Platforms for Personalized Polytherapeutics Discovery in COVID-19

Christopher Hopkins [1*], **Chidinma Onweni** [3], **Victoria Zambito** [3],
DeLisa Fairweather [2], **Kathryn McCormick** [1], **Hideki Ebihara** [3], **Thomas Caulfield** [2],
Yu Shrike Zhang [4] **and W. David Freeman** [2]

1 - *InVivo Biosystems,* Eugene, OR, United States
2 - *Mayo Clinic,* Jacksonville, FL, United States
3 - *Mayo Clinic,* Rochester, MN, United States
4 - *Brigham and Women's Hospital,* Harvard Medical School, Cambridge, MA, United States

Correspondence to Christopher Hopkins: chris.hopkins@invivobiosystems.com (C. Hopkins)
https://doi.org/10.1016/j.jmb.2021.166945
Edited by Eric O. Freed

Abstract

The COVID-19 pandemic entered its third and most intense to date wave of infections in November 2020. This perspective article describes how combination therapies (polytherapeutics) are a needed focus for helping battle the severity of complications from SARS-CoV-2 infection. It outlines the types of systems that are needed for fast and efficient combinatorial assessment of therapeutic candidates. Proposed are micro-physiological systems using human iPSC as a format for tissue-specific modeling of infection, the use of gene-humanized zebrafish and *C. elegans* for combinatorial drug screens due to the animals being addressable in liquid multi-well formats, and the use of engineered pseudo-typing systems to safely model infection in the transgenic animals and engineered tissue systems.

Perspective

During the years of 2020–21, the COVID-19 pandemic infected over 1% of the planet's human population.[1] Of the 113 million confirmed cases of COVID-19 by February 2021, 2.2% of the infected have died (2.5 million) (Figure 1). Without intervention we might be destined to follow in the footsteps of the 1918 influenza pandemic where 1/3 of the world's population was infected.[1] The grim forecast would indicate a potential for over 200 million to die in the next few years from SARS-CoV-2. In perspective, the number of deaths would be close to 2x the number of military and civilian casualties from World War I and II combined.[2,3] If we are to prevent this number of deaths we must find better ways to contain, manage, and treat this potentially devastating disease.

Infection Severity and Economic Burden. COVID-19 infection presents with a wide range of clinical effects which is not surprising based on diversity of the human population in terms of age, gender, ethnicity, and comorbidities. Presentation ranges from asymptomatic carriers to the severe and chronically debilitated.[4–6] Also there is a large fraction of symptomatic individuals who are increasingly recognized as so-called 'long haulers' or chronic COVID syndrome who have mild to moderate symptoms that last for months, with neurologic symptoms[7] and others manifestations.[8] The complications from COVID-19 comorbidities have overwhelming hospitals and rehabilitative care.[9] As a result, the severity and longevity of the disease is contributing to the high health care burden. Splitting COVID-19 infected into 5 categories (asymptomatic, mild, severe, long haulers and lethal),
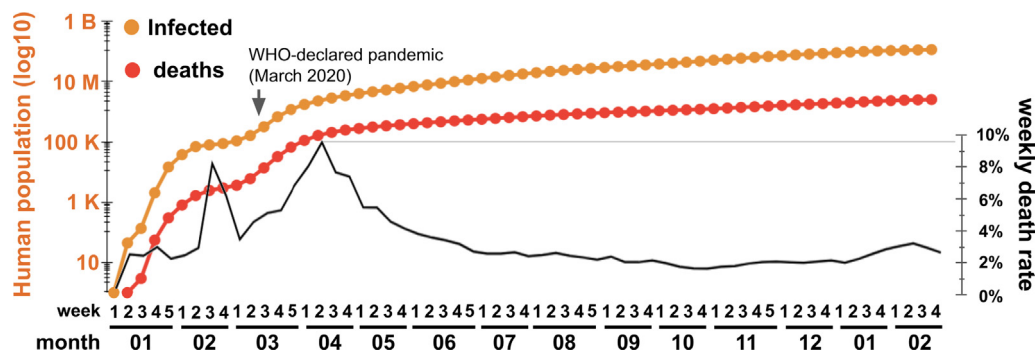
Figure 1. Rates for COVID-19 pandemic on infections and death rates. A log-linear plot shows the level of infections (orange) and deaths (red) since the first case was reported. Right axis is the weekly change in death rate (black line). Data source: https://covid19.who.int/.

healthcare system costs were estimated. To determine the direct healthcare costs to the asymptomatic and symptomatic categories, we first needed to estimate the societal load of asymptomatic carriers (persons testing positive but either not yet, or never, showing symptoms). Estimates vary widely from 5% to 80% depending on the source,[10] with credible sources for asymptomatic carrier load to be near 25% of the infected population.[11] Although the positive predictive value is poor for any individual tested in a large population where the COVID-19 incidence is low, the overall positive-testing population that is asymptomatic but infected is likely to still be close to 25%. The direct health care cost to these asymptomatic carriers is likely to be insignificant relative to the symptomatic infected, so the direct healthcare costs for the asymptomatic is assumed to be zero. Of the remaining 75% testing positive, they exhibit mild, severe or long-term symptoms. Half of the symptomatic are estimated to have "mild" symptoms that do not require hospitalization. While direct health care costs are lower and mainly associated with the cost of testing, the indirect economics or downtime from these individuals missing work and downstream productivity spans days to a few weeks. On the other extreme of the spectrum are the severe cases and critically ill who require hospitalization, who may require intensive care management for weeks and a few requiring months and lung transplantation. In the middle of this spectrum are long haulers whose full recovery from mild symptoms has yet to occur and may actually progress. The estimated distribution into these five classes results in mild cases as highest in frequency for infected populations (Figure 2(a)). Due to the daunting challenge of estimating world-wide healthcare costs, this article focuses on US healthcare costs as a country-specific example. The US is chosen because in November 2020, the US was reported to have the highest number of cumulative cases per population size (3.5%).[12] To facilitate intercountry comparisons, the rates per 100,000 were generated for 4 countries (Figure 2(b)). The US leads in

both the number of cases and infection rate. Compared to two countries with larger general populations, India and China, the infection numbers in the US are much larger. The country with infection numbers similar to the US is Brazil where the total number of deaths per 100,000 is just slightly higher than the US. However, the current 24 hour rate of death indicates that the US will soon take the lead in all 4 categories of Infection Numbers. To translate the infection burden into direct economic healthcare costs, it is assumed that the out-of-pocket burden to the healthcare system is low for victims experiencing mild symptoms and has been approximated at $500. The cost of an extended intensive care stay can range into the hundreds of thousands of dollars to even a million dollars. It is more difficult to estimate the financial burden for long haulers who experience chronic symptoms. For these symptomatic individuals, the estimated healthcare cost is $5000. Applying these estimates to the distribution of disease, a cumulative cost plot can be generated (Figure 2(c)). What is revealed is that the severe categories which contain only 10% of infected individuals leads to 70% of the financial burden. With the third wave of COVID-19 in the US being far higher than the previous two waves, the direct health care costs of the pandemic in this first year will likely reach 10 billion dollars. The indirect costs of this pandemic are likely to be high from impaired economic productivity of the U.S. workforce and might be orders of magnitude higher and will require further evaluation over time.

COVID-19 Vaccine. Decreasing the incidence and severity of SARS-CoV-2 infection will have a profound positive economic impact. For vaccine development, the Regulatory Affairs Professional Society is monitoring 50 clinical trials.[13] The first two vaccines that came out of trials are mRNA-based and efficacy appears to be near 90%.[14] Yet, there are reports of re-infection with different strains of COVID-19,[15,16] which is an established phenomenon for some coronavirus infections.[17–20] As a result, a significant concern is that, as SARS-CoV-2 spreads within the population, the known
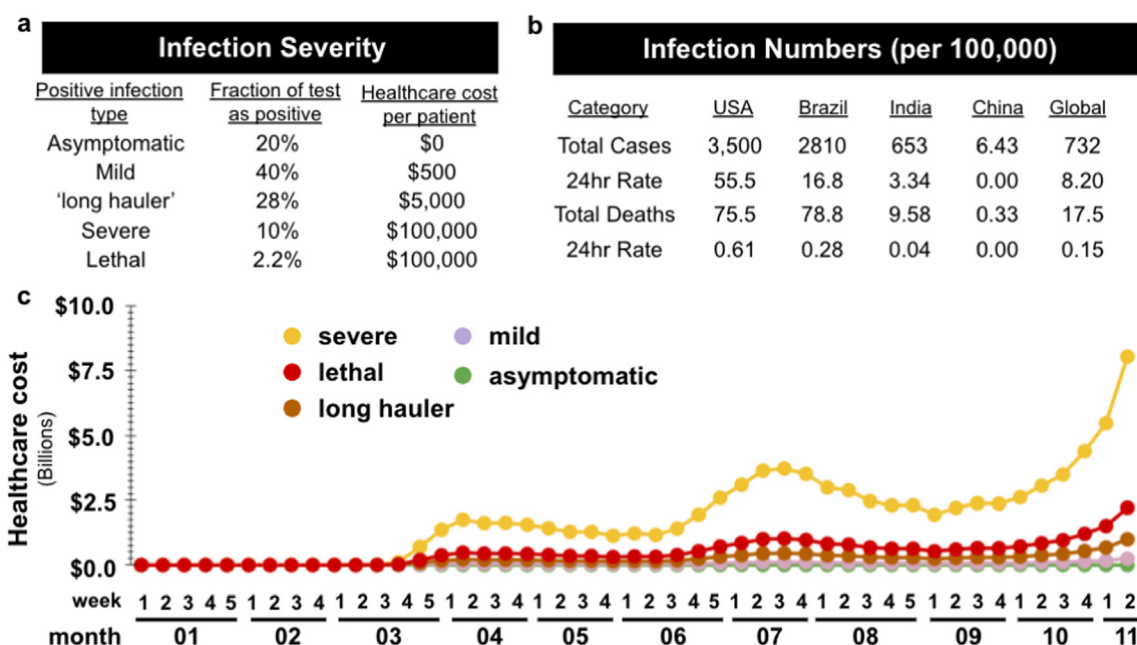
Figure 2. Infection type, load and healthcare costs of COVID-19 in the US. (a) Distribution of population and estimates for costs for 5 levels of COVID-19 infection severity. (b) 4 categories Accumulation and rates for infection by cases and deaths. (c) Estimate of accumulating healthcare costs in the US for the 5 levels of COVID-19 severity.

rare event of vaccine-resistant variants[21–24] will likely occur and lead to the virus becoming an endemic problem where these resistant strains arise.[25] One vaccine-to-fit-all is hopeful, but unlikely. A more likely scenario is that a new vaccine every year will be needed to target the most virulent strain of the season, similar to current influenza strategies. Successful vaccines will help stave off deaths, but society needs to quickly martial more resources and diverse approaches to minimize the severity of the pending loss of life. Masking, social distancing, and good hygiene are important practices to mitigate the spread of many viral diseases, but they do not disrupt the underlying molecular mechanisms to prevent infection.

Antivirals in Polytherapeutic Combinations. It is important to look to other diseases to provide guidance on therapeutic strategy. Using the HIV epidemic as a guide, we can see that combination therapies (polytherapeutics) were employed as effective measures that reduced disease mortality. An effective vaccine has not been developed for HIV and, as a result, antivirals are the mainstay of protection. As of 2019, HIV has infected 38 million people with 690,000 deaths from Acquired Immune Deficiency Syndrome (AIDS).[26] Because antivirals in multi-drug cocktail formulations have been highly successful in mitigating HIV progression to AIDS,[27,28] it may be possible to similarly manage COVID-19 by prophylactically reducing or preventing infection.[28] Yet, even when HIV infections are adequately managed in infected individuals, the risk of comorbidities remains high for heart, bone, liver, kidney, and neurological disease.[27,28] One of the first drug regimens used to treat HIV uses a daily oral dose with dual reverse transcriptase inhibitor cocktail (tenofovir disoproxil fumarate co-formulated with emtricitabine/TDF/FTC),[28] which was found to reduce infection by HIV as much as 86% in men who have sex with men[29] and 76% in heterosexual couples.[30] This prophylactic approach could be applied to SARS-CoV-2, where high risk, or susceptible, populations could be treated prior to infection and minimize symptomatic burden.

The development of a polytherapeutic approach to HIV has focused on inhibiting six main viral activities.[31] A similar prediction is that multi-drug targeted therapy could function prophylactically on SARS-CoV-2 to limit its infectivity. In HIV, the first type of molecules that are targeted are nucleic acid-based inhibitors of reverse transcriptase, which the virus needs to convert its mRNA into a DNA strand that can be integrated into the host genome. The second class of molecules that are targeted are non-nucleic acid-based scaffolds that interfere with reverse transcription of mRNA to DNA. The third are integrase inhibitors which prevent retroviral DNA from inserting into the host cell genome. The fourth are protease inhibitors that interfere with processing functional units of polypeptides. The fifth are fusion inhibitors that block fusion of viral particles to the plasma membrane. The sixth are co-receptor modulators that interfere with viral uptake. Applying this approach to SARS-CoV-2, the integrase inhibitors are not relevant because, unlike HIV,[32] coronavirus RNA does not need to be converted to DNA for integration into the host genome. Instead, its

infection cycle proceeds without genomic integration.[33] This viral strategy also makes the use of reverse transcriptase inhibitors irrelevant. However, inhibitors that are able to target the RNA-dependent RNA polymerase (RdRp) of coronaviruses would be useful.[33,34] The most promising anti-COVID-19 antiviral at the time of this writing is the nucleoside analog remdesivir targeting RdRp, which was found to be effective *in vitro*[33–35] and anecdotally.[36,37] As of November 17, 2020, remdesivir was the only FDA-approved drug for treatment of COVID-19,[38] and under Emergency Use Authorization (EUA) for mild to moderate COVID-19, the monoclonal antibody bamlanivimab can be used on patients at risk for developing severe COVID-19.[39]

Other Examples of Polytheraputics Successes. The use of combination therapy approaches are highly effective for some cancer treatments.[40,41] For instance, in treating pancreatic cancer, a four-drug cocktail (mFOLFIRINOX: folinic acid, 5-FU, irinotecan, and oxaliplatin) provided a near doubling of disease-free survival compared to monotherapy alone (gemcitabine).[42] Similarly, combined therapies in non-small cell lung cancer demonstrate higher efficacy and disease-free extension times.[43] In the infectious disease tuberculosis (TB), combination therapies (i.e., polytherapy) have been beneficial since the 1950s[44] particularly streptomycin combined with *para*-aminosalicylic acid. Current standard-of-care polytherapy for TB involves a four-drug regimen (isoniazid, rifampin, ethambutol, and pyrazinamide) in an 8-week intensive phase followed by an 18-week continuation phase (isoniazid and rifampin),[45] but this drug cocktail and regimen is ever changing due to drug resistance of the mycobacterium.[46,47]

Polytherapy Targeting COVID-19 Infection

Treatment. With the looming problem of COVID-19 infections becoming endemic worldwide, there is a pressing need for focused research efforts to provide novel therapeutic options. Most likely inhibiting SARS-CoV-2 infection will require a polytherapeutic approach to achieve maximum efficacy. Effective strategies will be multi-pronged and target multiple mechanisms inhibiting both viral activity and an overly aggressive host immune response. For example, a therapy targeting three pathways might involve 1) blocking viral entry of SARS-CoV-2 into host cells by preventing binding to ACE2, 2) blocking cytoplasmic entry via protease inhibitors that interfere with the TMPRSS2 protease activity needed for the virus's endosomal entry stage, and 3) preventing viral RNA replication by inhibiting RdRp polymerase, as has been demonstrated to be successful by the use of remdesivir. Other viral targets include the Mpro, CLpro, and PLpro proteases encoded by the virus that are needed

for processing the polyprotein after translation of the viral mRNA. On the host side, disease severity could be reduced by inhibiting overactive host responses. Disruption of the ACE2/S-protein interaction may be a 'double-edged sword'.[56] Viral binding to the receptor lowers ACE2 activity due to receptor internalization,[57] and antibody drugs targeting this interaction are starting to be used (casirivimab and zImdevimab). Yet, the potential negative consequences are an increase in hypertension and inflammation. RAS modulators (ARBs and ACEIs) are controversial for use in mediating the hypertensive/inflammatory consequence of COVID-19 because they upregulate ACE2.[58,59] Individuals using ACEIs/ARBs may be more susceptible to higher infectious load due to increased expression of ACE2 facilitating viral entry. Yet once infected, the negative consequences of unregulated RAS activity that can promote severe COVID-19, may overwhelm the negative consequences of facilitated viral entry.

The host immune response is known to mediate disease severity and comorbidity of presentation for most inflammatory diseases. A robust immune response aimed at eliminating the viral pathogen leads to an elevated interferons (IFNs), cytokines, chemokines, and cell-mediated innate response.[48,49] The genetic variation in the human population for the composition of cytokines and their receptors, among other immune factors, is likely to influence viral clearance and disease severity.[50] One viral infection strategy unique to SARS-CoV and SARS-CoV-2 is utilization of the renin-angiotensin system (RAS). Both viruses use angiotensin converting enzyme 2 (ACE2) as a cellular receptor it uses to get into the cell and make more virions. As discussed further below, ACE2 has an important role in regulating the hypertensive effects of angiotensin on RAS.[51,52] The virus binds ACE2 via its spike (S) protein,[52] which activates internalization of the virus into the cell. Blocking the binding interaction of the SARS-CoV-2 S-protein with ACE2 is not only a key target for vaccines but also for antiviral therapies. However, a drug would need to prevent viral interaction with ACE2 but not interfere with normal activity of ACE2, which may be technically difficult to achieve.

One way the body maintains normal homeostatic activity is through the production of angiotensin (Figure 3). First the liver makes angiotensinogen which is cleaved by kidney-derived renin to make angiotensin I. Angiotensin I is cleaved by ACE to make angiotensin II (ang II), which is then broken down by ACE2 to make angiotensin 1–7 (ang 1–7). The production of ang II occurs in a variety of tissues (heart, kidneys, endothelium, testes, gastrointestinal tract, and the lungs).[51,53] This stimulates 1) aldosterone production, 2) perfusion of the glomerulus, 3) blood vessel/ endothelium growth (angiogenesis), 4) cardiac myocytes growth/ cardiac remodeling, 5) proinflammatory response by
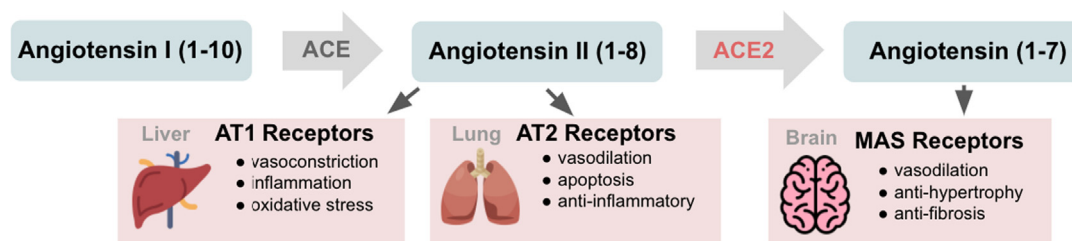
Figure 3. Renin-angiotensin system uses peptide signaling to regulate hypertension and inflammatory/stress response. Angiotensin I peptide is cleaved by ACE protease to produce angiotensin II, which is further cleaved by ACE2 to make angiotensin 1–7. Products of angiotensin cleavage create ligands for angiotensin receptors (AT1 type 1 and AT2 type 2) and the MAS receptor. Effects of native ligand interaction are pro-hypertensive (AT1 type 1 receptors) or anti-hypertensive (AT2 type 2 and MAS receptors).

increasing IL-2 and IL-6 production, and 6) increases blood pressure by causing vasoconstriction in the pulmonary vasculature. In order to maintain homeostasis (i.e., preventing the ang II from remaining in a continuously elevated state), the activity of ACE2 to make ang 1–7 from angII has counter-effects of being anti-hypertensive and anti-inflammatory via activation of AT2 type 2 and other similar-functioning receptors. Dysregulation of these pathways occurs in many diseases such as heart failure, chronic kidney disease, and diabetes.[53] Further, these diseases can be regulated by a variety of ACE inhibitors (ACEIs) and angiotensin receptor blockers (ARBs). Regarding COVID-19 infection, interfering with ACE2 function by viral-induced internalization is hypothesized to suppress ang 1–7 production leading to elevated blood pressure and further promoting inflammation due to elevated ang II and lung injury.[54,55]

Compatibility. For polytherapeutic prescriptions, and in drug compounding, the absorption, distribution, metabolism, excretion, and toxicity (ADME-tox) parameters of drug metabolism and interactions should be considered. For example, Trandolaprilat and Candesartan are well-matched for regimen timing (Tables 1 and 2) and they lack significant cytochrome P450 complications in the general population.[60] Animal studies indicate that remdesivir is an effective antiviral when administered either prophylactically or shortly after infection, but its oral bioavailability is poor and therefore must be administered via injection.[61] Nevertheless, the combination of remdesivir with ACEIs/ARBs could theoretically provide a synergistic effect on reducing viral replication in the host cell and viral-induced disruption of ACE2 activity being reduced. For prophylactic use in the general public, the ideal drug combination would have a matched oral bioavailability. Therefore there is a need to identify RdRp inhibitors with greater oral bioavailability.

Blocking Viral Infection Activity. In addition to the aforementioned drug combinations, additional targets for drug development could be developed to increase the combinatorial capacity. An obvious target is viral entry (Figure 4). For example,

blocking viral entry without disrupting normal ACE2 activity is desired. Antibodies as antivirals targeting the S-protein have the potential to exhibit this favorable effect. Another approach to blocking viral entry would be to introduce soluble ACE2 systemically to a COVID-19 patient. Recombinant/soluble ACE2 as a therapy for controlling hypertension is currently in clinical trials.[61,62] A possible polytherapy is the combination of remdesivir with recombinant ACE2, which has recently demonstrated improved therapeutic outcomes in kidney organoid infection model.[63] When recombinant ACE2 alone was delivered to a patient with severe COVID-19, an imbalance in RAS peptides was restored back to normal and ultimately the patient was discharged after removal from intubation and ventilatory assistance.[64] As a result, the ACE2 as a "decoy" molecule might prevent binding of the virus. It is possible that both a shift back to normal peptide balance in the RAS system and prevention of viral entry provided a synergistic benefit. Allosteric modulators (xanthenone and resorcinol-naphthalein) are known to stimulate ACE2 enzymatic activity and bind near the S-protein's receptor binding domain (RBD) interface with ACE2.[64,65] Unlike recombinant ACE2, these small molecules have increased capacity for co-formulation because of a higher propensity for oral bioavailability. Finding allosteric modulators that activate ACE2 but block S-protein interaction may inherently act as a "cloaking device" to hide the endogenous ACE2 receptor from the virus and promote a favorable physiological response. For the viral particles that bypass the cloaking, the boost in ACE2 activity from allosteric activation of all receptors can compensate for loss of activity occurring from SARS-CoV-2-induced ACE2 receptor internalization. The other obvious target for drug development is the RNA-dependent RNA polymerase (RdRp) used to replicate the genome. Although cross reactivity might occur to polymerases involved in RNA interference, targeting RdRp activity is now a proven therapeutic approach for COVID-19 with the emergency use approval by the FDA for remdesivir.[66] Modeling studies reveal

Table 1 Pharmacokinetic properties of ACEIs.

Drug	Half-life:	Average oral bioavailability:	Renal Excretion:	Protein binding:
Trandolapril	6–10 hrs (as prodrug or active trandolaprilat)	10%	33%	80%
Spirapril	0.8–1.6 hrs	50%	80%	86–91% (as active spiraprilat)
Ramipril	15 hrs (as active ramiprilat)	44% (as active ramiprilat)	60%	60–70% (both prodrug and active ramiprilat)
Quinapril	25 hrs (as active quinaliprat)	60% (prodrug)	96% (as active quinaliprat)	97% (as active quinaliprat)
Perindopril	3–10 hrs (as active perindoprilat)	25–30% (as active perindoprilat)	75%	60% (as active perindoprilat)
Moexipril	1 hr	15–20% (as active moexiprilat)	13%	50–70% (as active moexiprilat)
Lisinopril	12 hrs	25%	100%	0%
Fosinopril	12 hrs (as active fosinoprilat)	30%	44%	95%
Enalapril	35 hrs (as active enalaprilat)	60%	40% (as active enalaprilat)	50–60%
Captopril	1.9 hrs	75%	95%	25–30%
Benazepril	22 hrs (as active benazeprilat)	37%	33%	96.7% (both parent and active benazeprilat)

Table 2 Pharmacokinetic properties of ARBs.

Drug	Half-life:	Average oral bioavailability:	Renal excretion:	Protein binding:
Olemsartan	13 hrs	6%	35–50%	99%
Valsartan	6 hrs	25%	13%	95%
Azlisartan	11 hrs	60%	42%	99%
Candesartan	9 hrs	15%	33%	99%
Eprosartan	20 hrs	13%	7%	98%
Irbesartan	11–15 hrs	60–80%	20%	90%
Losartan	2 hrs	33%	35%	99%
Telmisartan	24 hrs	42–58%	less than 1%	99%

Figure 4. Viral replication and two current mechanisms targeted for therapeutic development. SARS-CoV-2 replication cycle has two current targets for intervention that have been identified. Viral ENTRY is blocked via vaccine-induced neutralizing antibody, recombinant ACE2, or allosteric modulators (xanthenone, resorcinolnaphthalein and diminazene aceturate). Genome REPLICATION is inhibited by use of RdRp inhibitors (remdesivir, ribavirin, sofosbuvir, galidesivir, and tenofovir).

that ribavirin, sofosbuvir, galidesivir, and tenofovir may also be effective in COVID-19 anti-viral treatments by targeting RdRp.[66] These alternatives to remdesivir are more attractive for use in polytherapeutic co-formulations because they have established oral bioavailability.

Inflammation. Anti-inflammatory drugs are an important polytherapy consideration for those with severe COVID-19. Dexamethasone is a corticosteroid that decreases inflammation and has been recommended by the world health organization (WHO) to be given orally or intravenously for the treatment of patients with severe and critical COVID-19.[68] Yet, there is also a recommendation against the use of corticosteroids in the treatment of patients with non-severe COVID-19.[68] Other anti-inflammatory medications for COVID-19 are under examination and recently the FDA issued permission for baricitinib to be used with remdesivir to treat severe to critically ill patients.[69] Reports of ibuprofen being contraindicated for COVID-19 infection have now been solidly refuted and a possible upregulation of ACE2 for increasing cellular infectivity is likely to be outweighed by the benefits of suppressing an overactive inflammatory response.[70] In the critically ill, drugs that block cytokine storm (e.g., tocilizumab) gave a 2-fold reduction in mortality despite higher superinfection occurrence.[71]

Preclinical Model Systems

Although current therapeutic approaches are succeeding in reducing overall mortality in COVID-19 patients and vaccines are rapidly becoming available, the full biological impact of the viral infection is only beginning to be understood. Developing polytherapeutic options will complement these efforts but require a better understanding of disease pathogenesis so that drug screening can be performed on appropriate preclinical models. A variety of new model options are needed, which can be combined with standard models to speed clinical deployment of polytherapeutic options.

Standard Models. Standard models can be differentiated into two types: cell lines and animal models. In cell lines, a Vero E6 line overexpressing TMPRSS2 allows the virus to replicate and SARS-CoV-2 particles can be isolated at high titer.[72] Yet, because cell lines are immortal and frequently aneuploid, among other issues, they do not represent normal physiology, and alter some aspects of the viral replication cycle. Primary cells offer a context with a more natural state, but their limited ability to proliferate often represents a significant hurdle to reliability and ease of use.[73] Induced pluripotent stem cells (iPSC) have the potential to be a platform to study COVID-19 infection. Like cell lines, they are highly proliferative, so large volumes of uniform starting material can be generated. Because iPSCs retain

normal genomic configuration, they can theoretically be induced in any cell type in the body.[74] Robust protocols are becoming available to create reproducible tissue types. For example, in cardiomyocyte generation, a variety of differentiation protocols have been developed[75] and over a dozen suppliers are offering cardiomyocyte generation kits. Tissues more relevant to COVID-19 infection (i.e., lung cell types) are in development[76–79] and may soon be commercially available as kits. As a result, advancements in tissue engineering may enable creation of physicochemical microenvironments that more closely mimic the natural environments found in the intact organism.

The use of animal models provides an enabling environment that can be engineered to be much closer to the human condition. In the past, mouse models have been central to understanding viral replication because of the similarity of the physiology and the immune response to that of humans.[80] Although a single animal model might not recapitulate all clinical disease hallmarks of COVID-19 in humans, animal models can recapitulate part of the COVID-19 disease presentation and pathology seen in humans.[81] According to the Preclinical Working Group of Accelerating COVID-19 Therapeutic Interventions and Vaccines (ACTIV), animal models for COVID-19 include mouse, hamster, ferret, guinea pig, and non-human primates.[81] Three mouse models have been developed in an attempt to better capture disease pathology: ACE2-transgenic strains, mouse-adapted virus, and adenoviral transduced ACE2 mice.[82–84] Because SARS-CoV-2 has poor binding affinity for the murine ACE2 receptor, transgenic mice expressing human ACE2 have been created to enable better infectivity with symptoms ranging from mild to lethal.[85] In a separate approach, serial passage has been used to select more virulent forms of SARS virus that mimic the mild symptoms observed in patients.[86,87] With these adaptations, mouse models enable recapitulation of the human symptoms and disease progression. Yet their use in rapid, high-throughput screening of drugs will be prohibitively expensive. More affordable proxies are needed for drug development.

While animal models often excel in resembling human physiology and immunology, the intrinsic genetic differences between mouse and humans can hinder smooth translation of the results. An example of this is found when examining toxicity, where interspecies mammalian concordance on compound toxicity is between 55–80%.[88,89] Conventional *in vitro* models solve some of these issues using human cell-based tests. Yet, most *in vitro* models lack the desired physiological complexity that allows accurate representation of human tissues and/or organs. For example, static cell culture monolayers have been used for a century now, but unfortunately they do not recapitulate the three-dimensional (3D) interactivity and multi-cellular dynamics that occur *in situ*. Any viral infection eval-

uated in monolayers likely differs significantly from the patient and corresponding pharmacokinetic (PK) and pharmacodynamic (PD) properties are often inaccurate. Multicellular spheroids are alternative *in vitro* models that provide the volumetric arrangement of cells and extracellular matrices (ECM).[90–92] However, they are still relatively simple in terms of proper structure and dynamics. As a result, the state of the art in preclinical models needs to evolve to more complex configurations that better mimic disease, while at the same time enable the performance of high-throughput assays which are a necessary requirement for polytherapy library screening.

Engineered Human Systems. A variety of tissue types can be created with bioengineered systems (lung, cardio, brain, vascular, kidney).[93] To bring a better understanding of disease pathology in the human context, bioengineering of tissue types is expected to be highly useful. Human organoids are biologically complex organ-like model systems that are derived from stem cells through the physiological developmental processes. An interesting example lies in recent research showing the use of kidney and vascular organoids to study SARS-CoV-2 infection and therapeutics testing.[94] The organoids are astrong mimetic to human organs, although they can still present certain limitations primarily including the lengthy production times (months), high expense, complex reagents, high variability and an inability to adequately mature them in many cases. Additionally, they are limited by an absence of systemic immune, hormone, and neural interactions. Addressing these limitations, engineering-based approaches have thus become attractive alternatives. An emerging area is the development of organ-on-a-chip devices, also termed microphysiological systems.[95] These systems are typically compartmentalized microfluidic devices that house relevant cell types in the appropriate configurations, where flows and mechanically active parameters can be introduced to model the dynamic processes that occur in the human body (Figure 5). More relevant, individual chip devices can be linked together in a single fluidic circulation in the way that the different organs connect naturally, further enabling more accurate PK/PD modeling through multi-organ interactions. Indeed, some of these platforms are being utilized for COVID research.[96]

Bioprinting. To bring even better contextualization, 3D bioprinting is a parallel effort in tissue model development, since it provides precise volumetric architectures by robotically patterning cells and extracellular matrix (ECM) at desired locations. Significantly, bioprinted tissue constructs may be further integrated with organ-on-a-chip devices to form 3D and dynamic organ systems that are anticipated to promote translational capacity when screening therapeutics (Figure 5).[97] Compared to organoids, the engi-
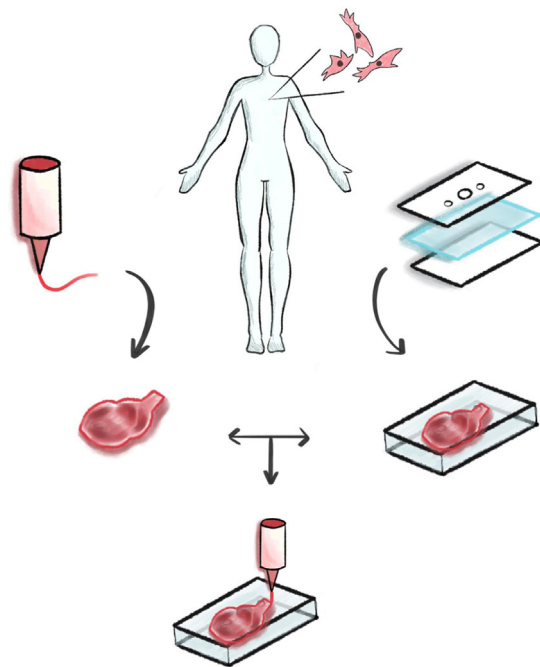

Figure 5. Engineering *in vitro* individualizable, human-based tissue models through bioprinting, organ-on-a-chip, and/or their combination. While bioprinting provides the volumetric architecture, the on-chip configuration further brings in the dynamic cues, which together allows the formation of biologically and (patho)physiologically relevant human-based tissue/organ models that are amenable to a range of therapeutics testing including those targeted toward COVID-19. Zhang et al., 2020, Bio-Design and Manufacturing, with permission.

neered, well-defined *in vitro* tissue models that involve bioprinting in lab-on-chip configurations feature much faster creation times and possibly higher-throughput in drug screening.[98]

Genetic Susceptibility. Pre-existing conditions are an important factor to consider in COVID-19. Heterogeneity in disease severity is in part due to underlying health conditions or comorbidities in COVID-19 patients such as hypertension and diabetes where RAS may promote pathophysiology in both conditions.[56] Estimates of the number of people with an underlying condition that have increased risk of developing severe COVID-19 are 1.7 billion persons (22%).[99] As a result, there is an urgent need to understand the pathophysiological consequences of comorbidities and to identify those with a rare disease that are at high risk of clinical deterioration. Therefore, a rare human disease may be the perfect physiologic model to better understand the pathogenesis of disease and generate more individualized therapeutic interventions for higher-risk COVID-19 groups. For example, patients with deficiencies in cellular chloride transport due to CFTR variants associated with cystic fibrosis (CF) are more prone to viral and bac-

terial infection.[100] Yet, because COVID-19 is a newly emergent disease, clear correlation of outcomes in SARS-CoV-2 infection in patients with CF are extremely limited,[101,102] yet the concern remains high for these patient groups.[103] Another rare disease group that might be negatively influenced by SARS-CoV-2 infection, are patents with Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL). CADASIL is caused by genetic lesions in the extracellular domain of the *NOTCH3* gene.[104] Like CF, accelerated disease progression in patients with CADASIL appears to be linked to influenza virus infection.[105] Yet the comorbidity of SARS-CoV-2 infection with *NOTCH3* pathogenic variations is only speculated to be associated with advancing CADASIL presentation.[106] Clear evidence is needed in order to support or refute a pathological association between COVID-19 infection and CADASIL. As a result, beter model systems are needed.

iPSCs as a Personalized Platform. Two approaches can be taken in iPSCs to model a patient's genetic predisposition to severe COVID-19 infection. In the first approach, the iPSCs are harvested from patient tissue (typically either skin fibroblasts or peripheral blood mononuclear cells (PBMCs).[107] Next, to enable clear indication that a genetic lesion is contributing to the disease condition in question, CRISPR-based gene-editing is performed to revert the variant in question back to the common wild-type allele.[108] This creates an isogenic control which, when differentiated into a tissue type, can be compared to the unmodified iPSC, similarly differentiated. In the second approach, a reference iPSC from healthy tissue is used as the starting material. The clinical variant of the patient is engineered into this cell using CRISPR gene-editing techniques. The original material becomes the isogenic control and the gene-edited cells become the patient avatar line. The second approach is more favorable from a disease modeling and drug discovery perspective because tissue differentiation protocols can be perfected on the reference and then be applied to the patient avatar line. Further, the hurdle of patient consent to access their tissues is avoided. Finally, the relative strengths in pathogenicity between patient avatars are also quantifiable without the need to worry about interference from background genetic modifiers that can occur between patient-derived tissues. Ideally the reference/avatar approach is deployed first and then, if interesting phenotype is detected, patient derived tissue is examined next. Uptake of virus in the derived tissue types can be expected to be highly revealing of the infection mechanisms of SARS-CoV-2 and other viruses. Further, the results could be a diagnostic test of the individual determining susceptibility to severe infection. Ultimately, these iPSC systems can be combined with bioprinting techniques to create microphysiological systems for analyzing COVID-19 infection biology and screening for therapeutics.

Alternative Animal Models. Although human tissue systems are desirable, modeling animals can be advantageous in throughput and cost. Mouse models can provide adequate recapitulation of patient phenotypes from COVID-19, but they have drawbacks in regards to performing high-throughput assays. Husbandry issues make it challenging to create 1000's of animals of near identical genetic composition. Whole animal assays in standard 96-well formats are not possible. As a result, automated dosing of 100,000 compound chemical libraries is not practical in mouse models. Alternative animal models that can thrive in a liquid matrix become ideal for scaling to high throughput screens. One animal that meets these prerequisites is the zebrafish. This animal easily allows developmental, in vivo assessment with high throughput. Fertilized embryos from a genetic cross are easily collected from the bottom of a tank and 100s of genetically similar animals can be examined for biological dysfunction. Injection of transgenes into the embryos allows discovery of chimerically modified animals that can be outcrossed to find uniform, genome-edited progeny. As a further enhancement, gene-humanization can be performed to introduce the human versions of conserved genes. When the human gene is found to replace the function of the fish orthologous gene, a significant degree of conserved biology has occurred. Next, clinical variants are installed and a battery of phenotypic tests are performed to elucidate their functional consequence. As an example applied to COVID-19 research, our team is creating an ACE2-humanized transgenic zebrafish for use as a high-throughput infection model for COVID-19 disease. The zebrafish model organism is becoming established for use in viral pathology research and can model host interferon response.[109] Applied to SARS-CoV infections, the RAS is highly conserved between humans and zebrafish, so establishing an infection model in zebrafish will aid understanding how the host factor response influences infectivity and disease severity in COVID-19. Exposure of SARS-CoV-2 to ACE2-humanized fish has yet to be validated for uptake and may require co-transformation with human *TMPRSS2* gene for generating efficient uptake, but if a zebrafish model susceptible to SARS-CoV-2 can be generated, a high-throughput system for examining viral infection and its biological effects will be possible. For instance ACEI/ARBs remain controversial for how they impact disease progression in SARS-CoV-2 infections and various combinations of drugs can be rapidly explored in the SARS-CoV-2 infection competent fish. Clinical variants can be installed in the humanized *ACE2* locus, or other genes, to

determine the role of preexisting conditions in increasing the likelihood of severe infection.

The *Caenorhabditis elegans* (*C. elegans*) nematode is another alternative animal model that fits the rapid and scalable liquid paradigm. Further it offers examination of the entire life cycle biology of a complex metazoan. Unlike zebrafish, which are naturally transparent only in the embryo stage, the nematode is transparent throughout its lifecycle. As a result, fluorescent reporters are easily examined in simple microplate assays throughout the animal's lifespan. Adding to these ideal properties for performing basic research, transgenic expression of human proteins is now becoming routine as a method to increase the genetic relevance of nematode translation to human biology. Variants can then be introduced in the human gene, allowing for functional data to be evaluated in a whole organism. In an applied example of the gene humanization technique, the human *STXBP1* coding sequence was inserted as gene replacement of the *unc-18* ortholog (Figure 6 (a)). A variety of clinical variants were installed in the humanized locus and tested for their effect on animal morphology and activity. A combined signal from 26 features was plotted for Benign, Pathogenic and Variant of Uncertain Significance (VUS). Using the boundaries between the signals for Benign vs. Pathogenic variants, 63% of VUS achieved PS3/BS3 assessment level for being either Likely Pathogenic or Likely Benign. Adapted to COVID-19 research, creation of a nematode expressing the human ACE2 receptor and TMPRSS2 co-factor could be used to explore

uptake of virus or pseudotyped viruses, enabling basic research into viral engagement and entry. This model could then be used to find novel, evolutionarily conserved host genes that either support or defend against viral infection, as was done for the Orsay virus which naturally infects nematodes.[110,111] Additionally, variants in the *ACE2* locus could be introduced via CRISPR, and phenotypic analysis could then be used to reveal whether rare or common variants in ACE2 confer susceptibility or resistance to SARS-CoV-2 infection. Lastly, the nematode is a well-established organism for the study of lifespan and healthspan, and could be used to examine basic biological questions about how SARS-CoV-2 infection impacts aging or aged individuals differently.

Pseudotyped Virus Systems. Working directly with SARS-CoV-2 requires biosafety level 3 (BSL3) facility. To reduce the hurdle and expense of BSL3 containment procedures, pseudotyped virus systems have been developed to enable study of viral activities at less expensive biocontainment levels. Typically these are virus chimeras using a component of deadly virus that is inserted in a relatively benign viral host, allowing the activity of the target virus to be studied at lower biosecurity levels (BSL2). For instance, commercial suppliers are offering lentiviral and vesicular stomatitis virus (VSV) that express the S-protein of SARS-CoV or SARS-Cov-2.[112] This allows easier study of virtual uptake in ACE2-expressing cells, which is useful for isolating antibodies as vaccine candidates or finding antivirals that interfere with viral entry. They are called pseu-

Figure 6. Humanized C. elegans nematode provides pathogenicity assessment in clinical variants. (a) Method of humanized animal generation involves replacing the native locus with a human version of the gene which then becomes a platform for installation of clinical variants. (b) Use of *STXBP1*-humanized animal for detecting pathogenicity in clinical variants involves application of a training set of 10 established Benign (green) and 10 established Pathogenic (red) which then provide definition of boundaries for pathogenic assessment of Variants of Uncertain Significance (VUS).

dotyped virus because their chimeric composition has a genomic insufficiency that prevents replication. The genome is missing a vital gene (typically a viral coat glycoprotein such as the S-protein that gives the corona halo to the coronavirus). Instead, the missing gene is provided as a second component, either as a plasmid or it is encoded in the genome of a stable cell line.[113] Infectious particles can be generated, but only replicate upon uptake if the infected cell expresses the missing glycoprotein. The pseudotyped virus cannot replicate in cells that do not produce the glycoprotein and therefore they are safer to handle in the laboratory. Typically, pseudotyped virus systems are developed in relatively benign viruses such as the Lentivirus system (Figure 7(a)). To create a system more amenable to study mechanisms of COVID-19 infection, a similar system can be envisioned using an infectious cDNA clone system for SARS-CoV-2. The coronavirus genome can be synthesized to contain all but the S and E structural proteins (Figure 7(b)), which has been demonstrated for SARS-CoV[114] and adapted for SARS-CoV-2.[115]

The E- and S-proteins are supplied exogenously either by a set of plasmids (Figure 7(c)) or by genomic integration into the host system (Figure 7 (d)). The resulting plasmid-based system can be co-transfected into a variety of cell types. If the transfected cell type expresses an appropriate receptor (ACE2) and processing proteins (TMPRSS2 and Cathepsin L) then infection can spread throughout the culture. In the genome-integrated method, the host system is genetically modified to express the E- and S-proteins. When the genetically-modified host system is exposed to coronavirus RNA replicon particles, derived from replication-incompetent or single-round infectious SARS-CoV-2, the infection spreads to all the tissues that express the required receptor (ACE2)

and the processing proteins (TMPRSS2 and Cathepsin L). The host system can range from cell lines, to iPSC-derived tissues, to model organism systems such as mouse, zebrafish, or *C. elegans*, where CRISPR-based gene-editing has simplified the process of creating genome-integrated transgenes. Because the RNA replicon genome of the virus is missing the coding sequence for the E- and S-protein, the pseudotyped viral particles created by the host system cannot spread the infection or productively replicate making it safer for researchers. As a result, the biosafety level drops to the much more affordable and easier to work with BSL2 level of containment. Care will need to be taken to recode the E- and S-protein transgenes so that a chance of spurious recombination is prevented and the original fully intact virus is not recreated. These pseudotyped virus systems will enable the development of the high-throughput assays that are necessary for screening large combinatorial libraries of drugs.

Concluding Remarks. As of February 2021, the COVID-19 pandemic is in its third and, so far, largest wave of infections. The direct cost to acutely ill patients is being measured in billions of dollars and indirect costs from chronically ill patients and other downstream psychological and economic costs may be orders of magnitude higher and it will take time to measure the final impact. Governing bodies around the world need to invest heavily in finding novel systems to increase our collective understanding of the pathogenesis of disease and to find new therapeutic options to treat the mild to severely ill. We have learned from other infectious diseases (HIV and TB) that drug combinations or polytherapeutics can be effective in attacking multiple mechanisms which could dramatically reduce viral load and the viral life cycle in SARS-CoV-2 infection. Developing a pandemic precision
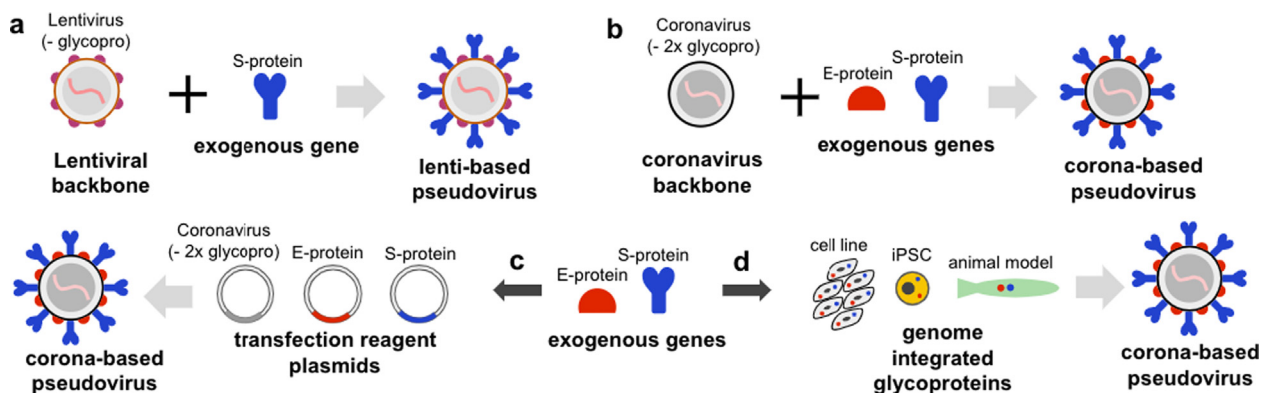

Figure 7. pseudotyped virus systems for elucidating viral mechanisms and host factor response. (a) Standard two-part pseudotyped virus system supplies the viral genome deficient in glycoprotein and an exogenously supplied glycoprotein (plasmid or genome-integrated gene). (b) SARS-CoV genome deficient in two glycoproteins with exogenous supply of the two missing glycoproteins. (c) Plasmid supply of exogenous glycoproteins can be used in transfection to create infectious pseudo virus particles that are incapable of infecting untransfected cells. (d) Exogenous glycoproteins integrated into the genome create a system where infection can spread via reinfection of neighboring cells and tissues.

platform with multiple approaches is needed to enable examination of the full therapeutic potential of large combinatorial libraries of drugs. We propose three areas of focus. The first is a need to create novel engineered tissue/organ systems that improve our understanding of organismal response to COVID-19 infection. The second is the use of humanized animal models that are compatible with liquid handling so that high-throughput assays can be developed. The third is the development of pseudotyped viral systems to enable study of coronavirus infection at an easier-to-manage BSL2 level of containment. Ultimately these novel systems will be used as screening platforms to find drug combinations that decrease the infectious burden associated with the COVID-19 pandemic.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Chris Hopkins: Conceptualization, Data curation, Visualization, Writing - original draft, Writing - review & editing. **Chidinma Onweni:** Writing - original draft, Writing - review & editing. **Victoria Zambito:** Writing - original draft, Writing - review & editing. **DeLisa Fairweather:** Writing - original draft, Writing - review & editing. **Kathryn McCormick:** Writing - original draft, Writing - review & editing. **Ebihara Hideki:** Writing - review & editing. **Thomas Caulfield:** Conceptualization, Writing - original draft, Writing - review & editing. **Yu Shrike Zhang:** Conceptualization, Visualization, Writing - original draft, Writing - review & editing. **W. David Freeman:** Conceptualization, Data curation, Writing - original draft, Writing - review & editing.

References

1. 1918 Pandemic (H1N1 virus). (2020). https://www.cdc.gov/flu/pandemic-resources/1918-pandemic-h1n1.html (accessed November 18, 2020).
2. Willmott, H.P., (2003). World War I. *DK Pub.,*.
3. Contributors to Wikimedia projects, World War II casualties, (2003). https://en.wikipedia.org/wiki/World_War_II_casualties (accessed December 6, 2020).
4. Rubin, R., (2020). As their numbers grow, COVID-19 "Long Haulers" stump experts. *JAMA*, **324**, 1381. https://doi.org/10.1001/jama.2020.17709.
5. Carfì, A., Bernabei, R., Landi, F., (2020). Gemelli against COVID-19 post-acute care study group, persistent symptoms in patients after acute COVID-19. *JAMA*, **324**, 603–605.
6. Caronna, E., Ballvé, A., Llauradó, A., Gallardo, V.J., María Ariton, D., Lallana, S., Maza, S.L., et al., (2020). Headache: A striking prodromal and persistent symptom, predictive of COVID-19 clinical evolution. *Cephalalgia*, **40**, 1410–1421.
7. Baig, A.M., (2020). Deleterious outcomes in long-hauler COVID-19: the effects of SARS-CoV-2 on the CNS in chronic COVID syndrome. *ACS Chem. Neurosci.,*. https://doi.org/10.1021/acschemneuro.0c00725.
8. Mandal, S., Barnett, J., Brill, S.E., Brown, J.S., Denneny, E.K., Hare, S.S., Heightman, M., Hillman, T.E., et al., (2020). Long-COVID": a cross-sectional study of persisting symptoms, biomarker and imaging abnormalities following hospitalisation for COVID-19. *Thorax,*. https://doi.org/10.1136/thoraxjnl-2020-215818.
9. Sheehy, L.M., (2020). Considerations for postacute rehabilitation for Survivors of COVID-19. *JMIR Public Health Surveill.*, **6**, e19462.
10. COVID-19: What proportion are asymptomatic? – The Centre for Evidence-Based Medicine. (n.d.). https://www.cebm.net/covid-19/covid-19-what-proportion-are-asymptomatic/ (accessed November 20, 2020).
11. Zhao, H., Lu, X., Deng, Y., Tang, Y., Lu, J., (2020). COVID-19: asymptomatic carrier transmission is an underestimated problem. *Epidemiol. Infect.*, **148**, e116
12. WHO Coronavirus Disease (COVID-19) Dashboard, (n.d.). https://covid19.who.int (accessed December 6, 2020).
13. COVID-19 vaccine tracker. (n.d.). https://www.raps.org/news-and-articles/news-articles/2020/3/covid-19-vaccine-tracker (accessed December 6, 2020).
14. Mishra, S. (2020). How mRNA vaccines from Pfizer and Moderna work, why they're a breakthrough and why they need to be kept so cold. Conversation. http://theconversation.com/how-mrna-vaccines-from-pfizer-and-moderna-work-why-theyre-a-breakthrough-and-why-they-need-to-be-kept-so-cold-150238 (accessed November 19, 2020).
15. Tillett, R.L., Sevinsky, J.R., Hartley, P.D., Kerwin, H., Crawford, N., Gorzalski, A., Laverdure, C., Verma, S.C., et al., (2020). Genomic evidence for reinfection with SARS-CoV-2: a case study. *Lancet Infect. Dis.,*. https://doi.org/10.1016/S1473-3099(20)30764-7.
16. To, K.K.-W., Hung, I.F.-N., Ip, J.D., Chu, A.W.-H., Chan, W.-M., Tam, A.R., Fong, C.H.-Y., Yuan, S., et al., (2020).

Coronavirus Disease 2019 (COVID-19) Re-infection by a phylogenetically distinct severe acute respiratory syndrome coronavirus 2 strain confirmed by whole genome sequencing. *Clin. Infect. Dis.*,. https://doi.org/10.1093/cid/ciaa1275.

17. CDC. (2020). Investigative Criteria for Suspected Cases of SARS-CoV-2 Reinfection (ICR). https://www.cdc.gov/coronavirus/2019-ncov/php/invest-criteria.html (accessed November 19, 2020).

18. Okar, L., Ahmad, R., Yassin, M.A., (2021). First report of COVID-19 reinfection in a patient with beta thalassemia major. *Clin. Case Rep.*, **9**, 861–865.

19. Wiersinga, W.J., de Bree, G.J., (2020). Covid-19 reinfections: what is the clinical relevance?. *Ned. Tijdschr. Geneeskd.*, **164** https://wwwncbi.nlm.nih.gov/pubmed/33332056.

20. Nainu, F., Abidin, R.S., Bahar, M.A., Frediansyah, A., Emran, T.B., Rabaan, A.A., Dhama, K., Harapan, H., (2020). SARS-CoV-2 reinfection and implications for vaccine development. *Hum. Vaccin. Immunother.*, **16**, 3061–3073.

21. Kennedy, D.A., Read, A.F., (2017). Why does drug resistance readily evolve but vaccine resistance does not?. *Proc. Biol. Sci.*, **284** https://doi.org/10.1098/rspb.2016.2562.

22. Weisblum, Y., Schmidt, F., Zhang, F., DaSilva, J., Poston, D., Lorenzi, J.C., Muecksch, F., Rutkowska, M., et al., (2020). Escape from neutralizing antibodies by SARS-CoV-2 spike protein variants. *Elife*, **9** https://doi.org/10.7554/eLife.61312.

23. Li, Q., Wu, J., Nie, J., Zhang, L., Hao, H., Liu, S., Zhao, C., Zhang, Q., et al., (2020). The impact of mutations in SARS-CoV-2 spike on viral infectivity and antigenicity. *Cell*, **182**, 1284–1294.e9.

24. Franzo, G., Legnardi, M., Tucciarone, C.M., Drigo, M., Martini, M., Cecchinato, M., (2019). Evolution of infectious bronchitis virus in the field after homologous vaccination introduction. *Vet. Res.*, **50**, 92.

25. Shaman, J., Galanti, M., (2020). Will SARS-CoV-2 become endemic?. *Science*, **370**, 527–529.

26. Deaths, (n.d.). https://www.who.int/gho/hiv/en/ (accessed November 19, 2020).

27. Ghosn, J., Taiwo, B., Seedat, S., Autran, B., Katlama, C., (2018). HIV. *Lancet*, **392**, 685–697.

28. Deeks, S.G., Overbaugh, J., Phillips, A., Buchbinder, S., (2015). HIV infection. *Nat. Rev. Dis. Primers.*, **1**, 15035.

29. McCormack, S., Dunn, D.T., Desai, M., Dolling, D.I., Gafos, M., Gilson, R., Sullivan, A.K., Clarke, A., et al., (2016). Pre-exposure prophylaxis to prevent the acquisition of HIV-1 infection (PROUD): effectiveness results from the pilot phase of a pragmatic open-label randomised trial. *Lancet*, **387**, 53–60.

30. Baeten, J.M., Donnell, D., Ndase, P., Mugo, N.R., Campbell, J.D., Wangisi, J., Tappero, J.W., Bukusi, E. A., et al., (2012). Antiretroviral prophylaxis for HIV prevention in heterosexual men and women. *N. Engl. J. Med.*, **367**, 399–410.

31. Arts, E.J., Hazuda, D.J., (2012). HIV-1 antiretroviral drug therapy. *Cold Spring Harb. Perspect. Med.*, **2**, a007161

32. Engelman, A., Cherepanov, P., (2012). The structural biology of HIV-1: mechanistic and therapeutic insights. *Nat. Rev. Microbiol.*, **10**, 279–290.

33. Masters, P.S., (2006). The molecular biology of coronaviruses. *Adv. Virus Res.*, **66**, 193–292.

34. Zhu, W., Chen, C.Z., Gorshkov, K., Xu, M., Lo, D.C., Zheng, W., (2020). RNA-dependent RNA polymerase as a target for COVID-19 drug discovery. *SLAS Discov.*,. 2472555220942123.

35. Gordon, C.J., Tchesnokov, E.P., Woolner, E., Perry, J.K., Feng, J.Y., Porter, D.P., Götte, M., (2020). Remdesivir is a direct-acting antiviral that inhibits RNA-dependent RNA polymerase from severe acute respiratory syndrome coronavirus 2 with high potency. *J. Biol. Chem.*, **295**, 6785–6797.

36. Grein, J., Ohmagari, N., Shin, D., Diaz, G., Asperges, E., Castagna, A., Feldt, T., Green, G., et al., (2020). Compassionate use of remdesivir for patients with severe Covid-19, N. Engl. J. Med., **382**, 2327–2336.

37. Antinori, S., Cossu, M.V., Ridolfo, A.L., Rech, R., Bonazzetti, C., Pagani, G., Gubertini, G., Coen, M., et al., (2020). Compassionate remdesivir treatment of severe Covid-19 pneumonia in intensive care unit (ICU) and Non-ICU patients: Clinical outcome and differences in post-treatment hospitalisation status. *Pharmacol. Res.*, **158**, 104899.

38. Antiviral Therapy, (n.d.). https://www.covid19treatmentguidelines.nih.gov/antiviral-therapy/ (accessed November 19, 2020).

39. Office of the Commissioner, Coronavirus (COVID-19) Update: FDA Authorizes Monoclonal Antibody for Treatment of COVID-19, (2020). https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-monoclonal-antibody-treatment-covid-19 (accessed December 14, 2020).

40. Chatterjee, N., Bivona, T.G., (2019). Polytherapy and targeted cancer drug resistance. *Trends Cancer Res.*, **5**, 170–182.

41. Datta, M., Coussens, L.M., Nishikawa, H., Hodi, F.S., Jain, R.K., (2019). Reprogramming the tumor microenvironment to improve immunotherapy: emerging strategies and combination therapies. *Am. Soc. Clin. Oncol. Educ. Book*, **39**, 165–174.

42. Lei, F., Xi, X., Batra, S.K., Bronich, T.K., (2019). Combination therapies and drug delivery platforms in combating pancreatic cancer. *J. Pharmacol. Exp. Ther.*, **370**, 682–694.

43. Zhang, C., Leighl, N.B., Wu, Y.-L., Zhong, W.-Z., (2019). Emerging therapies for non-small cell lung cancer. *J. Hematol. Oncol.*, **12**, 45.

44. Fox, W., Sutherland, I., (1956). A five-year assessment of patients in a controlled trial of streptomycin, para-aminosalicylic acid, and streptomycin plus para-aminosalicylic acid, in pulmonary tuberculosis. *Q. J. Med.*, **25**, 221–243.

45. Treatment for TB Disease. (2019). https://www.cdc.gov/tb/topic/treatment/tbdisease.htm (accessed November 22, 2020).

46. Kerantzas, C.A., Jacobs Jr, W.R., (2017). Origins of combination therapy for tuberculosis: lessons for future antimicrobial development and application. *MBio*, **8** https://doi.org/10.1128/mBio.01586-16.

47. Mase, S.R., Chorba, T., (2019). Treatment of drug-resistant tuberculosis. *Clin. Chest Med.*, **40**, 775–795.

48. Mayer-Barber, K.D., Yan, B., (2017). Clash of the Cytokine Titans: counter-regulation of interleukin-1 and

type I interferon-mediated inflammatory responses. *Cell. Mol. Immunol.*, **14**, 22–35.

49. Suprunenko, T., Hofer, M.J., (2016). The emerging role of interferon regulatory factor 9 in the antiviral host response and beyond. *Cytokine Growth Factor Rev.*, **29**, 35–43.

50. Tisoncik, J.R., Korth, M.J., Simmons, C.P., Farrar, J., Martin, T.R., Katze, M.G., (2012). Into the eye of the cytokine storm. *Microbiol. Mol. Biol. Rev.*, **76**, 16–32.

51. Jia, H.P., Look, D.C., Shi, L., Hickey, M., Pewe, L., Netland, J., Farzan, M., Wohlford-Lenane, C., et al., (2005). ACE2 receptor expression and severe acute respiratory syndrome coronavirus infection depend on differentiation of human airway epithelia. *J. Virol.*, **79**, 14614–14621.

52. Zhou, P., Yang, X.-L., Wang, X.-G., Hu, B., Zhang, L., Zhang, W., Si, H.-R., Zhu, Y., et al., (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*, **579**, 270–273.

53. Tikellis, C., Thomas, M.C., (2012). Angiotensin-Converting Enzyme 2 (ACE2) is a key modulator of the renin angiotensin system in health and disease. *Int. J. Pept.*, **2012**, 256294.

54. Zipeto, D., Palmeira, J. da F., Argañaraz, G.A., Argañaraz, E.R., (2020). ACE2/ADAM17/TMPRSS2 interplay may be the main risk factor for COVID-19. *Front. Immunol.*, **11**

55. Costa, L.B., Perez, L.G., Palmeira, V.A., Macedo, T., Cordeiro, E., Ribeiro, V.T., Lanza, K., Simões, A.C., et al., (2020). Insights on SARS-CoV-2 molecular interactions with the renin-angiotensin system. *Front. Cell Dev. Biol.*, **8**

56. Onweni, C.L., Zhang, Y.S., Caulfield, T., Hopkins, C.E., Fairweather, D.L., Freeman, W.D., (2020). ACEI/ARB therapy in COVID-19: the double-edged sword of ACE2 and SARS-CoV-2 viral docking. *Crit. Care*, **24**, 475.

57. Chung, M.K., Karnik, S., Saef, J., Bergmann, C., Barnard, J., Lederman, M.M., Tilton, J., Cheng, F., et al., (2020). SARS-CoV-2 and ACE2: The biology and clinical data settling the ARB and ACEI controversy. *EBioMedicine*, **58**, 102907.

58. Huang, M.-L., Li, X., Meng, Y., Xiao, B., Ma, Q., Ying, S.-S., Wu, P.-S., Zhang, Z.-S., (2010). Upregulation of angiotensin-converting enzyme (ACE) 2 in hepatic fibrosis by ACE inhibitors. *Clin. Exp. Pharmacol. Physiol.*, **37**, e1–e6.

59. Ferrario, C.M., Jessup, J., Chappell, M.C., Averill, D.B., Brosnihan, K.B., Tallant, E.A., Diz, D.I., Gallagher, P.E., (2005). Effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin-converting enzyme 2. *Circulation*, **111**, 2605–2610.

60. Flockhart, D.A., Tanus-Santos, J.E., (2002). Implications of cytochrome P450 interactions when prescribing medication for hypertension. *Arch. Intern. Med.*, **162**, 405–412.

61. Cao, Y.-C., Deng, Q.-X., Dai, S.-X., (2020). Remdesivir for severe acute respiratory syndrome coronavirus 2 causing COVID-19: An evaluation of the evidence. *Travel Med. Infect. Dis.*, **35**, 101647.

62. Hemnes, A.R., Rathinasabapathy, A., Austin, E.A., Brittain, E.L., Carrier, E.J., Chen, X., Fessel, J.P., Fike, C.D., et al., (2018). A potential therapeutic role for angiotensin-converting enzyme 2 in human pulmonary arterial hypertension. *Eur. Respir. J.*, **51** https://doi.org/10.1183/13993003.02638-2017.

63. Monteil, V., Dyczynski, M., Lauschke, V.M., Kwon, H., Wirnsberger, G., Youhanna, S., Zhang, H., Slutsky, A.S., et al., (2020). Human soluble ACE2 improves the effect of remdesivir in SARS-CoV-2 infection. *EMBO Mol. Med.*, e13426.

64. Zoufaly, A., Poglitsch, M., Aberle, J.H., Hoepler, W., Seitz, T., Traugott, M., Grieb, A., Pawelka, E., et al., (2020). Human recombinant soluble ACE2 in severe COVID-19. *Lancet Respir. Med.*, **8**, 1154–1158.

65. Hernández Prada, J.A., Ferreira, A.J., Katovich, M.J., Shenoy, V., Qi, Y., Santos, R.A.S., Castellano, R.K., Lampkins, A.J., et al., (2008). Structure-based identification of small-molecule angiotensin-converting enzyme 2 activators as novel antihypertensive agents. *Hypertension*, **51**, 1312–1317.

66. Office of the Commissioner. (2020). COVID-19 Update: FDA Broadens Emergency Use Authorization for Veklury (remdesivir) to Include All Hospitalized Patients for Treatment of COVID-19. https://www.fda.gov/news-events/press-announcements/covid-19-update-fda-broadens-emergency-use-authorization-veklury-remdesivir-include-all-hospitalized (accessed November 24, 2020).

67. Elfiky, A.A., (2020). Ribavirin, Remdesivir, Sofosbuvir, Galidesivir, and Tenofovir against SARS-CoV-2 RNA dependent RNA polymerase (RdRp): A molecular docking study. *Life Sci.*, **253**, 117592.

68. Corticosteroids for COVID-19. (n.d.). https://www.who.int/publications/i/item/WHO-2019-nCoV-Corticosteroids-2020.1 (accessed November 24, 2020).

69. Office of the Commissioner. (2020). Coronavirus (COVID-19) Update: FDA Authorizes Drug Combination for Treatment of COVID-19. https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-drug-combination-treatment-covid-19 (accessed November 24, 2020).

70. Moore, N., Carleton, B., Blin, P., Bosco-Levy, P., Droz, C., (2020). Does Ibuprofen Worsen COVID-19?. *Drug Saf.*, **43**, 611–614.

71. Somers, E.C., Eschenauer, G.A., Troost, J.P., Golob, J.L., Gandhi, T.N., Wang, L., Zhou, N., Petty, L.A., et al., (2020). Tocilizumab for treatment of mechanically ventilated patients with COVID-19. *medRxiv*, https://doi.org/10.1101/2020.05.29.20117358.

72. Matsuyama, S., Nao, N., Shirato, K., Kawase, M., Saito, S., Takayama, I., Nagata, N., Sekizuka, T., et al., (2020). Enhanced isolation of SARS-CoV-2 by TMPRSS2-expressing cells. *Proc. Natl. Acad. Sci. U. S. A.*, **117**, 7001–7003.

73. Kaur, G., Dufour, J.M., (2012). Cell lines: Valuable tools or useless artifacts. *Spermatogenesis*, **2**, 1–5.

74. Ellis, J. (2018) Transforming Research with Induced Pluripotent Stem Cells. http://www.biocompare.com/Editorial-Articles/353103-Transforming-Research-with-Induced-Pluripotent-Stem-Cells/ (accessed November 25, 2020).

75. Kishino, Y., Fujita, J., Tohyama, S., Okada, M., Tanosaki, S., Someya, S., Fukuda, K., (2020). Toward the realization of cardiac regenerative medicine using pluripotent stem cells. *Inflamm. Regen.*, **40**, 1–6.

76. Huang, S.X.L., Islam, M.N., O'Neill, J., Hu, Z., Yang, Y.-G., Chen, Y.-W., Mumau, M., Green, M.D., et al., (2014). Efficient generation of lung and airway epithelial cells from human pluripotent stem cells. *Nat. Biotechnol.*, **32**, 84–91.

77. Jacob, A., Morley, M., Hawkins, F., McCauley, K.B., Jean, J.C., Heins, H., Na, C.-L., Weaver, T.E., et al., (2017). Differentiation of human pluripotent stem cells into functional lung alveolar epithelial cells. *Cell Stem Cell*, **21**, 472–488.e10.

78. Tamò, L., Hibaoui, Y., Kallol, S., Alves, M.P., Albrecht, C., Hostettler, K.E., Feki, A., Rougier, J.-S., et al., (2018). Generation of an alveolar epithelial type II cell line from induced pluripotent stem cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **315**, L921–L932.

79. Abo, K.M., Ma, L., Matte, T., Huang, J., Alysandratos, K.D., Werder, R.B., Mithal, A., Beermann, M.L., et al., (2020). Human iPSC-derived alveolar and airway epithelial cells can be cultured at air-liquid interface and express SARS-CoV-2 host factors. *bioRxiv.*,. https://doi.org/10.1101/2020.06.03.132639.

80. Adachi, A., Miura, T., (2014). Animal model studies on viral infections. *Front. Microbiol.*, **5**, 672.

81. Hewitt, J.A., Lutz, C., Florence, W.C., Pitt, M.L.M., Rao, S., Rappaport, J., Haigwood, N.L., (2020). ACTIVating resources for the COVID-19 Pandemic, in vivo models for vaccines and therapeutics. *Cell Host Microbe.*, **28**, 646–659.

82. Muñoz-Fontela, C., Dowling, W.E., Funnell, S.G.P., Gsell, P.-S., Riveros-Balta, A.X., Albrecht, R.A., Andersen, H., Baric, R.S., et al., (2020). Animal models for COVID-19. *Nature*, **586**, 509–515.

83. Sun, J., Zhuang, Z., Zheng, J., Li, K., Wong, R.L.-Y., Liu, D., Huang, J., He, J., et al., (2020). Generation of a broadly useful model for COVID-19 pathogenesis, vaccination, and treatment. *Cell*, **182**, 734–743.e5.

84. Rathnasinghe, R., Strohmeier, S., Amanat, F., Gillespie, V.L., Krammer, F., García-Sastre, A., Coughlan, L., Schotsaert, M., et al., (2020). Comparison of transgenic and adenovirus hACE2 mouse models for SARS-CoV-2 infection. *Emerg. Microbes Infect.*, **9**, 2433–2445.

85. Lutz, C., Maher, L., Lee, C., Kang, W., (2020). COVID-19 preclinical models: human angiotensin-converting enzyme 2 transgenic mice. *Hum. Genomics.*, **14**, 20.

86. Dinnon 3rd, K.H., Leist, S.R., Schäfer, A., Edwards, C.E., Martinez, D.R., Montgomery, S.A., West, A., Yount Jr, B.L., et al., (2020). A mouse-adapted model of SARS-CoV-2 to test COVID-19 countermeasures. *Nature*, **586**, 560–566.

87. Leist, S.R., Dinnon 3rd, K.H., Schäfer, A., Tse, L.V., Okuda, K., Hou, Y.J., West, A., Edwards, C.E., et al., (2020). A mouse-adapted SARS-CoV-2 induces acute lung injury and mortality in standard laboratory mice. *Cell*, **183**, 1070–1085.e12.

88. Theunissen, P.T., Beken, S., Beyer, B., Breslin, W.J., Cappon, G.D., Chen, C.L., Chmielewski, G., de Schaepdrijver, L., et al., (2017). Comparing rat and rabbit embryo-fetal developmental toxicity data for 379 pharmaceuticals: on systemic dose and developmental effects. *Crit. Rev. Toxicol.*, **47**, 402–414.

89. Teixidó, E., Krupp, E., Amberg, A., Czich, A., Scholz, S., (2018). Species-specific developmental toxicity in rats and rabbits: Generation of a reference compound list for development of alternative testing approaches. *Reprod. Toxicol.*, **76**, 93–102.

90. Miller, A.J., Dye, B.R., Ferrer-Torres, D., Hill, D.R., Overeem, A.W., Shea, L.D., Spence, J.R., (2019). Generation of lung organoids from human pluripotent stem cells in vitro. *Nat. Protoc.*, **14**, 518–540.

91. Friedrich, J., Seidel, C., Ebner, R., Kunz-Schughart, L.A., (2009). Spheroid-based drug screen: considerations and practical approach. *Nat. Protoc.*, **4**, 309–324.

92. Dye, B.R., Hill, D.R., Ferguson, M.A.H., Tsai, Y.-H., Nagy, M.S., Dyal, R., Wells, J.M., Mayhew, C.N., et al., (2015). In vitro generation of human pluripotent stem cell derived lung organoids. *Elife*, **4** https://doi.org/10.7554/eLife.05098.

93. Skardal, A., Murphy, S.V., Devarasetty, M., Mead, I., Kang, H.-W., Seol, Y.-J., Zhang, Y.S., Shin, S.-R., et al., (2017). Multi-tissue interactions in an integrated three-tissue organ-on-a-chip platform. *Sci. Rep.*, **7** https://doi.org/10.1038/s41598-017-08879-x.

94. Monteil, V., Kwon, H., Prado, P., Hagelkrüys, A., Wimmer, R.A., Stahl, M., Leopoldi, A., Garreta, E., et al., (2020). Inhibition of SARS-CoV-2 infections in engineered human tissues using clinical-grade soluble human ACE2. *Cell*, **181**, 905–913.e7.

95. Zhang, Y.S. (n.d.). The Ultimate in Personalized Medicine: Your Body on a Chip. https://spectrum.ieee.org/biomedical/diagnostics/the-ultimate-in-personalized-medicine-your-body-on-a-chip (accessed November 25, 2020).

96. Tang, H., Abouleila, Y., Si, L., Ortega-Prieto, A.M., Mummery, C.L., Ingber, D.E., Mashaghi, A., (2020). Human organs-on-chips for virology. *Trends Microbiol.*, **28**, 934–946.

97. Parrish, J., Lim, K., Zhang, B., Radisic, M., Woodfield, T.B.F., (2019). New frontiers for biofabrication and bioreactor design in microphysiological system development. *Trends Biotechnol.*, **37**, 1327–1343.

98. Miri, A.K., Mirzaee, I., Hassan, S., Mesbah Oskui, S., Nieto, D., Khademhosseini, A., Zhang, Y.S., (2019). Effective bioprinting resolution in tissue model fabrication. *Lab Chip.*, **19**, 2019–2037.

99. Clark, A., Jit, M., Warren-Gash, C., Guthrie, B., Wang, H.H.X., Mercer, S.W., Sanderson, C., McKee, M., et al., (2020). Centre for the mathematical modelling of infectious diseases COVID-19 working group, Global, regional, and national estimates of the population at increased risk of severe COVID-19 due to underlying health conditions in a modelling study. *Lancet Glob Health.*, **8** (2020), e1003–e1017.

100. Bucher, J., Boelle, P.-Y., Hubert, D., Lebourgeois, M., Stremler, N., Durieu, I., Bremont, F., Deneuville, E., et al., (2016). Lessons from a French collaborative case-control study in cystic fibrosis patients during the 2009 A/H1N1 influenza pandemy. *BMC Infect. Dis.*, **16**, 55.

101. Colombo, C., Burgel, P.-R., Gartner, S., van Koningsbruggen-Rietschel, S., Naehrlich, L., Sermet-Gaudelus, I., Southern, K.W., (2020). Impact of COVID-19 on people with cystic fibrosis. *Lancet Respir. Med.*, **8**, e35–e36.

102. Cosgriff, R., Ahern, S., Bell, S.C., Brownlee, K., Burgel, P.-R., Byrnes, C., Corvol, H., Cheng, S.Y., et al., (2020). A multinational report to characterise SARS-CoV-2 infection in people with cystic fibrosis. *J. Cyst. Fibros.*, **19**, 355–358.

103. Manti, S., Parisi, G.F., Papale, M., Mulè, E., Aloisio, D., Rotolo, N., Leonardi, S., (2020). Cystic fibrosis: fighting together against coronavirus infection. *Front. Med.*, **7**, 307.

104. Wang, M.M., (2018). CADASIL. *Handb. Clin. Neurol.*, **148**, 733–743.

105. Mizutani, K., Sakurai, K., Mizuta, I., Mizuno, T., Yuasa, H., (2020). Multiple border-zone infarcts triggered by influenza a virus infection in a patient with cerebral autosomal dominant arteriopathy presenting with subcortical infarcts and leukoencephalopathy. *J. Stroke Cerebrovasc. Diseases*, **29**, https://doi.org/10.1016/j.jstrokecerebrovasdis.2020.104701 104701.

106. Williams, O.H., Mohideen, S., Sen, A., Martinovic, O., Hart, J., Brex, P.A., Sztriha, L.K., (2020). Multiple internal border zone infarcts in a patient with COVID-19 and CADASIL. *J. Neurol. Sci.*, **416**, https://doi.org/10.1016/j.jns.2020.116980 116980.

107. El Hokayem, J., Cukier, H.N., Dykxhoorn, D.M., (2016). Blood Derived Induced Pluripotent Stem Cells (iPSCs): benefits, challenges and the road ahead. *J. Alzheimers Dis. Parkinsonism.*, **6** https://doi.org/10.4172/2161-0460.1000275.

108. Skarnes, W.C., Pellegrino, E., McDonough, J.A., (2019). Improving homology-directed repair efficiency in human stem cells. *Methods*, **164–165**, 18–28.

109. Palha, N., Guivel-Benhassine, F., Briolat, V., Lutfalla, G., Sourisseau, M., Ellett, F., Wang, C.-H., Lieschke, G.J., Herbomel, P., Schwartz, O., Levraud, J.-P., et al., (2013). Real-time whole-body visualization of Chikungunya Virus infection and host interferon response in zebrafish. *PLoS Pathog.*, **9**, e1003619.

110. Jiang, H., Chen, K., Sandoval, L.E., Leung, C., Wang, D., (2017). An evolutionarily conserved pathway essential for orsay virus infection of caenorhabditis elegans. *mBio*, **8** https://doi.org/10.1128/mbio.00940-17.

111. Jiang, H., Leung, C., Tahan, S., Wang, D., (2019). Entry by multiple picornaviruses is dependent on a pathway that includes TNK2, WASL, and NCK1. *Elife*, **8** https://doi.org/10.7554/eLife.50276.

112. What is a Pseudovirus? (2020). https://www.news-medical.net/health/What-is-a-Pseudovirus.aspx (accessed November 26, 2020).

113. Li, Q., Liu, Q., Huang, W., Li, X., Wang, Y., (2018). Current status on the development of pseudoviruses for enveloped viruses. *Rev. Med. Virol.*, **28** https://doi.org/10.1002/rmv.1963.

114. Almazán, F., Sola, I., Zuñiga, S., Marquez-Jurado, S., Morales, L., Becares, M., Enjuanes, L., (2014). Coronavirus reverse genetic systems: infectious clones and replicons. *Virus Res.*, **189**, 262–270.

115. Zhang, Y., Song, W., Chen, S., Yuan, Z., Yi, Z., (2020). A bacterial artificial chromosome (BAC)-vectored noninfectious replicon of SARS-CoV-2. *Antiviral Res.*, **185**, 104974.