (PPE). Various methods of putting on and removing PPE may be acceptable; you should follow the guidelines of the Centers for Disease Control and Prevention (or CDC) and your institution.

This video demonstrates a procedure for putting on and removing one type of PPE that has been recommended by the CDC for use in U.S. hospitals to minimize the risk of exposure to infectious material during the care of patients with Covid-19.[3]

EQUIPMENT

Protection against exposure to SARS-CoV-2 includes both standard and transmission-based precautions. The PPE used to prevent exposure includes gloves, a gown, a respirator with a rating of N95 or higher, and a full face shield or goggles. When respirators are not available, face masks are an acceptable alternative. These recommendations may be updated during the Covid-19 pandemic as decisions about best practices change.

You will need a fluid-resistant gown or coveralls that should cover the body at least from the neck to the mid-calf and medical gloves that extend to cover the wrists of the gown. Gowns with integrated thumb hooks may help to secure the sleeves under the gloves.

If disposable N95 respirators are worn, they must be certified by the National Institute for Occupational Safety and Health (NIOSH) and fit-tested by occupational health officials. The respirator should be used along with a full face shield that protects the face and neck. Alternatively, a NIOSH-certified, powered air-purifying respirator (PAPR) can be used.

PROCEDURE FOR PUTTING ON PPE

Because you may be wearing PPE for an extended period of time, make sure that you are well hydrated and use the restroom before putting on PPE. If you wear

From the Department of Anesthesiology, Boston Medical Center, Boston. Address reprint requests to Dr. Ortega at the Department of Anesthesiology, Boston Medical Center, 750 Albany St., Boston, MA 02118, or at rafael.ortega@bmc.org.

This video was published on May 19, 2020, at NEJM.org.

N Engl J Med 2020;383:e105.
DOI: 10.1056/NEJMvcm2014809
Copyright © 2020 Massachusetts Medical Society.

The New England Journal of Medicine
Downloaded from nejm.org on June 27, 2020. For personal use only. No other uses without permission.
Copyright © 2020 Massachusetts Medical Society. All rights reserved.

prescription eyeglasses, make sure that they are positioned securely on your face. Prescription eyeglasses alone do not suffice as eye protection.

Put on the PPE when you are near the patient's room. Before donning PPE, it is advisable to change into scrubs, secure long hair, and remove personal items such as jewelry.

Before handling any PPE, wash your hands with soap and water or clean your hands with an alcohol-based hand sanitizer. Visually check the integrity of the equipment. If possible, have an observer watch you as you put on the PPE to make sure there are no breaches in technique.

If the gown has an opening for your head, pull the gown over your head through the opening. If the gown does not have such an opening, fasten it behind your neck. When using a respirator, cup the outside of the respirator in your dominant hand, holding the pliable nasal strip with your fingertips and letting the two straps hang freely around your hand. Bring the respirator to your face. Secure the lower strap around the back of your neck, and then secure the upper strap behind your head. Mold the pliable nasal strip around the bridge of your nose with both hands, without tenting it. Do not pinch the nosepiece with one hand.

Check the seal of the respirator. Begin by covering the respirator with your hands and inhaling deeply and quickly several times. The respirator should collapse slightly against your face when you inhale. Next, place your hands around the edges of the respirator and exhale to determine whether there are any air leaks (Fig. 1). If the respirator fails to collapse or if air leaks from the sides, remold the nasal strip and adjust the positioning of the respirator on your face. If you are still unable to obtain a complete seal, consider using a PAPR.

Figure 1. Testing for Air Leaks.

Put on the face shield, letting it rest on your forehead and securing the strap on the back of your head. If your hair is tied in a bun, make sure the strap is positioned in a manner that ensures that the strap will not slide up or down. Adjust the elastic strap if necessary, to ensure a snug fit. If you are wearing eyeglasses, make sure they are secure to minimize the need to readjust them during patient care.

Disinfect your hands with an alcohol-based hand sanitizer. Put on the gloves and extend the cuffs over the sleeves of the gown. You are now ready to enter the patient's room.

PROCEDURE FOR REMOVING PPE

Proper removal and disposal of contaminated PPE is the most difficult challenge in preventing exposure to pathogens. Careful attention is required. Whenever possible, have an observer watch you as you take off the PPE to make sure there are no breaches in technique. Removal of PPE should begin in the patient's room.

There is more than one technique for removing gloves. We describe the glove-in-glove technique (Fig. 2). First, remove your gloves by grasping the glove on one hand with the other hand. Grasping the exterior of the glove at the wrist, pull the glove off your hand, with the contaminated exterior folded inside. Hold the removed glove in your gloved hand. Slide a finger under the wristband of the remaining glove. Gently pull off the glove so that it is now inside-out, forming a bag for the other glove, and discard. Disinfect your hands.

Figure 2. Removal of Second Glove.

Remove your gown by first undoing the fastening at the waist. Someone may assist you in undoing the fastenings at the neck and the back of the gown. Grasp the shoulder area and peel the gown away from your body, turning the gown inside-out and wrapping it into a bundle. Only the interior of the gown should remain visible. Discard the gown and disinfect your hands.

You are now ready to leave the patient's room. Disinfect your hands again.

The New England Journal of Medicine
Downloaded from nejm.org on June 27, 2020. For personal use only. No other uses without permission.
Copyright © 2020 Massachusetts Medical Society. All rights reserved.

Once you have left the patient's room, remove the face shield (Fig. 3). It is particularly important to avoid contamination of the eyes and mucous membranes when removing facial PPE. Tilt your head forward and lift the shield by the strap. Lift it above and away from your head without touching the shield itself. If you wear prescription eyeglasses, make sure the glasses are not contaminated when you remove the PPE.

If you have used a nondisposable face shield, place it in the appropriate container for decontamination, following the guidelines at your institution. If you have used a disposable face shield, a waste receptacle should be available to discard such equipment.

Remove the N95 respirator (Fig. 4). To minimize the possibility of contamination, avoid contact with the respirator itself, touching only the straps. Tilt your head forward, grab the strap that is around your neck, and lift it over your head, allowing it to hang freely. Then bring the top strap over your head and use it to remove the respirator from your face. Discard the respirator or return it to a designated location for recycling, following the guidelines at your institution. Disinfect your hands.

Figure 3. Removal of Face Shield.

Figure 4. Removal of Respirator.

SUMMARY

When PPE is properly worn, removed, and discarded, it is effective in protecting both the person who wears it and those with whom that person comes into contact.

No potential conflict of interest relevant to this video was reported.
Disclosure forms provided by the authors are available with this video at NEJM.org.

REFERENCES

1. Mehra MR, Desai SS, Kuy S, Henry TD, Patel AN. Cardiovascular disease, drug therapy, and mortality in Covid-19. N Engl J Med. DOI: 10.1056/NEJMoa2007621.
2. van Doremalen N, Bushmaker T, Morris DH, et al. Aerosol and surface stability of SARS-CoV-2 as compared with SARS-CoV-1. N Engl J Med 2020;382:1564-7.
3. Centers for Disease Control and Prevention. Coronavirus disease 2019 (COVID-19): using personal protective equipment (PPE) (https://www.cdc.gov/coronavirus/2019-ncov/hcp/using-ppe.html).

Copyright © 2020 Massachusetts Medical Society.

The New England Journal of Medicine
Downloaded from nejm.org on June 27, 2020. For personal use only. No other uses without permission.
Copyright © 2020 Massachusetts Medical Society. All rights reserved.

Correctly fitted masks are an important tool to reduce airborne transmission of SARS-CoV-2, particularly in enclosed spaces, such as on this Moscow Metro train in Russia.

PHOTO: SERGEI FADEICHEV/TASS VIA GETTY IMAGES

Downloaded from http://science.sciencemag.org/ on June 25, 2020

VIEWPOINT: COVID-19

Reducing transmission of SARS-CoV-2

Masks and testing are necessary to combat asymptomatic spread in aerosols and droplets

By **Kimberly A. Prather[1], Chia C. Wang[2,3], Robert T. Schooley[4]**

Respiratory infections occur through the transmission of virus-containing droplets (>5 to 10 μm) and aerosols (≤5 μm) exhaled from infected individuals during breathing, speaking, coughing, and sneezing. Traditional respiratory disease control measures are designed to reduce transmission by droplets produced in the sneezes and coughs of infected individuals. However, a large proportion of the spread of coronavirus disease 2019 (COVID-19) appears to be occurring through airborne transmission of aerosols produced by asymptomatic individuals during breathing and speaking (*1*–*3*). Aerosols can accumulate, remain infectious in indoor air for hours, and be easily inhaled deep into the lungs. For society to resume, measures designed to reduce aerosol transmission must be implemented, including universal masking and regular, widespread testing to identify and isolate infected asymptomatic individuals.

Humans produce respiratory droplets ranging from 0.1 to 1000 μm. A competition between droplet size, inertia, gravity, and evaporation determines how far emitted droplets and aerosols will travel in air (*4*, *5*). Larger respiratory droplets will undergo gravitational settling faster than they evaporate, contaminating surfaces and leading to contact transmission. Smaller droplets and aerosols will evaporate faster than they can settle, are buoyant, and thus can be affected by air currents, which can transport them over longer distances. Thus, there are two

Published by AAAS

major respiratory virus transmission pathways: contact (direct or indirect between people and with contaminated surfaces) and airborne inhalation.

In addition to contributing to the extent of dispersal and mode of transmission, respiratory droplet size has been shown to affect the severity of disease. For example, influenza virus is more commonly contained in aerosols with sizes below 1 μm (submicron), which lead to more severe infection (*4*). In the case of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), it is possible that submicron virus-containing aerosols are being transferred deep into the alveolar region of the lungs, where immune responses seem to be temporarily bypassed. SARS-CoV-2 has been shown to replicate three times faster than SARS-CoV-1 and thus can rapidly spread to the pharynx, from which it can be shed before the innate immune response becomes activated and produces symptoms (*6*). By the time symptoms occur, the patient has transmitted the virus without knowing.

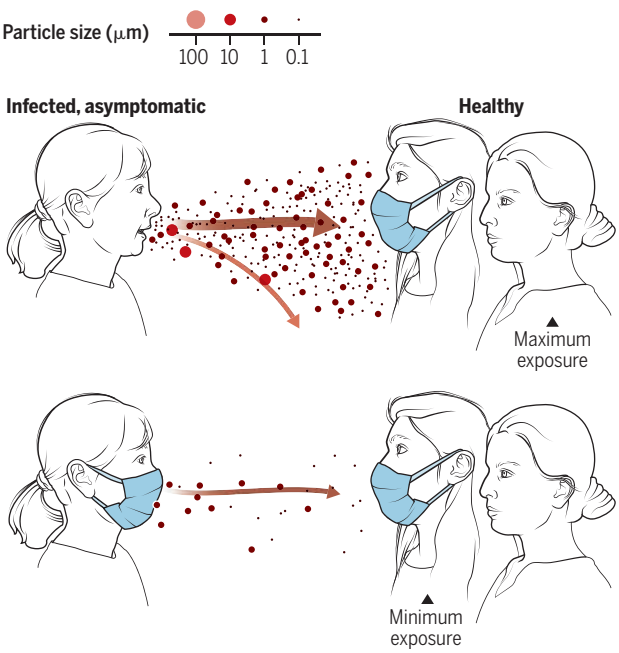
Identifying infected individuals to curb SARS-CoV-2 transmission is more challenging compared to SARS and other respiratory viruses because infected individuals can be highly contagious for several days, peaking on or before symptoms occur (*2, 7*). These "silent shedders" could be critical drivers of the enhanced spread of SARS-CoV-2. In Wuhan, China, it has been estimated that undiagnosed cases of COVID-19 infection, who were presumably asymptomatic, were responsible for up to 79% of viral infections (*3*). Therefore, regular, widespread testing is essential to identify and isolate infected asymptomatic individuals.

Airborne transmission was determined to play a role during the SARS outbreak in 2003 (*1, 4*). However, many countries have not yet acknowledged airborne transmission as a possible pathway for SARS-CoV-2 (*1*). Recent studies have shown that in addition to droplets, SARS-CoV-2 may also be transmitted through aerosols. A study in hospitals in Wuhan, China, found SARS-CoV-2 in aerosols further than 6 feet from patients, with higher concentrations detected in more crowded areas (*8*). Estimates using an average sputum viral load for SARS-CoV-2 indicate that 1 min of loud speaking could generate >1000 virion-containing aerosols (*9*). Assuming viral titers for infected super-emitters (with 100-fold higher viral load than average) yields an increase to more than 100,000 virions in emitted droplets per minute of speaking.

The U.S. Centers for Disease Control and Prevention (CDC) recommendations for social distancing of 6 feet and hand washing to reduce the spread of SARS-CoV-2 are based on studies of respiratory droplets carried out in the 1930s. These studies showed that large, ~100 μm droplets produced in coughs and sneezes quickly underwent gravitational settling (*1*). However, when these studies were conducted, the technology did not exist for detecting submicron aerosols. As a comparison, calculations predict that in still air, a 100-μm droplet will settle to the ground from 8 feet in 4.6 s, whereas a 1-μm aerosol particle will take 12.4 hours (*4*). Measurements now show that intense coughs and sneezes that propel larger droplets more than 20 feet can also create thousands of aerosols that can travel even further (*1*). Increasing evidence for SARS-CoV-2 suggests the 6 feet CDC recommendation is likely not enough under many indoor conditions, where aerosols can remain airborne for hours, accumulate over time, and follow airflows over distances further than 6 feet (*5, 10*).

Masks reduce airborne transmission

Infectious aerosol particles can be released during breathing and speaking by asymptomatic infected individuals. No masking maximizes exposure, whereas universal masking results in the least exposure.



GRAPHIC: V. ALTOUNIAN/*SCIENCE*

In outdoor environments, numerous factors will determine the concentrations and distance traveled, and whether respiratory viruses remain infectious in aerosols. Breezes and winds often occur and can transport infectious droplets and aerosols long distances. Asymptomatic individuals who are speaking while exercising can release infectious aerosols that can be picked up by airstreams (*10*). Viral concentrations will be more rapidly diluted outdoors, but few studies have been carried out on outdoor transmission of SARS-CoV-2. Additionally, SARS-CoV-2 can be inactivated by ultraviolet radiation in sunlight, and it is likely sensitive to ambient temperature and relative humidity, as well as the presence of atmospheric aerosols that occur in highly polluted areas. Viruses can attach to other particles such as dust and pollution, which can modify the aerodynamic characteristics and increase dispersion. Moreover, people living in areas with higher concentrations of air pollution have been shown to have higher severity of COVID-19 (*11*). Because respiratory viruses can remain airborne for prolonged periods before being inhaled by a potential host, studies are needed to characterize the factors leading to loss of infectivity over time in a variety of outdoor environments over a range of conditions

Given how little is known about the production and airborne behavior of infectious respiratory droplets, it is difficult to define a safe distance for social distancing. Assuming SARS-CoV-2 virions are contained in submicron aerosols, as is the case for influenza virus, a good comparison is exhaled cigarette smoke, which also contains submicron particles and will likely follow comparable flows and dilution patterns. The distance from a smoker at which one smells cigarette smoke indicates the distance in those surroundings at which one could inhale infectious aerosols. In an enclosed room with asymptomatic individuals, infectious aerosol concentrations can increase over time. Overall, the probability of becoming infected indoors will depend on the total amount of SARS-CoV-2 inhaled. Ultimately, the amount of ventilation, number of people, how long one visits an indoor facility, and activities that affect airflow will all modulate viral transmission pathways and exposure (*10*). For these reasons, it is important to wear properly fitted masks indoors even when 6 feet apart. Airborne transmission could account, in part, for the high secondary transmission rates to medical staff, as

[1]Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA 92037, USA. [2]Department of Chemistry, National Sun Yat-sen University, Kaohsiung, Taiwan 804, Republic of China. [3]Aerosol Science Research Center, National Sun Yat-Sen University, Kaohsiung, Taiwan 804, Republic of China. [4]Department of Medicine, Division of Infectious Diseases and Global Public Health, School of Medicine, University of California San Diego, La Jolla, CA 92093, USA. Email: kprather@ucsd.edu

Downloaded from http://science.sciencemag.org/ on June 27, 2020

Published by AAAS

well as major outbreaks in nursing facilities. The minimum dose of SARS-CoV-2 that leads to infection is unknown, but airborne transmission through aerosols has been documented for other respiratory viruses, including measles, SARS, and chickenpox (*4*).

Airborne spread from undiagnosed infections will continuously undermine the effectiveness of even the most vigorous testing, tracing, and social distancing programs. After evidence revealed that airborne transmission by asymptomatic individuals might be a key driver in the global spread of COVID-19, the CDC recommended the use of cloth face coverings. Masks provide a critical barrier, reducing the number of infectious viruses in exhaled breath, especially of asymptomatic people and those with mild symptoms (*12*) (see the figure). Surgical mask material reduces the likelihood and severity of COVID-19 by substantially reducing airborne viral concentrations (*13*). Masks can also protect uninfected individuals from SARS-CoV-2 aerosols and droplets (*13, 14*). Thus, it is particularly important to wear masks in locations with conditions that can accumulate high concentrations of viruses, such as health care settings, airplanes, restaurants, and other crowded places with reduced ventilation. The aerosol filtering efficiency of different materials, thicknesses, and layers used in properly fitted homemade masks was recently found to be similar to that of the medical masks that were tested (*14*). Thus, the option of universal masking is no longer held back by shortages.

From epidemiological data, places that have been most effective in reducing the spread of COVID-19 have implemented universal masking, including Taiwan, Japan, Hong Kong, Singapore, and South Korea. In the battle against COVID-19, Taiwan (population 24 million, first COVID-19 case 21 January 2020) did not implement a lockdown during the pandemic, yet maintained a low incidence of 441 cases and 7 deaths (as of 21 May 2020). By contrast, the state of New York (population ~20 million, first COVID case 1 March 2020), had a higher number of cases (353,000) and deaths (24,000). By quickly activating its epidemic response plan that was established after the SARS outbreak, the Taiwanese government enacted a set of proactive measures that successfully prevented the spread of SARS-CoV-2, including setting up a central epidemic command center in January, using technologies to detect and track infected patients and their close contacts, and perhaps most importantly, requesting people to wear masks in public places. The government also ensured the availability of medical masks by banning mask manufacturers from exporting them, implementing a system to ensure that every citizen could acquire masks at reasonable prices, and increasing the production of masks. In other countries, there have been widespread shortages of masks, resulting in most residents not having access to any form of medical mask (*15*). This striking difference in the availability and widespread adoption of wearing masks likely influenced the low number of COVID-19 cases.

Aerosol transmission of viruses must be acknowledged as a key factor leading to the spread of infectious respiratory diseases. Evidence suggests that SARS-CoV-2 is silently spreading in aerosols exhaled by highly contagious infected individuals with no symptoms. Owing to their smaller size, aerosols may lead to higher severity of COVID-19 because virus-containing aerosols penetrate more deeply into the lungs (*10*). It is essential that control measures be introduced to reduce aerosol transmission. A multidisciplinary approach is needed to address a wide range of factors that lead to the production and airborne transmission of respiratory viruses, including the minimum virus titer required to cause COVID-19; viral load emitted as a function of droplet size before, during, and after infection; viability of the virus indoors and outdoors; mechanisms of transmission; airborne concentrations; and spatial patterns. More studies of the filtering efficiency of different types of masks are also needed. COVID-19 has inspired research that is already leading to a better understanding of the importance of airborne transmission of respiratory disease. ■

REFERENCES AND NOTES

1. L. Morawska, *J. Cao, Environ. Int.* **139**, 105730 (2020).
2. E. L. Anderson *et al.*, *Risk Anal.* **40**, 902 (2020).
3. S. Asadi *et al.*, *Aerosol Sci. Technol.* **54**, 635 (2020).
4. R. Tellier *et al.*, *BMC Infect. Dis.* **19**, 101 (2019).
5. R. Mittal, R. Ni, J.-H. Seo, *J. Fluid Mech.* 10.1017/jfm.2020.330 (2020).
6. H. Chu *et al.*, *Clin. Infect. Dis.* 10.1093/cid/ciaa410 (2020).
7. X. He *et al.*, *Nat. Med.* **26**, 672 (2020).
8. Y. Liu *et al.*, *Nature* 10.1038/s41586-020-2271-3 (2020).
9. V. Stadnytskyi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 202006874 (2020).
10. G. Buonanno, L. Stabile, L. Morawska, *Environ. Int.* **141**, 105794 (2020).
11. E. Conticini, B. Frediani, D. Caro, *Environ. Pollut.* **261**, 114465 (2020).
12. N. H. L. Leung *et al.*, *Nat. Med.* **26**, 676 (2020).
13. J. F.-W. Chan *et al.*, *Clin. Infect. Dis.* 10.1093/cid/ciaa644 (2020).
14. A. Konda *et al.*, *ACS Nano* 10.1021/acsnano.0c03252 (2020).
15. C. C. Leung, T. H. Lam, K. K. Cheng, *Lancet* **395**, 945 (2020).

ACKNOWLEDGMENTS

The authors thank S. Strathdee, D. Petras, and L. Marr for helpful discussions. K.A.P. is supported by the NSF Center for Aerosol Impacts on Chemistry of the Environment (CHE1801971). R.T.S. is supported by the National Institute of Allergy and Infectious Diseases (R01 AI131424). C.C.W. is supported by the Ministry of Science and Technology (MOST 108-2113-M-110-003) and the Higher Education Sprout Project of the Ministry of Education, Taiwan, ROC.

Published online 27 May 2020
10.1126/science.abc6197

GENETICS

Evolution after genome duplication

Genetic interactions in yeast reveal factors governing duplicate gene retention and divergence

By **Ian M. Ehrenreich**

Genome duplication generates an extra copy of nearly all genes carried by an organism, providing a potential substrate for evolution. Although many duplicate genes will be eliminated after a genome duplication, those that are retained may evolve distinct functions over time. This process can be studied by characterizing the shared and divergent functions of duplicate genes in present-day organisms whose ancestors experienced genome duplication in the past. However, such work requires examining the functional relationships between each copy of a duplicated gene and the other genes in the genome. This is inherently difficult for duplicate genes because of their redundancy. However, on page 1445 of this issue, Kuzmin *et al.* (*1*) show that systematic analysis of di- and trigenic genetic interactions in budding yeast can overcome this challenge. With this approach, they discover general constraints that influence the retention and divergence of duplicate genes.

> **"...duplicate gene retention and divergence heavily depended on whether the different functions of these genes were entangled (i.e., unable to evolve independently)."**

In 1970, it was proposed that gene duplication provides the critical fuel for evolution (*2*). Owing to their functional redundancy, it was hypothesized that duplicate genes are able to evolve in ways that single-copy genes cannot. Over time, different

Downloaded from http://science.sciencemag.org/ on June 27, 2020

Published by AAAS

Science

Reducing transmission of SARS-CoV-2

Kimberly A. Prather, Chia C. Wang and Robert T. Schooley

Science **368** (6498), 1422-1424.
DOI: 10.1126/science.abc6197originally published online May 27, 2020

ARTICLE TOOLS http://science.sciencemag.org/content/368/6498/1422

RELATED CONTENT
http://stm.sciencemag.org/content/scitransmed/12/541/eabb5883.full
http://stm.sciencemag.org/content/scitransmed/11/499/eaat0360.full
http://stm.sciencemag.org/content/scitransmed/9/396/eaal3653.full
http://stm.sciencemag.org/content/scitransmed/12/534/eabb1469.full
file:/content

REFERENCES This article cites 9 articles, 0 of which you can access for free
http://science.sciencemag.org/content/368/6498/1422#BIBL

PERMISSIONS http://www.sciencemag.org/help/reprints-and-permissions

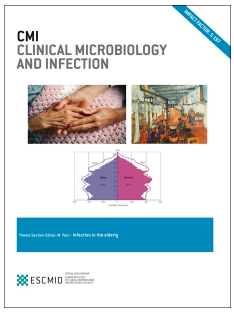
Use of this article is subject to the Terms of Service

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005. The title *Science* is a registered trademark of AAAS.

Copyright © 2020, American Association for the Advancement of Science

Downloaded from http://science.sciencemag.org/ on June 27, 2020

Journal Pre-proof



Large-scale implementation of pooled RNA extraction and RT-PCR for SARS-CoV-2 detection

Roni Ben-Ami, Agnes Klochendler, Matan Seidel, Tal Sido, Ori Gurel-Gurevich, Moran Yassour, Eran Meshorer, Gil Benedek, Irit Fogel, Esther Oiknine-Djian, Asaf Gertler, Zeev Rotstein, Bruno Lavi, Yuval Dor, Dana G. Wolf, Maayan Salton, Yotam Drier, The Hebrew University-Hadassah COVID-19 diagnosis team

PII: S1198-743X(20)30349-9

DOI: https://doi.org/10.1016/j.cmi.2020.06.009

Reference: CMI 2098

To appear in: *Clinical Microbiology and Infection*

Received Date: 9 May 2020

Revised Date: 6 June 2020

Accepted Date: 8 June 2020

Please cite this article as: Ben-Ami R, Klochendler A, Seidel M, Sido T, Gurel-Gurevich O, Yassour M, Meshorer E, Benedek G, Fogel I, Oiknine-Djian E, Gertler A, Rotstein Z, Lavi B, Dor Y, Wolf DG, Salton M, Drier Y, The Hebrew University-Hadassah COVID-19 diagnosis team, Large-scale implementation of pooled RNA extraction and RT-PCR for SARS-CoV-2 detection, *Clinical Microbiology and Infection*, https://doi.org/10.1016/j.cmi.2020.06.009.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 European Society of Clinical Microbiology and Infectious Diseases. Published by Elsevier Ltd. All rights reserved.

Journal Pre-proof

Large-scale implementation of pooled RNA extraction and RT-PCR for SARS-CoV-2 detection

Roni Ben-Ami[1,*], Agnes Klochendler[1,*], Matan Seidel[2,*], Tal Sido[3], Ori Gurel-Gurevich[4], Moran Yassour[5,6], Eran Meshorer[7], Gil Benedek[8], Irit Fogel[8], Esther Oiknine-Djian[8], Asaf Gertler[8], Zeev Rotstein[8], Bruno Lavi[8], Yuval Dor[1,#], Dana G. Wolf [8,9,#], Maayan Salton[10,#], Yotam Drier[9,#], The Hebrew University-Hadassah COVID-19 diagnosis team[11].

[1] Department of Developmental Biology and Cancer Research, IMRIC, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel.
[2] School of Mathematical Sciences, Tel Aviv University, Tel Aviv, Israel.
[3] Department of Mathematics, Bar-Ilan University, Ramat-Gan, Israel.
[4] Einstein Institute of Mathematics, The Hebrew University of Jerusalem, Jerusalem, Israel.
[5] Department of Microbiology and Molecular Genetics, IMRIC, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel.
[6] School of Computer Science and Engineering, The Hebrew University of Jerusalem, Jerusalem, Israel.
[7] Department of Genetics and Edmond and Lily Center for Brain Sciences (ELSC), The Hebrew University of Jerusalem, Jerusalem, Israel.
[8] Hadassah – Hebrew University Medical Center, Jerusalem, Israel.
[9] The Lautenberg Center for Immunology and Cancer Research, IMRIC, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel.
[10] Department of Biochemistry and Molecular Biology, IMRIC, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel.
[11] List of contributors in the acknowledgement section below.
[*] These authors contributed equally to the work.
[#] These authors contributed equally to the work. Corresponding authors e-mails: yuvald@ekmd.huji.ac.il, danaw@ekmd.huji.ac.il, maayan.salton@mail.huji.ac.il, yotam.drier@mail.huji.ac.il.

Abstract

Objectives: Testing for active SARS-CoV-2 infection is a fundamental tool in the public health measures taken to control the COVID-19 pandemic. Due to the overwhelming use of SARS-CoV-2 RT-PCR tests worldwide, availability of test kits has become a major bottleneck, while the need to increase testing throughput only rises. We aim to overcome these challenges by pooling samples together, performing RNA extraction and RT-PCR in pools.

Methods: We tested the efficiency and sensitivity of pooling strategies for RNA extraction and RT-PCR detection of SARS-CoV-2. We tested 184 samples both individually and in pools to estimate the effects of pooling. We further implemented Dorfman pooling with a pool size of 8 samples in large-scale clinical tests.

Results: We demonstrated pooling strategies that increase testing throughput while maintaining high sensitivity. A comparison of 184 samples tested individually and in pools of 8 samples, showed that test results were not significantly affected. Implementing the 8-sample Dorfman pooling to test 26,576 samples from asymptomatic individuals, we identified 31 (0.12%) SARS-CoV-2 positive samples, achieving a 7.3-fold increase in throughput.

Conclusions: Pooling approaches for SARS-CoV-2 testing allow a drastic increase in throughput while maintaining clinical sensitivity. We report the successful large-scale pooled screening of asymptomatic populations.

Journal Pre-proof

Introduction

An emerging novel severe acute respiratory syndrome-related coronavirus, SARS-CoV-2, is the virus behind the global COVID-19 pandemic. Among the foremost priorities to facilitate efficient public health interventions is a reliable and accessible diagnosis of an active SARS-CoV-2 infection. The standard laboratory diagnosis of COVID-19 involves three main steps, namely, viral inactivation and lysis of the nasopharyngeal swab sample, extraction (or purification) of viral RNA, and reverse transcription (RT)-PCR. Due to the rapid spread of the virus and the increasing demand for tests, the limited availability of test reagents, mainly RNA extraction kits, has become (and will likely continue to be) a major bottleneck as the pandemic expands [1,2].

Of particular importance is the ability to survey large asymptomatic populations- (1) to trace asymptomatic COVID-19 carriers which are otherwise difficult to identify and isolate; (2) to assure key personnel (e.g. healthcare personnel) are not contagious; (3) to screen high risk populations (such as nursing homes) to help protect them; (4) to accurately estimate the spread of infection and the effectiveness of community measures and social distancing; and (5) to allow and monitor a safe return to work. Efficient and higher-throughput diagnostic approaches are needed to support such efforts. While some of these applications (e.g. (4)) may be achieved with less sensitive detection approaches, most applications do require adhering to the current high standards of RT-PCR.

Several attempts to address this challenge were recently reported, and can be categorized into three major approaches. The first approach is to replace PCR-based methods by other direct diagnostic methods such as Loop-mediated Isothermal Amplification (LAMP) [3–6] and CRISPR based diagnostic tools [7–9]. The second approach involves serological surveys [10–13], and the third approach involves the improvement of the PCR methods capacity by optimization and automation [1,14,15] or by reducing the required number of tests via pooling samples together, known as group testing.

Group testing is a field of research in the intersection of mathematics, computer science and information theory, with applications in biology, communication and more. A group testing algorithm is a testing scheme directed

towards minimizing the number of tests conducted on a set of samples by using the ability to test pooled subsets of samples. If a pool of *n* samples tests negative, all samples must be negative, and therefore their status has been determined in only one test instead of *n* individual tests. Various group testing algorithms exist, with different assumptions and constraints [16,17]. While many such algorithms, most notably binary splitting, may be very efficient in theory, they might be unsuitable because of practical limitations. Some key constrains are (1) a limit on the number of stages due to the importance of delivering a test result quickly, exemplified by the urgent clinical context of COVID-19 diagnosis; (2) a limit on the ability to dilute samples and still safely identify a single positive sample in a pool; and (3) favorability of simple algorithms which may minimize human error in a laboratory setting.

While several pooling approaches for SARS-CoV-2 detection were recently suggested [2,18–22], these studies mostly discussed theoretical considerations. Here we describe and demonstrate practical pooling solutions that save time and reagents by performing RNA extraction and RT-PCR on pooled samples. We offer two such pooling approaches, based either on simple (Dorfman) pooling or matrix pooling [23,24], and demonstrate their efficiency and sensitivity in the daily reality of COVID-19.

Journal Pre-proof

Methods

Sample collection

At the Hadassah Medical Center (HMC), two distinct populations are tested for SARS-CoV-2 at present. First, we receive samples from symptomatic patients, from the hospital and from the community. In these samples, about 10% of SARS-CoV-2 tests are positive. Second, we receive samples from prospectively screened asymptomatic populations such as hospital employees and workers in essential industries. According to the Israeli Ministry of Health guidelines all samples were collected using a single swab for combined deep nasal and

Journal Pre-proof

oropharyngeal collection from the same patient. Nasopharyngeal swab samples were collected in 2ml Viral Transport Medium (VTM) or collected directly to 2ml Zymo lysis buffer.

RNA extraction and RT-PCR Detection for individual tests

For sample lysate preparation 220µL of sample VTM were added to 280µL Zymo lysis buffer. RNA was extracted using MagNA Pure 96 kit (Roche Lifesciences) using Roche platform and eluted in 60µL. 10µL of RNA was used in 30µL reaction using Real-Time Fluorescent RT-PCR kit (BGI). We followed kit instructions with thermocycler protocol: 1 cycle 50 °C 20 minutes; 1 cycle 95 °C 10 minutes; 40 cycles 95 °C 15 seconds; 60 °C 30 seconds. According to the clinical guidelines of the Israeli Ministry of Health at the time experiments were conducted, a sample was defined as *positive* if the viral genome was detected at threshold cycle (Ct) values ≤35, as *indeterminate* at Ct values >35 and ≤38, and as *negative* at Ct values >38.

Pooling approaches

The first pooling strategy is a simple two-stage testing algorithm known as Dorfman pooling [25]. In the first stage, the samples are divided into disjoint pools of *n* samples each, and each such pool is tested. A negative result implies that all samples in the pool are negative, while a positive result implies that at least one sample in the pool is positive. In the second stage, the samples of each pool that tested positive are individually tested.

To reduce the need to retest positive pools we have also tested a two-stage matrix pooling strategy [23,24], where n^2 samples are ordered in an $n \times n$ matrix. Each row and each column are pooled, resulting in $2n$ tests, $\frac{1}{2}n$ times less tests than individual testing. If either the number of positive rows or columns is one, the positive samples can be uniquely identified at the intersections of the positive rows and columns. Otherwise, if both the

Journal Pre-proof

number of positive rows and columns is greater than one, intersections of positive rows and columns will be retested individually.

Testing pooling strategies

Individual barcoded samples were received at the laboratory, inactivated by a lysis buffer and pooled on a Tecan liquid-handling robot. For matrix pool design we pooled equal volumes of sample lysate to a final volume of 450µL and used MagNA Pure 96 kit (Roche Lifesciences) using Roche platform. For 8 sample pools, we used QIAsymphony DSP Virus/Pathogen kit on a QIAsymphony platform. We pooled equal volumes of sample lysate to a final volume of 400µL. Positive pools were validated by individual tests as described above. Both Qiagen kits were used with Zymo lysis buffers, and therefore we skipped the lysis and Proteinase K step. RNA was eluted into 60µL; 10µL of RNA was used for a 30µL reaction using Real-Time Fluorescent RT-PCR kit (BGI). To reduce the risk of contamination, daily ultraviolet irradiation of RNA extraction robots was performed, and different rooms for processing before and after PCR were set up, without mixing personnel or machines between the two compartments.

Note that analysis of pool results requires close attention to indeterminate-result pools, as these may contain individual positive samples. Therefore, all pools detected with Ct ≤ 39 were retested (see **Table 2**, batch 3), while maintaining standard criteria for the individual tests when retesting.

Choosing pooling strategy and parameters

We define the *efficiency* of a pooling algorithm as the total number of samples divided by the expected number of tests conducted on them. We assume all samples are independent and identically distributed, and denote the probability of a sample to be positive by *p* (prevalence of detectable COVID-19 patients in the relevant

population) and the pool size by *n*. The efficiency of the algorithms described above depends on both *p* and *n*. The best theoretical efficiency is $(-plog_2(p) - (1-p)log_2(1-p))^{-1}$ [26]. The efficiency of Dorfman pooling is $(1 + \frac{1}{n} - (1-p)^n)^{-1}$ [25]. We chose a pool size of *n=8* samples as it allows low false negative rate (**Figure 1**) and high efficiency for a wide range of COVID-19 prevalence (**Table 1** and **Supplementary Table S1**). The prevalence of detectable COVID-19 in an asymptomatic population is estimated to be considerably below 1% [27], and indeed of the 26,576 samples tested in the present study only 0.12% were found positive. Therefore, efficiency is likely to be 5-7.5. For higher prevalence the efficiency of matrix pooling is somewhat higher (see **Table 1** and supplementary note). We provide a tool (https://github.com/matanseidel/pooling_optimization) to help choose the approach and pool size based on the prevalence.

Study Approval

These studies were part of the approved diagnosis optimization and validation procedures at the HMC, and therefore no additional Institutional Review Board approvals were required.

Results

A key requirement of pooled RNA extraction and RT-PCR tests is to retain sufficient sensitivity. Theoretically, pooling 8 samples should elevate the Ct of a single positive sample by 3 cycles. However, reproducibility of RNA extraction and RT-PCR might be affected by other factors. We therefore empirically tested assay sensitivity, when multiple negative samples and one positive sample were mixed at the lysate stage. We tested the pooling of 184 samples into 23 pools of 8 samples each, and also tested in parallel each sample individually. This approach yielded highly accurate results, with no loss of diagnostic assay sensitivity: each of the pools that contained one or more positive samples was found to be positive, and all the pools that contained only negative

Journal Pre-proof

samples were found to be negative (**Figure 1**). Of the 5 pools which contained one individual sample with an "indeterminate" result (in each pool), one was found to be negative per definitions of individual tests, but still with Ct < 39 that allows pool retesting.

In addition, we tested matrix pooling (see Methods) by pooling 75 samples into three *5 x 5* matrices, and identified all positive samples accurately (**Figure 2**). Importantly, the positive samples were detected in both the row and the column pools at a similar cycle in all three tested matrices, suggesting the pooling scheme is robust.

Given the successful validation of both pooling strategies, and the low prevalence in asymptomatic population, we have adopted a Dorfman pooling protocol of 1:8 and employed it for the routine testing of nasopharyngeal swab samples from screened asymptomatic healthcare personnel, employees of essential industries, and residents and employees of nursing homes.

In the first three batches run at the HMC (**Table 2**) we have tested 2,168 samples by pooling, using 311 RNA extraction and RT-PCR reactions (a mere 14% of kits that would have been used in the full individual testing, an increase of 7-fold in throughput). Among these samples, we have identified and individually validated five positive samples, corresponding to a rate of 0.23%. We have then implemented the method in a routine clinical diagnosis setting of asymptomatic populations, testing a total of 26,576 samples with 7.3-fold increase in throughput, identifying a total of 31 positives (0.12%).

Journal Pre-proof

Discussion

We demonstrate in a real-life situation the usefulness of pooled sampling starting at the early lysate stage. The simplicity of the method, similarity to currently approved procedures, and the fact that we do not require special sample handling or additional information make it easily adoptable on a large scale. This saves time, work and reagents, allowing a considerable throughput increase of clinical diagnostic labs and opening the door for efficient screening of large asymptomatic populations for the presence of SARS-CoV-2 infection.

Journal Pre-proof

An important consideration before implementing group testing is the expected rate of false positive and false negative results. Based on our experience with over 26,500 samples from asymptomatic individuals, we did not encounter any false positives in the pools (see **Figure 1** and **Table 2)**. False negatives are in principle more worrisome when testing in pools, because samples that failed at the RNA extraction step will be missed (while our individual testing includes amplification of a human transcript serving as an internal control for proper RNA extraction and RT-PCR of each sample). To define the magnitude of this potential problem, we examined a set of 13,781 individual tests done at our center, which were all expected to show a signal for a human gene serving as internal assay control. Amplification of the human gene failed in 52 samples (0.38%). Thus, we estimate that our current protocol of pooled sampling carries a risk of missing 0.38% of the positive samples. In a population of 1,000,000 individuals tested, of which 1,000 are positive (rate of 0.1%), this predicts that 4 positive individuals will be missed when using pools. We posit that this is a tolerable situation, particularly given the potentially much higher rates of false negative results due to swab sampling and other errors upstream. Large-scale implementation of the pooling scheme should be carefully done to assure pre-analytical influences (e.g. inadequate sampling, transport time, temperature influences) do not lead to significant loss of signal, which may further increase the risk of false negatives.

Increase in throughput applies to RNA extraction and RT-PCR, but not to viral inactivation and lysis or reporting of results. Reporting at the HMC is automated and was adapted to the pooling scheme and therefore does not require additional work. Viral inactivation and lysis typically take 30min while RNA extraction and RT-PCR 4-5 hours, and therefore 7.3-fold increase in efficiency translates to ~4.5 increase in efficiency of the entire workflow, or more if efficiency of viral inactivation is increased by other means (e.g. automation).

The increase in efficiency allowed the HMC to survey healthcare personnel and multiple nursing homes, and identify a nursing home with 16 positive individuals, helping to stop the spread at that center. Specifically, we have demonstrated that pooling lysates from 5 or 8 nasopharyngeal swab samples retains sufficient sensitivity of viral RNA detection, allowing identification of SARS-CoV-2-positive individuals, while increasing throughput 5-fold to 7.5-fold.

The prevalence of COVID-19 in the tested population is not always known, which could affect the optimal pool size. This could be addressed either by external estimates, such as a previous run of individual samples, rate of symptomatic patients, or alternative methods such as serological screening or wastewater titers monitoring [28,29]. Alternatively, it is possible to dynamically adapt pooling sizes, when the measured rate of positive samples is different than expected. Finally, some group testing algorithms (reviewed in [17]) estimate the number of positive samples while using a relatively small (logarithmic) number of tests, and may be adapted to clinical constraints and parameters. If samples are not independent, and we have information regarding their dependency, we can further improve efficiency by grouping together dependent samples, that is, samples that are likely all positives or all negatives, such as members of the same family, or samples that are likely to be all negative since they have a low risk profile. This will increase the number of negative pools, and therefore decrease the overall number of tests conducted. Future improvement of the sensitivity of the test, such as better sets of primers and improved sample collection will allow retaining sensitivity even when pooling a large number of sample lysates together. This will enable further improving efficiency, especially when prevalence is low, by increasing the pool size.

Journal Pre-proof

Transparency declaration

All authors disclose no conflicts of interest.

Acknowledgments

Yotam Drier is supported by an Alon Fellowship and the Concern Foundation / American Friends of the Hebrew University Young Professorship Award. The following people are members of the Hebrew University-Hadassah Medical School COVID-19 diagnosis team and have contributed to the development of the pooled testing pipeline: Agnes Klochendler, Amir Eden, Avihu Klar, Avraham Geldman, Ayelet Arbel, Ayelet Peretz, Batel

Journal Pre-proof

Shalom, Bracha Lea Ochana, Dana Avrahami-Tzfati, Daniel Neiman, Daniel Steinberg, Danny Ben Zvi, Etai shpigel, Gal Atlan, Hagar Klein, Hanna Chekroun, Haran Shani, Idit Hazan, Ihab Ansari, Itia Magenheim, Joshua Moss, Judith Magenheim, Liat Peretz, Libi Feigin, Malkiel Saraby, Maya Sherman, Mercedes Bentata, Mevaseret Avital, Miriam Kott, Moriah Peyser, Moriya Weitz, Moriyah Shacham, Myriam Grunewald, Naama Sasson, Nadav Wallis, Narmen Azazmeh, Netanel Tzarum, Ori Fridlich, Raz Sher, Reba Condiotti, Ron Refaeli, Roni Ben Ami, Roni Zaken Gallili, Ronny Helman, Shai Ofek, Shay Tzaban, Sheina Piyanzin, Shira Anzi, Shira Dagan, Sivan Lilenthal, Tal Sido, Tamar Licht, Tomer Friehmann, Yael Kaufman, Amir Pery, Ann Saada, Assaf Dekel, Avner Yeffet, Avraham Shaag, Ayelet Michael Gayego, Bracha-Lea Ochana, Ela Shay, Eliran Arbib, Hadil Onallah, Kerem Ben-Meir, Leonid Levinzon, Leonor Cohen-Daniel, Luba Natan, Marah Hamdan, Mila Rivkin, Mohammad Shwieki, Olesya Vorontsov, Rimma Barsuk, Rinat Abramovitch, Rita Gutorov, Salem Sirhan, Suhair Abdeen, Yulia Yachnin, Yutti Daitch.

Author contributions

Roni Ben-Ami, Agnes Klochendler and Matan Seidel contributed equally to this work. Yuval Dor, Dana Wolf, Maayan Salton and Yotam Drier contributed equally to this work. Concept and design: Eran Meshorer, Yuval Dor, Dana Wolf, Maayan Salton and Yotam Drier. Acquisition, analysis, or interpretation of data: Roni Ben-Ami, Agnes Klochendler, Matan Seidel, Tal Sido, Ori Gurel-Gurevich, Moran Yassour, Eran Meshorer, Yuval Dor, Dana Wolf, Maayan Salton and Yotam Drier. Software: Matan Seidel. Visualization: Tal Sido, Moran Yassour. Resources: The Hebrew University-Hadassah COVID-19 diagnosis team, Gil Benedek, Irit Fogel, Esther Oiknine-Djian, Asaf Gertler, Zeev Rotstein, Bruno Lavi, Yuval Dor, Dana Wolf. Writing original draft, review and editing: Yuval Dor, Dana Wolf, Maayan Salton and Yotam Drier. Supervision: Yuval Dor, Dana Wolf, Maayan Salton and Yotam Drier. All authors approved the final manuscript.

Journal Pre-proof

Figure captions

Figure 1: Pooling 8 lysates retains clinical sensitivity. Shown are results of 23 pooling experiments, with 8 lysates in each pool; 15 pools with positive samples indeed come up positive (pools 1-15), 3 pools without positive samples come up negative (pools 20, 21, 23), and 4 out of 5 pools containing a single indeterminate sample detected as indeterminate (pools 16, 17, 18, 19, 22); Pools containing 1-2 samples with low amount of SARS-CoV-2 are detected at a similar Ct (pools 9-18), showing clinical sensitivity is retained and the risk of false negatives is minimal. UD= Undetected.

Figure 2: Matrix pooling. (**a**) Scheme for 5x5 matrix pooling. 25 samples sorted in a 5x5 matrix and each row and each column is pooled into a total of 10 pools, on which RNA extraction, reverse transcription and qPCR are performed. In this illustration row B and column 3 are positive (black stars), hence sample B3 is the only positive sample. If more than one row and one column are positive then all samples in intersections need to be retested, as some may be negative. (**b**) Three 5x5 pool matrices were generated (30 pools from 75 lysates). Each matrix (25 lysates that were previously tested individually) included a single lysate positive for SARS-COV-2. As expected, only 6 pools (one row and one column per matrix) were positive for SARS-COV-2, while 24 pools had Ct >40 (Undetected). RT-PCR Ct values of positive pools were nearly identical in the column pool (green) and the row pool (blue), and similar to the values of the individual test of the positive sample (gray).

Journal Pre-proof

Journal Pre-proof

Prevalence (*p*)	0.1%	1%	2%	5%	10%	20%
Maximal theoretical efficiency	87.7	*12.4*	7.1	3.5	2.13	1.39
Dorfman *n*=8 efficiency	7.5	4.9	3.6	2.2	1.44	1.04
Matrix *n*=8 efficiency	4.0	3.9	3.6	2.6	1.68	1.05

Table 1: *Efficiency of Dorfman and matrix pooling with pool size n=8 compared with optimal efficiency.*

Journal Pre-proof

Journal Pre-proof

	No. of samples	No. of pools	No. of positive pools	Ct of positive pools	No. of positive individual samples in the positive pools	Ct of positive individual samples	No. of total tests	No. of tests saved, compared to single sample tests (%)
Pool batch 1	720	90	1	21.8	1	19.4	98	622 (86.4%)
Pool batch 2	720	90	0	-	-	-	90	630 (87.5%)
Pool batch 3	728	91	3 positives, 1 indeter-minate	25.39	2	22.05, 28.72	123	605 (83.1%)
				37.44	1	37.67*		
				35.26	1	34.66		
				38.67 indete-rminate	0	38.24 (determined as negative)		

Table 2: *Three pooled tests run at the Hadassah Medical Center**.** Ct- cycle threshold.*
** According to Hadassah Medical Center's protocol for indeterminate values, RT-PCR was repeated with a different kit, and eventually was determined as positive.*

Journal Pre-proof

References

[1] Fomsgaard AS, Rosenstierne MW. An alternative workflow for molecular detection of SARS-CoV-2 - escape from the NA extraction kit-shortage, Copenhagen, Denmark, March 2020. Euro Surveill 2020;25. https://doi.org/10.2807/1560-7917.ES.2020.25.14.2000398.

[2] Yelin I, Aharony N, Shaer-Tamar E, Argoetti A, Messer E, Berenbaum D, et al. Evaluation of COVID-19 RT-qPCR test in multi-sample pools. Infectious Diseases (except HIV/AIDS) 2020. https://doi.org/10.1101/2020.03.26.20039438.

[3] Zhang Y, Odiwuor N, Xiong J, Sun L, Nyaruaba RO, Wei H, et al. Rapid Molecular Detection of SARS-CoV-2 (COVID-19) Virus RNA Using Colorimetric LAMP. Infectious Diseases (except HIV/AIDS) 2020. https://doi.org/10.1101/2020.02.26.20028373.

[4] Yan C, Cui J, Huang L, Du B, Chen L, Xue G, et al. Rapid and visual detection of 2019 novel coronavirus (SARS-CoV-2) by a reverse transcription loop-mediated isothermal amplification assay. Clinical Microbiology and Infection. 2020 Apr 7; Available from: http://dx.doi.org/10.1016/j.cmi.2020.04.001

[5] Park G-S, Ku K, Baek S-H, Kim S-J, Kim SI, Kim B-T, et al. Development of Reverse Transcription Loop-mediated Isothermal Amplification (RT-LAMP) Assays Targeting SARS-CoV-2. J Mol Diagn 2020. https://doi.org/10.1016/j.jmoldx.2020.03.006.

[6] Ben-Assa N, Naddaf R, Gefen T, Capucha T, Hajjo H, Mandelbaum N, et al. SARS-CoV-2 On-the-Spot Virus Detection Directly From Patients. Public and Global Health 2020. https://doi.org/10.1101/2020.04.22.20072389.

[7] Ai J-W, Zhang Y, Zhang H-C, Xu T, Zhang W-H. Era of molecular diagnosis for pathogen identification of unexplained pneumonia, lessons to be learned. Emerg Microbes Infect 2020;9:597–600.

[8] Lucia C, Federico P-B, Alejandra GC. An ultrasensitive, rapid, and portable coronavirus SARS-CoV-2 sequence detection method based on CRISPR-Cas12. bioRxiv 2020:2020.02.29.971127. https://doi.org/10.1101/2020.02.29.971127.

[9] Broughton JP, Deng X, Yu G, Fasching CL, Singh J, Streithorst J, et al. Rapid Detection of 2019 Novel Coronavirus SARS-CoV-2 Using a CRISPR-based DETECTR Lateral Flow Assay.

Journal Pre-proof

Infectious Diseases (except HIV/AIDS) 2020. https://doi.org/10.1101/2020.03.06.20032334.

[10] Guo L, Ren L, Yang S, Xiao M, Chang D, Yang F, et al. Profiling Early Humoral Response to Diagnose Novel Coronavirus Disease (COVID-19). Clin Infect Dis 2020. https://doi.org/10.1093/cid/ciaa310.

[11] Xiao S-Y, Wu Y, Liu H. Evolving status of the 2019 novel coronavirus infection: Proposal of conventional serologic assays for disease diagnosis and infection monitoring. J Med Virol 2020;92:464–7.

[12] Zhang W, Du R-H, Li B, Zheng X-S, Yang X-L, Hu B, et al. Molecular and serological investigation of 2019-nCoV infected patients: implication of multiple shedding routes. Emerg Microbes Infect 2020;9:386–9.

[13] Lassaunière R, Frische A, Harboe ZB, Nielsen ACY, Fomsgaard A, Krogfelt KA, et al. Evaluation of nine commercial SARS-CoV-2 immunoassays. Infectious Diseases (except HIV/AIDS) 2020. https://doi.org/10.1101/2020.04.09.20056325.

[14] Barra GB, Santa Rita TH, Mesquita PG, Jacomo RH, Nery LFA. Analytical sensibility and specificity of two RT-qPCR protocols for SARS-CoV-2 detection performed in an automated workflow. Infectious Diseases (except HIV/AIDS) 2020. https://doi.org/10.1101/2020.03.07.20032326.

[15] Kalikiri MKR, Hasan MR, Mirza F, Xaba T, Tang P, Lorenz S. High-throughput extraction of SARS-CoV-2 RNA from nasopharyngeal swabs using solid-phase reverse immobilization beads. Infectious Diseases (except HIV/AIDS) 2020. https://doi.org/10.1101/2020.04.08.20055731.

[16] Du D, Hwang FK, Hwang F. Combinatorial Group Testing and Its Applications. World Scientific; 2000.

[17] Aldridge M, Johnson O, Scarlett J. Group Testing: An Information Theory Perspective. Foundations and Trends® in Communications and Information Theory 2019;15:196–392.

[18] Sinnott-Armstrong N, Klein D, Hickey B. Evaluation of Group Testing for SARS-CoV-2 RNA. Infectious Diseases (except HIV/AIDS) 2020. https://doi.org/10.1101/2020.03.27.20043968.

[19] Shani-Narkiss H, Gilday OD, Yayon N, Landau ID. Efficient and Practical Sample Pooling High-Throughput PCR Diagnosis of COVID-19. Public and Global Health 2020.

https://doi.org/10.1101/2020.04.06.20052159.

[20] Deckert A, Bärnighausen T & Kyei N. Pooled-sample analysis strategies for COVID-19 mass testing: a simulation study. Bull World Health Organ. E-pub: 2 April 2020. doi:http://dx.doi.org/10.2471/BLT.20.257188

[21] Shental N, Levy S, Skorniakov S, Wuvshet V, Shemer-Avni Y, Porgador A, et al. Efficient high throughput SARS-CoV-2 testing to detect asymptomatic carriers. Infectious Diseases (except HIV/AIDS) 2020. https://doi.org/10.1101/2020.04.14.20064618.

[22] Gollier C, Gossner O. Group Testing against COVID-19. Center for Research in Economics and Statistics; 2020.

[23] Kwiatkowski TJ Jr, Zoghbi HY, Ledbetter SA, Ellison KA, Chinault AC. Rapid identification of yeast artificial chromosome clones by matrix pooling and crude lysate PCR. Nucleic Acids Res 1990;18:7191–2.

[24] Barillot E, Lacroix B, Cohen D. Theoretical analysis of library screening using a N-dimensional pooling strategy. Nucleic Acids Res 1991;19:6241–7.

[25] Dorfman R. The Detection of Defective Members of Large Populations. Ann Math Stat 1943;14:436–40.

[26] T. Li, C. L. Chan, W. Huang, T. Kaced and S. Jaggi, "Group testing with prior statistics," 2014 IEEE International Symposium on Information Theory, Honolulu, HI, 2014, pp. 2346-2350, doi: https://doi.org/10.1109/ISIT.2014.6875253.

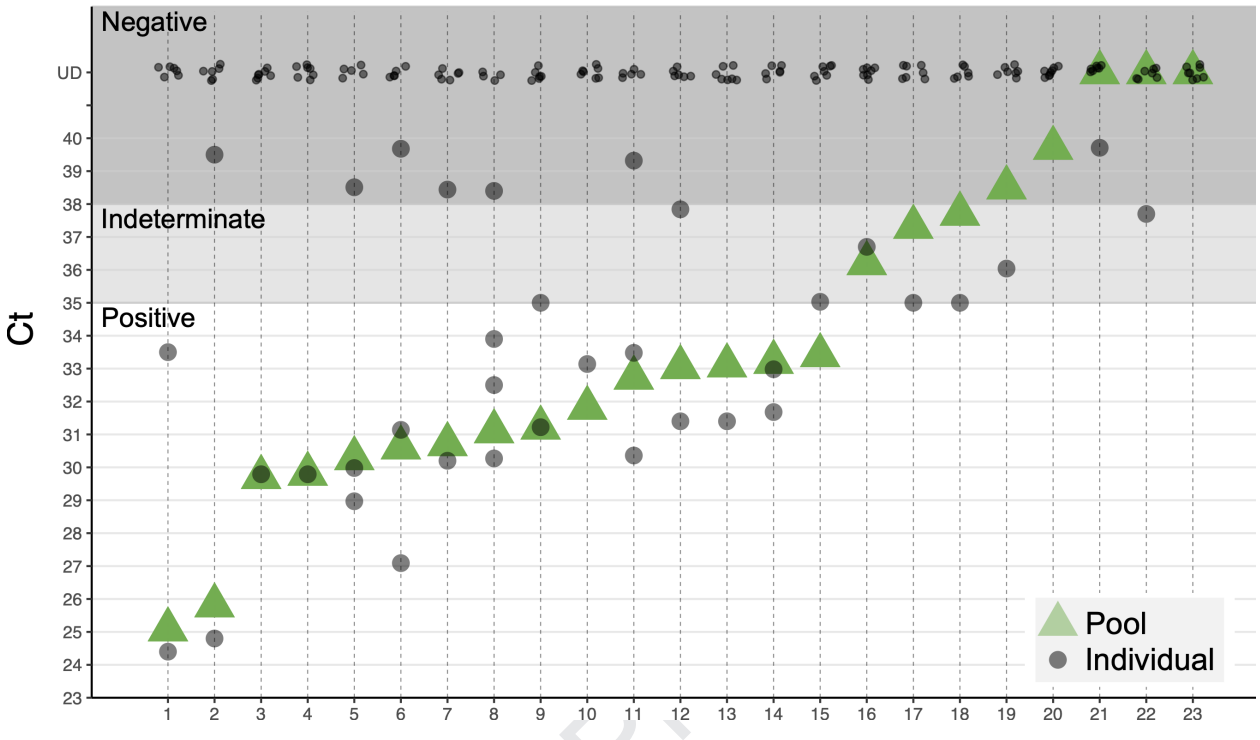
[27] Lavezzo E, Franchin E, Ciavarella C, Cuomo-Dannenburg G, Barzon L, Del Vecchio C, et al. Suppression of COVID-19 outbreak in the municipality of Vo, Italy. Epidemiology 2020. https://doi.org/10.1101/2020.04.17.20053157.

[28] Randazzo W, Truchado P, Cuevas-Ferrando E, Simón P, Allende A, Sánchez G. SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area. Water Res 2020;181:115942.

[29] Orive G, Lertxundi U, Barcelo D. Early SARS-CoV-2 outbreak detection by sewage-based epidemiology. Sci Total Environ 2020;732:139298.

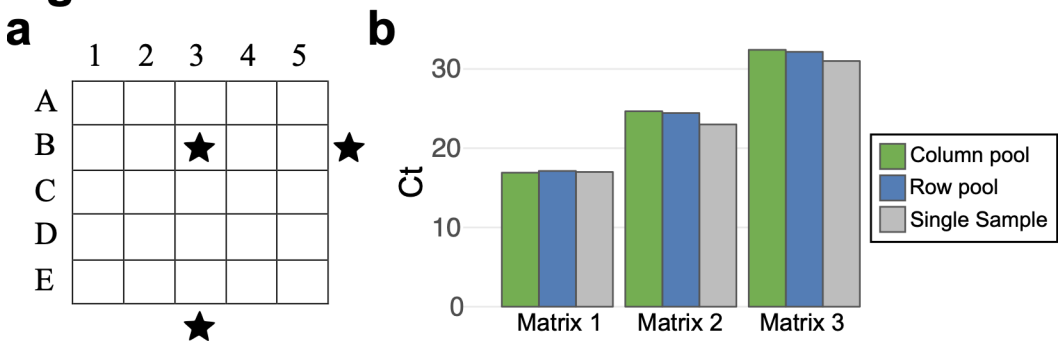
Journal Pre-proof

Figure 1



Journal Pre-proof

Figure 2



Journal Pre-proof

An In Vitro Microneutralization Assay for SARS-CoV-2 Serology and Drug Screening

Fatima Amanat,[1,2,7] Kris M. White,[2,3,7] Lisa Miorin,[2,3,7] Shirin Strohmeier,[2] Meagan McMahon,[2] Philip Meade,[1,2] Wen-Chun Liu,[2,3] Randy A. Albrecht,[2,3] Viviana Simon,[2,3,5] Luis Martinez-Sobrido,[4] Thomas Moran,[2] Adolfo García-Sastre,[2,3,5,6] and Florian Krammer[2,8]

[1]Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, New York, New York
[2]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, New York
[3]Global Health Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, New York, New York
[4]Texas Biomedical Research Institute, San Antonio, Texas
[5]Division of Infectious Diseases, Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, New York
[6]Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, New York
[7]These authors contributed equally
[8]Corresponding author: *florian.krammer@mssm.edu*; all other authors can be reached at *fatima.amanat@icahn.mssm.edu*

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in the city of Wuhan, Hubei Province, China, in late 2019. Since then, the virus has spread globally and caused a pandemic. Assays that can measure the antiviral activity of antibodies or antiviral compounds are needed for SARS-CoV-2 vaccine and drug development. Here, we describe in detail a microneutralization assay, which can be used to assess in a quantitative manner if antibodies or drugs can block entry and/or replication of SARS-CoV-2 in vitro. © 2020 Wiley Periodicals LLC.

Basic Protocol 1: Microneutralization assay to test inhibition of virus by antibodies (purified antibodies or serum/plasma)
Basic Protocol 2: Screening of anti-SARS-CoV-2 compounds in vitro
Support Protocol: SARS-CoV-2 propagation

Keywords: antivirals • COVID-19 • COVID19 • medium-throughput screening • microneutralization • neutralization • SARS-CoV-2

How to cite this article:
Amanat, F., White, K. M., Miorin, L., Strohmeier, S., McMahon, M., Meade, P., Liu, W.-C., Albrecht, R. A., Simon, V., Martinez-Sobrido, L., Moran, T., García-Sastre, A., & Krammer, F. (2020). An in vitro microneutralization assay for SARS-CoV-2 serology and drug screening. *Current Protocols in Microbiology*, *58,* e108. doi: 10.1002/cpmc.108

INTRODUCTION

Several "common cold" seasonal coronaviruses circulate in the human population, but in late 2019, a novel coronavirus surfaced in China that was later classified as a beta-coronavirus and named severe acute respiratory syndrome coronavirus 2

CURRENT PROTOCOLS
A Wiley Brand
Current Protocols in Microbiology e108, Volume 58
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpmc.108
© 2020 Wiley Periodicals LLC

(SARS-CoV-2; Wu et al., 2020). SARS-CoV-2 is closely related to SARS CoV (now renamed SARS-CoV-1), which caused an epidemic from 2002-2004 in 26 countries. As of today, millions of cases of SARS-CoV-2 have been reported across the globe. Currently, no vaccines and only limited therapeutics are available as treatment for COronaVIrus Disease 2019 (COVID-19).

Both SARS-CoV-1 and SARS-CoV-2 use angiotensin-converting enzyme 2 (ACE2) as their receptor for attachment to host cells (Letko, Marzi, & Munster, 2020). The viral protein that interacts with ACE2 is the spike protein, a large trimeric glycoprotein (Wrapp et al., 2020). The part of the spike protein that interacts with the receptor is referred to as the receptor binding domain (RBD), and this domain is one of the major targets of neutralizing antibodies (Walls et al., 2020). Several studies have already demonstrated that individuals who recover from SARS-CoV-2 infection seroconvert and produce high levels of antibodies against the spike protein, as measured by binding assays (Amanat et al., and Okba et al., 2020). Initial results show that the induced antibodies also neutralize the virus efficiently, but large-scale serology studies to correlate results from binding assays and neutralization assays are needed. In addition, there is a dire need for robust assays to screen for antiviral drugs that inhibit the replication of SARS-CoV-2 in vitro. Several types of neutralization assays have been applied for SARS-CoV-2. The most common assay used is the plaque reduction neutralization test (PRNT). PRNTs require an agarose overlay, which is time-consuming, and it is difficult to perform PRNTs at higher throughput in a Biosafety Level 3 (BSL-3) laboratory. In addition, pseudotyped virus particle entry inhibition assays have been developed which can be used at higher throughput and at lower biosafety levels. However, they do not employ authentic virus and are, therefore, always just an approximation of "real" virus neutralization. In addition, they can only be used to test entry inhibitors, but not other types of drugs. The microneutralization assays described here are performed in a 96-well format, which allows for medium throughput. The readout for these assays is based on staining of the virus nucleoprotein (NP), which provides quantitative assessment of inhibitory concentration and does not rely on cumbersome and subjective visual inspection of cytopathic effect. Basic Protocol 1 describes the use of the assay for testing sera, plasma, or monoclonal antibodies, while Basic Protocol 2 describes the use of the assay in the context of drug screening.

Biosafety cautions

SARS-CoV-2 is a BSL-3 pathogen. Follow all appropriate guidelines for the use and handling of pathogenic microorganisms (*https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html*). Also see Current Protocols article: Burnett, Lunn, & Coico (2009).

Human-derived material such as serum and plasma samples can potentially contain infectious SARS-CoV2, as well as other blood-borne viral pathogens. Heat inactivation at 56°C for 1 hr is recommended prior to use. Follow all appropriate guidelines and regulations for the use and handling of human-derived materials.

BASIC PROTOCOL 1 MICRONEUTRALIZATION ASSAY TO TEST INHIBITION OF VIRUS BY ANTIBODIES (PURIFIED ANTIBODIES OR SERUM/PLASMA)

This protocol can be used to assess the extent to which antibodies are able to neutralize SARS-CoV-2 in vitro. Serum or plasma samples from any species, as well as monoclonal antibodies (mAbs), can be used in this assay. This assay is similar to a PRNT, but it can be performed in a 96-well cell culture plate and allows for a higher throughput compared to a standard PRNT. The virus that we have used in the assay is SARS-CoV-2 isolate USA-WA1/2020 (BEI Resources NR-52281), but local isolates can be used as well. The cell type described here is Vero.E6, which has been shown to be permissive

for SARS-CoV-2, but the assay can be adapted to other cell lines (Harcourt et al., 2020). Heat inactivation of serum or plasma at 56˚C for 1 hr is recommended to minimize the effect of complement on the cells, as well as to mitigate biosafety risks. Positive and negative controls should be used at all times. For serology, naïve or pre-pandemic sera or plasma can be used as negative controls, while convalescent patient sera or sera from immunized/infected animals can be used as positive controls. For mAbs, the negative control should be a non-SARS-CoV-2 antibody of the same isotype as the tested mAbs. Any available neutralizing mAb or antiserum can be used as positive control (Amanat et al., 2020). We also preferably perform the assays with serum/plasma/mAb in the overlay at the same concentration as the initial infection, since we believe that this better reflects physiological conditions. However, the assay can be modified to have antibody only in the initial virus-antibody incubation period or only in the overlay, to study entry inhibition only or inhibition of virus spread within the culture only, respectively.

The amount of virus used for the assay is an important variable. Virus titers can be established using different protocols (see Current Protocols article: Mendoza, Manguiat, Wood, & Drebot, 2020). In any case, a virus dose that leads to robust staining needs to be used, and approximately 10,000 $TCID_{50}$/ml are used in this assay. Another crucial reagent is the staining antibody. Here, we describe an antibody against the nucleoprotein (NP), since this protein is abundant within infected cells. However, mAbs or antisera targeting other viral proteins might be used as well. Antisera raised against the whole virus might also be useful for cell staining . We perform our data analysis in GraphPad Prism, but any other similar data analysis software can be used. Preparation of the cell plates for the assay and the final staining steps may be performed outside the BSL-3 facility. Any work with replication-competent SARS-CoV2 needs to be performed inside a BSL-3 facility using appropriate personal protective equipment (PPE). Plates may only be taken out of the BSL-3 facility once the virus has been inactivated.

Definitions

BSA = bovine serum albumin
BSL = Biosafety Level
cDMEM = complete Dulbecco's modified Eagle Medium
CPE = cytopathic effect
DMEM = Dulbecco's modified Eagle Medium
FBS = fetal bovine serum
HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP = horseradish peroxidase
ID_{50} = 50% inhibitory dilution
MEM = minimal essential medium
MNA = microneutralization assay
NP = nucleoprotein
OPD = *o*-phenylenediamine dihydrochloride
RT = room temperature
$TCID_{50}$ = 50% tissue culture infectious dose
WFI = water for injection

Materials

Vero.E6 cells (ATCC #CRL-1586)
Complete DMEM medium (cDMEM; see recipe)
Universal mycoplasma detection kit (ATCC 30-1012K)
2× MEM (see recipe)
Fetal bovine serum (FBS; Corning, cat. no. 35011CV)
Water for injection for cell culture (WFI; Gibco, cat. no. A1287301)

SARS-CoV-2 isolate USA-WA1/2020 (BEI Resources, cat. no. NR-52281, or similar)
Serum samples for testing
10% formaldehyde (Polysciences, cat. no. 04018-1)
Phosphate-buffered saline (PBS; Gibco, cat. no. 10010-023, or equivalent)
Triton X-100 (Fisher Bioreagents, cat. no. BP151-100)
Non-fat milk (AmericanBio, cat. no. AB10109-01000, or equivalent)
Mouse anti-SARS NP antibody, e.g., 1C7 (an in-house mAb, provided by Dr. Thomas Moran, *Thomas.Moran@mssm.edu*)
Anti-mouse IgG HRP (Rockland, cat. no. 610-4302)
SIGMAFAST™ OPD (Sigma-Aldrich, cat. no. P9187)
3 M hydrochloric acid (Fisher Scientific, cat. no. S25856)

Class II biological safety cabinet
96-well cell culture plate (Corning, cat. no. 3595)
Light microscope
Polypropylene sterile conical tubes:
15-ml (Denville Scientific, cat. no. C1018P or equivalent)
50-ml (Fisher Denville Scientific, cat. no. C1060P or equivalent)
1.5-ml microcentrifuge tubes (Denville, cat. no. C2170 or equivalent)
Pipet-Aid
Sterile, serological pipettes:
5-ml (Falcon, cat. no. 356543 or equivalent)
10-ml (Falcon, cat. no. 357551 or equivalent)
25-ml (Falcon, cat. no. 357535 or equivalent)
50-ml (Falcon, cat. no. 356550 or equivalent)
Micropipettes
Micropipette tips:
20-µl barrier tips (Denville Scientific, cat. no. P1121 or equivalent)
200-µl barrier tips (Denville Scientific, cat. no. P1122 or equivalent)
200-µl tips (USA Scientific, cat. no. 1111-1700 or equivalent)
1000-µl barrier tips (Denville Scientific, cat. no. P1126 or equivalent)
Sterile reservoirs (Fisher Scientific, cat. no. 07-200-127 or equivalent)
Biotek SynergyH1 Microplate Reader or equivalent
GraphPad Prism 7 (or equivalent software)

Determining the 50% tissue culture infectious dose ($TCID_{50}$)

1. Maintain Vero.E6 cells in culture using cDMEM.

   Cells should be mycoplasma free and should also have high viability (>95%) when used in this assay.

2. The day before infection, seed 20,000 cells per well in 96-well cell culture plates.

   This can be performed outside of the BSL-3 facility, and the cells can be transferred into the facility on the day of virus infection.

3. The next day, prepare 10-fold dilutions of the virus in 1× MEM supplemented with 2% FBS (1× MEM/2% FBS). 1× MEM/2% FBS is prepared by combining equal amounts of 2× MEM and WFI, plus 2% FBS.
   100 µl of the original virus stock can be added to 900 µl of 1× MEM/2% FBS, and this would serve as the 10^{-1} dilution. A 10-fold dilution series is then prepared from the 10^{-1} dilution. Dilutions up to 10^{-8} are usually sufficient to determine the $TCID_{50}$. The dilutions of the virus are added to the plate in a horizontal manner, and row H is used as a control row with addition of only 1× MEM/2% FBS but no virus (no-virus control). Row H is used as a reference to check CPE and assess the CPE

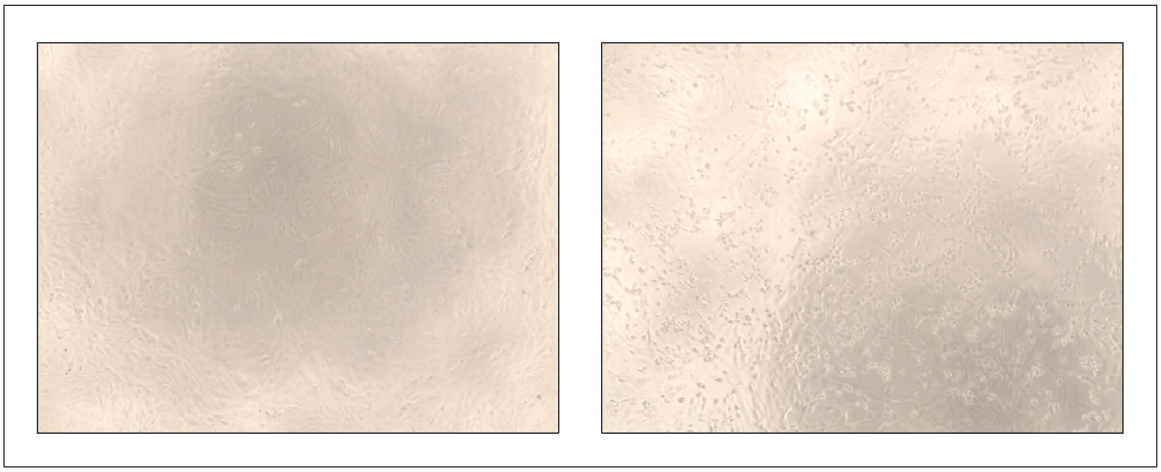


Figure 1 CPE induced by SARS-CoV-2 in Vero.E6 cells. The left panel shows healthy, uninfected Vero.E6 cells forming a monolayer. The right panel shows CPE after infection with SARS-CoV-2 (e.g., rounded up cells, halo around cells, etc.).

in infected cells. Be sure to prepare enough volume of each dilution for six to eight replicates.

4. Check the confluency of the cells under a light microscope. Cells should be between 90% and 100% confluence. Infection efficiency may be lower if overconfluent cells are used. Remove all of the medium from the wells of the 96-well cell culture plate and add 100 µl/well of each respective virus dilution to the plate. 100 µl per well of 1× MEM/2% FBS without virus should be added to a few rows or columns, and these wells can serve as "no-virus" controls. Place the plate at 37°C for 1 hr.

   Here and below, pipettes tips should be changed for every virus transfer, since reusing the same tips will impact the $TCID_{50}$. Add the liquid to the side of the well to avoid disturbing the cell monolayer.

5. After 1 hr of viral absorption, remove the virus inoculum from every well. Also remove the medium from "no-virus" control wells. Add 200 µl of 1× MEM/2% FBS to all the wells.

6. Incubate the cells for 3 days at 37°C.

7. On day 3 post infection, check the cells under the microscope for CPE (Fig. 1). Wells with the highest amount of virus should show strong CPE, and this effect should decrease as the virus is diluted further. "No-virus" controls can be used as reference for no infection.

8. Make a note of the number of wells that are positive for each dilution and calculate the $TCID_{50}$/ml using the Reed & Muench method (Ramakrishnan et al., 2016).

Performing the microneutralization assay (MNA)

9. Maintain Vero.E6 cells in culture using cDMEM.

   Cells should be mycoplasma free and should also have high viability when used in this assay.

10. The day before infection, seed 20,000 cells per well in a 96-well cell culture plate as described in step 2.

11. Prepare serum dilutions in empty, sterile 96-well cell culture plates using 1× MEM/2% FBS. 1× MEM/2% FBS is prepared by adding equal amounts of 2× MEM and WFI, plus 2% FBS.

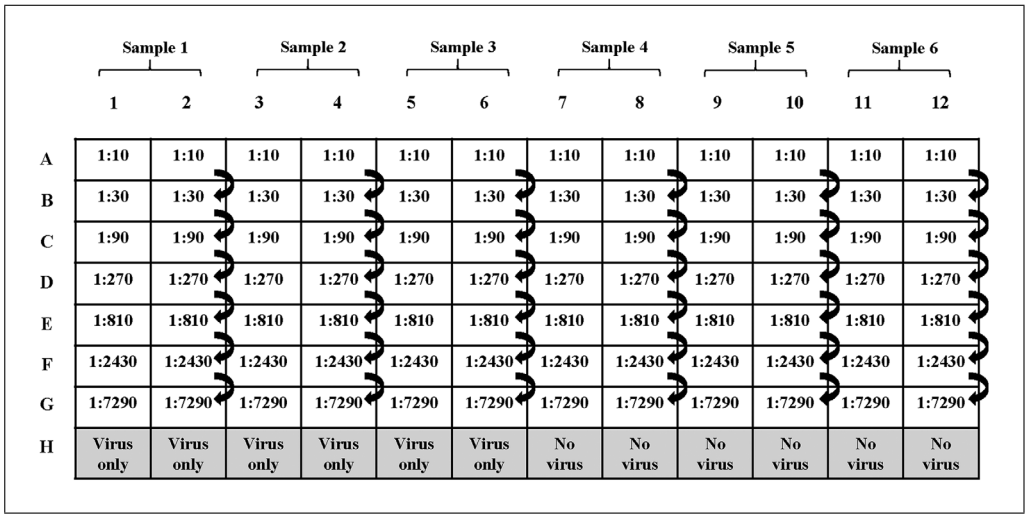


Figure 2 Plate layout for microneutralization assay. Indicated dilutions are suggestions for testing serum samples. Other dilution series or concentrations (for mAbs) might be used.

Serum/plasma samples from humans or animals infected with SARS-CoV-2 should be heat-inactivated at 56°C for 1 hr prior to use. Heat-inactivation of serum/plasma samples will ensure that any complement present in the sample is inactived, and it also increases safety since heat inactivation might inactivate infectious virus present in samples. Each sample is tested in duplicate.

12. Add 270 µl of 1× MEM/2% FBS to row A and add 200 µl of 1× MEM/2% FBS to row B-G. Add 30 µl of each respective sample to row A and pipet up and down several times to mix. Once all the samples are added, transfer 100 µl from row A to B and mix 5-10 times using the multichannel pipette. Discard tips. Load new tips on to the multi-channel pipette and transfer 100 µl from row B to row C, then mix 5-10 times again. Repeat this process until row G is reached. At row G, discard the 100 µl so each column has 200 µl remaining. Row H is a control row. This plate is called plate A. Refer to Figure 2 for plate layout.

13. Obtain a new 96-well cell culture plate and transfer 80 µl of each respective dilution to the new plate. Ensure that the order of the samples and dilutions is preserved in the new plate (referred to as plate B). Dilutions and preparation of plate A and plate B can all be performed in at BSL-2.

14. Handle SARS-CoV-2 in a Biosafety Level 3 (BSL-3) laboratory in a biosafety cabinet. Prepare 10,000 $TCID_{50}$/ml of SARS-CoV-2 in 1× MEM/2% FBS. Add 80 µl of the virus to each well in plate B except wells H7-H12. Add 80 µl of 1× MEM/2% FBS to rows H7-H12. Close the lid of the 96-well plate and tap gently with the palm of the hand to mix the serum with the virus. Each well should now have a total of 160 µl (80 µl of serum dilution plus 80 µl of virus). Incubate for 1 hr at room temperature. It is not necessary to rock the plate.

15. After the incubation time is over, take the Vero.E6 cells in the 96-well cell culture plate (step 9) and remove the medium using a multi-channel pipette or an aspirator.

    The medium should be removed carefully such that the tip does not touch the cells or the cells are not dislodged in any way.

16. Add 120 µl of serum-virus mixture from plate B to the cells. Ensure that the order of the serum dilutions is preserved across all plates. Tips do not need to be changed if the serum-virus mixture is moved from the bottom of the plate to the top (i.e.,

starting from row H and ending at row A). Place the cells at 37°C in a humidified incubator with 5% CO_2 for 1 hr.

Infection was done with only 60 μl of virus, amounting to 600 $TCID_{50}$ per well. Amounts of virus can be varied depending on how long the cells are to be kept in the incubator for infection.

600 $TCID_{50}$ per well has given us reasonable staining as well as reasonable sensitivity. However, the amount of virus per well can be further optimized.

17. After 1 hr, tilt the plate with one hand and remove all the virus-serum mixture using a multi-channel pipette. Ensure that all the medium is removed. Next, add 100 μl from each well of plate A to the cells (the time that the cells are without liquid should be kept as a minimum; otherwise they may dry out and die). Finally, add 100 μl per well of 1× MEM/2% FBS. Tips do not need to be changed if the wells are not touched with the tips.

18. Incubate the cells for 48 hr.

    Plates A and plate B can be discarded.

19. Forty-eight hours later, check for cytopathic effect, as cells that did not receive any virus should be healthy while wells H1-H6 should show signs of infection. Remove all medium from each well containing cells, and add 100 μl of 10% formaldehyde.

    SARS-CoV-2 needs to be inactivated for 24 hr at 4°C.

Staining protocol

20. After inactivation of virus for 24 hr, the plate can be taken out of the BSL-3 facility and staining can be performed in a BSL-2 facility inside a biosafety cabinet.

    CAUTION: *Before proceeding with this option, please discuss how inactivation needs to be tested and proven with your biosafety officer, since local regulations may differ. Some institutions may not allow bringing material out of the BSL-3 containment facility, and staining will need to be performed within the BSL-3 in these cases. Even after inactivation, plates should be handled at BSL-2 in a biosafety cabinet in order to reduce residual risk.*

21. Remove formaldehyde from the cells carefully using a multi-channel pipette. Care should be taken not to touch the cells with the tip or dislodge the cells in any way.

    Fixation with 10% formaldehyde ensures that all cells are cross-linked onto the wells, but cells can be inspected under the microscope to ensure that they have not detached.

22. Wash cells by addition of 200 μl of phosphate-buffered saline (PBS) to each well. Aspirate using a multi-channel aspirator or remove the PBS with a multi-channel pipette.

23. Prepare fresh permeabilization solution by adding 500 μl of Triton X-100 to 500 ml of PBS (0.1% PBS/Triton). Mix vigorously. Add 150 μl per well of 0.1% PBS/Triton and incubate the cells with this solution for 15 min at RT.

24. Once the incubation time is over, wash the cells with 200 μl per well of PBS. Remove all the PBS and add 100 μl/well of PBS supplemented with 3% non-fat milk. Incubate cells for 1 hr at RT.

25. Next, prepare primary antibody solution in PBS supplemented with 1% non-fat milk. 1 μg/ml of 1C7 (mouse anti-SARS NP—or equivalent) mAb was used for our assay, provided by Dr. Thomas Moran. Add 100 μl/well of primary antibody solution and incubate for 1 hr at RT.

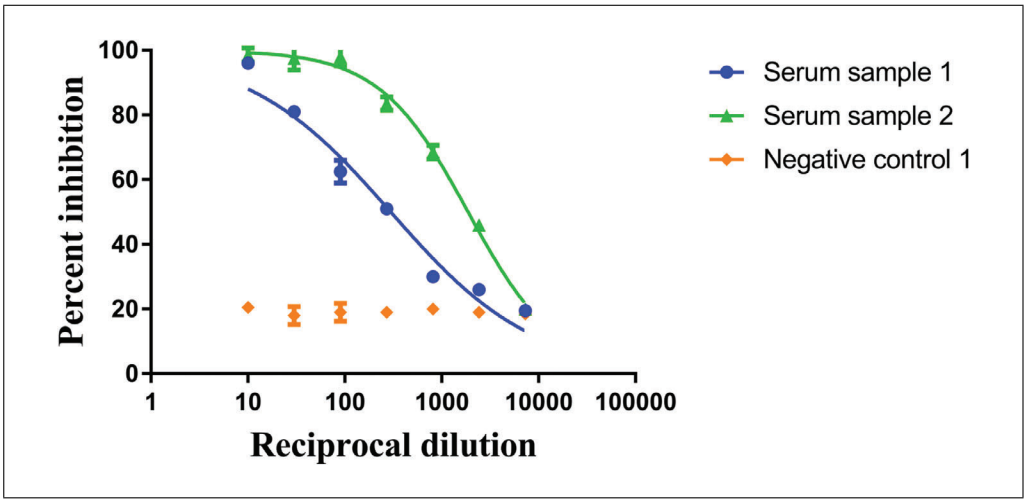


Figure 3 Analysis of data as reciprocal dilution of serum and percent inhibition of virus (ID_{50} of serum sample 1 is 1:298 and serum sample 2 is 1:1873).

26. Next, wash each well twice with 200 µl of PBS. To remove all the PBS, gently tap the plate on some paper towels to ensure that no primary antibody is left on the plate. Prepare secondary antibody solution, anti-mouse IgG HRP, in PBS supplemented with 1% non-fat milk at a dilution of 1:3000. Add 100 µl/well and incubate for 1 hr at RT.

27. Finally, wash each well twice with 200 µl/well of PBS. Dry the plate by gently tapping on paper towels. Prepare developing solution (SIGMAFAST™ OPD) according to the manufacturer instructions.

28. Add 100 µl of developing solution per well and wait 10-12 min.

29. Stop the reaction by adding 50 µl/well of 3 M hydrochloric acid.

30. Measure the optical density at 490 nanometers on a Biotek SynergyH1 Microplate Reader (or equivalent) and record data. Average the "virus-only" wells and separately average all the "no-virus" wells. The formula to calculate percent inhibition at each well is 100 − ((X-average of 'no virus' wells)/(average of 'virus only' wells-average of 'no virus' wells)*100), where X is the read for each well. Non-linear regression curve fit analysis over the dilution curve can be performed to calculate ID_{50}. The top and bottom constraints are set at 100% and 0% (Fig. 3).

BASIC PROTOCOL 2

SCREENING OF ANTI-SARS-CoV-2 COMPOUNDS IN VITRO

This protocol can be used to assess the ability of a compound to inhibit SARS-CoV-2 infection at any stage of the viral life cycle (e.g., entry, replication, assembly, egress and spread). Repurposing of existing drugs already approved for the treatment of other diseases represents one of the most rapid strategies for the identification of compounds targeting SARS-CoV-2 (Gordon et al., 2020). However, any compound predicted to have antiviral activity could be tested using this assay. Here, the antiviral activity of the compound is evaluated based on its ability to inhibit SARS-CoV-2 replication as assessed by immunostaining for the viral NP. Specifically, Vero.E6 cells are pre-incubated with each compound dilution for 2 hr, followed by a low-MOI infection to allow multi-cycle replication. 48 hr post-infection, cells are fixed and stained prior to automated imaging and analysis. The toxicity of the test compound is determined in parallel by performing an MTT assay or any other commercial cell viability assay. The ratio of IC_{50} calculated from the antiviral data over the CC_{50} calculated from the cytotoxicity data will yield the selectivity index of a compound, indicating how selective

it is for inhibiting viral replication compared to its impact on cell death. Importantly, when performing the screening, standard positive and negative controls should be included so that the results can be compared between different assays. Positive controls could be previously established—for example, SARS-CoV-2 inhibitors such as remdesivir, which has an expected IC_{50} of ~500 nM in this assay (Bouhaddou et al., submitted). Negative controls should include diluent-only treatment (e.g., DMSO). Of note, these conditions have been optimized for infection of Vero.E6 cells, but could be adapted to other cell lines that can support SARS-CoV-2 infection. In addition, time-of-addition and drug combination studies can also be performed with similar assay settings.

Definitions

BSA = bovine serum albumin
CC_{50} = 50% cytotoxic concentration
cDMEM = complete Dulbecco's modified Eagle Medium
DAPI = 4′,6-diamidino-2-phenylindole
DMEM = Dulbecco's modified Eagle Medium
DMSO = dimethyl sulfoxide
FBS = fetal bovine serum
HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC_{50} = 50% inhibitory concentration
MOI = multiplicity of infection
MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
$TCID_{50}$ = 50% tissue culture infectious dose

Materials

Vero.E6 cells (ATCC #CRL-1586)
Universal mycoplasma detection kit (ATCC 30-1012K)
Complete DMEM medium (cDMEM; see recipe)
Viral growth medium (DMEM with 2% FBS; see recipe)
SARS-CoV-2 isolate USA-WA1/2020 (BEI Resources, cat. no. NR-52281, or similar)
Compound library
DMSO (Corning, cat. no. 25-950-CQC)
10% formaldehyde (Polysciences, cat. no. 04018-1)
Anti-SARS nucleoprotein antibody (mAb 1C7, produced in house)
Donkey anti-mouse IgG (H+L) highly cross-absorbed secondary antibody, Alexa Fluor Plus 488 (Invitrogen, cat. no. A32766 or equivalent)
4′,6-Diamidine-2′-phenylindole (DAPI; e.g., Sigma-Aldrich)
Phosphate-buffered saline (PBS; Gibco, cat. no. 10010-023, or equivalent)
Bovine serum albumin (BSA; MP Biomedicals, cat. no. 08810063)
Cell Proliferation Kit I (MTT assay, Roche, cat. no. 11465007001) or similar cytotoxicity assay.

CO_2 incubator
96-well cell culture plate (Corning, cat. no. 3595).
96-well deep well (2-ml) plates (Corning, cat. no. 3960, or equivalent)
Plate sealing film, adhesive (BioRad, cat. no. MSB1001)
Polypropylene sterile conical tubes:
  15-ml (Denville Scientific, cat. no. C1018P or equivalent)
  50-ml (Fisher Denville Scientific, cat. no. C1060P or equivalent)
Pipet-Aid

Sterile, serological pipettes:
5-ml (Falcon, cat. no. 356543 or equivalent)
10-ml (Falcon, cat. no. 357551 or equivalent)
25-ml (Falcon, cat. no. 357535 or equivalent)
50-ml (Falcon, cat. no. 356550 or equivalent)
Single-channel and multi-channel micropipettes
Micropipette tips:
20-µl barrier tips (Denville Scientific, cat. no. P1121 or equivalent)
200-µl barrier tips (Denville Scientific, cat. no. P1122 or equivalent)
200-µl tips (USA Scientific, cat. no. 1111-1700 or equivalent)
1000-µl barrier tips (Denville Scientific, cat. no. P1126 or equivalent)
Sterile reservoirs (Fisher Scientific, cat. no. 07-200-127 or equivalent)
1.5-ml microcentrifuge tubes (Denville, cat. no. C2170 or equivalent)
Celigo 96-well plates (Greiner, cat. no. 655090)
Plate cytometer (Celigo) or laser-scanning cytometer (Acumen)

Additional reagents and equipment for $TCID_{50}$ assay and immunostaining (Basic Protocol 1)

Performing the SARS-CoV2 antiviral assay

1. Maintain Vero.E6 cells in culture using cDMEM.

2. The day before infection, seed 2000 cells per well in a 96-well cell culture plate. The low number of cells per well is important for the accuracy of both our immunofluorescent antiviral readout and the cytotoxicity assay used. Three compounds can be tested per 96-well plate, and separate plates are required to test antiviral and cytotoxic activities. This and all following steps until step 6 can be performed outside of a BSL-3 facility, and the cells and dilutions can be transferred into the facility on the day of virus infection.

3. Prepare compound dilutions in empty, sterile 2-ml 96-well deep well plates using 1× DMEM supplemented with 2% FBS (viral growth medium).

   If compounds to be tested are dissolved in DMSO (common), medium supplemented with a matching percentage of DMSO should be prepared to perform dilutions.

4. Add 1100 µl of 1× DMEM (without DMSO) supplemented with 2% FBS to columns 1 and 7, and add 750 µl of 1× DMEM (containing DMSO if necessary) supplemented with 2% FBS to columns 2-6 and 8-12. Add 50% more compound than your selected maximum dose to columns 1 and 7 (2 compounds tested per row, 16 total compounds diluted per deep well plate), e.g., 3.3 µl of a 10 mM stock for concentration of 30 µM, which will dilute to a final maximum concentration of 20 µM when 50 µl of virus is added. Based on this compound example, the DMSO concentration for medium in columns 2-6 and 8-12 would be 0.3% (do not exceed 0.5% DMSO, due to toxicity concerns). Once all the compounds are added, pipet column 1 up and down 20 times to mix, transfer 375 µl from column 1 to column 2, and mix 5-10 times using the multi-channel pipette. Discard tips. Load new tips to the multi-channel pipet, transfer 375 µl from column 2 to column 3, and mix 5-10 times again. Repeat this process until column 6 is reached. Repeat for columns 7-12. If the entire deep-well plate is used, this will result 16 compounds with six 3-fold serial dilutions of each (final concentrations of 20-80 nM for this example).

   This plate is called plate X, refer to Figure 4 for plate layout.

5. Take the Vero.E6 cells seeded the previous day in the two 96-well cell culture plates (one for antiviral, one for cytotoxicity) and remove the medium using a multi-channel pipette or an aspirator.

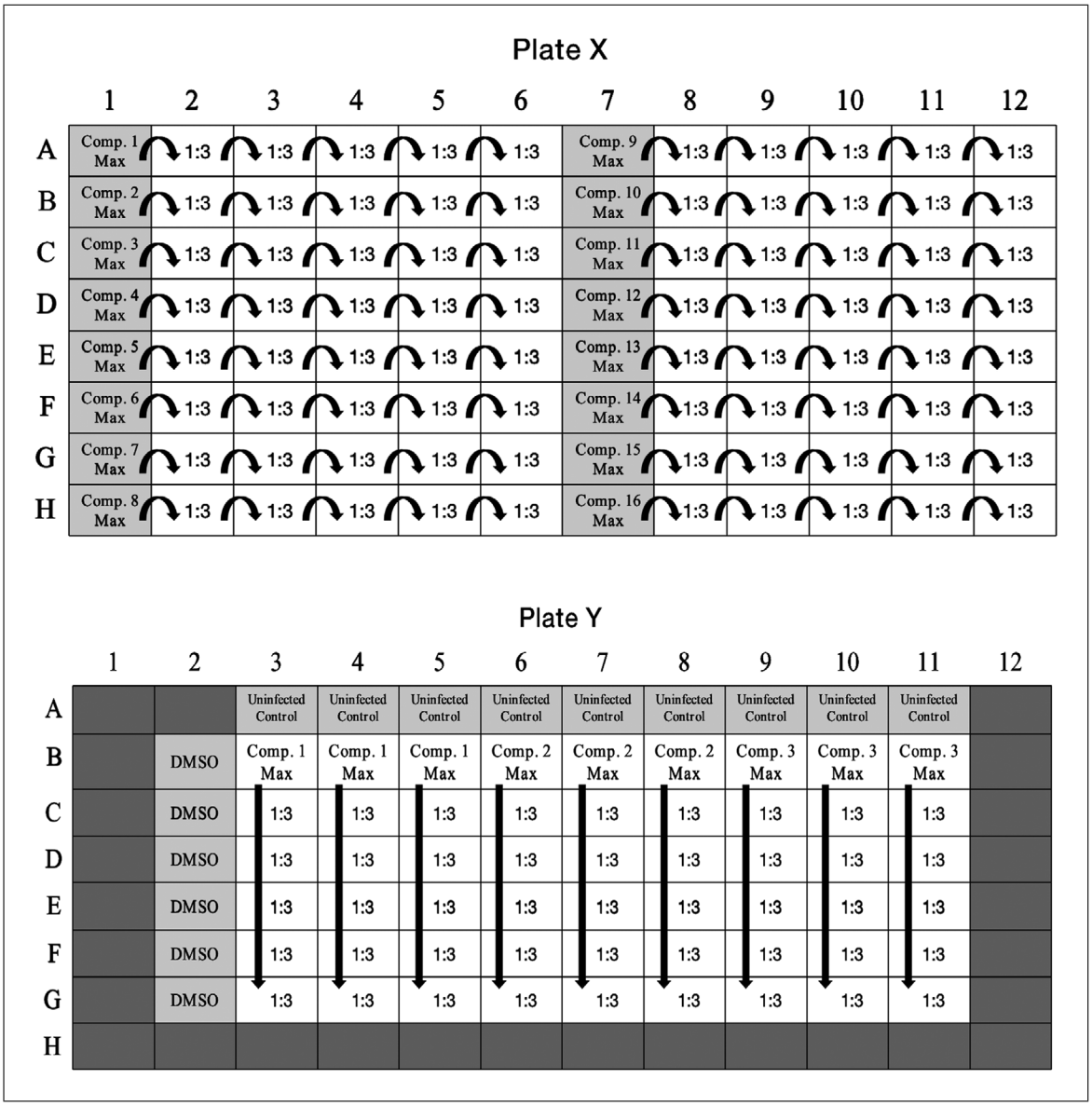


Figure 4 Plate X and plate Y layout for the antiviral assay.

The medium should be removed carefully such that the tip does not touch the cells and the cells are not dislodged in any way. Transfer 100 µl of each respective dilution to the Vero.E6 plates; there should be enough volume (750 µl) in the deep-well plate for triplicates for both antiviral and cytotoxicity testing. Using the same dilution series for both antiviral and cytotoxic assays will produce reliable matching data sets, and these should be performed at the same time. Three compounds and six DMSO control wells can be tested per 96-well plate (plate format Y, see Fig. 4). The outer wells should be avoided due to the potential for medium evaporation to alter compound concentrations. These outer wells should be filled with medium and cells to be used as uninfected controls for viral detection through immunostaining. The cells should be incubated with compound for 2 hr prior to infection as a standard, although this time frame can be adjusted for specific experiments.

6. Handle SARS-CoV-2 in a BSL-3 laboratory in a biosafety cabinet. Prepare 2000 $TCID_{50}$/ml of authentic SARS-CoV-2 (SARS-CoV-2 isolate USA-WA1/2020; see Basic Protocol 1, steps 1-8, for determination of $TCID_{50}$) in 1× DMEM supplemented with 2% FBS. Add 50 µl (100 $TCID_{50}$ per well or 0.025 MOI) of

the virus to the 100 µl of compound containing medium in each well of plate Y except the outer wells, pipet up and down gently 2-3 times to mix each well (important for even viral infection and compound concentration across the plates). Add 50 µl of 1× DMEM with 2% FBS to the sample wells of the cytotoxicity plate to adjust compound concentrations to match the antiviral assay. Each inner well should now have a total of 150 µl. Incubate for 48 hr at 37°C in a humidified incubator with 5% CO_2.

All work for testing cytotoxicity can be performed outside the BSL-3 laboratory, e.g., at BSL-2, since no infectious virus is involved.

7. After the incubation time is complete, remove the infected Vero.E6 cells in the 96-well cell culture plates from the incubator under BSL-3 conditions. Remove the medium using a multi-channel pipette.
   Optionally, viral titers can be quantified from the removed medium using $TCID_{50}$ assay (see Basic Protocol 1) or plaque assay if desired. Fix the Vero.E6 cells by adding 200 µl of 4% formaldehyde (dilute from 10%) in PBS to each well of the 96-well plate. Incubate in 4% formaldehyde for 24 hr at room temperature prior to removing plates from the BSL-3 laboratory.

8. Once the plates have been removed from the BSL-3 laboratory, proceed to immunostain the fixed cells with SARS-CoV2 specific antibodies as outlined in Basic Protocol 1, steps 20-30, with the following modifications:
   Use the antibodies listed in the Basic Protocol 2 materials list for this protocol. Briefly, after three washes with 200 µl of PBS, incubate the cells with a secondary antibody solution containing anti-mouse IgG and DAPI in PBS supplemented with 0.5% BSA at a dilution of 1:2000. Add 100 µl/well and incubate for 45 min at RT. Wash three times for 5 min in PBS with gentle shaking. A plate cytometer (Celigo) or laser scanning cytometer (Acumen) can be used to assess infected cells over total cells for highly accurate quantification of viral infection. The DMSO control should be normalized to 100% infection for comparison with compound treated wells.

9. Assay the uninfected Vero.E6 cell cytotoxicity controls for cell viability with the MTT assay or any other commercial cell viability assay according to the manufacturer's instructions.

   Cytotoxicity measurements should be matched in incubation time with your antiviral assay.

10. Calculate the IC_{50} and CC_{50} for each compound using Prism software as described in Basic Protocol 1.

SUPPORT PROTOCOL

SARS-CoV-2 PROPAGATION

The SARS-CoV2 USA-WAS1/2020 viral strain used for the optimization of the assays described here has been propagated using the Vero.E6 cell line, as it was previously shown that the virus can replicate to high titer in this cell line (*https://www.biorxiv.org/content/10.1101/2020.03.02.972935v1.full.pdf*). Stocks are generated by infecting a confluent monolayer of cells at a low multiplicity of infection to avoid the generation of defective interfering particles that can lower the titer of the viral preparation. Importantly, always work with low-passage-number stocks that are fully sequenced, to avoid selecting mutants that are better adapted to grow in the cell line used for virus amplification.

Materials

Vero.E6 cells (ATCC CRL-1586)
Vero.E6 growth medium: cDMEM (see recipe)
SARS-CoV-2 isolate USA-WA1/2020, (BEI Resources NR-52281 or similar)
Viral growth medium (DMEM with 2% FBS; see recipe)

Class II biological safety cabinet
75-cm^2 tissue culture flasks with filter caps (size varies according to the volume of stock needed)
Counting chamber or automatic cell counter
CO_2 incubator
Polypropylene sterile conical tubes
15-ml (Denville Scientific, cat. no. C1018P or equivalent)
50-ml (Fisher Denville Scientific, cat. no. C1060P or equivalent)
Pipet-Aid
Sterile, serological pipettes
5-ml (Falcon, cat. no. 356543 or equivalent)
10-ml (Falcon, cat. no. 357551 or equivalent)
Tabletop centrifuge, 4°C
2.0-ml cryotubes with O-ring

Additional reagents and equipment for $TCID_{50}$ assay (Basic Protocol 1, steps 1-8)

1. Maintain Vero.E6 cells in culture using cDMEM (also see Basic Protocol 1).
2. The day before infection seed 4×10^6 Vero.E6 cells into 75-cm^2 flasks and incubate in cDMEM for 24 hr. Make sure to prepare at least three flasks: one for virus propagation, one to be used as uninfected control, and the third one for counting the cells. Cells should be ~90% confluent at the time of infection.

   This step can be performed outside of the BSL-3 facility, and the cells can be transferred into the facility on the day of virus infection.

3. On the day of the inoculation, use one of the flasks to count the cells (e.g., using a counting chamber or an automatic cell counter) and calculate the volume of viral inoculum required to infect at multiplicity of infection of 0.001. When using Vero.E6, a confluent T-75 flask can yield about 8×10^6 cells. Remove the medium, wash once gently in PBS, and infect cells in 5 ml of viral growth medium for 1 hr at 37°C and 5% CO_2, rocking the flask every 15 min to spread the viral inoculum evenly across the monolayer.
4. After the 1-hr adsorption, add 5 ml of viral growth medium and incubate the flask at 37°C. Observe cell culture daily and follow the development of cytopathic effects. Typically, within 3 or 4 days, cells will become rounded-up and will detach from the monolayer. Harvest the virus when the cytopathic effect has progressed to 80%.
5. Clarify the medium by centrifugation in a 50-ml conical tube for 10 min at 1300 × *g*, 4°C.
6. Aliquot pooled supernatants into labeled 2.0-ml O-ring cryotubes and store samples at −80°C until further use.
7. Determine virus stock titer by plaque assay or $TCID_{50}$ following the protocol described in Basic Protocol 1.

REAGENTS AND SOLUTIONS

Complete DMEM medium (cDMEM; used in Basic Protocol 1 and 2)

880 ml of DMEM (Gibco, cat. no. 11995-065, or equivalent)
10 ml of penicillin-streptomycin (Gibco, cat. no. 15140122)
10 ml of HEPES buffer (Gibco, cat. no. 15630080)
100 ml of FBS (Corning, cat. no. 35011CV)
Filter using a 0.22-μm Stericup filter (MilliporeSigma, cat. no. S2GPU05RE)
Store up to 4 weeks at 4°C

MEM, 2× (used in Basic Protocol 1)

200 ml of 10× MEM (Gibco, cat. no 11430030)
20 ml of penicillin-streptomycin (Gibco, cat. no. 15140122)
20 ml of HEPES buffer (Gibco, cat. no. 15630080)
20 ml of L-glutamine (Gibco, cat. no. 25030081)
32 ml of 7.5% sodium bicarbonate (Gibco, cat. no. 25080094)
12 ml of 35% BSA (MP Biomedicals, cat. no. 08810063)
696 ml of water for injection for cell culture (WFI; Gibco, cat. no. A1287301)
Filter using a 0.22-μm Stericup filter (MilliporeSigma, cat. no. S2GPU05RE)
Store up to 4 weeks at 4°C.

This medium will be used to prepare 1× MEM/2% FBS by mixing equal amounts of WFI and 2× MEM.

Viral growth medium (DMEM with 2% FBS; Basic Protocol 2)

880 ml of (DMEM Gibco, cat. no. 11995-065, or equivalent)
10 ml of penicillin-streptomycin (Gibco, cat. no. 15140122)
10 ml of HEPES buffer (Gibco, cat. no. 15630080)
20 ml of FBS (Corning, cat. no. 35011CV)
Filter using a 0.22-μm Stericup filter (MilliporeSigma, cat. no. S2GPU05RE)
Store up to 4 weeks at 4°C

COMMENTARY

Background Information

The microneutralization assay that is described here has been adapted from established protocols from work with other viruses such as influenza virus (Amanat, Meade, Strohmeier, & Krammer, 2019). The assay has a medium throughput, and more samples can be analyzed as compared to PRNTs. Compared to RBD-ACE2 inhibition assays, the MNA described here will also detect neutralizing antibodies binding to epitopes outside of the RBD. Different virus isolates can be used, and the assay can likely be adapted for staining antibodies other than mAbs against NP (e.g., polyclonal sera, antibodies targeting S or M, etc.). The assay should be optimized based on the level of infection that is observed in each respective cell line used. Viral input can be varied as well. in order to make the assay more or less sensitive.

The antiviral assay described here has also been adapted from previous work with influenza virus. The immunostaining output is medium throughput and can be used to comprehensively analyze the antiviral and cytotoxic activity of many more compounds than can be done using $TCID_{50}$ or plaque assay. The time course of infection, cell line used, viral strain and MOI, and antibody used can be altered to adapt this assay to answer many different questions related to the antiviral activity of a compound.

Critical Parameters and Troubleshooting

Cells that are used for the microneutralization assay in Basic Protocol 1 should be healthy, and viability should be checked periodically. The cell count should be performed with care to ensure that cells are not overconfluent on the day of the assay. The cells should also be mycoplasma free. The $TCID_{50}$ measurement of each stock should be performed with care, as each virus stock will be used numerous times for several microneutralization assays. Most importantly, the virus should be sequenced every few passages to ensure that no major mutations have occurred from the passaging of virus in cell culture. The microneutralization assay can be performed with serum/plasma or purified antibodies. If antibodies are to be used, a trial run should be performed to assess the appropriate concentrations that should be tested.

The immunostaining antiviral assay described in Basic Protocol 2 has a consistently lower sensitivity than an assay that detects infectious viral particles produced in the supernatant ($TCID_{50}$/plaque assay). The IC_{50} determined for any particular compound in this assay will typically be 3- to 5-fold higher than the aforementioned assays. Therefore, this antiviral assay is well suited for medium-throughput screening of potential antivirals,

and we suggest confirming top hits using the mentioned classical virology methods. The DAPI counterstain can be used as a proxy for toxicity in infected cells, to be paired with the MTT assay performed in uninfected cells. If the majority of DAPI staining is lost in a compound-treated well, that compound concentration should be excluded from your final IC_{50} calculation.

Time Considerations

The $TCID_{50}$ is only performed once for each viral stock. The $TCID_{50}$ takes 3 days in total, and the microneutralization should only take 4 days, as the cells are fixed 2 days after the assay has been performed. On day 3, plates should be stained immediately, as leaving the cells in formaldehyde can negatively impact the staining. Thus, the whole microneutralization assay and data generation are completed within 4 days.

Compounds are typically added 2 hr prior to SARS-CoV-2 infection, although this can be adjusted based on the proposed mechanism of action of the compound or on the hypothesis being tested. The antiviral assay takes 3 days total from infection to staining, scanning, and data analysis. The compound should always be incubated on the cells for the same amount of time between antiviral and cytotoxicity assays.

Acknowledgments

This work was partially supported by the NIAID Centers of Excellence for Influenza Research and Surveillance (CEIRS) contract HHSN272201400008C (F.K., A.G.-S.), Collaborative Influenza Vaccine Innovation Centers (CIVIC) contract 75N93019C00051 (F.K., A.G.-S.), DARPA project HR0011-19-2-0020 (A.G.-S.), and the generous support of the JPB foundation, the Open Philanthropy Project (#2020-215611 to A.G.-S.), and other philanthropic donations.

Conflict of Interest Statement

Mount Sinai has licensed serological assays to commercial entities and has filed for patent protection for serological assays.

Literature Cited

Amanat, F., & Krammer, F. (2020). Sars-Cov-2 vaccines: Status report. *Immunity*, *52*(4), 583–589. doi: 10.1016/j.immuni.2020.03.007.

Amanat, F., Meade, P., Strohmeier, S., & Krammer, F. (2019). Cross-reactive antibodies binding to H4 hemagglutinin protect against a lethal H4n6 influenza virus challenge in the mouse model. *Emerging Microbes & Infections*, *8*(1), 155–168. doi: 10.1080/22221751.2018.1564369.

Amanat, F., Stadlbauer, D., Strohmeier, S., Nguyen, T. H. O., Chromikova, V., McMahon, M., … Krammer, F. (2020). A serological assay to detect Sars-Cov-2 seroconversion in humans. *Nature Medicine*. doi: 10.1038/s41591-020-0913-5 [Online ahead of print].

Bouhaddou, M., Memon, D., Meyer White, K. M., Rezelj, V. V., Marrero, M. C., … Kroger, N. J. The global phosphorylation landscape of SARS-CoV-2 infection. Submitted for publication.

Burnett, L. C., Lunn, G., & Coico, R. (2009). Biosafety: Guidelines for working with pathogenic and infectious microorganisms. *Current Protocols in Microbiology*, *13*, 1A.1.1–1A.1.14. doi: 10.1002/9780471729259.mc01a01s13.

Gordon, D. E., Jang, G. M., Bouhaddou, M., Xu, J., Obernier, K., White, K. M., … Krogan, N. J. (2020). A Sars-Cov-2 protein interaction map reveals targets for drug repurposing. *Nature*. doi: 10.1038/s41586-020-2286-9 [Online ahead of print].

Harcourt, J., Tamin, A., Lu, X., Kamili, S., Sakthivel, S. K., Murray, J., … Thornburg, N. J. (2020). Isolation and characterization of Sars-Cov-2 from the first Us Covid-19 patient. *bioRxiv*, doi: 10.1101/2020.03.02.972935.

Letko, M., Marzi, A., & Munster, V. (2020). Functional assessment of cell entry and receptor usage for Sars-Cov-2 and other lineage b betacoronaviruses. *Nature Microbiology*, *5*(4), 562–569. doi: 10.1038/s41564-020-0688-y.

Mendoza, E. J., Manguiat, K., Wood, H., & Drebot, M. (2020). Two detailed plaque assay protocols for the quantification of infectious Sars-Cov-2. *Current Protocols in Microbiology*, *57*(1), ecpmc105. doi: 10.1002/cpmc.105.

Okba, N. M. A., Muller, M. A., Li, W., Wang, C., Geurts van Kessel, C. H., Corman, V. M., … Haagmans, B. L. (2020). Severe acute respiratory syndrome coronavirus 2-specific antibody responses in coronavirus disease 2019 patients. *Emerging Infectious Diseases*. *26*(7). doi: 10.3201/eid2607.200841 [Online ahead of print].

Ramakrishnan, M. A. (2016). Determination of 50% endpoint titer using a simple formula. *World Journal of Virology*, *5*(2), 85–86. doi: 10.5501/wjv.v5.i2.85.

Walls, A. C., Park, Y. J., Tortorici, M. A., Wall, A., McGuire, A. T., & Veesler, D. (2020). Structure, function, and antigenicity of the Sars-Cov-2 spike glycoprotein. *Cell*, *181*(2), 281–292 e6. doi: 10.1016/j.cell.2020.02.058.

Wrapp, D., Wang, N., Corbett, K. S., Goldsmith, J. A., Hsieh, C. L., Abiona, O., … McLellan, J. S. (2020). Cryo-Em structure of the 2019-Ncov spike in the prefusion conformation. *Science*, *367*(6483), 1260–1263. doi: 10.1126/science.abb2507.

Wu, F., Zhao, S., Yu, B., Chen, Y. M., Wang, W., Song, Z. G., … Zhang, Y. Z. (2020). A new coronavirus associated with human respiratory disease in China. *Nature*, *579*(7798), 265–269. doi: 10.1038/s41586-020-2008-3.

settings and this is achieved by supporting both R&D and access activities for a wide range of diseases, including COVID-19. .FIND has several clinical research projects to evaluate multiple new diagnostic tests against published Target Product Profiles that have been defined through consensus processes. These studies are for diagnostic products developed by private sector companies who provide access to know-how, equipment/reagents, and contribute through unrestricted donations as per FIND policy and external SAC review.

Devy Emperador: is employed by FIND with funding from DFID and KFW. FIND is a global non-for profit product development partnership and WHO Diagnostic Collaboration Centre. It is FIND's role to accelerate access to high quality diagnostic tools for low resource settings and this is achieved by supporting both R&D and access activities for a wide range of diseases, including COVID-19. .FIND has several clinical research projects to evaluate multiple new diagnostic tests against published Target Product Profiles that have been defined through consensus processes. These studies are for diagnostic products developed by private sector companies who provide access to know-how, equipment/reagents, and contribute through unrestricted donations as per FIND policy and external SAC review.

Lotty Hooft: none known

Mariska MG Leeflang: none known

Ann Van den Bruel: none known

SOURCES OF SUPPORT

Internal sources

- Liverpool School of Tropical Medicine, UK
- University of Birmingham, UK

External sources

- Department for International Development, UK

  Project number: 300342-104
- National Institute for Health Research (NIHR), UK
- NIHR Birmingham Biomedical Research Centre at the University Hospitals Birmingham NHS Foundation Trust and the University of Birmingham, UK

DIFFERENCES BETWEEN PROTOCOL AND REVIEW

As we explained in the review, due to poor reporting, we were unable to identify studies that evaluated the test in patients who were symptomatic (active disease) separately from those who had recovered from their symptoms (convalescent). Our stratification of results according to days since onset of symptoms will in part be related to these categorisations.

We planned to check the following websites for eligible index tests, however these did not prove to be very accessible or easy to use and, after initial review, were not further considered:

- National Institute for Health Research (NIHR) Innovation Observatory (www.io.nihr.ac.uk/)
- www.rapidmicrobiology.com/test-method/testing-for-the-wuhan-coronavirus-a-k-a-covid-19-sars-cov-2-and-2019-ncov

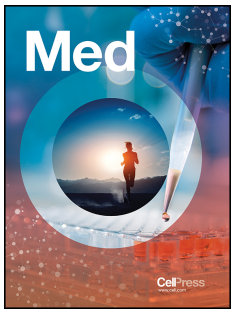
We planned to check the following evidence repository for additional eligible studies however, the EPPI-Centre and Norwegian Institute of Public Health resources proved to be more accessible therefore we decided to prioritise our other sources of evidence.

- Meta-evidence (meta-evidence.co.uk/the-role-of-evidence-synthesis-in-covid19/)

QUADAS-2 (Whiting 2011), item "Was there an appropriate interval between index test(s) and reference standard?" was dropped from assessment because for antibody tests, the body's immune response to SARS-CoV-2 infection tends to increase over time such that the time between confirmation of the presence of SARS-CoV-2 an the index test is less relevant than the time from symptom onset to the application of the index test.

Copyright © 2020 The Authors. Cochrane Database of Systematic Reviews published by John Wiley & Sons, Ltd. on behalf of The Cochrane Collaboration.

Journal Pre-proof



When two pandemics meet: Why is obesity associated with increased COVID-19 mortality?

Sam M. Lockhart, Stephen O'Rahilly

PII: S2666-6340(20)30010-6

DOI: https://doi.org/10.1016/j.medj.2020.06.005

Reference: MEDJ 10

To appear in: *Med*

Please cite this article as: Lockhart, S.M., O'Rahilly, S., When two pandemics meet: Why is obesity associated with increased COVID-19 mortality?, *Med* (2020), doi: https://doi.org/10.1016/j.medj.2020.06.005.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Crown Copyright © 2020 Published by Elsevier Inc.

Journal Pre-proof

When two pandemics meet: Why is obesity associated with increased COVID-19 mortality?

Sam M. Lockhart and Stephen O'Rahilly[1]

MRC Metabolic Diseases Unit, Wellcome Trust-Medical Research Council Institute of Metabolic Science and NIHR Cambridge Biomedical Research Centre, University of Cambridge, Cambridge CB2 0QQ, UK

[1] To whom correspondence should be addressed so104@medschl.cam.ac.uk

Journal Pre-proof

Journal Pre-proof

Abstract

A growing body of evidence indicates that obesity is strongly and independently associated with adverse outcomes of COVID-19 including death. By combining emerging knowledge of the pathological processes involved in COVID-19 with insights into the mechanisms underlying the adverse health consequences of obesity, we present some hypotheses regarding the deleterious impact of obesity on the course of COVID-19. These hypotheses are testable and could guide therapeutic and preventive interventions. As obesity is now almost ubiquitous and no vaccine for COVID-19 is currently available, even a modest reduction in the impact of obesity on mortality and morbidity from this viral infection could have profound consequences for public health.

Journal Pre-proof

Journal Pre-proof

Introduction

Emerging evidence suggests that people with obesity are at increased risk of mortality from Coronavirus Disease 2019 (COVID-19) but the mechanisms underlying this are poorly understood. An improved understanding of the pathophysiological intersection of COVID-19 and obesity should help guide preventive and therapeutic strategies for this vulnerable group. Here we summarise existing knowledge regarding the pathophysiology of COVID-19 and consider how its various components might be exacerbated by the presence of obesity. We end by suggesting some experiments which could inform public health interventions and/or approaches to therapy.

The strong association of obesity with adverse outcomes in COVID-19 is real and relatively specific to a subset of viral pneumonias.

Soon after the emergence of COVID-19 there was a flurry of reports from hospitals around the world drawing attention to an apparent excess of obese patients among those ventilated [5, 10, 12, 45, 62]. More recently, preprints have appeared which report much larger and more rigorous epidemiological investigations. OpenSAFELY examined 5683 COVID-19 deaths in the UK and related these to pre-existing potential risk factors documented in over 17 million electronic health records [72]. As in all studies to date, age was the most important pre-existing risk factor, but the effect of obesity was highly significant and graded according to the severity of the obesity. The hazard ratio (adjusted for ethnicity) for death for those with Class III obesity (Body Mass Index (BMI) >40kg/m2) was as high as 2.28 (1.96-2.65). The ISARIC study of 16,749 COVID-19 related admissions to Intensive Care Units in the UK reported a lower hazard ratio of 1.37 (1.16-1.63) associated with clinician-reported obesity [19]. It should be noted, however, that BMI was not reported in this study and reliance on clinical diagnosis is known to seriously underdiagnose obesity [53].

In an analysis of COVID-19 mortality in over 300,000 patients with diabetes, obesity was associated with mortality in both type 1 (T1D) and type 2 diabetes (T2D) [36]. Taken together with myriad smaller studies it seems increasingly clear that obesity does indeed increase the risk of mortality and of requiring admission to Intensive Care in people infected with SARS-CoV-2. In contrast to worse outcomes once an obese person is infected, there is currently no evidence that obesity has a significant impact on the risk of becoming infected by the virus in the first place.

Is there something about infection with the SARS-CoV-2 virus that interacts so adversely with the obese state, or does being obese have a similar impact on other forms of viral pneumonia? Although obesity has been associated with an increased risk of hospitalisation in seasonal influenza, a study of almost 10,000 cases of seasonal influenza in the USA did not find any evidence of obesity as a risk factor for requiring mechanical ventilation or death [6]. In contrast, it seems clear that during the 2009 H1N1 influenza pandemic, which largely spared the partly immune elderly, obesity was a strong risk factor for adverse outcomes [51]. The role of obesity in severity of SARS-CoV-1 and MERS-CoV, other pandemic coronavirus infections with poor outcomes, has not been thoroughly examined. The Acute Respiratory Distress Syndrome (ARDS) has some pathophysiological similarities to COVID-19 pneumonia. While obesity has been reported to increase the risk of developing ARDS of a variety of aetiologies [32], it has been reported to be associated with increased survival rates, something that has come to be known as the ARDS obesity paradox [67]. Thus, the association of obesity with worse outcomes in acute lung infection or widespread alveolar damage of other types, appears to be strongest and most consistent with COVID-19 and pandemic H1N1 influenza.

What are obese patients with COVID-19 dying from?

Journal Pre-proof

The majority of COVID-19 patients die having required artificial ventilation for hypoxemic respiratory failure due to COVID-19 pneumonia [59]. Emerging post-mortem histopathology of the COVID 19 lung offers insights into the underlying pathophysiology. Briefly, there is evidence of diffuse alveolar damage, as in other forms of viral pneumonia, but sometimes this is patchy [2, 48]. What is striking, and shared to a degree with the pathology of pandemic H1N1 influenza [2], is the extent of pulmonary capillary microangiopathy which is considerable and near universal, at least in some series [9]. Complement deposition has also been observed in the endothelium in association with the formation of microthrombi [44]. This suggests that COVID-19 may lead to a state of alveolar hypoperfusion due to a microthrombotic pulmonary angiopathy. The frequent finding of elevated levels of fibrin D-dimers in a large proportion of hospitalised patients is consistent with a thrombotic process, as is the frequent occurrence of venous thrombosis and pulmonary emboli during the course of the illness [16, 35]. The clinical characteristics of COVID-19 pneumonia are still being defined but in early reports from European centres a substantial proportion of ventilated patients were reported to have preserved pulmonary compliance with well aerated lungs, suggesting that hypoxia is being driven by microvascular dysfunction [29, 30]. Reports of CT based lung perfusion imaging supports this [40]. However, a subsequent larger study from the USA described a cohort of patients with respiratory mechanics more in keeping with classical ARDS [24]. Finally, patients who are seriously ill with COVID-19 have evidence of high levels of inflammation with high CRP and circulating pro-inflammatory cytokines [11]. Indeed, it has been suggested that a hyperinflammatory response, occurring downstream of a vigorous activation of either adaptive or innate immunity, or both, may drive the underlying pathophysiological process [47] and IL-6 antagonists are being trialled in severely ill patients [65].

Pathophysiological mechanisms mediating the adverse effects of obesity

Obesity is associated with a wide range of adverse health outcomes with diverse underlying pathogenic processes. For some, e.g. sleep apnoea and reflux oesophagitis, the expanded mass of adipose tissue itself is directly and mechanically contributing to the disease. T2D is one of the commonest sequelae of obesity. An increase in circulating insulin levels in both fasting and post-prandial state is one of the earliest metabolic disturbances associated with obesity and it is due to impaired insulin action, principally in liver and skeletal muscle [57]. This “insulin resistance” clearly predisposes to developing T2D, which ensues when beta cell compensation fails.

The mechanism whereby chronic over-nutrition leads to insulin resistance appears to primarily involve not the expanded adipose tissue itself, but the additional excess nutrient that is stored ectopically in the major insulin responsive tissues, muscle and fat [41]. An alternative hypothesis suggests that adipose tissue inflammation contributes directly to insulin resistance in obesity. Inflammation undoubtedly occurs in obesity however it has less compelling underpinning support from human genetics or human pharmacology [52].

How might the metabolic state of obesity intersect with and exacerbate pathological mechanisms in COVID-19?

Enhanced production of cytokines. A corollary of storing excess fat in non-adipose tissue is that the adipose tissue has reached or is reaching the limits of its ability to store fat safely. Thus, in adipose tissue biopsies from obese, insulin resistant people, one frequently sees an excess of dead and dying adipocytes, often accompanied by an excess of infiltrating macrophages, usually arranged in crown-like structures [13]. These macrophages are activated and contribute to the production of a systemic

Journal Pre-proof

pro-inflammatory state, characterised by increases in circulating levels of cytokines such as TNFα, IL6 and IL1β [46, 66]. Lipotoxic damage to other cells such as hepatocytes can also contribute to the enhanced inflammatory state. If increased inflammation contributes to alveolar damage, then this provides an obvious potential route whereby the metabolic risk factors could drive increased mortality.

Altered adipose tissue hormones Adipose tissue expansion not only results in elaboration of inflammatory cytokines, but also changes the profile of secreted hormones. A key signature of insulin resistance is an increase in the ratio of circulating leptin and adiponectin [24]. Obesity is associated with higher circulating leptin and lower circulating adiponectin. There is some literature associating high leptin levels with pulmonary inflammation but this is not, as yet, compelling (24, 25). There is, however, a growing body of evidence more securely implicating adiponectin as an anti-inflammatory agent [61]. Notably, adiponectin-deficient mice develop inflammation of the pulmonary vasculature [63] and are predisposed to experimental acute lung injury [39] suggesting that the hypoadiponectinemia frequently seen in obesity could facilitate an exaggerated inflammatory response directed to pulmonary capillaries. In addition to being lower in obesity and most insulin resistant states it is worth noting that adiponectin levels have been reported to be significantly lower in many of the COVID-19 "at risk" groups e.g. Male < Females [20] and South Asians < White Europeans [1, 20]. Perhaps most interesting is the finding that, at equivalent levels of body fat, black people also tend to have lower levels of adiponectin than white people despite having no more insulin resistance and a lower propensity to store fat ectopically [8]. However, it should be noted adiponectin levels tend to rise after the age of 70 [3, 14], and old age is by far the biggest risk factor for COVID-19 mortality. However, it is possible that different causal pathways may mediate the risk of age vs obesity on COVID-19 severity.

Complement components Gralinski et al. [33] recently reported that mice lacking C3, the central component of the complement system, were protected against severe disease when infected with a mouse adapted SARS-CoV-1 virus. The role of complement in human COVID-19 has not yet been well studied but immunohistological examination of lungs and skin lesions from affected patients show deposition of components of the alternative and lectin complement pathways [44]. Moreover, the N-protein of SARS-CoV-2 can activate the lectin pathway [28] and aberrant activation of complement is clearly implicated in a subgroup of thrombotic microangiopathies suggesting complement could play a causal role in the microthrombosis observed in COVID-19 [7].

Adipocytes are a major source of several of the components of the complement system complement proteins [71]. Levels of some of these (e.g. C3, C3a, CFD and Properdin) are increased with increasing adiposity [74]. Circulating levels of C3 are positively associated with insulin resistance, independent of adiposity [73]. Given the existence of amplification loops in the complement pathway it is conceivable that modest elevations of complement components in obesity could serve as a nidus for microthrombosis and/or pathological inflammation and mediate poor outcomes in obesity, as has been suggested by others [31].

Thrombosis Venous thromboembolism rates are much higher in patients with severe COVID-19 than historical critically ill controls and there is growing evidence of high rates of thrombotic microangiopathy in severe COVID-19 [16, 35]. Obesity is an established risk factor for arterial and venous thrombosis and dysfunction of the endothelium, platelets, fibrinolytic system and the clotting cascade have all been implicated [70]. For example, Plasminogen Activation Inhibitor-1 (PAI-1) is secreted from adipose tissue, associated with insulin resistance and likely contributes to thrombotic risk in obesity by impairing fibrinolysis [23]. In addition, obesity is associated with increased thromboxane metabolites and mean platelet volume (both validated indices of platelet activation)

Journal Pre-proof

that normalise with weight loss [15, 18]. Notably, obesity is a robust risk factor for the development of thrombocytopenic thrombogenic purpura [69] with one group suggesting increased circulating antibodies to ADAMTS13 in the obese [42, 76].

Vasculature The role of the vasculature, particularly the endothelium, in the pathogenesis of COVID-19 has recently been highlighted [34, 64]. In a comprehensive analysis of ACE2 (the SARS-CoV-2 receptor) expression in the human vasculature the highest expression was found in the pericytes of heart and brain (but not the lung) with little in endothelial cells [34]. It was proposed that microvascular dysfunction associated with obesity or type 2 diabetes could permit viral passage across the endothelium to infect pericytes, with their dysfunction promoting subsequent endothelial activation and microthrombosis [34]. The effects of diabetes on endothelial barrier function is well established [56] and there is evidence from studies of large animals that endothelial permeability is increased in obesity [27].

Dysfunction of the systemic microcirculation is well described in obesity and the metabolic syndrome [68]. While the effects of obesity on the pulmonary circulation are less studied, there is emerging evidence of a pulmonary vascular dysfunction associated with obesity. In a rodent model of obesity pulmonary resistance vessels were resistant to agonist and hypoxia induced vasoconstriction *ex vivo* compared to lean controls [50]. If the vasoconstrictive response to hypoxia is impaired in the human pulmonary vasculature then this could potentially exacerbate shunting in COVID19 pneumonia, thus contributing to hypoxia.

Alveolus The key functional unit of the lung is the alveolar-capillary unit. Key cells include type 1 pneumocytes (AT1) separated from capillary endothelial cells by a fused basement membrane and the less numerous type 2 pneumocytes (AT2) that produce surfactant and serve as alveolar progenitors. ACE2 is the proposed receptor for SARS-CoV-2 and in the alveolus it is expressed predominantly (if not solely) by AT2 [34]. Critical to gas exchange and pulmonary function, the alveolar capillary unit is the primary site of injury in COVID-19. Understanding how obesity interacts with pre-morbid alveolar function and injury may guide pre-emptive therapeutic intervention.

Circulating Surfactant proteins A and D have been shown to be increased in patients with obesity and Type 2 Diabetes [21, 43], assuming these proteins are expressed only in the lung and secreted to the apical membrane, and this suggests that obesity may affect the integrity of the alveolus. The science of ectopic fat has largely focused on the liver, muscle and heart, where a large body of evidence clearly describes the adverse consequences to these tissues of a chronic excess of intracellular lipid. More recently, however, work is emerging suggesting that, in states of over-nutrition, ectopic lipid can appear in cells of the pulmonary alveolus resulting in ultrastructural abnormalities and altered surfactant production [25]. Genetic enhancement of endogenous lipid synthesis specifically in mouse AT2 cells results in alveolar inflammation [54]. Remarkably, AT2 cells of aged mice were noted to demonstrate similar gene expression changes to these mice and also exhibited increased lipid content [4] suggesting that “fatty lung” could potentially be a common causal pathway whereby both obesity and age worsen COVID-19 pathology. Similarly, genetic deletion of the lipid sensor Liver X Receptor (LXR) resulted in accumulation of lipid in type 2 pneumocytes and, subsequently, pulmonary inflammation and foam cell accumulation [17].

Some testable hypotheses and their potential implications for interventions

Insulin Resistance, not fat mass, is key to the link between Obesity and poor COVID Outcomes

If true, this is important, as even short-term low calorie diets can improve insulin sensitivity within days [38]. Human genetics should ultimately come to our aid here as meta-analysis of Genome Wide

Journal Pre-proof

SNP data from COVID victims throughout the world can be undertaken to examine whether the genetic risk scores for insulin resistance are better predictors of outcome than those for obesity *per se*. In the meantime, animal models of SARS infection might be able to provide some early information through the examination of effects of insulin-lowering and insulin-sensitising medications. Some commentators have argued that as it is difficult for obese patients to attain normal weight then there is not much that can be done given the rapid spread of the COVID-19 pandemic. However, if improving insulin sensitivity reduces risk then even a modest amount of caloric restriction, combined with physical activity and perhaps an insulin sensitising/lowering drug such as metformin, may provide a way of reducing risk of death for the large number of at risk obese people

Low circulating levels of adiponectin predispose to aggressive pulmonary inflammation and explain why obese people fare worse with COVID-19

Again, human genetics will be able to help us test this hypothesis as there are genetic instruments which explain quite a high proportion of the variance in serum adiponectin [58]. Agonists of the nuclear receptor PPARγ, such as the thiazolidinedione class of drugs rapidly and markedly increase circulating levels of adiponectin. Examination of the effects of PPARγ agonists on disease outcome in obese animal models of COVID-19 could provide helpful insights. Pioglitazone is licensed for us in T2D worldwide and cheap generic formulations are now available for large scale clinical trials.

Ectopic lipid in alveolar Type 2 cells influences the extent of alveolar damage due to COVID-19

SARS-CoV-2 causes pneumonia by first entering the AT2 through ACE2 which is abundantly expressed on their surface. These cells are lipid rich, storing polar lipids in lamellar bodies, and their structure, and possibly function, are influenced by diet and obesity, at least in animal models [25, 55, 75]. Experiments should be undertaken to examine the effects of lipid content of cells on ACE2 expression, viral uptake, replication and release. Some viruses e.g. Hepatitis C seems entirely reliant on intracellular fat droplets to facilitate its movement around a cell [49]. Viral infections of cells frequently lead to a rapid switch from oxidative phosphorylation to aerobic glycolysis, the so called Warburg effect [60]. Ectopic lipid in cells elsewhere is known to be associated with metabolic inflexibility [26], the inability to shift rapidly between fat and carbohydrate metabolism. Might AT2 cells that have excess lipid be less able to switch to aerobic glycolysis and thus be more prone to cell death during viral infection? Indeed, in mice, diet-induced obesity is associated with downregulation of fatty acid synthase (*Fasn)* in lung and genetic deletion of *Fasn* in AT2 cells impairs induction of glycolysis in response to hyperoxic stress *in vitro* and predisposes to acute lung injury in mice [55]. Though unproven, it is likely that ectopic lipid in lung will start to reduce quickly after people go into negative energy balance, so that modest changes in diet and exercise may be have benefit.

Conclusion

In summary, we have applied insights into the pathophysiology of the adverse consequences of obesity and emerging evidence regarding the pathological mechanisms in COVID-19 to suggest possible routes whereby obesity can exacerbate the tissue damage associated with infection by the SARS-CoV-2 virus. These hypotheses suggest several tractable experiments in cells, animals and humans, some of which we are undertaking and which we encourage others to pursue. Obesity is a notoriously difficult condition to "cure" and this may explain why widespread public health messaging about weight loss in the obese as a preventive measure to reduce COVID-19 mortality has not been vigorously pursued. If obesity is exerting its effects on COVID-19 outcome through its metabolic sequelae, such as insulin resistance, then those abnormalities start to improve very

rapidly when energy intake drops below energy expenditure. In addition to its effects on energy expenditure, regular physical activity, even of moderate intensity and duration can also improve insulin sensitivity and lower circulating insulin levels [37]. The potential implications for unintended adverse consequences of intense COVID-19 "lockdown" strategies that limit opportunities for exercise are obvious.

Given how rapidly large trials of a wide variety of pharmacological agents in COVID-19 are currently being undertaken (some with a rather tenuous rationale [22]) it should be possible to consider undertaking trials of simple interventions in people with obesity either before or immediately after the onset of COVID-19 symptoms. These could involve diet and exercise intentions that do not aim for unrealistic amounts of weight loss but would be designed to ameliorate insulin resistance. These interventions could be supplemented by drugs that assist in modest weight loss and lower circulating insulin, such as metformin or SGLT2 inhibitors, or agents that improve insulin sensitivity, reduce ectopic lipid and increase circulating adiponectin, such as pioglitazone. Such approaches would also be applicable to T2D, another condition which predisposes to increased mortality from COVID-19 [19, 36, 72]. In the majority of T2D cases, obesity precedes and contributes to the development of diabetes through inducing compensatory hyperinsulinemia, necessitated by insulin resistance, which eventually exhausts the ability of genetically vulnerable pancreatic beta cells to maintain insulin production. There is evidence that, in both T1D and T2D, the level of glycaemia is related to COVID-19 outcomes [36, 77]. We urgently need to know if the intensification of glycaemic control using an approach which sensitises patients to insulin would provide benefits to the COVID-19 infected T2D patient that are greater than those achieve by approaches that increase levels of circulating insulin, either through exogenous injection or the stimulation of endogenous secretion.

Obesity affects a very large proportion of the population of most developed and developing countries. Understanding the nature of the link between chronic positive caloric imbalance and COVID-19 pathology could provide novel avenues to reduce the death toll produced by this dangerous new viral infection. Funding agencies will need to foster the interdisciplinary approaches that will be required to respond to this new biomedical challenge which lies at the intersection between traditional disciplines.

Acknowledgements

S.M.L is supported by an Academic Clinical Fellowship from The National Institute for Health Research (NIHR). S.O'R is supported by the Wellcome Trust (WT 095515/Z/11/Z), the MRC Metabolic Disease Unit (MC_UU_00014/1), and NIHR Cambridge Biomedical Research Centre and NIHR Rare Disease Translational Research Collaboration.

Disclosures

S.O.R. is an employee of the University of Cambridge and has provided remunerated consultancy services to the following pharmaceutical and biotechnology companies Pfizer, AstraZeneca, Novo-Nordisk, GSK and ERX. S.M.L. has no relevant conflicts of interest to declare.

References

1. Abate, N., Chandalia, M., Snell, P.G., and Grundy, S.M. (2004). Adipose tissue metabolites and insulin resistance in nondiabetic Asian Indian men. J Clin Endocrinol Metab *89*, 2750-2755.
2. Ackermann, M., Verleden, S.E., Kuehnel, M., Haverich, A., Welte, T., Laenger, F., Vanstapel, A., Werlein, C., Stark, H., Tzankov, A*., et al.* (2020). Pulmonary Vascular Endothelialitis, Thrombosis, and Angiogenesis in Covid-19. New England Journal of Medicine.

Journal Pre-proof

3. Adamczak, M., Rzepka, E., Chudek, J., and Wiecek, A. (2005). Ageing and plasma adiponectin concentration in apparently healthy males and females. Clin Endocrinol (Oxf) *62*, 114-118.
4. Angelidis, I., Simon, L.M., Fernandez, I.E., Strunz, M., Mayr, C.H., Greiffo, F.R., Tsitsiridis, G., Ansari, M., Graf, E., Strom, T.M*., et al.* (2019). An atlas of the aging lung mapped by single cell transcriptomics and deep tissue proteomics. Nat Commun *10*, 963.
5. Bhatraju, P.K., Ghassemieh, B.J., Nichols, M., Kim, R., Jerome, K.R., Nalla, A.K., Greninger, A.L., Pipavath, S., Wurfel, M.M., Evans, L*., et al.* (2020). Covid-19 in Critically Ill Patients in the Seattle Region - Case Series. N Engl J Med *382*, 2012-2022.
6. Braun, E.S., Crawford, F.W., Desai, M.M., Meek, J., Kirley, P.D., Miller, L., Anderson, E.J., Oni, O., Ryan, P., Lynfield, R*., et al.* (2015). Obesity not associated with severity among hospitalized adults with seasonal influenza virus infection. Infection *43*, 569-575.
7. Brocklebank, V., Wood, K.M., and Kavanagh, D. (2018). Thrombotic Microangiopathy and the Kidney. Clin J Am Soc Nephrol *13*, 300-317.
8. Bush, N.C., Darnell, B.E., Oster, R.A., Goran, M.I., and Gower, B.A. (2005). Adiponectin is lower among African Americans and is independently related to insulin sensitivity in children and adolescents. Diabetes *54*, 2772-2778.
9. Carsana, L., Sonzogni, A., Nasr, A., Rossi, R., Pellegrinelli, A., Zerbi, P., Rech, R., Colombo, R., Antinori, S., Corbellino, M*., et al.* (2020). Pulmonary post-mortem findings in a large series of COVID-19 cases from Northern Italy. medRxiv, 2020.2004.2019.20054262.
10. Caussy, C., Wallet, F., Laville, M., and Disse, E. (2020). Obesity is Associated with Severe Forms of COVID-19. Obesity (Silver Spring).
11. Chen, G., Wu, D., Guo, W., Cao, Y., Huang, D., Wang, H., Wang, T., Zhang, X., Chen, H., Yu, H*., et al.* (2020). Clinical and immunological features of severe and moderate coronavirus disease 2019. J Clin Invest *130*, 2620-2629.
12. Chen, Q., Zheng, Z., Zhang, C., Zhang, X., Wu, H., Wang, J., Wang, S., and Zheng, C. (2020). Clinical characteristics of 145 patients with corona virus disease 2019 (COVID-19) in Taizhou, Zhejiang, China. Infection.
13. Cinti, S., Mitchell, G., Barbatelli, G., Murano, I., Ceresi, E., Faloia, E., Wang, S., Fortier, M., Greenberg, A.S., and Obin, M.S. (2005). Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res *46*, 2347-2355.
14. Cnop, M., Havel, P.J., Utzschneider, K.M., Carr, D.B., Sinha, M.K., Boyko, E.J., Retzlaff, B.M., Knopp, R.H., Brunzell, J.D., and Kahn, S.E. (2003). Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex. Diabetologia *46*, 459-469.
15. Coban, E., Yilmaz, A., and Sari, R. (2007). The effect of weight loss on the mean platelet volume in obese patients. Platelets *18*, 212-216.
16. Connors, J.M., and Levy, J.H. (2020). COVID-19 and its implications for thrombosis and anticoagulation. Blood.
17. Dai, Y.B., Miao, Y.F., Wu, W.F., Li, Y., D'Errico, F., Su, W., Burns, A.R., Huang, B., Maneix, L., Warner, M*., et al.* (2016). Ablation of Liver X receptors α and β leads to spontaneous peripheral squamous cell lung cancer in mice. Proc Natl Acad Sci U S A *113*, 7614-7619.
18. Davì, G., Guagnano, M.T., Ciabattoni, G., Basili, S., Falco, A., Marinopiccoli, M., Nutini, M., Sensi, S., and Patrono, C. (2002). Platelet activation in obese women: role of inflammation and oxidant stress. Jama *288*, 2008-2014.
19. Docherty, A.B., Harrison, E.M., Green, C.A., Hardwick, H.E., Pius, R., Norman, L., Holden, K.A., Read, J.M., Dondelinger, F., Carson, G*., et al.* (2020). Features of 16,749 hospitalised UK patients with COVID-19 using the ISARIC WHO Clinical Characterisation Protocol. medRxiv, 2020.2004.2023.20076042.
20. Duncan, B.B., Schmidt, M.I., Pankow, J.S., Bang, H., Couper, D., Ballantyne, C.M., Hoogeveen, R.C., and Heiss, G. (2004). Adiponectin and the development of type 2 diabetes: the atherosclerosis risk in communities study. Diabetes *53*, 2473-2478.

Journal Pre-proof

21. Fernández-Real, J.M., Chico, B., Shiratori, M., Nara, Y., Takahashi, H., and Ricart, W. (2008). Circulating surfactant protein A (SP-A), a marker of lung injury, is associated with insulin resistance. Diabetes Care *31*, 958-963.
22. Ferner, R.E., and Aronson, J.K. (2020). Chloroquine and hydroxychloroquine in covid-19. Bmj *369*, m1432.
23. Festa, A., D'Agostino, R., Mykkänen, L., Tracy, R.P., Zaccaro, D.J., Hales, C.N., and Haffner, S.M. (1999). Relative contribution of insulin and its precursors to fibrinogen and PAI-1 in a large population with different states of glucose tolerance. The Insulin Resistance Atherosclerosis Study (IRAS). Arterioscler Thromb Vasc Biol *19*, 562-568.
24. Finucane, F.M., Luan, J., Wareham, N.J., Sharp, S.J., O'Rahilly, S., Balkau, B., Flyvbjerg, A., Walker, M., Højlund, K., Nolan, J.J*., et al.* (2009). Correlation of the leptin:adiponectin ratio with measures of insulin resistance in non-diabetic individuals. Diabetologia *52*, 2345-2349.
25. Foster, D.J., Ravikumar, P., Bellotto, D.J., Unger, R.H., and Hsia, C.C. (2010). Fatty diabetic lung: altered alveolar structure and surfactant protein expression. Am J Physiol Lung Cell Mol Physiol *298*, L392-403.
26. Galgani, J.E., Moro, C., and Ravussin, E. (2008). Metabolic flexibility and insulin resistance. Am J Physiol Endocrinol Metab *295*, E1009-1017.
27. Galili, O., Versari, D., Sattler, K.J., Olson, M.L., Mannheim, D., McConnell, J.P., Chade, A.R., Lerman, L.O., and Lerman, A. (2007). Early experimental obesity is associated with coronary endothelial dysfunction and oxidative stress. Am J Physiol Heart Circ Physiol *292*, H904-911.
28. Gao, T., Hu, M., Zhang, X., Li, H., Zhu, L., Liu, H., Dong, Q., Zhang, Z., Wang, Z., Hu, Y*., et al.* (2020). Highly pathogenic coronavirus N protein aggravates lung injury by MASP-2-mediated complement over-activation. medRxiv, 2020.2003.2029.20041962.
29. Gattinoni, L., Chiumello, D., Caironi, P., Busana, M., Romitti, F., Brazzi, L., and Camporota, L. (2020). COVID-19 pneumonia: different respiratory treatments for different phenotypes? Intensive Care Med.
30. Gattinoni, L., Coppola, S., Cressoni, M., Busana, M., Rossi, S., and Chiumello, D. (2020). COVID-19 Does Not Lead to a "Typical" Acute Respiratory Distress Syndrome. Am J Respir Crit Care Med *201*, 1299-1300.
31. Gavriilaki, E., and Brodsky, R.A. (2020). Severe COVID-19 infection and thrombotic microangiopathy: success doesn't come easily. Br J Haematol.
32. Gong, M.N., Bajwa, E.K., Thompson, B.T., and Christiani, D.C. (2010). Body mass index is associated with the development of acute respiratory distress syndrome. Thorax *65*, 44-50.
33. Gralinski, L.E., Sheahan, T.P., Morrison, T.E., Menachery, V.D., Jensen, K., Leist, S.R., Whitmore, A., Heise, M.T., and Baric, R.S. (2018). Complement Activation Contributes to Severe Acute Respiratory Syndrome Coronavirus Pathogenesis. mBio *9*.
34. He, L., Mäe, M.A., Sun, Y., Muhl, L., Nahar, K., Liébanas, E.V., Fagerlund, M.J., Oldner, A., Liu, J., Genové, G*., et al.* (2020). Pericyte-specific vascular expression of SARS-CoV-2 receptor ACE2 – implications for microvascular inflammation and hypercoagulopathy in COVID-19 patients. bioRxiv, 2020.2005.2011.088500.
35. Helms, J., Tacquard, C., Severac, F., Leonard-Lorant, I., Ohana, M., Delabranche, X., Merdji, H., Clere-Jehl, R., Schenck, M., Fagot Gandet, F*., et al.* (2020). High risk of thrombosis in patients with severe SARS-CoV-2 infection: a multicenter prospective cohort study. Intensive Care Med.
36. Holman, N.K., Peter. Kar, Partha. O'Keefe, Jackie. Curley, Matt. Weaver, Andy. Barron, Emma. Bakhai, Chirag. Khunti, Kamlesh. Wareham, Nick. Sattar, Naveed. Young, Bob. Valabhji, Johnathan. (2019). Type 1 and Type 2 diabetes and COVID-19 related mortality in England: a cohort study in people with diabetes (NHS England).
37. Houmard, J.A., Tanner, C.J., Slentz, C.A., Duscha, B.D., McCartney, J.S., and Kraus, W.E. (2004). Effect of the volume and intensity of exercise training on insulin sensitivity. J Appl Physiol (1985) *96*, 101-106.

38. Kirk, E., Reeds, D.N., Finck, B.N., Mayurranjan, S.M., Patterson, B.W., and Klein, S. (2009). Dietary fat and carbohydrates differentially alter insulin sensitivity during caloric restriction. Gastroenterology *136*, 1552-1560.

39. Konter, J.M., Parker, J.L., Baez, E., Li, S.Z., Ranscht, B., Denzel, M., Little, F.F., Nakamura, K., Ouchi, N., Fine, A.*, et al.* (2012). Adiponectin attenuates lipopolysaccharide-induced acute lung injury through suppression of endothelial cell activation. J Immunol *188*, 854-863.

40. Lang, M., Som, A., Mendoza, D.P., Flores, E.J., Reid, N., Carey, D., Li, M.D., Witkin, A., Rodriguez-Lopez, J.M., Shepard, J.O.*, et al.* (2020). Hypoxaemia related to COVID-19: vascular and perfusion abnormalities on dual-energy CT. Lancet Infect Dis.

41. Langenberg, C., and Lotta, L.A. (2018). Genomic insights into the causes of type 2 diabetes. Lancet *391*, 2463-2474.

42. Lombardi, A.M., Fabris, R., Scarda, A., Zanato, V., Dal Prà, C., Scarparo, P., Vettore, S., Granzotto, M., Berti De Marinis, G., Foletto, M.*, et al.* (2012). Presence of anti-ADAMTS13 antibodies in obesity. Eur J Clin Invest *42*, 1197-1204.

43. López-Cano, C., Lecube, A., García-Ramírez, M., Muñoz, X., Sánchez, E., Seminario, A., Hernández, M., Ciudin, A., Gutiérrez, L., Hernández, C.*, et al.* (2017). Serum Surfactant Protein D as a Biomarker for Measuring Lung Involvement in Obese Patients With Type 2 Diabetes. J Clin Endocrinol Metab *102*, 4109-4116.

44. Magro, C., Mulvey, J.J., Berlin, D., Nuovo, G., Salvatore, S., Harp, J., Baxter-Stoltzfus, A., and Laurence, J. (2020). Complement associated microvascular injury and thrombosis in the pathogenesis of severe COVID-19 infection: a report of five cases. Transl Res.

45. Mahase, E. (2020). Covid-19: most patients require mechanical ventilation in first 24 hours of critical care. Bmj *368*, m1201.

46. Marques-Vidal, P., Bastardot, F., von Känel, R., Paccaud, F., Preisig, M., Waeber, G., and Vollenweider, P. (2013). Association between circulating cytokine levels, diabetes and insulin resistance in a population-based sample (CoLaus study). Clin Endocrinol (Oxf) *78*, 232-241.

47. Mehta, P., McAuley, D.F., Brown, M., Sanchez, E., Tattersall, R.S., Manson, J.J., and HLH Across Speciality Collaboration, U.K. (2020). COVID-19: consider cytokine storm syndromes and immunosuppression. Lancet *395*, 1033-1034.

48. Menter, T., Haslbauer, J.D., Nienhold, R., Savic, S., Hopfer, H., Deigendesch, N., Frank, S., Turek, D., Willi, N., Pargger, H.*, et al.* (2020). Post-mortem examination of COVID19 patients reveals diffuse alveolar damage with severe capillary congestion and variegated findings of lungs and other organs suggesting vascular dysfunction. Histopathology.

49. Miyanari, Y., Atsuzawa, K., Usuda, N., Watashi, K., Hishiki, T., Zayas, M., Bartenschlager, R., Wakita, T., Hijikata, M., and Shimotohno, K. (2007). The lipid droplet is an important organelle for hepatitis C virus production. Nat Cell Biol *9*, 1089-1097.

50. Moral-Sanz, J., Menendez, C., Moreno, L., Moreno, E., Cogolludo, A., and Perez-Vizcaino, F. (2011). Pulmonary arterial dysfunction in insulin resistant obese Zucker rats. Respir Res *12*, 51.

51. Morgan, O.W., Bramley, A., Fowlkes, A., Freedman, D.S., Taylor, T.H., Gargiullo, P., Belay, B., Jain, S., Cox, C., Kamimoto, L.*, et al.* (2010). Morbid obesity as a risk factor for hospitalization and death due to 2009 pandemic influenza A(H1N1) disease. PLoS One *5*, e9694.

52. O'Rahilly, S. (2016). Harveian Oration 2016: Some observations on the causes and consequences of obesity. Clin Med (Lond) *16*, 551-564.

53. Pantalone, K.M., Hobbs, T.M., Chagin, K.M., Kong, S.X., Wells, B.J., Kattan, M.W., Bouchard, J., Sakurada, B., Milinovich, A., Weng, W.*, et al.* (2017). Prevalence and recognition of obesity and its associated comorbidities: cross-sectional analysis of electronic health record data from a large US integrated health system. BMJ Open *7*, e017583.

54. Plantier, L., Besnard, V., Xu, Y., Ikegami, M., Wert, S.E., Hunt, A.N., Postle, A.D., and Whitsett, J.A. (2012). Activation of sterol-response element-binding proteins (SREBP) in alveolar type II cells enhances lipogenesis causing pulmonary lipotoxicity. J Biol Chem *287*, 10099-10114.

55. Plataki, M., Fan, L., Sanchez, E., Huang, Z., Torres, L.K., Imamura, M., Zhu, Y., Cohen, D.E., Cloonan, S.M., and Choi, A.M. (2019). Fatty acid synthase downregulation contributes to acute lung injury in murine diet-induced obesity. JCI Insight *5*.
56. Rask-Madsen, C., and King, G.L. (2013). Vascular complications of diabetes: mechanisms of injury and protective factors. Cell Metab *17*, 20-33.
57. Reaven, G.M. (1988). Banting lecture 1988. Role of insulin resistance in human disease. Diabetes *37*, 1595-1607.
58. Richards, J.B., Waterworth, D., O'Rahilly, S., Hivert, M.F., Loos, R.J., Perry, J.R., Tanaka, T., Timpson, N.J., Semple, R.K., Soranzo, N.*, et al.* (2009). A genome-wide association study reveals variants in ARL15 that influence adiponectin levels. PLoS Genet *5*, e1000768.
59. Richardson, S., Hirsch, J.S., Narasimhan, M., Crawford, J.M., McGinn, T., Davidson, K.W., Barnaby, D.P., Becker, L.B., Chelico, J.D., Cohen, S.L.*, et al.* (2020). Presenting Characteristics, Comorbidities, and Outcomes Among 5700 Patients Hospitalized With COVID-19 in the New York City Area. JAMA.
60. Sanchez, E.L., and Lagunoff, M. (2015). Viral activation of cellular metabolism. Virology *479-480*, 609-618.
61. Scherer, P.E. (2019). The many secret lives of adipocytes: implications for diabetes. Diabetologia *62*, 223-232.
62. Simonnet, A., Chetboun, M., Poissy, J., Raverdy, V., Noulette, J., Duhamel, A., Labreuche, J., Mathieu, D., Pattou, F., and Jourdain, M. (2020). High prevalence of obesity in severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) requiring invasive mechanical ventilation. Obesity (Silver Spring).
63. Summer, R., Fiack, C.A., Ikeda, Y., Sato, K., Dwyer, D., Ouchi, N., Fine, A., Farber, H.W., and Walsh, K. (2009). Adiponectin deficiency: a model of pulmonary hypertension associated with pulmonary vascular disease. Am J Physiol Lung Cell Mol Physiol *297*, L432-438.
64. Teuwen, L.-A., Geldhof, V., Pasut, A., and Carmeliet, P. (2020). COVID-19: the vasculature unleashed. Nature Reviews Immunology.
65. Toniati, P., Piva, S., Cattalini, M., Garrafa, E., Regola, F., Castelli, F., Franceschini, F., Focà, E., Andreoli, L., Latronico, N.*, et al.* (2020). Tocilizumab for the treatment of severe COVID-19 pneumonia with hyperinflammatory syndrome and acute respiratory failure: A single center study of 100 patients in Brescia, Italy. Autoimmun Rev, 102568.
66. Um, J.Y., Chung, H.S., Song, M.Y., Shin, H.D., and Kim, H.M. (2004). Association of interleukin-1beta gene polymorphism with body mass index in women. Clin Chem *50*, 647-650.
67. Umbrello, M., Fumagalli, J., Pesenti, A., and Chiumello, D. (2019). Pathophysiology and Management of Acute Respiratory Distress Syndrome in Obese Patients. Semin Respir Crit Care Med *40*, 40-56.
68. van der Heijden, D.J., van Leeuwen, M.A.H., Janssens, G.N., Lenzen, M.J., van de Ven, P.M., Eringa, E.C., and van Royen, N. (2017). Body Mass Index Is Associated With Microvascular Endothelial Dysfunction in Patients With Treated Metabolic Risk Factors and Suspected Coronary Artery Disease. J Am Heart Assoc *6*.
69. Vesely, S.K., George, J.N., Lämmle, B., Studt, J.D., Alberio, L., El-Harake, M.A., and Raskob, G.E. (2003). ADAMTS13 activity in thrombotic thrombocytopenic purpura-hemolytic uremic syndrome: relation to presenting features and clinical outcomes in a prospective cohort of 142 patients. Blood *102*, 60-68.
70. Vilahur, G., Ben-Aicha, S., and Badimon, L. (2017). New insights into the role of adipose tissue in thrombosis. Cardiovasc Res *113*, 1046-1054.
71. Vlaicu, S.I., Tatomir, A., Boodhoo, D., Vesa, S., Mircea, P.A., and Rus, H. (2016). The role of complement system in adipose tissue-related inflammation. Immunol Res *64*, 653-664.
72. Williamson, E., Walker, A.J., Bhaskaran, K.J., Bacon, S., Bates, C., Morton, C.E., Curtis, H.J., Mehrkar, A., Evans, D., Inglesby, P.*, et al.* (2020). OpenSAFELY: factors associated with COVID-19-

Journal Pre-proof

related hospital death in the linked electronic health records of 17 million adult NHS patients. medRxiv, 2020.2005.2006.20092999.
73. Wlazlo, N., van Greevenbroek, M.M., Ferreira, I., Feskens, E.J., van der Kallen, C.J., Schalkwijk, C.G., Bravenboer, B., and Stehouwer, C.D. (2014). Complement factor 3 is associated with insulin resistance and with incident type 2 diabetes over a 7-year follow-up period: the CODAM Study. Diabetes Care *37*, 1900-1909.
74. Xin, Y., Hertle, E., van der Kallen, C.J.H., Schalkwijk, C.G., Stehouwer, C.D.A., and van Greevenbroek, M.M.J. (2018). Longitudinal associations of the alternative and terminal pathways of complement activation with adiposity: The CODAM study. Obes Res Clin Pract *12*, 286-292.
75. Yilmaz, C., Ravikumar, P., Gyawali, D., Iyer, R., Unger, R.H., and Hsia, C.C. (2015). Alveolar-capillary adaptation to chronic hypoxia in the fatty lung. Acta Physiol (Oxf) *213*, 933-946.
76. Zanato, V., Lombardi, A.M., Busetto, L., Prà, C.D., Foletto, M., Prevedello, L., De Marinis, G.B., Fabris, F., Vettor, R., and Fabris, R. (2017). Weight loss reduces anti-ADAMTS13 autoantibodies and improves inflammatory and coagulative parameters in obese patients. Endocrine *56*, 521-527.
77. Zhu, L., She, Z.G., Cheng, X., Qin, J.J., Zhang, X.J., Cai, J., Lei, F., Wang, H., Xie, J., Wang, W*., et al.* (2020). Association of Blood Glucose Control and Outcomes in Patients with COVID-19 and Pre-existing Type 2 Diabetes. Cell Metab.

Figure Legend

Obesity exacerbates COVID-19: Potential Mechanisms

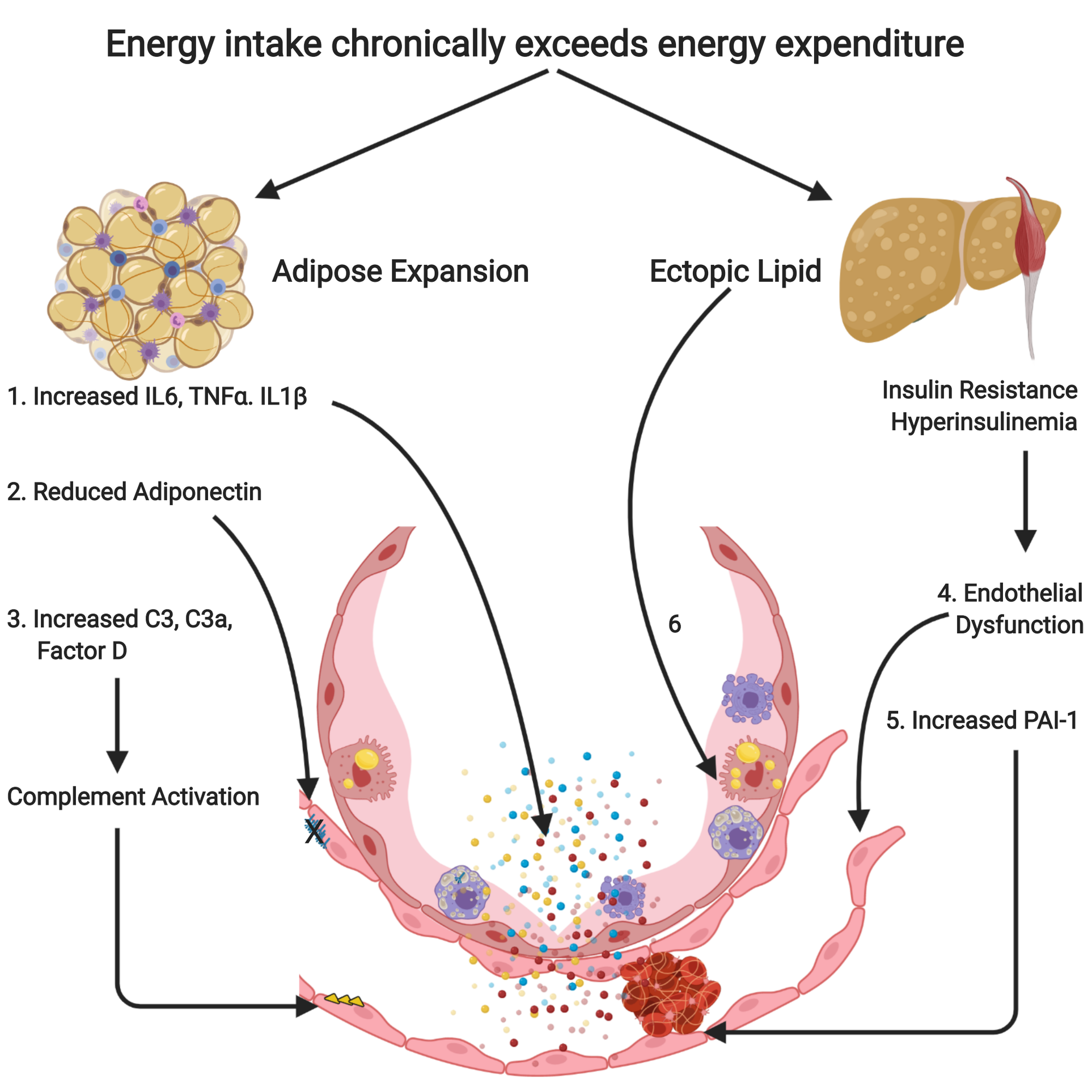
Obesity is a disorder of energy balance that ensues when energy intake exceeds expenditure. Adipose tissue expansion occurs to safely store excess energy safely in triglyceride rich lipid droplets. This process is associated with adipose tissue inflammation and elaboration of pro-inflammatory cytokines, increased components of the complement system and altered adipose tissue hormones. 1) Increased inflammatory cytokines are secreted into the systemic circulation and can act on the alveolar capillary unit to potentiate the inflammatory response to SARS-CoV-2 infection. 2) Adipose tissue expansion is associated with a reduction in Adiponectin secretion from the adipose tissue that is that at least partly driven by systemic insulin resistance. Mouse studies suggest adiponectin is abundant in the pulmonary endothelium in the healthy lung and adiponectin deficiency results in pulmonary vascular inflammation and pre-disposes to experimental lung injury. 3) Increases in circulating complement components elaborated from adipose tissue occur in expanded adipose and in association with insulin resistance and could pre-dispose to complement activation and subsequent thrombotic microangiopathy. When the capacity for adipose tissue to expand is exceeded lipid is deposited in other organs. Lipid deposition in skeletal muscle and liver likely plays a causal role in the development of insulin resistance and hyperinsulinemia. 4) Systemic insulin resistance is associated with endothelial dysfunction that may pre-dispose to thrombosis and contribute to lung injury via vascular inflammation and enhanced endothelial permeability. 5) Insulin resistance is robustly associated with increased plasminogen activator inhibitor-1 (PAI-1) which impairs fibrinolysis and may contribute to risk of thrombosis in COVID-19. 6). Finally, ectopic lipid may actually be directly deposited in type 2 pneumocytes pre-disposing to lung injury in SARS-CoV-2 infection.

Journal Pre-proof

eTOC Blurb

Obesity is associated with increased COVID-19 related mortality. Lockhart and O'Rahilly review the pathophysiological mechanisms that may underlie this link and converge on a set of testable hypotheses to guide investigation of the effects of obesity on COVID-19.

Journal Pre-proof


Energy intake chronically exceeds energy expenditure
Adipose Expansion
Ectopic Lipid
Insulin Resistance
Hyperinsulinemia
1. Increased IL6, TNFα. IL1β
2. Reduced Adiponectin
3. Increased C3, C3a,
Factor D
Complement Activation
6
4. Endothelial
Dysfunction
5. Increased PAI-1

JAMA Neurology | Original Investigation

Clinical Characteristics and Outcomes in Patients With Coronavirus Disease 2019 and Multiple Sclerosis

Céline Louapre, MD; Nicolas Collongues, MD; Bruno Stankoff, MD; Claire Giannesini, MD; Caroline Papeix, MD; Caroline Bensa, MD; Romain Deschamps, MD; Alain Créange, MD; Abir Wahab, MD; Jean Pelletier, MD; Olivier Heinzlef, MD; Pierre Labauge, MD; Laurent Guilloton, MD; Guido Ahle, MD; Mathilde Goudot, MD; Kevin Bigaut, MD; David-Axel Laplaud, MD; Sandra Vukusic, MD; Catherine Lubetzki, MD; Jérôme De Sèze, MD; for the Covisep investigators

IMPORTANCE Risk factors associated with the severity of coronavirus disease 2019 (COVID-19) in patients with multiple sclerosis (MS) are unknown. Disease-modifying therapies (DMTs) may modify the risk of developing a severe COVID-19 infection, beside identified risk factors such as age and comorbidities.

OBJECTIVE To describe the clinical characteristics and outcomes in patients with MS and COVID-19 and identify factors associated with COVID-19 severity.

DESIGN, SETTING, AND PARTICIPANTS The Covisep registry is a multicenter, retrospective, observational cohort study conducted in MS expert centers and general hospitals and with neurologists collaborating with MS expert centers and members of the Société Francophone de la Sclérose en Plaques. The study included patients with MS presenting with a confirmed or highly suspected diagnosis of COVID-19 between March 1, 2020, and May 21, 2020.

EXPOSURES COVID-19 diagnosed with a polymerase chain reaction test on a nasopharyngeal swab, thoracic computed tomography, or typical symptoms.

MAIN OUTCOMES AND MEASURES The main outcome was COVID-19 severity assessed on a 7-point ordinal scale (ranging from 1 [not hospitalized with no limitations on activities] to 7 [death]) with a cutoff at 3 (hospitalized and not requiring supplemental oxygen). We collected demographics, neurological history, Expanded Disability Severity Scale score (EDSS; ranging from 0 to 10, with cutoffs at 3 and 6), comorbidities, COVID-19 characteristics, and outcomes. Univariate and multivariate logistic regression models were used to estimate the association of collected variables with COVID-19 outcomes.

RESULTS A total of 347 patients (mean [SD] age, 44.6 [12.8] years, 249 women; mean [SD] disease duration, 13.5 [10.0] years) were analyzed. Seventy-three patients (21.0%) had a COVID-19 severity score of 3 or more, and 12 patients (3.5%) died of COVID-19. The median EDSS was 2.0 (range, 0-9.5), and 284 patients (81.8%) were receiving DMT. There was a higher proportion of patients with a COVID-19 severity score of 3 or more among patients with no DMT relative to patients receiving DMTs (46.0% vs 15.5%; $P < .001$). Multivariate logistic regression models determined that age (odds ratio per 10 years: 1.9 [95% CI, 1.4-2.5]), EDSS (OR for EDSS ≥6, 6.3 [95% CI. 2.8-14.4]), and obesity (OR, 3.0 [95% CI, 1.0-8.7]) were independent risk factors for a COVID-19 severity score of 3 or more (indicating hospitalization or higher severity). The EDSS was associated with the highest variability of COVID-19 severe outcome (R^2, 0.2), followed by age (R^2, 0.06) and obesity (R^2, 0.01).

CONCLUSIONS AND RELEVANCE In this registry-based cohort study of patients with MS, age, EDSS, and obesity were independent risk factors for severe COVID-19; there was no association found between DMTs exposure and COVID-19 severity. The identification of these risk factors should provide the rationale for an individual strategy regarding clinical management of patients with MS during the COVID-19 pandemic.

JAMA Neurol. doi:10.1001/jamaneurol.2020.2581
Published online June 26, 2020.

Supplemental content

Author Affiliations: Author affiliations are listed at the end of this article.

Group Information: A complete list of the members of the Covisep investigators appears at the end of this article.

Corresponding Author: Céline Louapre, MD, Institut du Cerveau et de la Moelle Épinière, Assistance Publique des Hôpitaux de Paris, Hôpital de la Pitié Salpêtrière, Sorbonne Université, Institut National de la Santé et de la Recherche Médicale, Clinical Investigation Center Neuroscience Unité Mixte De Recherche S 1127, Centre National de la Recherche Scientifique Unité Mixte De Recherche 7225, F-75013, Paris, France (celine.louapre@aphp.fr).

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a University of Exeter User on 06/27/2020

In December 2019, the first cases of a new coronavirus infection (coronavirus disease 2019 [COVID-19]) attributable to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) appeared in Wuhan, China. The virus has spread rapidly, reaching 225 countries and more than 5 million people as of May 21, 2020. In France, by the same date, 181 000 patients had tested positive for COVID-19 and more than 28 000 patients had died of COVID-19. In the pandemic context, populations at risk of developing severe forms of COVID-19 have begun to be identified.[1]

Patients with multiple sclerosis (MS) represent a population of particular interest in the pandemic context because most of them are treated with immunosuppressants. On March 13, 2020, the Multiple Sclerosis International Federation issued recommendations regarding the risk of COVID-19 in patients with MS, with a specific statement regarding disease-modifying therapies (DMTs), indicating that "some MS medications might increase the likelihood of developing complications from a COVID-19 infection but this risk needs to be balanced with the risks of stopping treatment."[2] In statements from national MS societies and published editorials,[3,4] it is recommended to consider delaying DMT initiation for those treatments with the highest presumed infectious risk or delaying the completion of a cure for sequential DMTs. The attitude toward DMTs in the case of proven COVID-19 infection is to consider temporarily stopping immunosuppressive DMTs. Expert panels have proposed a stratification of the risk of treatments according to known systemic immunosuppression.[3,4]

In this context, we have initiated a French registry enabling the collection of highly suspect and proven COVID-19 in patients with MS. The purpose of this registry was to determine the characteristics of COVID-19 in this population and identify the risk factors for developing a severe form of this infection.

Key Points

Question What are the risk factors for developing a severe form of coronavirus disease 2019 (COVID-19) in patients with multiple sclerosis (MS)?

Findings In this cohort study of 347 patients with MS, risk factors for severe forms of COVID-19 were neurological disability, age, and obesity, but no association was found between disease-modifying therapies exposure and COVID-19 severity.

Meaning The identification of these risk factors could provide a rationale for an individual strategy of clinical management in patients with MS during the COVID-19 pandemic.

Methods

Data Collection

We conducted a multicenter, retrospective, observational cohort study of patients with MS with confirmed or highly suspected COVID-19 diagnosis (Covisep registry). All French MS expert centers (Centre de Ressources et de Compétences-Sclérose en Plaques), part of the French Clinical Research Infrastructure Network for Multiple Sclerosis (which is part of Institut National de la Santé et de la Recherche Médicale), and general hospitals and neurologists collaborating with the Centre de Ressources et de Compétences-Sclérose en Plaques or members of the French MS society (Société Francophone de la Sclérose en Plaques) participated in the collection of data.

The study followed the Strengthening Reporting of Observational Studies in Epidemiology (STROBE) guidelines. The study received approval from the ethics committee of Sorbonne University and is registered at the Institut National des Données de Santé for the use of confidential electronically processed patient data. The study protocol is registered on ClinicalTrials.gov (NCT04355611). Patients included were informed about the objective of the study, and the collection of nonopposition (consent) to the use of medical data was carried out according to French law, good clinical practice, and the General Data Protection Regulation. Using an electronic clinical record form on RedCap (https://www.project-redcap.org/), study data were collected from medical records of patients who were diagnosed with COVID-19 between March 1, 2020, and May 21, 2020.

Population of Interest

Inclusion criteria were MS and at least 1 of the following 4 criteria: (1) a biologically confirmed COVID-19 diagnosis based on a positive result of a SARS-CoV-2 polymerase chain reaction (PCR) test on a nasopharyngeal swab; (2) typical thoracic computed tomography (CT) abnormalities (ground-glass opacities) in epidemic areas; (3) anosmia or ageusia of sudden onset in the absence of rhinitis or nasal obstruction; or (4) COVID-19–typical symptoms (triad of cough, fever, and asthenia) in an epidemic zone of COVID-19. The noninclusion criterion was the patient's opposition to the use of his or her medical data.

Definition of Study End Points

We collected patient demographics, current DMT use, Expanded Disability Severity Scale score (EDSS) before COVID-19, and comorbidities and the presence of known lymphopenia before infection. Regarding COVID-19, we collected symptoms and diagnostic data (PCR for SARS-CoV-2 or chest CT). The primary end point was the participant's clinical status at the most severe point of their COVID-19 course (on a 7-point ordinal scale[5]), referred as the *COVID-19 severity score*, where 1 indicated the patient was not hospitalized and had no limitations on activities; 2, the patient was not hospitalized and had limitation on activities; 3, the patient was hospitalized and did not require supplemental oxygen; 4, the patient was hospitalized and required supplemental oxygen; 5, the patient was hospitalized and receiving noninvasive ventilation or high-flow oxygen; 6, the patient was hospitalized and receiving invasive mechanical ventilation or extracorporeal membrane oxygenation; or 7, death.

Statistical Analysis

Descriptive statistics were performed on demographic and clinical variables. Demographic data were compared between patients with COVID-19 severity scores of 1 or 2 and pa-

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a University of Exeter User on 06/27/2020

tients with COVID-19 severity scores of 3 or more (ie, hospitalization or death from COVID-19). Group comparisons were performed using the Mann-Whitney *U* test for numerical and ordinal variables and the Fisher test or χ^2 test when appropriate for categorical variables. Any 2-sided $P < .05$ was considered statistically significant.

Univariate logistic regression models were performed on identified variables to assess their association with COVID-19 outcome (severity scores, 1-2 vs ≥3). Age was treated as a continuous variable, and EDSS was segmented into 3 bins (<3, 3-5.5, and ≥6). Disease-modifying therapies were categorized according to systemic infection risk (no risk: interferon beta and glatiramer; low risk: teriflunomide, dimethylfumarate, natalizumab, and other drugs [mycophenolate mofetil, methotrexate, and cyclophosphamide]; intermediate or high risk: fingolimod, anti-CD20 therapies, cladribine, and alemtuzumab) as proposed in a previous study.[4] Subsequently, a multivariate logistic regression model was performed to determine which variables are independently associated with severe COVID-19 (severity score ≥3). Variable selection was done through backward elimination and forward selection by minimizing Akaike information criterion. Results are expressed as odds ratios (ORs) and 95% CIs. McFadden pseudo-R^2 values were calculated for each variable to quantify the proportion of the total variability of the outcome that is accounted for by the model. Data analyses were performed in Python (Python Software Foundation) using Pandas package version 1.0.3, Scipy version 1.4.1, and Statsmodels version 0.11.1.

Results

Study Population

As of May 21, 2020, 348 patients with MS were in the Covisep registry. Among 46 patients with a negative SARS-CoV-2 PCR result, 10 patients had a chest CT showing typical lesions for COVID-19 and 1 patient had further SARS-CoV-2-positive serology results. For the remaining patients with negative SARS-CoV-2 PCR results, 34 of 35 were confirmed to have a highly suspicious COVID-19 with a typical medical history. One patient had an uncertain COVID-19 diagnosis and was excluded from the analysis. Ultimately, 347 patients with MS were included.

Demographics and Clinical Characteristics

Demographics and clinical characteristics of the cohort are presented in the **Table**. Briefly, their mean (SD) age was 44.6 (12.8) years, 249 of 347 were women, and the mean (SD) disease duration was 13.5 (10.0) years.

Seventy-three patients had a COVID-19 severity score of 3 or more. Patients presented with typical symptoms for COVID-19. Notably, fever (temperature more than 38 °C) and dyspnea were more frequent in patients with severe COVID-19 (67 of 73 [91.8%] and 54 of 73 [74.0%], respectively) than patients who were not hospitalized (193 of 274 [70.4%] and 108 of 274 [39.4%] respectively; $P < .001$), while anosmia/ageusia and headache were more common in patients who were not hospitalized (135 of 274 [49.3%] and 164 of 274 [59.8%],

Table. Demographic and Disease Characteristics of Patients With Multiple Sclerosis Diagnosed With Coronavirus Disease 2019 in the Covisep Registry, as of May 21, 2020

Characteristic	No. (%)
No. of patients with MS	347
Demographics	
Age, mean (SD), y	44.6 (12.8)
Sex ratio, female:male	249:98
Disease duration, mean (SD), y	13.5 (10.0)
Disease course, No.	
Clinically isolated syndrome	6
Remitting-relapsing multiple sclerosis	276
Secondary progressive multiple sclerosis	48
Primary progressive multiple sclerosis	17
Expanded Disability Severity Score, median (range)	2.0 (0.0-9.5)
Disease-modifying therapies	
Interferon beta	20 (5.8)
Glatiramer	33 (9.5)
Teriflunomide	33 (9.5)
Dimethylfumarate	35 (10.1)
Natalizumab	57 (16.4)
Fingolimod	42 (12.1)
Ocrelizumab	38 (11.0)
Rituximab	17 (4.9)
Cladribine	3 (0.9)
Alemtuzumab	1 (0.3)
Other[a]	5 (1.4)
None	63 (18.2)
Lymphopenia before COVID-19 onset, No./No. tested (%)	
No lymphopenia	128/187 (68.4)
Lymphopenia	59/187 (31.6)
Lymphopenia, excluding patients receiving fingolimod	34/157 (21.6)
Lymphopenia grade, No.	
1	10
2	21
3	23
4	2
Comorbid conditions	
Cardiovascular disease	23 (6.6)
Pulmonary disease	15 (4.3)
Diabetes	16 (4.6)
Obesity (BMI>30)	24 (6.9)
Smoking	33 (9.5)
COVID-19 diagnosis, No./No. tested (%)	
Positive SARS-CoV-2 polymerase chain reaction result	146/191 (76.4)
Ground-glass opacity on thoracic computed tomography scan	62/93 (66.7)
COVID-19 symptoms	
Asthenia	290 (83.6)
Fever	260 (74.9)
Cough	266 (76.7)
Anosmia/ageusia	150 (43.2)
Headache	180 (51.9)
Dyspnea	162 (46.7)
Digestive disorders	88 (25.4)
Dizziness	54 (15.6)

(continued)

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a University of Exeter User on 06/27/2020

Table. Demographic and Disease Characteristics of Patients With Multiple Sclerosis Diagnosed With Coronavirus Disease 2019 in the Covisep Registry, as of May 21, 2020 (continued)

Characteristic	No. (%)
Inclusion criteria	
Positive SARS-CoV-2 polymerase chain reaction	146 (42.1)
Ground-glass opacity on thoracic computed tomography scan, without fulfilling above criteria	18 (5.2)
Anosmia/ageusia, without fulfilling above criteria	91 (26.2)
Typical COVID-19 symptoms, without fulfilling above criteria	92 (26.5)

Abbreviations: BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

[a] Other treatments for patients with multiple sclerosis: mycophenolate mofetil (n = 3), cyclophosphamide (n = 1), and methotrexate (n = 1).

respectively) vs patients who were hospitalized (15 of 73 [20.5%] and 16 of 73 [21.9%], respectively; $P < .001$).

The distribution of clinical severity for COVID-19 according to the different DMTs is presented in **Figure 1**. The ages and EDSSs of patients in each DMT group are shown in eFigure 1 in the Supplement.

Variables Associated With COVID-19 Severity

Patients with COVID-19 severity scores of 3 or more were more frequently male compared with patients with COVID-19 who were not hospitalized (female:male sex ratio: 1.4 vs 3.0; $P = .008$). Age and EDSS differences between groups and clinical course distribution are represented in **Figure 2**. Patients with COVID-19 severity scores of 3 or more were older (mean [SD] age, 55.0 [13.7] years vs 41.9 [11.0] years; $P < .001$), had higher EDSS (median EDSS, 6.0 vs 2.0; $P < .001$), and more frequently had a progressive course (36 of 73 [49.3%] vs 29 of 274 [10.6%]; $P < .001$) compared with patients with COVID-19 who were not hospitalized.

The presence of at least 1 comorbidity was associated with an increased risk of severe COVID-19. The proportion of patients with at least 1 comorbidity was lower in the nonhospitalized group vs the severe COVID-19 group (75 of 274 [27.4%] vs 38 of 73 [52.1%], respectively; $P < .001$). Comorbidities associated with a COVID-19 severity score of 3 or more were diabetes (9 of 73 [12.3%] vs 7 of 274 [2.5%]; $P = .002$), cardiovascular comorbidity (13 of 73 [17.8%] vs 10 of 274 [3.6%]; $P < .001$), pulmonary comorbidity (7 of 73 [9.6%] vs 8 of 274 [2.9%]; $P = .02$), and obesity (10 of 73 [13.7%] vs 14 of 274 [5.1%]; $P = .02$).

There was a higher proportion of patients with severe COVID-19 among patients with no DMTs relative to patients who were receiving DMTs (29 of 63 [46.0%] vs 44 of 284 [15.5%]; $P < .001$). Among patients with DMTs (n = 284), there was a difference in COVID-19 outcome between DMT groups, with a higher proportion of patients with severe COVID-19 in the moderate-risk or high-risk DMT group (24 of 101 [23.8%] vs 17 of 130 [13.1%] in the low-risk DMT group and 3 of 53 [5.7%] in the no-risk DMT group; $P = .007$). Disease-modifying treatments were stopped or postponed (in the case of sequential treatment) in 77 patients (27.1%), but DMT modification was not associated with COVID-19 outcome.

Among 187 patients for whom lymphocyte counts were available before COVID-19 onset, we did not find an association between lymphopenia and severe COVID-19. However, if we excluded patients taking fingolimod because of this drug's mechanism of action, lymphopenia was more frequent in patients with COVID-19 severity scores of 3 or more (22 of 57 [38.6%] vs 12 of 100 [12.0%]; $P < .001$).

Univariate and Multivariate Logistic Regression Models of Severe COVID-19

To estimate the risk factors associated with COVID-19 severity scores of 3 or more, we calculated the odds ratios (ORs) from univariate logistic regression models (**Figure 3**A). Greater age (OR per 10 years, 2.49 [95% CI, 1.92-3.22]), male sex (OR, 2.11 [95% CI, 1.23-3.63]), comorbidities (OR, 2.93 [95% CI, 1.73-4.99]), progressive disease (OR, 8.22 [95% CI, 4.52-14.96]), and higher EDSS (ORs, EDSS 3-5.5, 6.28 [95% CI, 2.97-13.26]; EDSS ≥6, 16.10 [95% CI, 7.72-33.58]) were associated with increased risk of severe COVID-19, while DMTs were associated with lower risks of severe COVID-19 (interferon or glatiramer acetate: OR, 0.07 [95% CI, 0.02-0.25]; teriflunomide, dimethylfumarate, natalizumab, or other drugs: OR, 0.18 [95% CI, 0.09-0.36]; alemtuzumab, cladribine, fingolimod, ocrelizumab, or rituximab: OR, 0.37 [95% CI, 0.19-0.72]). In the multivariate logistic regression model, age, male, obesity, cardiac comorbidity, and EDSS were retained in the model as independent variables associated with severe COVID-19 (Figure 3B). Among these, higher age (OR per 10 years, 1.9 [95% CI, 1.4-2.5]), an EDSS of 6 or more (OR, 6.3 [95% CI, 2.8-14.4]), and obesity (OR, 3.0 [95% CI, 1.03-8.7]) were independent variables associated with severe COVID-19 (severity score ≥3). The EDSS showed the highest variability of COVID-19 severe outcome (R^2, 0.20), followed by age (additional R^2, 0.06) and obesity (additional R^2, 0.01) (Figure 3C).

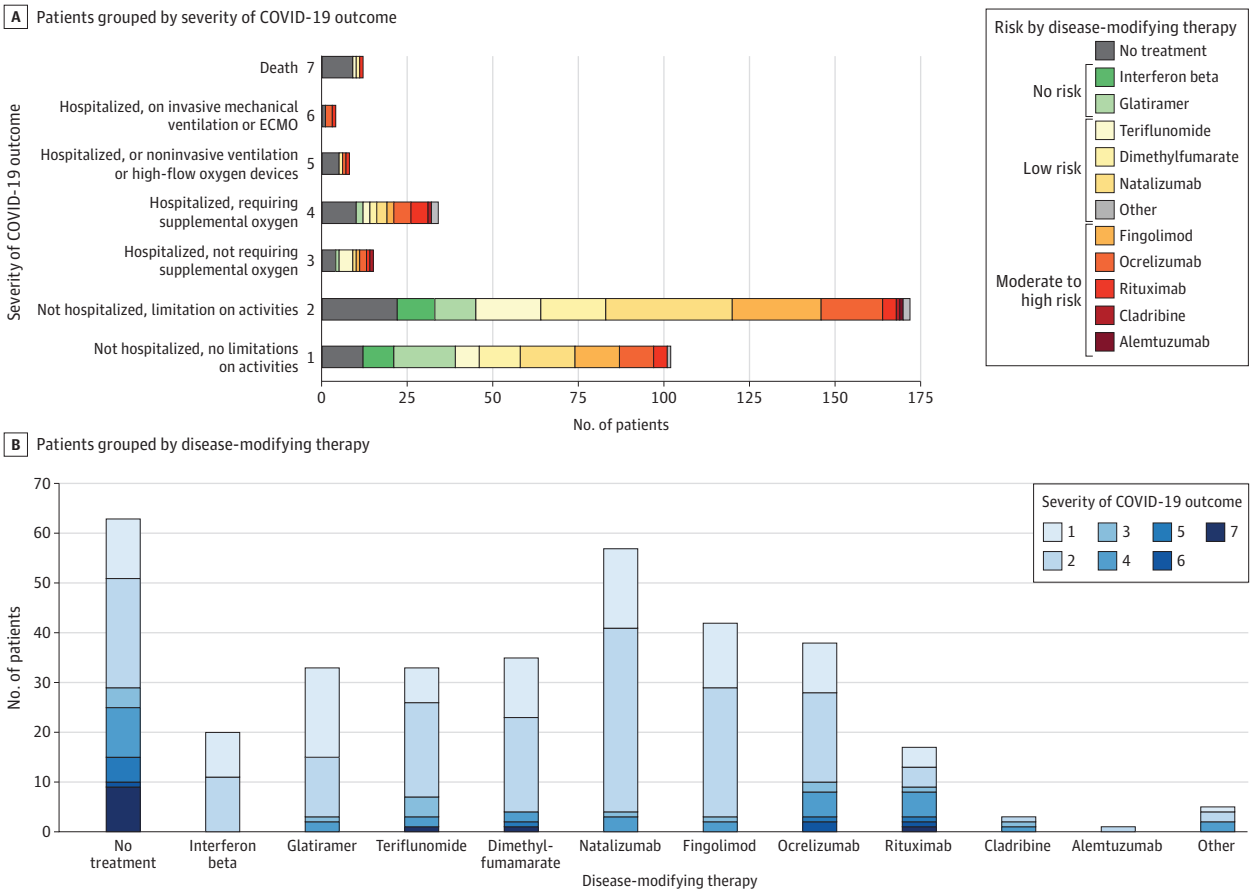
A cutoff score of 7 for EDSS provided similar results (eFigure 2 in the Supplement). As a sensitivity analysis, when including only patients with positive SARS-CoV-2 PCR results (n = 146), a higher age (OR per 10 years, 1.6 [95% CI, 1.1-2.3]) and an EDSS of 6 or more (OR, 7.0 [95% CI, 2.3-21.2]) remained independent variables associated with severe COVID-19.

We performed a post hoc analysis with a threshold of 4 for COVID-19 severity score (hospitalized, requiring supplemental oxygen, or higher severity). We obtained similar results at univariate analysis (eFigure 3 in the Supplement). In the multivariate logistic regression model, age, male sex, obesity, cardiac comorbidity, and EDSSs were retained as independent variables associated with severe COVID-19 (eFigure 3 in the Supplement). A higher age (OR per 10 years, 2.1 [95% CI, 1.5-3.0]), EDSS of 6 or more (OR, 8.3 [95% CI, 3.1-22.3]), male sex (OR, 2.6 [95% CI, 1.2-5.3]), and obesity (OR, 5.2 [95% CI, 1.6-16.5]) were the independent variables associated with severe COVID-19 (severity score ≥4). The EDSS showed the highest variability of COVID-19 severe outcome (R^2, 0.23), followed by age (additional R^2, 0.08), obesity (R^2, 0.02), and male sex (R^2, 0.01) (eFigure 3 in the Supplement).

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a University of Exeter User on 06/27/2020

Figure 1. Clinical Condition Rating at Nadir and Disease-Modifying Therapies in Patients With Multiple Sclerosis



A, Patients were grouped according to coronavirus disease 2019 (COVID-19) severity score (from 1 [not hospitalized with no limitations on activities] to 7 [death]). B, Patients were grouped according to disease-modifying therapy. Systemic immunosuppression risk was adapted from Brownlee et al.[3] Other treatments used by patients with multiple sclerosis included mycophenolate mofetil (n = 3), cyclophosphamide (n = 1), and methotrexate (n = 1).

Clinical Description of Patients With Highest COVID-19 Severity

Twelve patients in the cohort (3.5%) died of COVID-19, with most of these patients having a progressive form of the disease and a high EDSS. Four patients were hospitalized in intensive care units. One relatively young patient was in intensive care under mechanical ventilation for 3 weeks and is now in rehabilitation with a tracheotomy. Another patient was admitted to an intensive care unit 10 days after symptom onset. Both patients were receiving ocrelizumab and had obesity as a comorbidity. A third patient with primary progressive MS was in intensive care under mechanical ventilation for 8 days and has since been discharged from the hospital. This person was taking rituximab, had an EDSS of 5.5, and had no known comorbidities. A fourth patient, who had relapsing-remitting MS with no DMT, was admitted in intensive care under mechanical ventilation.

Figure 4 displays an associative plot between age, EDSS, and COVID-19 severity of individuals from the cohort. A description of demographics and clinical characteristics of patients with the most severe COVID-19 outcome (score 6: under mechanical ventilation; score 7: death) is provided in eTable 1 in the Supplement.

Fatality Rate in Patients With Multiple Sclerosis Hospitalized for COVID-19

We provide in eTable 2 in the Supplement the numbers of patients, stratified by age and sex, and the death rate among patients who were hospitalized. As a reference, we have included the death rate among patients hospitalized for COVID-19 in the overall population in France.[6] Although no statistical analysis can be performed because of the low number of patients who died in this cohort, we observed a higher fatality rate among patients who were hospitalized for most age groups in patients with MS compared with reference estimates.

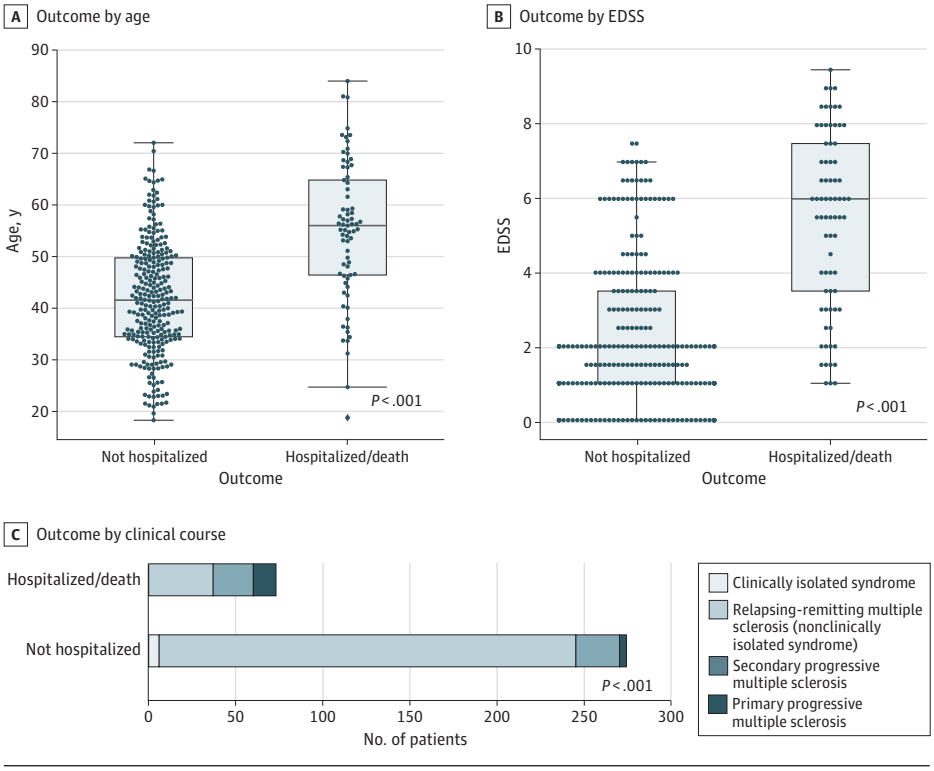
Discussion

We found COVID-19 symptom profiles to be consistent with that described in the general population. In this cohort, fever and dyspnea were more common in patients hospitalized for

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a University of Exeter User on 06/27/2020

Figure 2. Characteristics of Patients Not Hospitalized for Coronavirus Disease 2019 (COVID-19) and Patients Who Were Hospitalized With or Died of COVID-19



A, Age. B, Expanded Disability Severity Scale (EDSS) score. C, Clinical course. The *P* values were obtained from Mann-Whitney *U* tests for age and EDSS group differences and χ^2 tests for clinical course distribution differences.

COVID-19. Similarly, in a series of 201 patients with COVID-19 pneumonia, high fever was associated with the development of acute respiratory distress syndrome.[7] Headache and anosmia were more frequently associated with ambulatory forms in our cohort, in line with a recent article[8] reporting that anosmia was strongly and independently associated with outpatient care.

Importantly, we identified in univariate analyses risk factors for COVID-19 severity: age, EDSS, progressive course of MS, and male sex. Comorbidities were also identified as risk factors, including cardiovascular and pulmonary diseases, diabetes, and obesity. These comorbidities were most frequently found in a large series of patients hospitalized in an intensive care unit for COVID-19 in New York, New York.[9]

The presence of DMT was associated with a lower risk of hospitalization in univariate analysis. Among patients who were hospitalized or died, there was an increased proportion of patients without treatment compared with the patients who were not hospitalized. Given the interactions between age, disease course, EDSS, and exposure to DMTs, it is crucial to carry out a multivariate analysis to estimate the independent outcome of each of these variables. Indeed, DMTs are more frequently prescribed in younger patients, those with a relapsing form of MS, and those with a lower level of disability. In multivariate analyses, only the following variables were independent risk factors of severe form of COVID-19: age, EDSS, and obesity. Additionally, the multivariate analysis carried out with a threshold of severity of COVID-19 of 4 or more found an independent outcome of male sex as risk factor for severe COVID-19, beside age, obesity, and EDSS.

In this cohort, the exposure to DMTs and the level of immunosuppression were not factors that independently modified the risk of developing a severe form of COVID-19. Caution should be exercised when interpreting this result, because it is possible that a larger cohort could identify a subgroup of patients with 1 or more DMTs that modify the risk of COVID-19. Therefore, the pooling of registries and/or replication of results in other cohorts from several countries will be important to reinforce this result. In our population, 2 patients with relapsing-remitting MS who were receiving ocrelizumab have developed acute respiratory distress syndrome. Although these 2 patients also had obesity, it cannot be excluded that their DMTs may have increased their susceptibility to COVID-19 severity. In the literature, cases of patients with MS and COVID-19 who were receiving ocrelizumab were reported, with 1 being asymptomatic[10] and the other having a favorable outcome.[11] Serological studies following COVID-19 infection in these patients will be crucial to determine the characteristics of the immune response to COVID-19, depending on the different DMTs and in particular on anti-CD20 therapies, for which lower immune response to vaccination has been reported.[12]

It is not surprising to identify age and obesity as independent risk factors for COVID-19 severity. Age was identified in the earliest published study[13] as a major risk factor for the severity of COVID-19 infection. More recently, obesity has also been identified as an independent risk factor.[14]

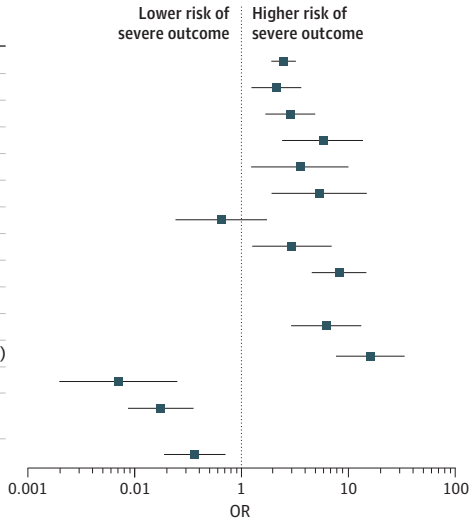
© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a University of Exeter User on 06/27/2020

Figure 3. Risk Factors of Severe Coronavirus Disease 2019 (COVID-19)

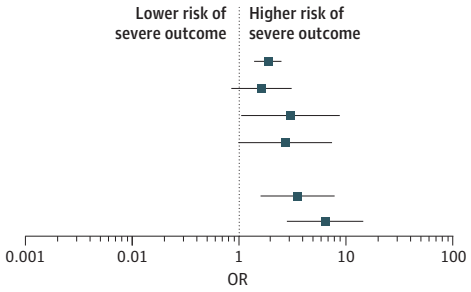
A Univariate analysis

Group	OR (95% CI)
Age per 10 y	2.49 (1.92-3.22)
Male	2.11 (1.23-3.63)
≥1 Comorbidity	2.93 (1.73-4.99)
Cardiac comorbidity	5.72 (2.39-13.66)
Pulmonary comorbidity	3.53 (1.23-10.07)
Diabetes	5.36 (1.93-14.95)
Smoking	0.65 (0.24-1.74)
Obesity	2.95 (1.25-6.94)
Progressive course	8.22 (4.52-14.96)
EDSS <3	NA
EDSS 3-5.5	6.28 (2.97-13.26)
EDSS ≥6	16.10 (7.72-33.58)
Interferon or glatiramer acetate	0.07 (0.02-0.25)
Teriflunomide, dimethlyfumarate, natalizumab, or other	0.18 (0.09-0.36)
Alemtuzumab, cladribine, fingolimod, ocrelizumab, or rituximab	0.37 (0.19-0.72)

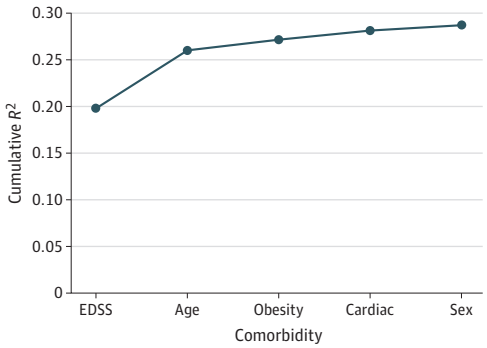


B Multivariate analysis

Group	OR (95% CI)
Age per 10 y	1.85 (1.39-2.46)
Male	1.61 (0.83-3.11)
Obesity	2.99 (1.03-8.70)
Cardiac comorbidity	2.68 (0.97-7.40)
EDSS <3	NA
EDSS 3-5.5	3.48 (1.55-7.84)
EDSS ≥6	6.33 (2.78-14.39)



C Cumulative variability of severe COVID-19



Severe COVID-19 was defined as a severity score of 3 or more. A, Univariate analysis. B, Multivariate analysis. Results are expressed as odds ratios (ORs) with 95% CIs. C, Cumulative variability (McFadden R^2) of severe COVID-19 accounted for each variable retained in the multiple logistic regression model. Numbers of patients per category: men, 98; with 1 or more comorbidity, 113; with obesity, 24; with cardiac comorbidity, 23; with an Expanded Disability Severity Status (EDSS) score less than 3, 194; with an EDSS of 3 to 5.5, 75; with an EDSS of 6 or more, 69; without disease-modifying therapy, 63; receiving interferon or glatiramer acetate, 53; receiving teriflunomide, dimethylfumarate, natalizumab, or other drugs, 130; and receiving fingolimod, ocrelizumab, rituximab, cladribine, or alemtuzumab, 101. NA indicates not applicable.

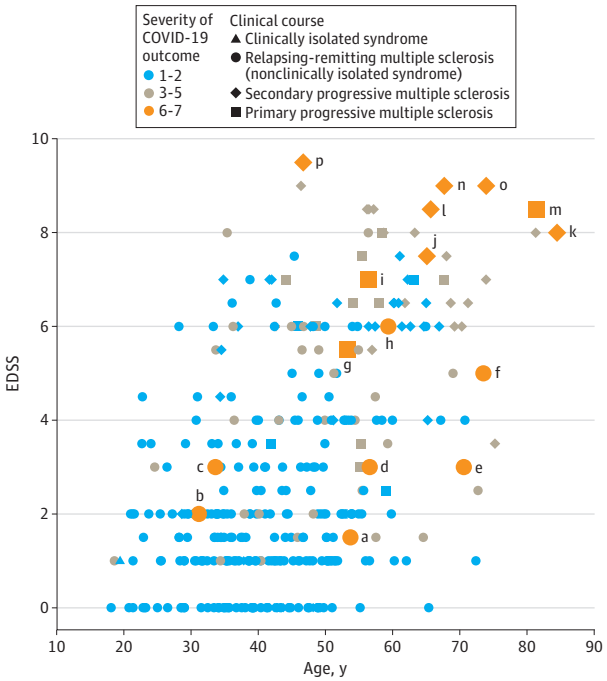
The association between COVID-19 severity and the level of neurological disability assessed by EDSS in this cohort has not been previously reported, because there are (to our knowledge) currently no data available on the severity of COVID-19 in patients with preexisting neurological disabilities. Respiratory dysfunction is common in patients with MS and EDSS of 7 or more and is linked to expiratory muscles and cough impairment.[15] With the EDSS being the independent variable with the highest explained variability on COVID-19 severity, this finding should lead to strengthening and sustaining precautions and barrier measures to limit the risk of COVID-19 infection in patients with high disability.

The overall mortality rate of COVID-19 in the cohort was 3.5%. The case-fatality ratio was estimated at 0.44% in mainland China in the age group of 40 to 49 years.[16] A recent study estimated the infection fatality ratio in France as 0.7% (95% CI, 0.4%-1.0%), including asymptomatic cases.[6] In our cohort, only 2 patients without symptoms were diagnosed as positive for SARS-CoV-2 by PCR swab, and most cases were reported because of typical symptoms for COVID-19. There-

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a University of Exeter User on 06/27/2020

Figure 4. Plot Associating Expanded Disability Severity Scale (EDSS) Score, Age, and Coronavirus Disease 2019 (COVID-19) Severity Score



Letters *a* to *p* refer to case reports of patients in need of mechanical ventilation or extracorporeal membrane oxygenation, or dead of COVID-19, as detailed in eTable 1 in the Supplement.

fore, we cannot infer an infection fatality ratio from this cohort. Instead, the fatality rate among patients who were hospitalized is a relevant measure at this stage of the outbreak. The fatality rate among patients who were hospitalized was higher for most age groups in patients with MS compared with the general population in France, suggesting an increased risk of death in patients with MS compared with the general population, which needs to be confirmed with large epidemiological studies.

Few data are available on the risk of COVID-19 in other autoimmune diseases in which patients are frequently treated by immunosuppressants. The preliminary results of the Global Rheumatology Alliance registry show a hospitalization rate of 35% (vs 21% in our population) and a death rate of 5% (vs 3.5% in our cohort) in 110 patients with various autoimmune pathologies.[17] The identification of risk factors associated with COVID-19 severity in this population will be particularly relevant compared with our cohort.

Limitations

Limitations may include a potential referral bias toward severe COVID-19 or patients receiving DMTs who may have been more concerned about COVID-19 symptoms than patients who are untreated. It is also important to note that some immunosuppressive treatments, notably cladribine and alemtuzumab, are not commonly used in France, so it is not possible to analyze specifically whether these treatments could increase the risk associated with COVID-19.

Conclusions

COVID-19 has significantly changed medical practice for several weeks, and it is likely to remain an issue for months. In patients with MS, we identified disability, age, and obesity as the main risk factors for COVID-19 severity. Our data do not support an increased risk of severe outcome associated with DMTs, which should reinforce the recommendation of not stopping current DMTs and not delaying treatment initiation in patients who have higher disease inflammatory activity, risk for relapses, or subsequent disability. Conversely, patients with high EDSS and older age are at highest risk of severe COVID-19. Given the importance of these 2 variables on COVID-19 outcomes, the identification of a potential outcome of certain DMTs on COVID-19 risk would require a large international effort to collect data and a specific focus on patients with less advanced disease that also considers known comorbidities for COVID-19.

ARTICLE INFORMATION

Accepted for Publication: May 28, 2020.

Published Online: June 26, 2020. doi:10.1001/jamaneurol.2020.2581

Author Affiliations: Institut du Cerveau et de la Moelle Épinière, Assistance Publique des Hôpitaux de Paris, Hôpital de la Pitié Salpêtrière, Sorbonne Université, Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, Clinical Investigation Center Neuroscience, Paris, France (Louapre, Stankoff, Papeix, Lubetzki); Service de Neurologie, Clinical Investigation Center Institut National de la Santé et de la Recherche Médicale 1434, Centre Hospitalier Universitaire de Strasbourg, Strasbourg, France (Collongues, Bigaut, De Sèze); Hôpital St Antoine, Assistance Publique des Hôpitaux de Paris, Paris, France (Stankoff, Giannesini); Département de Neurologie, Hôpital Fondation Adolphe de Rothschild, Paris, France (Bensa, Deschamps); Service de Neurologie, Centre de Ressources et de Compétences–Sclérose en Plaques, Assistance Publique des Hôpitaux de Paris, Groupe Hospitalier Henri Mondor, Université Paris-Est Créteil, Créteil, France (Créange, Wahab); Service de Neurologie, Pôle de Neurosciences Cliniques, Assistance Publique–Hôpitaux de Marseille, Hôpital de la Timone, Aix Marseille Université, Marseille, France (Pelletier); Département de Neurologie, Centre Hospitalier de Poissy, St Germain, France (Heinzlef); Département de Neurologie, Centre Hospitalier Universitaire de Montpellier, Montpellier, France (Labauge); Association des Neurologues Libéraux de Langue Française, Bergerac, France (Guilloton); Département de Neurologie, Hôpitaux Civils de Colmar, Colmar, France (Ahle); Service de Neurologie, Groupe Hospitalier de la Region de Mulhouse, Mulhouse, France (Goudot); Centre de Recherche en Transplantation et Immunologie–Institut National de la Santé et de la Recherche Médicale U1064, Service Neurologie, Clinical Investigation Center 1413, Centre Hospitalier Universitaire Nantes, Nantes, France (Laplaud); Hôpital Neurologique Pierre Wertheimer, Hospices Civils de Lyon, Lyon/Bron, France (Vukusic).

Author Contributions: Dr Louapre had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Concept and design: Collongues, Papeix, Pelletier, Vukusic, de Seze.
Acquisition, analysis, or interpretation of data: All authors.
Drafting of the manuscript: Louapre, Collongues, Pelletier, Labauge, Vukusic, de Seze.
Critical revision of the manuscript for important intellectual content: Collongues, Stankoff, Giannesini, Papeix, Bensa, Deschamps, Créange, Wahab, Pelletier, Heinzlef, Guilloton, Ahle, Goudot, Bigaut, Laplaud, Vukusic, Lubetzki, de Seze.
Statistical analysis: Louapre.
Administrative, technical, or material support: Créange, Wahab, Labauge, Ahle, Laplaud, Vukusic.
Supervision: Collongues, Stankoff, Pelletier,

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a University of Exeter User on 06/27/2020

Heinzlef, Guilloton, Ahle, Vukusic, Lubetzki, de Seze.

Conflict of Interest Disclosures: Dr Louapre has received consulting or travel fees from Biogen, Novartis, Roche, Sanofi, Teva and Merck Serono, outside the submitted work. Dr Collongues serves on scientific advisory boards for and has received honoraria from Biogen Idec, Merck Serono, Sanofi-Genzyme, Bayer Schering Pharma and Alexion Pharmaceutical, and personal fees and nonfinancial support from Roche, Biogen, Novartis, Sanofi, and MedDay outside the submitted work outside the submitted work. Dr Stankoff has received fees for advisory boards and lectures from Genzyme, Novartis, Teva, and Biogen and research support from Roche, Sanofi-Genzyme, and Merck-Serono outside the submitted work. Dr Papeix has received consulting and lecturing fees and travel grants from Biogen, Genzyme, Novartis, Merck, Roche, Sanofi, and Teva Pharma outside the submitted work. Dr Bensa has received consulting fees for advisory boards from Biogen, Novartis, Roche, and Sanofi Genzyme outside the submitted work. Dr Deschamps has received travel grants from Biogen outside the submitted work. Dr Créange has received departmental research grants from Biogen, GeNeuro, MedDay, Novartis, Octapharma, and Roche; completed expert testimony with Biogen, Novartis, GeNeuro, and Roche; and received grants and personal fees from MedDay outside the submitted work. Dr Wahab has received expert testimony fees from Sanofi-Genzyme and Roche and travel grants from Biogen and Roche outside the submitted work. Dr Pelletier has received consulting or travel fees from Biogen, Novartis, Roche, Sanofi, Teva, MedDay, and Merck Serono outside the submitted work. Dr Heinzlef has received consulting or travel fees from Biogen, Novartis, Roche, Sanofi, Teva, Merck Serono, and MedDay outside the submitted work. Dr Guilloton has received consulting or travel fees from Biogen, Merck Serono, Novartis, Sanofi, and Teva outside the submitted work. Dr Ahle has received consulting or travel fees from AbbVie, Biogen, Novartis, Roche, and Sanofi outside the submitted work and reported grants from Roche, Novartis, Biogen, and Sanofi during the conduct of the study. Dr Bigaut has received travel grants from Biogen Idec and Sanofi-Genzyme outside the submitted work. Dr Laplaud has received grants from the ARSEP Foundation and MedDay; personal fees from Biogen, Sanofi-Genzyme, Merck, Celgene, Roche, MedDay, and Teva; grants and personal fees from Novartis; and consulting and lecturing fees, travel grants, and unconditional research support from Biogen, Genzyme, Novartis, Merck Serono, Roche, Sanofi-Aventis, and Teva Pharma outside the submitted work. Dr Vukusic has received grants, personal fees and nonfinancial support from Biogen, Merck, Novartis, Sanofi-Genzyme, Roche, Teva, Celgene, and MedDay and grants from Novartis, Roche, Sanofi, and Teva outside the submitted work. Dr Lubetzki reports grants and personal fees from Biogen and personal fees from Merck-Serono, Roche, Rewind, and Ipsen outside the submitted work. Dr De Sèze has received consulting fees from Biogen, Roche, Novartis, Teva, Cellgen, Jansen, and Sanofi-Genzyme and contracted research from Novartis and Sanofi-Genzyme outside the submitted work.

Funding/Support: This study did not receive specific funding. The French Clinical Research Infrastructure Network for Multiple Sclerosis is supported by a grant provided by the French State and handled by the Agence Nationale de la Recherche, within the framework of the Investments for the Future program, and support from the Fondation d'Aide pour la Recherche sur la Sclerose en Plaques (ARSEP). Paris Brain Institute Clinical Research Infrastructure Network is supported by a grant provided by the French State and handled by the Agence Nationale de la Recherche, within the framework of the Institut Hospitalo-Universitaire program. The Observatoire Français de la Sclérose en Plaques is supported by a grant provided by the French State and handled by the Agence Nationale de la Recherche, within the framework of the Investments for the Future program (ANR-10-COHO-002), the Eugène Devic EDMUS Foundation Against Multiple Sclerosis, and the ARSEP Foundation.

Role of the Funder/Sponsor: The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Group Information: Covisep Investigators: Fayçal Derouiche, MD, Mulhouse Neurological Office, Mulhouse, France; Ayman Tourbah, MD, Assistance Publique des Hôpitaux de Paris, Hôpital Raymond Poincaré, Garches, France; Guillaume Mathey, MD, Nancy University Hospital, Nancy, France; Marie Théaudin, MD, Lausanne University Hospital and University of Lausanne, Lausanne, Switzerland; François Sellal, MD, Colmar Hospital, Colmar, France; Marie-Hélène Dugay, MD, Colmar Hospital, Colmar, France; Helene Zéphir, MD, Lille University Hospital, Lille, France; Patrick Vermersch, MD, Lille University Hospital, Lille, France; Françoise Durand-Dubief, MD, Lyon University Hospital, Lyon, France; Romain Marignier, MD, Lyon University Hospital, Lyon, France; Géraldine Androdias-Condemine, MD, Lyon University Hospital, Lyon, France; Julie Pique, MD, Lyon University Hospital, Lyon, France; Pékès Codjia, MD, Lyon University Hospital, Lyon, France; Caroline Tilikete, MD, Lyon University Hospital, Lyon, France; Véronique Marcaud, MD, Paris Saint-Joseph Hospital, Paris, France; Christine Lebrun-Frenay, MD, Nice University Hospital, Nice, France; Mikael Cohen, MD, Nice University Hospital, Nice, France; Aurelian Ungureanu, MD, Assistance Publique des Hôpitaux de Paris, Saint-Antoine Hospital, Paris, France; Elisabeth Maillart, MD, Assistance Publique des Hôpitaux de Paris, Pitié-Salpêtrière Hospital, Paris, France; Ysoline Beigneux, MD, Assistance Publique des Hôpitaux de Paris, Pitié-Salpêtrière Hospital, Paris, France; Thomas Roux, MD, Assistance Publique des Hôpitaux de Paris, Pitié-Salpêtrière Hospital, Paris, France; Jean-Christophe Corvol, MD, Assistance Publique des Hôpitaux de Paris, Pitié-Salpêtrière Hospital, Paris Brain Institute, Paris, France; Amandine Bordet, MSc, Assistance Publique des Hôpitaux de Paris, Pitié-Salpêtrière Hospital, Paris Brain Institute, Paris, France; Yanica Mathieu, PhD, French Clinical Research Infrastructure Network for Multiple Sclerosis, Paris Brain Institute, Assistance Publique des Hôpitaux de Paris, Paris, France; Frédérique Le Breton, MD, Assistance Publique des Hôpitaux de Paris, Tenon Hospital, Paris, France; Dalia Dimitri Boulos, MD, Assistance Publique des Hôpitaux de Paris, Bicêtre Hospital, Kremlin-Bicêtre, France; Olivier Gout, MD, Fondation Ophtalmologique Adolphe de Rothschild, Paris, France; Antoine Guéguen, MD, Fondation Ophtalmologique Adolphe de Rothschild, Paris, France; Antoine Moulignier, MD, Fondation Ophtalmologique Adolphe de Rothschild, Paris, France; Marine Boudot, MD, Fondation Ophtalmologique Adolphe de Rothschild, Paris, France; Audrey Chardain, MD, Vincennes Neurological Office, Vincennes, France; Sarah Coulette, MD, Fondation Ophtalmologique Adolphe de Rothschild, Paris, France; Eric Manchon, MD, Gonesse Hospital, Gonesse, France; Samar S. Ayache, MD, Assistance Publique des Hôpitaux de Paris, Henri Mondor Hospital, Créteil, France; Thibault Moreau, MD, Dijon University Hospital, Dijon, France; Pierre-Yves Garcia, MD, Compiegne Noyon Hospital, Compiègne, France; Deiva Kumaran, MD Assistance Publique des Hôpitaux de Paris, Bicêtre Hospital, Kremlin Bicêtre, France; Giovanni Castelnovo, MD, Nîmes University Hospital, Nîmes, France; Eric Thouvenot, MD, Nîmes University Hospital, Nîmes, France; Frederic Taithe, MD, Clermont-Ferrand University Hospital, Clermont-Ferrand, France; Julien Poupart, MD, Groupe Hospitalier Institut Catholique de Lille, Lille, France; Arnaud Kwiatkowski, MD, Groupe Hospitalier Institut Catholique de Lille, Lille, France; Gilles Defer, MD, Caen University Hospital, Caen, France; Nathalie Derache, MD, Caen University Hospital, Caen, France; Pierre Branger, MD, Caen University Hospital, Caen, France; Damien Biotti, MD, Toulouse University Hospital, Toulouse, France; Jonathan Ciron, MD, Toulouse University Hospital, Toulouse, France; Christine Clerc, MD, Montbeliard Neurological office, Montbeliard, France; Mathieu Vaillant, MD, Grenoble University Hospital, Grenoble, France; Laurent Magy, MD, Limoges University Hospital, Limoges, France; Alexis Montcuquet, MD, Limoges University Hospital, Limoges, France; Philippe Kerschen, MD, Luxembourg Hospital, Luxembourg City, Luxembourg; Marc Coustans, MD, Quimper Hospital, Quimper, France; Anne-Marie Guennoc, MD, Tours University Hospital, Tours, France; Bruno Brochet, MD, Bordeaux University Hospital, Bordeaux, France; Jean-Christophe Ouallet, MD, Bordeaux University Hospital, Bordeaux, France; Aurélie Ruet, MD, Bordeaux University Hospital, Bordeaux, France; Cécile Dulau, MD, Bordeaux University Hospital, Bordeaux, France; Sandrine Wiertlewski, MD, Nantes University Hospital, Nantes, France; Eric Berger, MD, Besançon Hospital, Besançon, France; Dan Buch, MD, Assistance Publique des Hôpitaux de Paris, Lariboisière Hospital, Paris, France; Bertrand Bourre, MD, Rouen University Hospital, Rouen, France; Maud Pallix-Guiot, MD, Orleans Hospital, Orleans, France; Aude Maurousset, MD, Tours University Hospital, Tours, France; Bertrand Audoin, MD, Assistance Publique des Hôpitaux de Marseille, Marseille, France; Audrey Rico, MD, Assistance Publique des Hôpitaux de Marseille, Marseille, France; Adil Maarouf, MD, Assistance Publique des Hôpitaux de Marseille, Marseille, France; Gilles Edan, MD, Rennes University Hospital, Rennes, France; Jérémie Papassin, MD, Chambéry Hospital, Chambéry, France; Dorothée Videt, MD, Vannes Hospital, Vannes, France.

Additional Contributions: We thank Assistance Publique des Hôpitaux de Paris and French Clinical Research Infrastructure Network for Multiple Sclerosis for the support in regulatory aspects of the study, Amandine Bordet, MSc, Assistance

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a University of Exeter User on 06/27/2020

Publique des Hôpitaux de Paris, Pitié-Salpêtrière Hospital, Paris Brain Institute, and Yanica Mathieu, PhD, French Clinical Research Infrastructure Network for Multiple Sclerosis, Paris Brain Institute, Assistance Publique des Hôpitaux de Paris, for setting up the electronic clinical record form and the data management. We thank the Société Francophone de la Sclérose en Plaques, the Observatoire Français de la Sclérose en Plaques group, all the Covisep investigators, clinical research staff, and the patients for helping to collect these data. The named individuals were not compensated for their help.

REFERENCES

1. Zhou F, Yu T, Du R, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet*. 2020;395(10229):1054-1062. doi:10.1016/S0140-6736(20)30566-3

2. Multiple Sclerosis International Federation. The coronavirus and MS—updated global advice. Published April 13, 2020. Updated June 18, 2020. Accessed June 19, 2020. https://www.msif.org/news/2020/02/10/the-coronavirus-and-ms-what-you-need-to-know/.

3. Brownlee W, Bourdette D, Broadley S, Killestein J, Ciccarelli O. Treating multiple sclerosis and neuromyelitis optica spectrum disorder during the COVID-19 pandemic. *Neurology*. 2020;94(22):949-952. doi:10.1212/WNL.0000000000009507

4. Giovannoni G, Hawkes C, Lechner-Scott J, Levy M, Waubant E, Gold J. The COVID-19 pandemic and the use of MS disease-modifying therapies. *Mult Scler Relat Disord*. 2020;39:102073. doi:10.1016/j.msard.2020.102073

5. Cao B, Wang Y, Wen D, et al. A trial of lopinavir-ritonavir in adults hospitalized with severe COVID-19. *N Engl J Med*. 2020;382(19):1787-1799. doi:10.1056/NEJMoa2001282

6. Salje H, Tran Kiem C, Lefrancq N, et al. Estimating the burden of SARS-CoV-2 in France. *Science*. 2020;eabc3517. doi:10.1126/science.abc3517

7. Wu C, Chen X, Cai Y, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. *JAMA Intern Med*. 2020. Published online March 13, 2020. doi:10.1001/jamainternmed.2020.0994

8. Yan CH, Faraji F, Prajapati DP, Ostrander BT, DeConde AS. Self-reported olfactory loss associates with outpatient clinical course in COVID-19. *Int Forum Allergy Rhinol*. 2020. doi:10.1002/alr.22592

9. Richardson S, Hirsch JS, Narasimhan M, et al; and the Northwell COVID-19 Research Consortium. Presenting characteristics, comorbidities, and outcomes among 5700 patients hospitalized with COVID-19 in the New York City area. *JAMA*. 2020. doi:10.1001/jama.2020.6775

10. Louapre C, Maillart E, Roux T, et al. Patients with MS treated with immunosuppressive agents: across the COVID-19 spectrum. *Rev Neurol (Paris)*. 2020;176(6):523-525. doi:10.1016/j.neurol.2020.04.009

11. Novi G, Mikulska M, Briano F, et al. COVID-19 in a MS patient treated with ocrelizumab: does immunosuppression have a protective role? *Mult Scler Relat Disord*. 2020;42:102120. doi:10.1016/j.msard.2020.102120

12. Stokmaier D, Winthrop K, Chognot C, et al Effect of ocrelizumab on vaccine responses in patients with multiple sclerosis. *Neurology.* 2018; 90(15 supplement).

13. Chen N, Zhou M, Dong X, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet*. 2020;395(10223):507-513. doi:10.1016/S0140-6736(20)30211-7

14. Zheng KI, Gao F, Wang XB, et al. Letter to the editor: obesity as a risk factor for greater severity of COVID-19 in patients with metabolic associated fatty liver disease. *Metabolism*. 2020;108:154244. doi:10.1016/j.metabol.2020.154244

15. Levy J, Bensmail D, Brotier-Chomienne A, et al. Respiratory impairment in multiple sclerosis: a study of respiratory function in wheelchair-bound patients. *Eur J Neurol*. 2017;24(3):497-502. doi:10.1111/ene.13231

16. Verity R, Okell LC, Dorigatti I, et al. Estimates of the severity of coronavirus disease 2019: a model-based analysis. *Lancet Infect Dis*. 2020;20(6):669-677. doi:10.1016/S1473-3099(20)30243-7

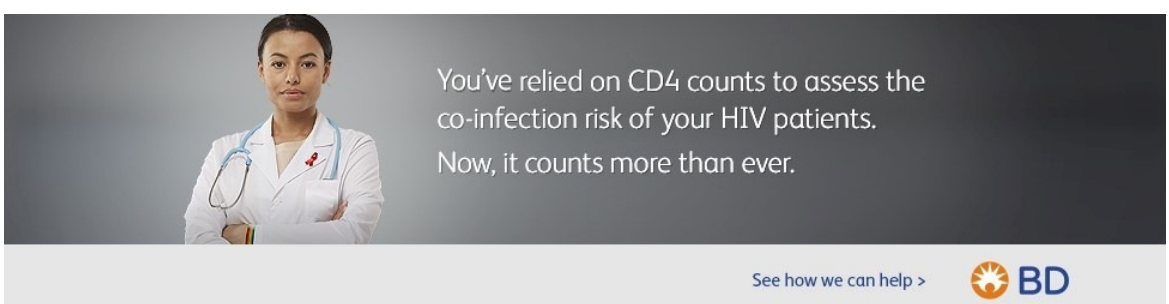
17. Gianfrancesco MA, Hyrich KL, Gossec L, et al; COVID-19 Global Rheumatology Alliance Steering Committee. Rheumatic disease and COVID-19: initial data from the COVID-19 Global Rheumatology Alliance provider registries. *Lancet Rheumatol*. 2020;2(5):E250-E253. doi:10.1016/S2665-9913(20)30095-3

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a University of Exeter User on 06/27/2020

High-dose but not low-dose corticosteroids potentially delay viral shedding of patients with COVID-19

Sijia Li, Xinyu Song, Zhigang Hu



Downloaded from https://academic.oup.com/cid/article-abstract/doi/10.1093/cid/ciaa829/5863128 by guest on 27 June 2020

High-dose but not low-dose corticosteroids potentially delay viral shedding of patients with COVID-19

Sijia Li*[1,2] , Zhigang Hu[# 1,2] , Xinyu Song[# 1,2]

1. Department of Respiratory and Critical Care Medicine ,The first College of Clinical Medicine science, China Three Gorges University, Yichang 443003, People's Republic of China

2. Department of Respiratory and Critical Care Medicine, Yichang Central People's Hospital Yichang 443003, People's Republic of China

Zhigang Hu and Xinyu Song are listed as co-corresponding author.

Corresponding author:

Zhigang Hu, MD Department of Respiratory medicine, the first College of Clinical medicine science, Three Gorges University, NO. 183 Yiling Road, Yichang 443003, People's Republic of China. E-mail: hxq910813@163.com

Phone: 0717-6486930 ORCID ID:https://orcid.org/0000-0003-3421-0674

© The Author(s) 2020. Published by Oxford University Press for the Infectious Diseases Society of America. All rights reserved. For permissions, e-mail: journals.permissions@oup.com.

Accepted Manuscript

Downloaded from https://academic.oup.com/cid/article-abstract/doi/10.1093/cid/ciaa829/5863128 by guest on 27 June 2020

Dear Editors:

Considering cytokine storm secondary to SAR-Cov-2 infection, some patients with COVID-19 received the treatment of systemic corticosteroids, especially severe and critical patients. Systemic corticosteroid (methylprednisolone, <1-2 mg/kg, 3-5 days) is recommended as adjuvant therapy of COVID-19 in China [1]. We read with interest the recent study by Xu et al [2] who determined risk factor associated with prolonged viral shedding in patients with COVID-19. They reported that use of corticosteroids was associated with higher probability of late viral RNA clearance (≥15 days after illness onset) in unvariate logistic regression analysis (P = 0.025), but not shown in multivariate logistic regression analysis (OR= 1.38, 95%CI: 0.53, 3.65, P = 0.519).

Systemic corticosteroids always was controversy in treating viral pneumonia. A newly published meta-analysis demonstrated that corticosteroids treatment was associated with longer length of stay, higher probability of bacterial infection and mortality among patients with coronavirus pneumonia [3]. In addition, whether systemic corticosteroids delay viral clearance is another topic priority. The first randomized controlled trial about corticosteroids and viral clearance observed that patients with early use of hydrocortisone harbored higher plasma SARS-CoV viral load and longer time of viral shedding than those without hydrocortisone [4]. During the outbreak of Middle East respiratory syndrome(MERS), 48.9% critical patients received the treatment of corticosteroids in Saudi Arabian and early use (< 7 days

Accepted Manuscript

Downloaded from https://academic.oup.com/cid/article-abstract/doi/10.1093/cid/ciaa829/5863128 by guest on 27 June 2020

after hospital) of high-dose (methylprednisolone equivalent dose > 60 mg/d, aHR=0.26, 95%CI : 0.09, 0.77, P =0.015) and lose-dose corticosteroids (≤ 60 mg/d, aHR=0.41, 95%CI : 0.99, 0.88, P =0.022) also delayed viral shedding compared with no use of corticosteroids [5]. High-dose corticosteroids seemingly were more likely to prolong viral clearance of MERS. Ogimi and the colleagues [6] further suggested that high-dose steroids (> 1 mg/kg) were associated with prolonged shedding of human coronavirus than low-dose steroids (≤ 1 mg/kg). Actually, a dose-response manner about corticosteroid and viral shedding also was shown in influenza viral pneumonia. Two studies from Cao et al [7] and Boudreault et al [8] also observed that prolonged shedding of influenza viral was found in high-dose corticosteroids, but not shown in low-dose corticosteroids.

We collected more than 60 variables from 206 patients with COVID-19 to assess risk factors of long-term (> 30 days) positive SARS-CoV-2 and viral shedding. Least absolute shrinkage and selection operator (LASSO) analysis effectively resolve the colinearity of high-dimensional data and performed tuning parameter selection used 10-fold cross-validation [9]. LASSO analysis with logistic regression model indicated no impact of corticosteroids on long-term positive SARS-CoV-2. However, LASSO analysis with Cox regression model and restricted mean survival time analysis demonstrated that high-dose (80mg/d, aHR= 0.67, 95%CI: 0.46 - 0.96, P = 0.031) but not low-dose corticosteroids (40mg/d, aHR= 0.72, 95%CI: 0.48 - 1.08, P = 0.11) potentially delayed viral shedding of patients with COVID-19.

Accepted Manuscript

Downloaded from https://academic.oup.com/cid/article-abstract/doi/10.1093/cid/ciaa829/5863128 by guest on 27 June 2020

Our study suggested that the effect of corticosteroids on viral shedding maybe a dose-response manner in Cox regression analysis. In addition, high-dose but not low-dose corticosteroids were found to potentially increase the mortality of patients with severe COVID-19 [10]. Therefore, use of high-dose corticosteroids should be extreme caution in treating COVID-19.

Accepted Manuscript

Downloaded from https://academic.oup.com/cid/article-abstract/doi/10.1093/cid/ciaa829/5863128 by guest on 27 June 2020

Contributions: All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Ethics approval: All patients gave written informed consent and the study was approved by the ethics committee of Yichang Central People's Hospital.

Funding: The work was supported by Natural Science Foundation of Hubei province (No.2018CFB643).

Conflict of interest

This was not an industry supported study. The authors have declared that there are no conflicts of interest.

Accepted Manuscript

Downloaded from https://academic.oup.com/cid/article-abstract/doi/10.1093/cid/ciaa829/5863128 by guest on 27 June 2020

References

1. National Health Commission of the People's Republic of China. The notice of launching guideline on diagnosis and treatment of the novel coronavirus pneumonia (NCP). Revised version of the 5th edition. http://www.nhc.gov.cn/xcs/zhengcwj/202002/d4b895337e19445f8d728fcaf1e3e13a.shtml (accessed Feb 8, 2020; in Chinese).

2. Xu K, Chen Y, Yuan J, et al. Factors associated with prolonged viral RNA shedding in patients with COVID-19.Clin Infect Dis 2020;:

3. Yang Z,Liu J,Zhou Y,et al.The effect of corticosteroid treatment on patients with coronavirus infection: a systematic review and meta-analysis.J Infect 2020;:

4. Lee N, Allen Chan KC, Hui DS, et al. Effects of early corticosteroid treatment on plasma SARS-associated Coronavirus RNA concentrations in adult patients. J Clin Virol 2004; 31: 304-9.

5. Arabi YM, Mandourah Y, Al-Hameed F, et al. Corticosteroid Therapy for Critically Ill Patients with Middle East Respiratory Syndrome. Am J Respir Crit Care Med 2018; 197: 757-67.

6. Ogimi C, Greninger AL, Waghmare AA, et al. Prolonged Shedding of Human Coronavirus in Hematopoietic Cell Transplant Recipients: Risk Factors and Viral Genome Evolution. J Infect Dis. 2017;216:203-9.

Accepted Manuscript

Downloaded from https://academic.oup.com/cid/article-abstract/doi/10.1093/cid/ciaa829/5863128 by guest on 27 June 2020

7. Cao B, Gao H, Zhou B, Deng X, Hu C, Deng C, et al. Adjuvant corticosteroid treatment in adults with inflfluenza A (H7N9) viral pneumonia. Crit Care Med 2016;44(6):e318–28 PMID: 26934144.

8. Boudreault AA, Xie H, Leisenring W, et al. Impact of corticosteroid treatment and antiviral therapy on clinical outcomes in hematopoietic cell transplant patients infected with influenza virus. Biol Blood Marrow Transplant. 2011;17:979-86.

9. Avalos M,Adroher ND,Lagarde E,et al.Prescription-drug-related risk in driving: comparing conventional and lasso shrinkage logistic regressions.Epidemiology 2012;23:706-712.

10. Li X, Xu S, Yu M, et al. Risk factors for severity and mortality in adult COVID-19 inpatients in Wuhan.J Allergy Clin Immunol 2020;:

Accepted Manuscript

Downloaded from https://academic.oup.com/cid/article-abstract/doi/10.1093/cid/ciaa829/5863128 by guest on 27 June 2020