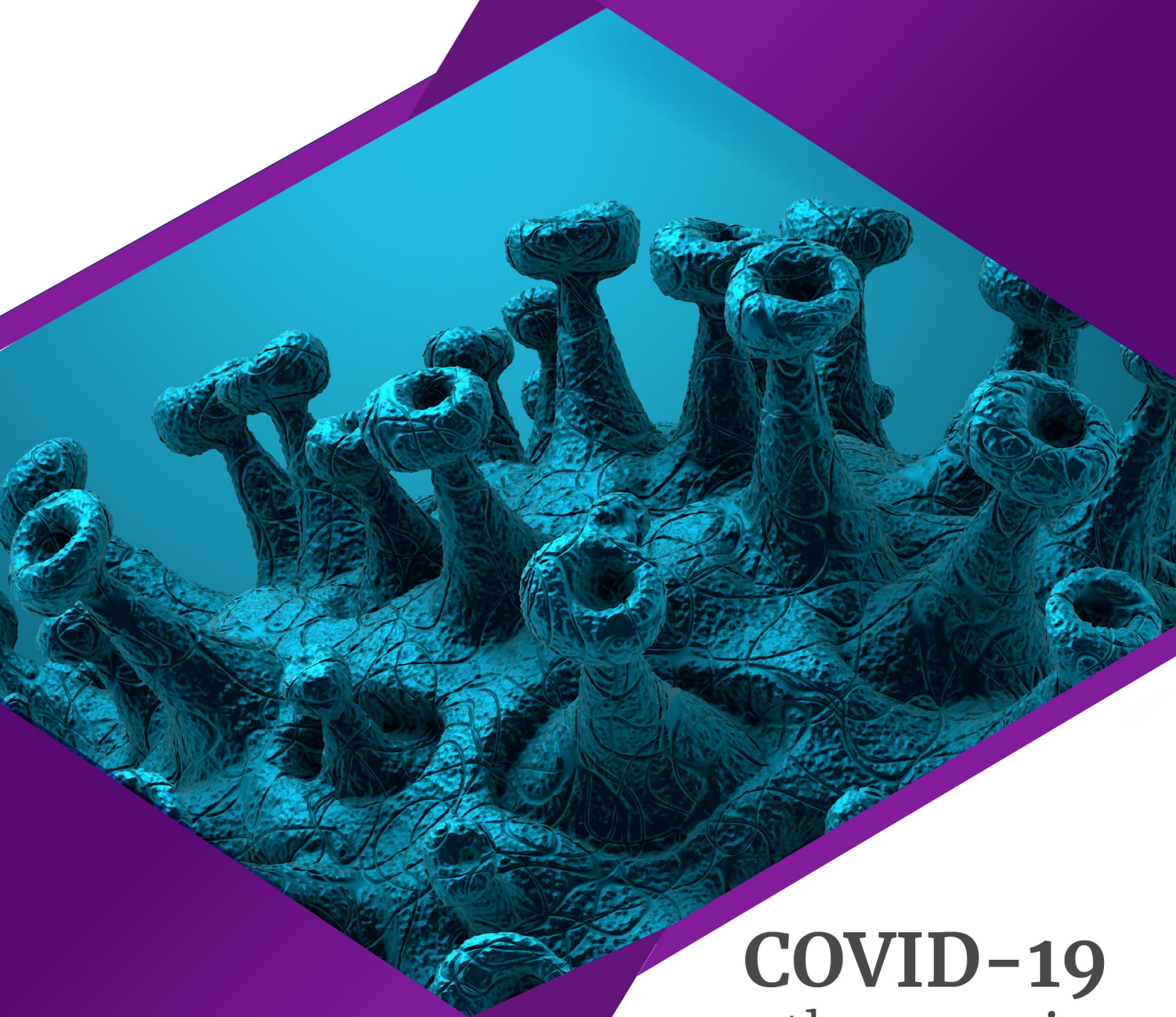
INFECTIOUS DISEASES HUB



COVID-19
pathogenesis, prevention & treatment

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FOREWORD

The COVID-19 pandemic has put virology, a field of research that has been neglected for years, back on the map. Virologists have been called upon to answer fundamental questions, including: how does SARS-CoV-2 enter host cells? How does disease develop? How can hosts be protected and infection prevented? How can symptoms be relieved and treated?

To answer these fundamental questions, microplate-based assays and microplate readers have become an essential part of the modern-day virologists' toolkit. They provide objective and quantitative data for monitoring viral infection in real-time in living cells for days, through to the screening of thousands of anti-viral compounds a day in high-throughput.

Microplate-based assays and microplate readers have broadened the range of methods for virus detection, analysis of interactions and biological responses; increasing efficiency and data quality in virology labs, and have provided crucial insights during the COVID-19 pandemic in pathogenesis, prevention and treatment of SARS-CoV-2 infection,

In this eBook, discover how microplate-based assays and microplate readers are enabling novel insights for:

- Understanding the molecular and cellular mechanisms of infection
- Identifying and quantifying the presence of a virus through detection of DNA or RNA
- Identifying new compounds that can prevent infection through anti-viral drug screening

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The 'omic' era, viral fitness assays and SARS-CoV-2: 60s with Alfredo Castello

Senior Lecturer at the MRC-University of Glasgow Centre for Virus Research (UK), Alfredo Castello, speaks to Infectious Diseases Hub about the current landscape of virology research, the emerging 'omic' era and the novel insights he has gained from the use of microplate-based assays and plate readers.

Castello completed his PhD studies in CMBSO (Madrid, Spain) in 2009, focusing on virus-host interactions. He then completed his Postdoc at EMBL (Germany) in 2014 on the topic of discovery of the cellular RNA-bound proteome. From 2014–2020, he was a Principal investigator at the University of Oxford (UK), conducting research into protein-RNA interactions in virus-infected cells. In 2020, he moved to his current role at the University of Glasgow.

Alfredo Castello

Q) In what way is virology research changing today and how does this change affect your work?

In the past, the field of virology has mainly been driven by candidate-focused molecular virology techniques. Most research was limited to the 'knowns' and new discoveries were slower and would occur over long time frames. With the emergence of the 'omic' era, the ground rules have changed, and we can answer global questions in one go. For example, before it was possible to ask questions such as: does protein X interact with viral RNA? Now we can totally change the approach and ask: which proteins interact with viral RNA? This implies near-unbiased answers with expected and unexpected results. Hence, the discovery potential of all research areas, including virology, has massively improved with 'omic' technologies.

Q) Why was the viral fitness assay developed, what problem was it trying to solve?

One of the problems of 'omic' approaches is the volume of data that one obtains. We move from individual proteins to systems, and it is difficult to then assess which of those candidates actually have a functional role. This is what happened to us. We developed several approaches to discover which cellular proteins engage with viral RNA in infected cells as they can play critical roles in the viral life cycle. We applied it to different viruses including SARS-CoV-2, HIV-1, Sindbis virus and others.

We got a long list of proteins that we are likely to play central roles in virus infection, as they form part of the ribonucleoproteins of different viruses. However, we didn't have formal evidence of their functional importance. The question was, how can we test dozens of candidates in one go?

Moreover, most of the fitness assays take snapshots of the 'race' at the beginning and end, but lack information about what happens in the middle. As kinetics are critical in virus infection, we wanted to develop a method that would provide near real-time information of the whole race. This would allow us to distinguish different phenotypes, such as delays, inhibition, full suppression, or enhancements.

Hence, when we discovered that the CLARIOstar microplate reader had not only high sensitivity and flexibility for fluorescence measurements, but also counted with an atmospheric control unit as an accessory, we thought it was the perfect platform for such an assay. Indeed, it worked extremely well in our pioneering study (Garcia-Moreno *et al*, 2019) and we now use it routinely as our first line viral fitness assay. We have tested its value in different cell lines and with different viruses.

Q) How can this assay help with the COVID-19 pandemic?

We have identified the complement of cellular proteins that interact with SARS-CoV-2 RNA

The 'omic' era, viral fitness assays and SARS-CoV-2: 60s with Alfredo Castello

(Kamel *et al,* Mol Cell 2021). We now aim to test their roles in the viral life cycle and explore their potential as therapeutic targets. For example, we have identified hundreds of compounds already available that target these cellular proteins and we plan to test these drugs with our viral fitness assay. For this, we already have a fluorescent SARS-CoV-2 variant and a CLARIOstar plate reader placed in a biosafety laboratory class 3. We hope to be able to conduct this drug screen soon to provide new therapeutic targets to combat vaccine-resistant variants.

Q) What novel insights has this approach enabled?

We have discovered new host factors that are involved in virus infection, several of which play pivotal roles in the infection of several viruses and hold potential as therapeutic targets. We have also performed limited drug screens and found compounds able to inhibit virus infection. While this is very exciting, I think that this is just the beginning and we will exploit our viral fitness assay to test large libraries of available drugs in the future.

Q) Your work focusses mainly on understanding how viruses exploit cellular molecules for their own sake. Where do you see the advantages of this approach for the prevention or limitation of infectious diseases?

Viruses evolve fast and in the presence of a selective pressure, such as a drug targeting a viral protein, resistant strains are expected to arise. In order to minimize the chances of the emergence of drug-resistant variants, retroviral therapies simultaneously employ a cocktail of three drugs targeting several viral proteins. Getting multiple drugs for a given virus requires substantial inversion and time and it is unlikely that we will get such combinations for all viruses that are human threats.

Moreover, new viruses can emerge, and we need first line compounds that we can use in such circumstances. As host cells are less prone to mutation when compared to viruses, drugs targeting the host cell are expected to be more stable treatments. Moreover, as viruses tend to hijack a shared pool of cellular factors, inhibitors targeting them may hold broad-range effects, which is something available for bacteria but lacking for viruses. As a drawback, host-targeting antivirals may have secondary effects, but treatment for acute viral infections is short-term and the chances of co-lateral damages limited. We should not forget that many of the pills that we typically take, such as aspirin, are ultimately targeting cellular proteins.

Q) What role do microplate readers play in modern virology?

As mentioned above, for us they are an opportunity to measure viral gene expression throughout the infection with high sensitivity and accuracy. They are a necessary tool for the transition from large datasets (e.g. drug libraries) to a candidate-focused approach. Combined with gene editing systems, lentiviral vectors to overexpress or knock down cellular proteins, and drug libraries; they take phenotypic profiling approaches to another level. For me, it is amazing that the experiments that took me months during my PhD can be performed now in a shorter time frame and for dozens of proteins, drugs and viruses simultaneously.

Flexible microplate reader solutions for virology research

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Commentary

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Future MICROBIOLOGY

Glimpses into evolutionary trajectories of SARS-CoV-2: emerging variants and potential immune evasion routes

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"The fact that a host APOBEC-like editing process appears to be driving much of sequence change in SARS-CoV-2 has profound implications for its short- and long-term evolution."

Tweetable abstract: An opinion on the coronaviruses' evolution paradoxes, the continuing adaptation of the SARS-CoV-2 in humans following the zoonotic transmission, and clues into escape routes from host immune responses.

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Keywords: adaptation • APOBEC • coronavirus • COVID-19 • genetic diversity • genome evolution • immune escape • SARS-CoV-2 variants of concern • spike • vaccines

Paradoxes in the evolution of coronaviruses

The genomes of most RNA viruses are confined to average sizes of 10 kb, partly because of their high mutation rates that, in the absence of proofreading mechanisms, may result in the accumulation of deleterious mutations that would inactivate the virus (error–catastrophe hypothesis) [1,2]. In this low-complexity 'Eigen trap' with few RNA-protein (RNP) elements, the diversity generated by the typical errors of 1–2 mutations per nucleotide site per replication round by the RNA-dependent RNA polymerases during viral RNA synthesis provides the virus population the capacity to adapt to diverse ecological niches and conditions, such as the dynamic environment of different anatomic sites in infected individuals and the hosts' immune responses [3]. Accordingly, even modestly increased replication fidelity has been shown to lead to reduced viral fitness *in vitro* and pathogenicity in animal models, although low-fidelity RNA viruses with higher mutation rates, may also display compromised fitness *in vivo* [4]. Hence, a finely balanced mixture of genetic diversity, on one hand, on which selection can act, possibly actuating episodic evolution affecting only specific domains at times as required for adaptation, and genome stability, on the other, to ensure that structural constraints are not violated, seems to be required for survival and is thus selected through evolution [4].

Up until the characterization of the 41.1 kb genome of planarian secretory cell nidovirus [5], roni- and coronaviruses were considered to be at the upper end of the RNA virus genome size range (26.3–31.7 kb) and complexity among positive-sense ssRNA viruses. The acquisition of a 3'–5' proofreading exoribonuclease (nsp14-ExoN) that was implicated in controlling RNA replication fidelity and avoidance of the accumulation of excessive numbers of deleterious mutations that would lead to a dramatic loss in fitness, is thought to have contributed to genome expansion. Thus, the ancestor of these large nidoviruses arose, exemplified by the 20.2 kb genome of the first insect (mosquito)-borne nidovirus, Nam Dinh virus [3]. The discoveries of Nam Dinh virus and planarian secretory cell nidovirus provided the missing evolutionary links in the transition from small to large nidoviruses, concomitantly

starting to bridge, in essence, the daunting gap for the proposed evolution of contemporary DNA–RNP-based life from primordial RNP entities as primitive life radiated and dominated the earth.

Despite their large and complex genomes, the high plasticity and recombination-driven adaptive capacity of coronaviruses is strong as shown by the frequent host-switching events through the exploitation of different cellular receptors by the spike protein [6–8]; and by the emergence, presumably from bats, late in 2019 in Hubei Province, China, and rapid spread among the human population worldwide, of SARS-CoV-2 [9].

The adaptation of SARS-CoV-2 in humans following the zoonotic transmission

The pandemic SARS-CoV-2 that causes COVID-19, has claimed more than 2.9 million lives globally. Although geographically defined clusters that recapitulated the early routes of international spread were described, the remarkably low virus diversity recorded in the early phases of the pandemic (with a midrange substitution rate of 3×10^{-4} substitutions/site/year), complicated epidemiological analyses [10]. C→U transitions comprised about half of sequence changes, with an eightfold base frequency normalized directional asymmetry between C→U and U→C substitutions. Of note, similarly elevated ratios were also observed in the other epidemic coronaviruses that emerged recently in evolutionary time (SARS-CoV-1 in 2002 and MERS-CoV in 2012), while decreasing ratios were found in the four endemic human coronaviruses (HCoV-NL63, -OC43, -229E and -HKU1) [10].

Contrary to initial expectations of a slow evolutionary pace of SARS-CoV-2, a number of genomic alterations have been recorded in recent months, as the adaptation of this novel coronavirus in humans continues. The first such change, the D614G amino acid substitution in the spike protein, which prevailed globally soon after its surfacing in February 2020 [11], was shown to be associated with increased viral transmission through a shifted conformation toward a favored human angiotensin-converting enzyme 2 binding-competent state; however, this gain in infectivity likely came at a cost to the neutralization properties of the virus [12,13]. Additional 'variants of concern' (VOC), due to their suggested impact on transmission and virulence, are continuously recorded [14].

In September 2020, a lineage that arose in the UK was found to harbor a constellation of 23 genomic mutations, including some amino acid-altering mutations in the spike protein [14]. Increased transmissibility arguably characterizes this designated as 'B.1.1.7' (20I/501Y.V1/B.1.1.7) lineage, based on the rapid displacement of previously circulating viruses in the region where it first appeared in southeast England, and its consequent association with an increased R_e and elevated viral RNA levels in nasopharyngeal washes, as measured by PCR or RNA sequencing [14]. However, discerning between the action of positive selection and the momentum of the founder effect of a virus already exceedingly transmissible among humans, is very difficult. B.1.1.7 variants are certainly fit or reproductively successful, but they are not necessarily more transmissible biologically.

Additional variants that share some of the B.1.1.7 mutations, have been detected in Brazil (P.1/20J/501Y.V3/B.1.1.248), South Africa (20H/501Y.V2/B.1.351), California (20C/S:452R;/B.1.429) and many other locations around the world [14]. Experiments measuring infectious virus in animal models, or humans for that matter, which would provide solid proof of increased transmissibility, have not been conducted yet for any of these variants. Their potential functional effect on disease severity also remains uncertain at present. According to a recent report by the New and Emerging Respiratory Virus Threats Advisory Group, VOC B.1.1.7 is associated at 40–50% confidence with an increased risk of death compared with non-VOC [15]. Genomic surveillance efforts should be intensified globally to monitor for the emergence of variants and the characterization of their biological properties, especially pertaining to immune evasion, as vaccine rollout and mass vaccination of the world's population continues at a, perhaps unavoidably, slow rate.

The selection pressure to escape from host immune responses

Host immune responses after natural infection or immunization, can exert selection pressures on the virus that will likely result in the further exploitation of sequence space in search of escape routes. The correlates of immunity to – and protection from – SARS-CoV-2 are not fully understood yet. However, following the impressive interim efficacy results of candidate vaccines in late phase clinical trials as well as the preliminary real-world vaccination results particularly from Israel, people around the world are anxiously anticipating their distribution, asking emphatically whether they will suffice to end this pandemic. Thus, one of the most pressing questions at present is: will the virus mutate to escape the selection pressure from host immune responses, and, moreover, are any SARS-CoV-2 variants already resistant to licensed vaccines?

Pseudotyped viruses bearing the SARS-CoV-2 spike glycoprotein from the B.1.1.7 lineage were efficiently neutralized by sera from recipients of Moderna's (mRNA-1273) vaccine; in contrast, approximately 6.4-fold reduced

neutralization titers were found against the South African B.1.351 lineage, prompting Moderna to announce the advancement of a modified vaccine (mRNA-1273.351) to prevent severe COVID-19 from this lineage, too [16]. Neutralization of sera from recipients of the Pfizer/BioNTech's (BNT162b2) mRNA vaccine to viruses engineered with selected changes from both lineages (deletion of amino acids 69/70, N501Y and D614G for B.1.1.7 and E484K + N501Y + D614G for B.1.351, respectively) was not found to be compromised [17]. Johnson & Johnson's recently authorized single-shot Ad26.COV2.S COVID-19 vaccine appears to be effective against the B.1.351 variant [18], while AZD1222, the other adenovirus-based vaccine by Oxford–AstraZeneca, does not work well against the South African variant [19].

In a recent study, Weisblum *et al.* [20] provide clues to potential immune evasion routes using a recombinant chimeric VSV/SARS-CoV-2 reporter virus system. With this system, escape mutants from antibody neutralization, which is thought to be key for the protection of the population, can be rapidly generated and assessed. Functional SARS-CoV-2 spike protein variants with resistance-conferring mutations to monoclonal antibodies or convalescent plasma were thus shown to be readily selected *in vitro* [20]. The resistance mutations mapped to the receptor binding domain and N-terminal domain. Importantly, but perhaps not surprisingly, escape mutants to commonly elicited neutralizing antibodies can already be detected at low frequencies in circulating SARS-CoV-2 populations [20].

A granular view of potential antibody escape pathways is presented by a recently released preprint that emphasizes that individual variation should be anticipated in antibody-mediated virus evolution [21]. To define the profile of antibody escape to the SARS-CoV-2 spike protein using COVID-19 convalescent plasma, an approach that comprehensively addresses the effect of all possible mutations on binding to a protein of interest, phage deep mutational scanning (DMS), was used. The fusion peptide and linker region, upstream of the heptad repeat region 2, were the two regions where antibody binding was common, although escape mutations varied within these immunodominant regions [21]. Individual variation was also evident in less commonly targeted epitopes.

The large proportion of sequence change in SARS-CoV-2 found to comprise C→U hypermutation points to the direction of RNA-editing processes acting within the infected cell as the most plausible explanation. Mourier *et al.* [22] recently reviewed three such human defense mechanisms and their potential implications on SARS-CoV-2 evolution: APOBEC, ROS and ADAR.

APOBEC, typically considered an antiviral mechanism against retroviruses, may also mediate antiviral functions against RNA viruses, since it catalyzes cytosine deamination to uracil in foreign ssDNA and RNA [22]. Extensive C-to-U mutations, the genomic context of which was enriched for APOBEC target sites [23], have been observed in SARS-CoV-2 since the early phases of the pandemic [24,25]. Interestingly, only viruses regularly infecting tissues with high expression of APOBEC and other antiviral proteins exhibited CpG-depletion and U-rich genomes [26]. The study by Simmonds published in late June reported that about half of the observed nonsynonymous mutations in SARS-CoV-2 were the result of C-to-U changes [10], while, according to Mourier *et al.* [22], that frequency was 36.9% as of 2 October 2020, by comparing approximately 80,000 assembled consensus genomes to the SARS-CoV-2 reference genome (MN908947.1). Of note, C→U transitions occurred preferentially in both 5' U/A and 3' U/A flanking sequence contexts that are comparable to favored motifs of human APOBEC3 proteins [10]. The evolutionary trajectory of SARS-CoV-2 may be severely restricted as a consequence of the potential depletion of alanine, histidine, glutamine, proline and threonine codons due to the progressive loss of genomic cytosines [27].

Another line of host defenses against viral infections involve ROS that may lead to virus mutagenesis and inactivation through the oxidation of proteins, lipids and nucleic acids [28]. In particular, guanine may be oxidized by ROS to 7,8-dihydro-8-oxo-2'-deoxyguanine (oxoguanine) that can readily base pair with adenine, leading to G-to-T transversions [29]. G-to-U, as well as C-to-A changes, have been hypothesized to be associated with the mutagenic activity of ROS [30].

A-to-G changes may stem from the deamination by ADAR of adenine to inosine (I) that pairs with cytosine. A chief controller of cytoplasmic innate immunity, *ADAR1*, the first of the three human *ADAR* genes, targets dsRNA which can arise during the replication-transcription process of positive-sense ssRNA viruses, including SARS-CoV-2 [31]. Its two isoforms exhibit different expression patterns: ADAR1p110 is constitutively expressed in most tissues, while ADAR1p150 is localized in the nucleus and released to operate in the cytoplasm upon stimulation by interferon [32]. *ADAR1* is also an important regulator of self-tolerance, since unedited dsRNA is interpreted as nonself, leading to the activation of the innate immune sensing response via MDA5 [33].

Both ADAR and APOBEC were found as frequent interactors with SARS-CoV-2 RNA by Schmidt *et al.* [34], who used RNA antisense purification and mass spectrometry in their study in infected human cells. Sequence analyses also showed a role for ADAR-mediated editing of the viral genome [23,35], with a note of caution that

detected variation could be due to artifacts introduced during sequencing [35]. Indeed, a few other studies found no evidence of ADAR1 activity acting on SARS-CoV-2 [36,37]. However, the mutations created by ADAR1 do not match those observed in SARS-CoV-2 or other coronaviruses; in fact, the excess of C→U transitions contradicts those induced by ADAR1 [10].

The fact that a host APOBEC-like editing process appears to be driving much of sequence change in SARS-CoV-2 has profound implications for its short- and long-term evolution. Prolonged C→U hypermutation could lead to low G+C contents and base asymmetries in the long run, as observed in bat-derived and endemic human coronaviruses [10]. Will the mutational journey of SARS-CoV-2 that started in a hostile human cellular environment lead to a tolerated symbiosis and, if so, how soon could that be in evolutionary time? For now, the novel coronavirus represents an intriguing paradigm with respect to its diversification in sequence space that stems predominantly from biased, convergent, and context-dependent mutations in addition to neutral changes, rendering the tracking of its evolutionary trajectory and description of its fitness landscape challenging for molecular epidemiology investigations.

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Future MICROBIOLOGY

Editorial

Herd immunity: could it bring the COVID-19 pandemic to an end?

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"To date, rinderpest is the only infectious disease that has been eradicated by engendering herd immunity sufficient to lead to its extinction in the wild."

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The first comprehensive definition and detailed discussion of herd immunity appeared in 1929 in the first edition of the classic text, Topley and Wilson's 'The Principles of Bacteriology and Immunity' [1]. The definition was broad, encompassing the innate resistance of a species to infection by a particular pathogen; herd habits and environmental and ecological factors promoting nonspecific immunity; as well as resistance acquired following infection or after immunization. They pointed out that freedom from a disease is not synonymous with specific immunity, saying that "*there is little doubt that the English herd, as such, is immune to plague and to typhus*" because plague rats are very rare and lousiness is very uncommon, but "*that is quite clear that the individuals who compose the English herd enjoy this immunity only so long as they remain within it.*" Nowadays, it is likely that it would have been considered wise to discuss this phenomenon with respect to the herds of other UK nations; the outbreak of yellow fever in Swansea in 1865 [2] would have been appropriate. The 29 cases with a fatality rate of 68% were transmitted by *Aedes aegypti* that had travelled from Cuba on the copper ore-carrying ship *Hecla*. This mosquito cannot breed in Wales, or anywhere else in the UK, because the winters are too cold, so it is not possible for the virus to become established.

As soon as it was coined, the use of the term 'herd' to designate human communities was criticized. Dudley [3] mounted a vigorous defence, also in 1929. He pointed out that it was first popularized by psychologists with the phrase 'herd instinct', and that biologically there is little fundamental difference between a herd of deer, a herd of swine, or a herd of *Homo sapiens*. Presciently, he also discussed the subherd of antivaccinationists. "*They form a very difficult administrative problem. They are perfectly honest in their convictions, but their power of dissociation and rationalization is so great that they often seem to the saner members of the herd to be absolutely unscrupulous and dishonest, whereas really they are only completely inaccessible to logic. . . making the most absurd accusations against those who dare to differ from them.*"

Considerations this year about COVID-19 control have in general used the term 'herd immunity' with a much narrower meaning than Topley's and Wilson's. They carry the implication of a level of specific immunity against the virus that will reduce the effective reproductive rate R_0 to <1, so that the virus will not be able to maintain itself and continue to spread, a situation which should eventually result in its elimination. The calculation underpinning this view is [4]:

$$p_c = 1 - (1/R_0) \quad \text{(Eq. 1)}$$

In which p_c is the proportion of the population that is immune, either as a consequence of an infection or by immunization with a vaccine or both [4]. It is reasonable to suppose that the term was used in this sense by Dr David Halpern, a government scientific advisor, when he said on 11 March 2020 on the BBC Today programe that "*You will want to protect those at risk groups so that they basically do not catch the disease and by the time that they come out of their cocooning, herd immunity has been achieved in the rest of the population.*" This strategy has been endorsed

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by the Great Barrington Declaration signed on 4 October 2020 by some infectious disease epidemiologists and public health scientists [5]: "*We know that all populations will eventually reach herd immunity, in other words, the point at which the rate of new infections is stable and that this can be assisted by (but is not dependent upon) a vaccine. Our goal should, therefore, be to minimize mortality and social harm until we reach herd immunity. The most compassionate approach that balances the risks and benefits of reaching herd immunity, is to allow those who are at minimal risk of death to live their lives normally to build up immunity to the virus through natural infection, while better protecting those who are at highest risk.*" Other scientists and healthcare professionals disagree profoundly with this strategy. Their views have been outlined in the John Snow Memorandum, first published on 14 October 2020 [6]: "*The arrival of a second wave and the realization of the challenges ahead has led to renewed interest in a so-called herd immunity approach, which suggests allowing a large uncontrolled outbreak in the low-risk population, while protecting the vulnerable. Proponents suggest this would lead to the development of infection-acquired population immunity in the low-risk population, which will eventually protect the vulnerable. This is a dangerous fallacy unsupported by scientific evidence. . . uncontrolled transmission in younger people risks significant morbidity and mortality across the whole population, furthermore, there is no evidence for lasting protective immunity to SARS-CoV-2 following natural infection.*"

A wide range of estimates of the herd immunity threshold for COVID-19 have been published. Formula (1) above assumes that everyone in the population is equally likely to become infected. However, there is abundant evidence that this is not the case. Social activity levels and age-related effects mean that the population is heterogeneous regarding the likelihood of becoming infected. Together with the large number of asymptomatic cases, these things make estimating the threshold a difficult task at this time [7,8].

It is often claimed that the eradication of smallpox testifies to the success of a herd immunity policy. It does not. The comment of Anderson and May [4] in this regard is appropriate: "*Too many of the putative facts known to public health planners rest on enthusiastic retelling of plausible tales, rather than on controlled experiments or careful analysis of data.*" On the basis of estimated values of its effective reproductive rate they calculated the degree of herd immunity required for smallpox eradication(p_c) to be 70–80%. Before 1967, the WHO smallpox eradication programe was defined in terms of the number of vaccinations performed: "*It has been demonstrated that eradication of smallpox from an endemic area can be accomplished by vaccinating 80% of a population within a period of 4–5 years.*" However, it was found that outbreaks still occurred in districts where the 80% goal had been reached; in 1973 the goal had been achieved in India, but in that year it had 88,114 cases. Continued virus transmission in mass vaccinated communities was strongly associated with high population densities [9]. Accordingly, the WHO began to move to and implement a surveillance–containment strategy, finding that this was effective in controlling transmission, even when vaccination coverage was much less than 80%.

To date, rinderpest is the only infectious disease that has been eradicated by engendering herd immunity sufficient to lead to its extinction in the wild. Related to measles, it causes a disease in cattle and buffalo often with high morbidity and mortality. Historically, attempts to control it in Britain have an uncanny similarity to current events. Imported from Europe in 1865, it killed at least 420,000 cows [10]. Experts were hired. A Royal Commission (equivalent to a public inquiry today) was established. There was a debate about disinfectants. The contacts of infected animals were slaughtered. Railway transit stopped and fairs and markets were closed. Some members of the Commission dissented from these measures, saying that cattle movement stoppage, "*would involve an interference with the course of trade at variance with our national habits; and it would demand sacrifices from large numbers of people, who are removed from the presence of the disease.*" Legislation giving effect to control measures was hurried through the House of Commons in a manner that was described as savoring of despotism [11].

The UN Food and Agriculture Organization and the World Organization for Animal Health declared the global eradication of rinderpest in 2011. Absolutely central to this success was TRCV, the tissue culture rinderpest vaccine, which protected against all rinderpest variants, provided life-long immunity, was never associated with any adverse reactions, gave immunity after a single dose and in its ThermoVax form was thermostable, with a shelf life of 30 days outside the cold-chain, holding a maximum titre for 14 days at 45°C [12].

It is too early to tell whether any of the COVID-19 vaccines under development will possess all or even just some, of these properties. To block the transmission of SARS-CoV-2 by creating the necessary levels of herd immunity, much depends on vaccine efficacy and the duration of vaccine protection. For a vaccine with 100% efficacy that gives life-long protection, and taking into account prelockdown values of R_o of between 2.5 and 3.5, the level of herd immunity needed to block virus transmission is about 60–72% [13]. Less efficacious vaccines and ones that only give a short duration of protection will require a higher proportion of the population to be immunized

to interrupt virus transmission and repeated vaccinations may be required, particularly if virus variants to which current vaccines offer poor protection become common.

However effective COVID-19 vaccines turn out to be, it is likely in most countries that the first batches will mainly be used to protect groups of individuals with the highest case fatality rates, in other words, those older than 70 years. It seems likely that herd immunity strategies will have to wait; giving time, perhaps, to counter the influence of the antivax sub herd and to address the issues that cause vaccine hesitancy.

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Perspective

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Mechanisms of infection by SARS-CoV-2, inflammation and potential links with the microbiome

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The pandemic SARS coronavirus 2 utilizes efficient mechanisms to establish infection and evade the immune system. Established infection leads to severe inflammation in susceptible patients, the main hallmark of progression to severe coronavirus disease (COVID-19). Knowledge of the mechanisms of disease has expanded rapidly. As inflammation emerges as the central pathophysiological feature in COVID-19, elucidating how the immune system, lungs and gut communicate and interact with microbial components of the ecological niches that conform the human microbiome will shed light on how inflammation and disease progression are promoted. Studying the microbiome in COVID-19 could allow scientists to identify novel approaches to prevent severe inflammation by targeting components of the human microbiome. Innovation in the aforementioned is needed to combat this pandemic.

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Human coronaviruses (HCoVs) were first isolated from patients with the common cold in the 1960s [1–3]. Seven HCoVs known to cause disease in humans have since been identified: HCoV-229E, HCoV-NL63, HCoV-OC43, HCoV-HKU1, the SARS coronavirus (SARS-CoV), the Middle East respiratory syndrome coronavirus and the novel SARS-CoV-2 [4]. The latter was identified after a spike in cases of pneumonia of unknown etiology in Wuhan, Hubei Province, China during December 2019 and was initially named novel coronavirus (2019-nCoV) [5,6]. The virus was renamed SARS-CoV-2 according to the International Committee on Taxonomy of Viruses classification criteria due to its genomic closeness to SARS-CoV; the disease caused by this virus was named coronavirus disease (COVID-19) according to the WHO criteria for naming emerging diseases [7]. SARS-CoV-2 belongs to the genera *Betacoronavirus* and shares a different degree of genomic similarity with the other two epidemic coronaviruses: SARS-CoV (~79%) and Middle East respiratory syndrome coronavirus (~50%) [8].

COVID-19 has caused considerable morbidity and mortality worldwide and has become the central phenomenon that is shaping our current societies. Human-to-human transmission is the main route of spread of the virus, mainly through direct contact, respiratory droplets and aerosols [9–12]. Management of COVID-19 has been extremely challenging due to its high infectivity, lack of effective therapeutics and potentially small groups of individuals (i.e., asymptomatic or mild disease) rapidly spreading the disease [13–17]. Although research describing COVID-19 and the mechanisms of infection by SARS-CoV-2 and its pathogenesis has expanded rapidly, there is still much to be learnt. Important gaps in knowledge which remain to be elucidated are the dynamic and complex interactions between the virus and the host's immune system, as well as the potential interspecies communications occurring

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between ecological niches encompassing distinct microorganisms in both healthy individuals and persons living with chronic diseases, and how these interactions could determine or modulate disease progression and outcomes.

In this review, we describe recent insights into these topics, as well as remaining questions whose answers will allow us to understand how interactions between the virus, the immune system and microbial components could possibly be related to disease states in patients with COVID-19, as well as existing studies of the microbiome in patients with COVID-19.

SARS-CoV-2 utilizes efficient mechanisms to establish infection & evade the innate immune response

The human angiotensin-converting enzyme 2 (hACE2) receptor has extensively been shown to be the primary receptor for SARS-CoV-2 through recognition of its 'spike' (S) glycoprotein, with subsequent priming by the transmembrane serine protease 2 and lysosomal cathepsins [5,18–21]. This surface protein has a novel furin-cleavage site between the S1 and S2 subunits [18], which resembles a homologous domain in the human epithelial sodium channel α-subunit [22]. Since the SARS-CoV-2 S protein is highly glycosylated and remains mostly in a closed prefusion conformation [23], pre-activation of the S protein by furin protease is thought to be an essential step to expose its receptor binding domain [21,24]. The pathogenesis of SARS-CoV-2 infection has been extensively reviewed elsewhere [25].

The hACE2 receptor has a high expression in the small intestine, testis, kidneys, heart, thyroid and adipose tissue; medium expression, in the lungs and liver; and low-to-no expression, in most cells and organs of the immune system (blood cells, spleen, bone marrow and blood vessels [unspecified]) [26]. Even when similar replication of SARS-CoV and SARS-CoV-2 has been found in the lower respiratory tract [27], SARS-CoV-2 replicates 100-fold more efficiently under the conditions encountered in the upper respiratory tract (i.e., 33°C) [28].

The interferon (IFN) response includes type I (IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω), type II (IFN-γ) and type III (IFN-λ) IFNs, with type I and III IFNs being the first major line of defense against viruses [29]. The upper respiratory tract elicits greater type I and III IFN responses after viral infection when compared with bronchial epithelial cells [30]. However, SARS-CoV-2 induces only suboptimal early expression of type I, II and III IFN [27].

A highly efficient mechanism of entry to the cells, which involves the S protein remaining most of the time in a conformation which allows antigen occultation, with subsequent pre-activation of its receptor binding domain by furin before binding to hACE2 would explain why SARS-CoV-2 is highly successful at evading the innate immune system while achieving early accelerated replication. Other potential mechanisms of evasion of the innate immune system by SARS-CoV-2 have been reviewed elsewhere [31].

Immune-mediated injury is the predominant pathophysiological driver in severe & critical COVID-19

Even though many of the symptoms of COVID-19 could partly be attributed to viral tropism as mentioned earlier, a subset of clinical features is apparently due to immune-mediated mechanisms. Namely, thromboembolic events and multiorgan failure are considered complications of COVID-19 rather than direct effects of the virus, which only patients who progress to severe and critical COVID-19 develop. The delimitation between clinical manifestations caused predominantly by the virus and the complications due to immune dysregulation is evident since most symptoms and pneumonia have their onset in the first 6 days after symptom onset in most patients, whereas complications start to occur between days 7–8, coinciding with the time viral RNA starts to decline (Figure 1) [32–36].

It has been hypothesized that SARS-CoV-2 could disseminate to other organs via secondary viremia, targeting susceptible organs and causing direct damage [13] after successful replication in cells of the respiratory tract and evasion of innate immune defenses. However, damage to different organs is likely immune mediated as supported by the characteristic inflammatory storm described in patients who progress to severe and critical COVID-19 [37,38]. Two distinctive features have been noticed in these patients: progressive increases in inflammation and an unusual hypercoagulable state. Determinants of progression to an unregulated inflammatory state have not been completely elucidated yet, although the clinical characteristics of these patients and recent experimental studies have started to uncover some of the key elements.

Progression to severe and critical COVID-19 is known to occur more frequently in patients with increasing ages and significant comorbidities, most of which involve some degree of dysregulation of the renin–angiotensin–

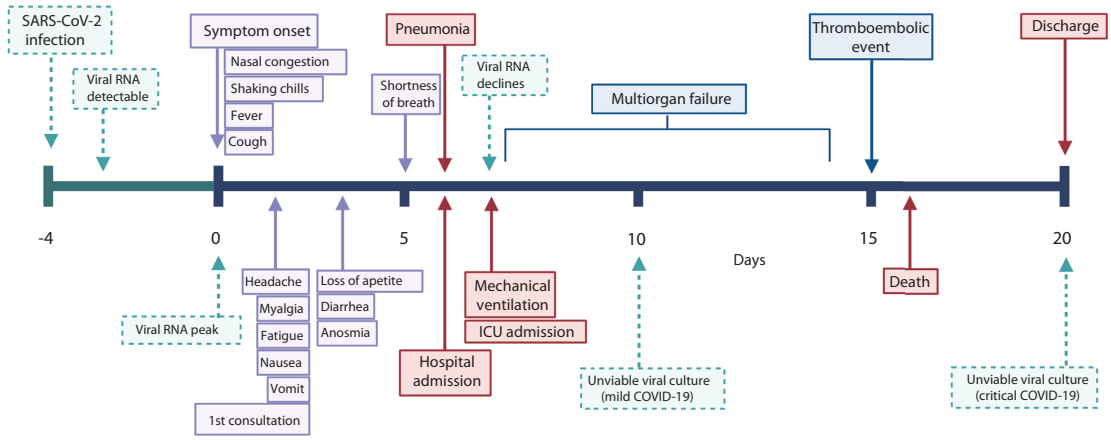

Figure 1. Time from onset of symptoms to specific events of interest in patients with symptomatic coronavirus disease. This timeline describes the time (mean days) at which different outcomes and other events of interest (solid boxes) occur in patients with COVID-19, including those who either experience mild-to-moderate disease or progress to severe-to-critical COVID-19. Events related to the dynamics of the virus (dashed boxes) include onset of infection, incubation period (dashed horizontal line), detection of viral ribonucleic acid (RNA) in respiratory specimens and the last day at which viable viral cultures have been obtained from patients with COVID-19. Figure created with BioRender.com.
COVID-19: Coronavirus disease.

aldosterone system (RAAS), inflammation or both [39–43]. An adequate early immune response and optimal regulation of RAAS could be the key early features that prevent progression.

Type I and II IFNs induce greater expression of the hACE2 receptor in epithelial surfaces, and patients with inflammatory states involving upregulated IFNs (including COVID-19) are known to express high levels of hACE2 [44–46]. High basal expression of hACE2 in patients susceptible to COVID-19 could initially put them at increased risk through greater availability of hACE2. However, increased expression of hACE2 following robust early IFN responses in both healthy and diseased individuals would be important to guarantee proper regulation of angiotensin II, which is thought to be an important element in initiating and perpetuating the hyperinflammatory state in COVID-19 through initial hyperactivation of the NF-κB pathway [47]. Early type I and III IFN responses have been related to successful resolution of the infection, while late and sustained type I and III IFN responses are related to disease progression, contributing to the inflammatory storm [29,48].

Individuals who progress to the immune-mediated injury phase present decreased lymphocyte counts and significant elevation of neutrophils; the neutrophil-to-lymphocyte ratio is an early predictor of progression to severe and critical disease [42,49]. Coagulation, inflammatory and organ damage markers are significantly elevated in patients with severe and critical COVID-19: C-reactive protein, procalcitonin, ferritin, erythrocyte sedimentation rate, IL-6, IL-2, IL-7, IL-8, IL-9, IL-10, CXCL10, MCP1, MIP1A, TNF-α, bilirubin, aspartate transaminase (AST), alanine transaminase (ALT) and lactate dehydrogenase (LDH) [39,50]. Increased ferritin, neutrophil-to-lymphocyte ratio, IL-6 and D-dimer are associated to increased mortality, while decreases in B cells, T cells and NK cells were characteristically noted in severe COVID-19 at presentation [51,52]. Longitudinal comparison of lymphocyte subpopulations in patients with mild and severe disease showed marked decreases in $CD3^+$, $CD8^+$ and $CD4^+$ T cells in severe COVID-19, while no significant differences in the trajectories of B cells and NK cells were observed in the 16 day follow-up [53]. In this same study, IL-2 and IFN-γ peaked and subsequently declined in patients with severe disease, while IL-10 and IL-6 showed a sustained elevation with respect to patients with mild disease.

Functional exhaustion of NK and $CD8^+$ T cells with increased expression of NKG2, which were found to recover in convalescent patients, suggests that immune disturbance occurs early in the disease as a combination of both direct and bystander effects [54]. The finding that most patients who undergo mild-to-moderate disease produce neutralizing antibodies and specific T-cell responses [55,56], and early evidence of memory cells [57] add to the statement that an adequately mounted early immune response leads to resolution of the infection while

generating specific and memory responses to the virus. However, the extent of different memory cells throughout the organs of the immune system and duration of memory remain to be determined.

Pathological studies in patients who died after developing critical disease have revealed diffuse alveolar damage, hyaline membrane, alveolar wall thickening and infiltration with macrophages, mild-to-moderate mononuclear response, viral cytopathic effects, significant diffuse hemorrhage, small vessel thrombi with surrounding $CD4^+$ cells, deep venous thrombosis in some cases, and degenerated neutrophils, which could represent neutrophil extracellular traps [52,58–60]. These findings are consistent with the important immune dysregulation occurring in critical COVID-19, which together with the high pro-inflammatory cytokine levels and macrophage and monocytic infiltration resemble the macrophage activation syndrome [37], and reflect the important hypercoagulable state in critical COVID-19, which has been referred to as 'thromboinflammation' due to high correlation between IL-6 levels, fibrinogen and histopathologic findings [61].

The main features of hypercoagulability in critical COVID-19 are normal-to-prolonged prothrombin time and activated partial thromboplastin time, elevated D-dimer, and increased fibrinogen [62]. Tang *et al.* reported that 71.4% of nonsurvivors and 0.6% of survivors had evidence of overt deep intravascular coagulation; more patients exhibited latent deep intravascular coagulation characterized by a hypercoagulable state, as demonstrated by fibrin thrombus [63].

Pro-inflammatory cytokines are known to contribute to hypercoagulation; IL-6 contributes mainly through increased fibrinogen production, which is known to be an acute phase reactant [64]. Certain fibrinogen polymorphisms are related to greater concentrations in response to IL-6 [65], which could partially account for the varying frequencies in thrombotic complications encountered in COVID-19 patients. IL-1β and IL-8 promote rapid clot formation through increased fibrin-cross-linking and cellular clot components (more pronounced effect by IL-8), while IL-6 only mildly affects clot conformation [66].

The clinical course of COVID-19 can be divided into three phases (viremia, acute and severe or recovery phase), under two different scenarios (with or without interventions) [13]. The first scenario (Figure 2A) reflects the natural course of patients who progress to severe and critical COVID-19. The second scenario represents the timing of interventions (i.e., antivirals, immunoglobulin and low-molecular-weight heparin) which could potentially alter the course of the disease and prevent progression (Figure 2B). Patients with significant risk factors could require more interventions than immunocompetent individuals to avoid progression to severe and critical disease.

SARS-CoV-2 productively infects human enterocytes

Zhang and colleagues [67] noted that diarrhea accounted for a notable proportion of COVID-19 patients, ranging from 8.0 to 12.9%; high levels of hACE2 mRNA and protein expression were detected in the small intestinal enterocytes. In 2003, postmortem studies after SARS-CoV infection established the important viral tropism for intestinal cells (small and large intestine) where productive viral replication occurred, with subsequent accumulation of virions in the endoplasmic reticulum and shedding of the virus through its apical membrane. SARS-CoV-2 is known to cause less gastrointestinal manifestations than SARS-CoV, which could be partly explained by greater replication kinetics of SARS-CoV in intestinal cell lines [20]. However, SARS-CoV-2 has been shown to infect and productively replicate in human small intestinal organoids, subsequently altering gene expression, increasing cytokine production, promoting IFN-regulated genes and increasing hACE2 expression [46]. Thus, infection of intestinal cells could play an important role in promoting the inflammatory storm in COVID-19, mainly by promoting pro-inflammatory cytokines and through late and prolonged induction of IFNs.

Gut microbiome

The trillions of microbial cells colonizing the human body were long ignored by the scientific community, but in recent years research in the field has rapidly expanded and we have learnt that microbes are a central metabolic hub, promoting in many cases physiological homeostasis and immune functions through a close symbiotic relationship with the host [68–70]. The gut microbiome has received most attention. Recently, fungi have been recognized as important components of the human microbiome, and their role in health and diseases is increasingly being studied [71,72]. In addition, during physiological states and after disturbances in the gut microbiome (i.e., single course of antibiotics), fungal species may overtake immune modulation tasks commonly done by bacteria, thereby preventing mucosal damage and *vice versa*. Viruses are also key components of the human microbiome (human virome) [73,74].

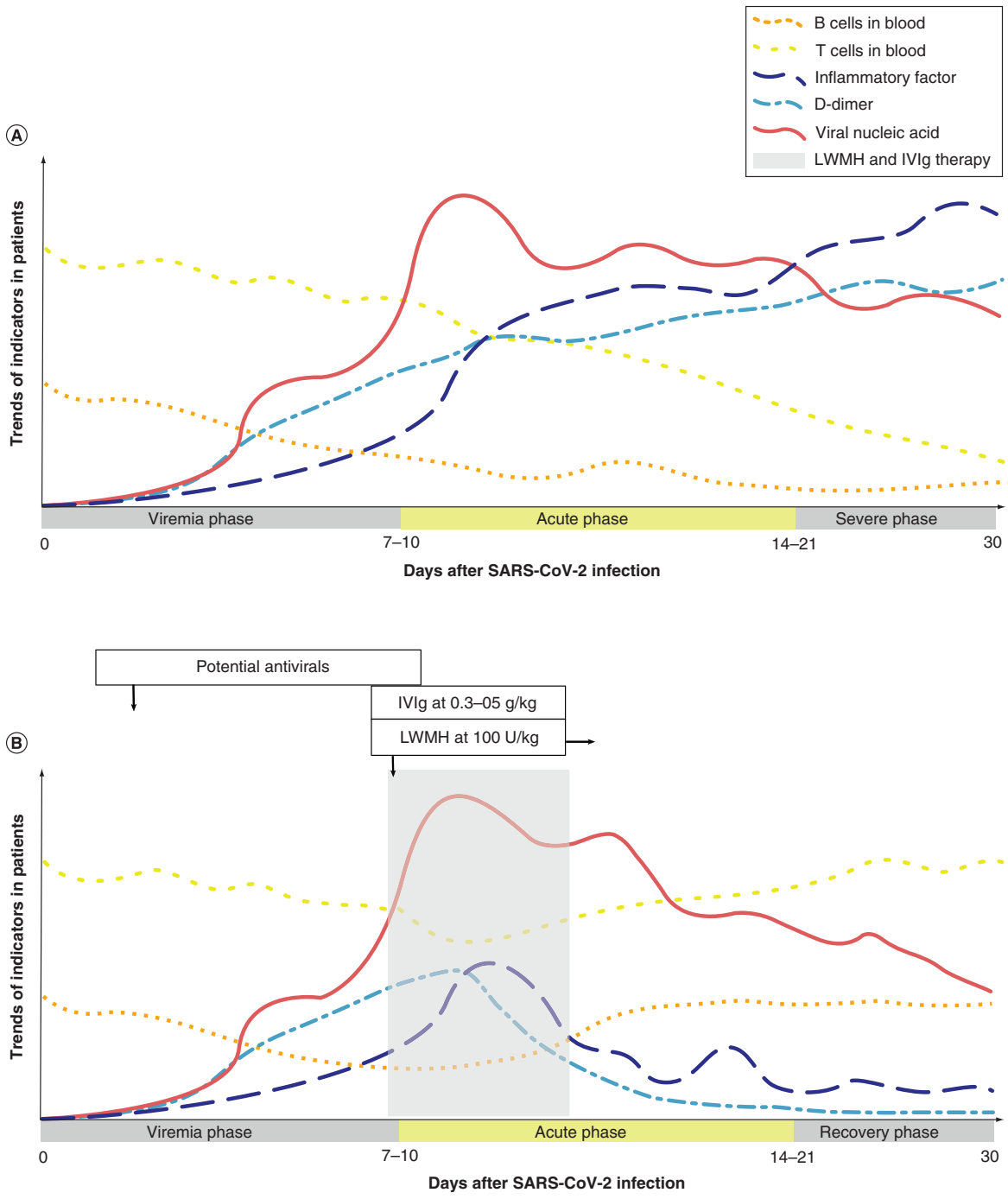

Figure 2. Hypothetical trajectories of selected parameters in coronavirus disease. The trend of T cells, B cells, inflammatory factors, D-dimer and viral load (y-axis) are graphed with respect to disease duration in days after SARS-CoV-2 infection (X-axis); disease course is divided into three phases (viremia, acute and severe/recovery). **(A)** Trajectories in patients reaching severe phase without specific interventions. **(B)** Trajectories in patients reaching recovery phase after LWMH and high dose IVIg therapy. The shaded areas represent the recommended intervention time for LMWH and IVIg.
IVIg: Intravenous immunoglobulin; LMWH: Low-molecular-weight heparin; SARS-CoV-2: SARS coronavirus 2.
Modified with permission from [13].

The gut microbiome spans beyond the GI tract, mediating a variety of intercommunications between the gut, enteric nervous system and the brain [75]; perturbations in the gut microbiota (dysbiosis) contribute to the initiation of pro-inflammatory signaling. For instance, dysbiosis has been found to increase translocation of bacterial lipopolysaccharide (LPS) due to increased permeability of the intestinal epithelial barrier, leading to obesity and insulin resistance [76].

Novel ways by which microbial microenvironments are shaped in the human body have started to emerge. For instance, small molecules (21–24 nucleotides) of RNA named miRNAs [77], are known to modify the gut microbiota in patients with colorectal cancer, while also altering gene expression in cancer cells, thus suggesting bilateral communication via these molecules between bacteria and human cells [78,79]. Intestinal epithelial cells have been shown to produce miRNAs which are delivered through their apical membrane to the intestinal lumen inside extracellular vesicles, and are able to enter bacteria (*Fusobacterium nucleatum* and *Escherichia coli*), altering gene expression and promoting their growth [80]. Different miRNA profiles produced by noncolitogenic and colitogenic microbiotas are associated with intestinal inflammation in the latter [81]. Interestingly, miRNAs contained in ginger exosomes after its ingestion have been shown to shape the microbiota (increased *Lactobacillaceae* and *Bacteroidales*, and diminished *Clostridiaceae*) in both humans and mice, while also altering gene expression in *Lactobacillus rhamnosus* [82]. The miRNAs contained in extracellular vesicles travelling through the bloodstream have been studied as biomarkers of inflammation and prognostic factors in patients with acute myocardial infarction [83,84], although their potential as mediators of inflammation and shapers of the microbiota at distant sites has not been studied yet.

Dysbiosis in individuals at risk for severe & critical COVID-19

Aging and comorbidities such as hypertension, cardiovascular disease, diabetes, obesity, chronic respiratory diseases, neurologic diseases and immunosuppression are significant risk factors for severe and critical COVID-19 [40,85]. The presence of obesity-associated fatty liver disease was associated with an approximately sixfold increased risk of severe COVID-19 (unadjusted odds ratio: 5.77; 95% CI: 1.19–27.91; p = 0.029), even after adjusting for age, sex, smoking, diabetes, hypertension and dyslipidemia (adjusted odds ratio: 6.32; 95% CI: 1.16–34.54; p = 0.033) [86].

Although the precise composition of the healthy gut microbiota is not known, it is evident that microbial diversity adds to the host's health. Obesity, hypertension and diabetes are diseases extensively associated to dysbiosis, in which increased intestinal permeability and chronic inflammation occur [87,88]. A systemic, low-grade inflammatory state is a hallmark of obesity and the metabolic syndrome. A wide range of inflammatory markers, namely C-reactive protein and pro-inflammatory cytokines are strongly associated with adiposity [89].

Multiple lines of evidence implicate gut dysbiosis as a key modulator of immune signaling in the context of metabolic diseases. LPS binds to toll-like receptors in mucosal and peripheral tissues, thereby initiating pro-inflammatory signaling [90,91]. Studies in both human and murine models have linked the obesity phenotype to endotoxemia, characterized by elevated LPS plasma levels [76,92]. Baseline levels of LPS were 20% higher in patients with obesity or glucose intolerance, whereas those with Type 2 diabetes had levels 125% higher than healthy subjects [93]. On one hand, pathogenic strains that dominate in gut dysbiosis are a rich source of LPS that could enter the circulation and initiate an immune response. On the other hand, there is strong evidence of the essential role of the gut microbiome in maintaining the integrity of the epithelial barrier; its alteration would allow increased intestinal translocation of endotoxins [94].

Microbial ecological niches are mostly limited by the external mucus layer of epithelia in the gut, interacting with the environment in the lumen and having important roles in the metabolism of components of the diet. The inner mucus layer serves as a barrier between epithelial cells and microorganisms, including potential pathogens. Symbiotic bacteria impede the proliferation of exogenous bacteria that could damage the host [95].

Microbial interactions within the gut–lung axis

The lung microbiome has recently been recognized as a cornerstone in the physiopathology of numerous respiratory diseases [96]. The predominant bacterial phyla in lungs are the same as the ones in the gut, mainly Firmicutes and Bacteroidetes, followed by Proteobacteria and Actinobacteria [97]. From birth and throughout the entire life span, a close correlation between the composition of the gut and lung microbiome exists, suggesting a host-wide network [98]. Conversely, modifications in the lung microbiota after antimicrobial exposure may affect the gut microbiota composition [99]. Interactions between the lung microbiome and immunity are also a two-way process; major inflammatory events in the lungs can morbidly alter the composition of the lung microbiota [100].

Intestinal permeability, translocation, inflammation & COVID-19

As mentioned in the previous section, the long-reaching immunologic impact of the gut microbiome in other organs and systems (i.e., lungs and immune system) is increasingly recognized [101]. The mesenteric lymphatic system shapes the crucial road network allowing intercommunications between the lungs and the intestine. This way, microorganisms, microbial fragments (i.e., LPS) and metabolites (i.e., short chain fatty acids) may cross the intestinal mucosal barrier and reach the lung, thereby modulating the lung immune response [102,103].

Particularly important players in this long-reaching immune interaction are gut segmented filamentous bacteria, which are commensal bacteria that colonize the ileum in most animals, including humans, and modulate the immune system [104]. Segmented filamentous bacteria regulate $CD4^+$ T-cell differentiation toward the Th17 phenotype, implied in the response to pulmonary fungal infections and manifestations of autoimmune diseases in the lung [105,106]. Recently, innate lymphoid cells, which are involved in tissue repair, have been shown to be recruited from the gut to the lungs in response to inflammatory signals upon IL-25 [107]. Of note, both Th17 and innate immune cells are major sources of second order cytokines, required for effective early microbial clearance in the lungs [108].

As mentioned earlier, SARS-CoV-2 can infect cells that express high levels of hACE2 and transmembrane serine protease 2, a common feature between the lungs, esophagus, small intestine, and to a lesser extent, the large intestine. Although the specific mechanisms involved in the pathogenesis of diarrhea in COVID-19 are not entirely known, viral infection likely alters intestinal permeability, resulting in enterocyte malabsorption [86]. Intestinal inflammation, as reflected by increased calprotectin levels, correlates with the presence of diarrhea in COVID-19 [109].

Alternatively, SARS-CoV-2 could enter and shape components of the gut microbiome through glycan receptor binding via the S glycoprotein, in a similar fashion to its proven entry to common pathogens of the lungs (*Streptococcus pneumoniae* and *Pseudomonas aeruginosa*) [110]. The significance of SARS-CoV-2 entry to microorganisms of the gut and lung microbiomes remains to be studied.

Elucidating the basis of diarrhea in COVID-19 is important since patients with diarrhea had a higher need for ventilatory support (26.4 vs 8.2%; $p = 0.004$) and intensive care (49.0 vs 11.8%; $p < 0.001$), suggesting increased severity, although no correlation with the mortality rate was found [86].

Gut microbiome & severity of infection in COVID-19

The small intestine comprises the largest organ with immune functions in humans, and we have discussed how the gut microenvironment is able to affect multiple organs and systems, including the lungs.

Researchers from Hong Kong have longitudinally characterized the bacterial [111] and fungal [112] composition of the gut microbiome in hospitalized patients through the entire spectrum of symptomatic COVID-19 (mild-to-critical), compared with non-SARS-CoV-2 pneumonia and healthy controls. Higher numbers of opportunistic bacteria (*Clostridium hathewayi*, *Actinomyces viscosus* and *Bacteroides nordii*) and fungi (*Candida albicans*, *C. auris* and *Aspergillus flavus*) were found in patients with COVID-19, while patients who received antibiotics were depleted in bacterial symbionts (*Fecalibacterium prausnitzii*, *Lachnospiraceae bacterium* 5_1_63FAA, *Eubacterium rectale*, *Ruminococcus obeum* and *Dorea formicigenerans*). Dysbiosis was found to persist throughout hospitalization, even after resolution of symptoms and a negative throat swab reverse-transcriptase polymerase chain reaction (RT-PCR) for SARS-CoV-2; fungal opportunistic pathogens also persisted (*A. flavus* and *A. niger*). Predominance of certain Firmicutes bacteria, which are negatively associated with expression of ACE2 in murine models, was also negatively correlated with severity of COVID-19. The opposite was true for other Firmicutes bacteria that are associated with greater expression of ACE2. Higher expression of hACE2 in normal human tissues has also been noted to occur under the presence of certain microbial and immunological signatures [113], which further supports the possible link between COVID-19 progression and the microbiome.

In a cross-sectional study of hospitalized patients with mild-to-severe COVID-19, gut microbial composition of patients had a low diversity compared with healthy controls, a finding similar to patients with Influenza A H1N1 infection [114]. Relative abundance of *Streptococcus* and *Escherichia/Shigella* was higher both in patients with COVID-19 and influenza. Patients with COVID-19 had predominance of *Streptococcus*, *Rothia*, *Veillonella*, *Erysipelatoclostridium* and *Actinomyces*. The authors of this study created two prediction models to distinguish COVID-19 from influenza A H1N1 infection and from healthy controls, by using gut microbial components as biomarkers, finding an overall good performance. However, this model was not validated and does not distinguish between mild and severe disease due to similar microbial compositions in both groups at admission.

Another prospective study of 15 hospitalized patients with COVID-19 found that viral infection in the GI tract occurs even in the absence of gastrointestinal symptoms and after resolution of all symptoms [115]. Interestingly, active replication of SARS-CoV-2 and transcriptional activity were correlated with shifts in the microbiota with greater abundance of opportunistic bacteria (*Collinsella aerofaciens*, *C. tanakaei*, *S. infantis* and *Morganella morganii*), while low signatures of SARS-CoV-2 infection in the gut was associated to higher presence of short-chain fatty acid producing bacteria (*Parabacteroides merdae*, *B. stercoris*, *Alistipes onderdonkii* and *L. bacterium* 1_1_57FAA).

Other studies have implied inflammation as a determinant factor of the intestinal microbiome in COVID-19, which could in turn accentuate dysregulation of the immune function: reductions in *Bifidobacterium*, *Lactobacillus* and *Eubacterium*, while significant increases in *Corynebacterium*, *Actinobacterium* and *Ruthenibacterium* (Firmicutes) were detected; fungi not commonly found in healthy subjects were also found (*Aspergillus* and *Kluyveromyces*) [116,117]. Changes in the intestinal microbiota in the context of patients with severe disease are thought to reflect the prominent inflammatory state.

Patients who develop acute respiratory distress syndrome (ARDS) have shifts in the pulmonary microbiota toward a richer composition in usual components of the gastrointestinal microbiome (*Enterobacteriaceae* and *Bacteriodetes*) [118]. Even when gastrointestinal pathogens have not been encountered in respiratory samples of patients with ARDS and SARS-CoV-2 infection, patients with COVID-19 have diminished microbial diversity in their lung microbiome, with increased abundance of opportunistic pathogens (Candida and other viruses), and transcriptional patterns that are associated with inflammation pathways [119].

Search strategy

We searched MEDLINE and EMBASE through OVID, PubMed, BioRxiv and MedRxiv for research on COVID-19 published until 30 October 2020. We used the publicly available COVID-19 Living Evidence on COVID-19 dataset [120]. Search terms for the first search strategy related to inflammation were: ('severe acute respiratory syndrome coronavirus 2' [supplementary concept] OR 'COVID-19' [supplementary concept] OR 'coronavirus' OR 'HCoV' OR 'nCoV' OR '2019 nCoV' OR 'covid' OR 'covid19' OR 'severe acute respiratory syndrome coronavirus 2' OR 'SARS-CoV-2' OR 'SARS-CoV 2' OR 'SARS coronavirus 2') AND ('inflammation' OR 'immunity' OR 'immune system' OR 'adaptive immunity' OR 'innate immune system' OR 'cytokine storm' OR 'macrophage activation syndrome' OR 'interferon'). Search terms for the second search strategy related to the microbiome were: ('severe acute respiratory syndrome coronavirus 2' [supplementary concept] OR 'COVID-19' [supplementary concept] OR 'coronavirus' OR 'HCoV' OR 'nCoV' OR '2019 nCoV' OR 'covid' OR 'covid19' OR 'severe acute respiratory syndrome coronavirus 2' OR 'SARS-CoV-2' OR 'SARS-CoV 2' OR 'SARS coronavirus 2') AND ('microbiome' OR 'microbiota' OR 'human microbiome' OR 'lung microbiome' OR 'respiratory microbiome' OR 'gastrointestinal microbiome' OR 'gut microbiome'). Studies were chosen regardless of language, provided an abstract in English was available, and if considered relevant by consensus of at least two authors.

For studies published prior to 2020, no specific search strategy was conducted and references were chosen at the authors' best judgment to provide educational context to readers, and may thus be biased toward the authors' opinions in this perspective article.

Conclusion

Knowledge on the mechanisms at the molecular, cellular and systems levels by which SARS-CoV-2 causes disease in humans have been studied and communicated in an incredibly expedite manner never seen before. SARS-CoV-2 enters a wide variety of human cells in an extraordinarily efficient way which allows the virus to evade the immune system initially, thereby impeding robust type I and III IFN responses much needed to eliminate the virus. Dysregulation of RAAS and late type I and III IFN overactivation contribute to progression of the disease in susceptible individuals, although other mechanisms leading to severe and critical COVID-19 are most likely uncovered at this moment. Potential infection of cells of the immune system and direct damage to these cells by SARS-CoV-2 remains to be studied to understand how the immune response could be directly affected by the virus.

We have discussed some ways by which the microbiota could possibly promote inflammation in individuals with COVID-19. We have mentioned bacterial translocation and the presence of LPS in the bloodstream as a potential promoter of inflammation, which could contribute in a similar way to the inflammatory storm which occurs in sepsis. miRNAs are also potential promoters of systemic inflammation which could be acting in different ways. For example, established dysbiosis in patients with chronic diseases could be the set point allocating these

patients at increased risk of developing greater inflammation. Studying dietary and lifestyle habits in persons with comorbidities before COVID-19 infection and looking for differences in these groups could be an interesting way of establishing this connection. Cohorts of patients with chronic diseases in whom the microbiota composition has been prospectively studied could serve as the perfect group to study how dysbiosis affects predisposition to COVID-19 severity and outcomes.

The gut and lungs are robustly intercommunicated through pathways such as the mesenteric lymphatic system, although the bloodstream could also serve as another highway communicating the lungs and gut through small molecules such as bacterial metabolites or miRNAs, which are likely to be able to travel long distances in the body since they are contained inside extracellular vesicles. What happens in the lung can have consequences in the gut and *vice versa*, while intercommunication through the immune system is undoubted.

Interestingly, SARS-CoV-2 has been shown to enter bacteria through rhamnosylated epitopes which are present in some pathogenic bacteria. Exploring the expression of these epitopes in components of both healthy and dysbiotic microbiomes could be the first step toward understanding if SARS-CoV-2 is able to shape the microbiota by directly interacting with their components. The same could apply to fungal components of the microbiome.

To date, only the microbiota (taxonomy of microbial components) has been studied in the context of COVID-19. Studies aiming to describe the metagenomics and transcriptomics of microbial components in the context of COVID-19 will allow to start characterizing the extent to which the microbiome affects or is affected by COVID-19.

Finally, answering these questions could allow the scientific community to develop ingenious ways to ameliorate disproportionate inflammation, prevent disease progression or improve patient outcomes.

Future perspective

A great number of scientists from multiple backgrounds have united efforts and redirected their attention toward rapid characterization of SARS-CoV-2 and the disease it causes in humans (COVID-19). This is unprecedented, since no biologic phenomena had ever drawn this amount of attention. Discoveries in this topic have occurred in an impressively accelerated way, and the pace at which breakthroughs are occurring suggests that it will not be long until we have precise characterizations of the virus, its pathophysiology and available therapeutics, including vaccines, to combat this pandemic. Characterization of the interplays between the lungs, gut, immune system and the human microbiome will aid to have a greater understanding of how these elements could determine disease progression or modulate responses to infection. This will open new venues toward developing innovative interventions for COVID-19.

Author contributions

MM Aguirre García supervised and contributed to all sections of the manuscript. J Mancilla-Galindo and N Ávila-Vanzzini contributed to the initial sections of the manuscript. M Paredes-Paredes, J Mancilla-Galindo and A Zamudio Tiburcio contributed to the final sections of the manuscript. J Mancilla-Galindo assembled the figures. All authors read and approved the final manuscript.

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Executive summary

- Coronavirus disease (COVID-19), the pandemic caused by the SARS coronavirus 2 (SARS-CoV-2), has challenged current societies due to rapid spread and lack of therapeutics.

SARS-CoV-2 utilizes efficient mechanisms to establish infection & evade the innate immune response

- The mechanisms involved include antigen hiding, pre-activation of its spike protein and avid recognition of its receptor, the human angiotensin-converting enzyme 2. This allows the virus to initially prevent adequate interferon (IFN) responses.

Immune-mediated injury is the predominant pathophysiological driver in severe & critical COVID-19

- Early IFN responses have been related to successful resolution of the infection, whereas late and sustained IFN responses are related to disease progression.
- Unregulated angiotensin II could promote inflammation pathways, especially in individuals with diseases involving alterations in the renin–angiotensin–aldosterone system.
- Progressive increases in inflammation and hypercoagulability are two main hallmarks of severe and critical COVID-19.
- Clinical, pathologic and experimental studies have revealed the central role of inflammation in COVID-19 pathophysiology.
- Early interventions in patients with risk factors could stop progression to severe and critical COVID-19.

SARS-CoV-2 productively infects human enterocytes

- SARS-CoV-2 productively infects human enterocytes, altering gene expression, cytokine production, IFN pathways and human angiotensin-converting enzyme 2 expression.

Gut microbiome

- The components of the human microbiome comprise different microorganisms (i.e., bacteria, viruses and fungi).
- miRNAs are 21–24 RNA molecules frequently encountered inside extracellular vesicles. miRNAs produced by the intestinal epithelium and from diet are able to shape the gut microbiome, while certain miRNA profiles promote shifting to a microbiome associated with decreased or increased intestinal inflammation.

Microbial interactions within the gut–lung axis

- The gut–lung axis results from complex interactions between the different microbial components of both the gut and lung niches, and the immune system. Events occurring in the lungs are able to alter the gut and *vice versa*.

Intestinal permeability, translocation, inflammation & COVID-19

- Microorganisms, microbial fragments (i.e., lipopolysaccharide) and metabolites (i.e., short-chain fatty acids) may cross the intestinal mucosal barrier and reach the lung through the mesenteric lymphatic system and bloodstream.
- Diarrhea is an important manifestation of COVID-19, which correlates with inflammation and disease severity. This symptom is likely a result of malabsorption, although SARS-CoV-2 is able to enter microorganisms with rhamnosylated epitopes, which leaves an open question for possible direct effects in microbial components of the microbiota.

Gut microbiome & severity of infection in COVID-19

- Few studies have investigated the role of the microbiome in COVID-19. In patients without diarrhea, dysbiosis was related to disease severity.
- In acute respiratory distress syndrome, the lung microbiota shifts toward a richer composition in habitual intestinal microorganisms.
- Studies with translational approaches are needed to better understand COVID-19 and possible interventions.

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Studying the molecular mechanism of viral replication in real time using the CLARIOstar *Plus* with ACU

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- **A fluorescent-based approach for near real-time measurement of viral gene expression**
- **Can be used to study virtually any virus in a 96- or 384- well format**
- **High reproducibility and sensitivity make it suitable for genetic and drug screens**

Introduction

Viruses are obligate intracellular pathogens that can infect all the living organisms. They not only assume an outmost medical relevance due to their connection with several human diseases, but they also have a major economic impact given the costs required for medical treatments and for protecting cattle and crops. Viruses encode a limited number of proteins, and they thus interact and hijack host cellular machineries to promote their replication and spread. Therefore, understanding host-virus interactions become crucial to uncover the molecular mechanisms underpinning infection, and for the development of new treatments. With the emergence of 'omics' in the last decade, known host-virus interactions have been massively expanded, calling for novel approaches to test the importance of cellular proteins in virus infection in a systematic way. Moreover, the discovery of antivirals often relies on the screen of large libraries of compounds, which also poses a technical challenge. Hence, to test the importance of cellular proteins in virus infection and to discover new antivirals, robust and efficient high-throughput methods are required to measure viral fitness without using expensive reagents such as luciferin.
Here we describe a method to analyse viral gene expression in near real time using fluorescence as a proxy for infection. This assay can discover not only if a given condition inhibits or enhances virus infection, but also provides insights into its effects on the kinetics of infection.

Assay Principle

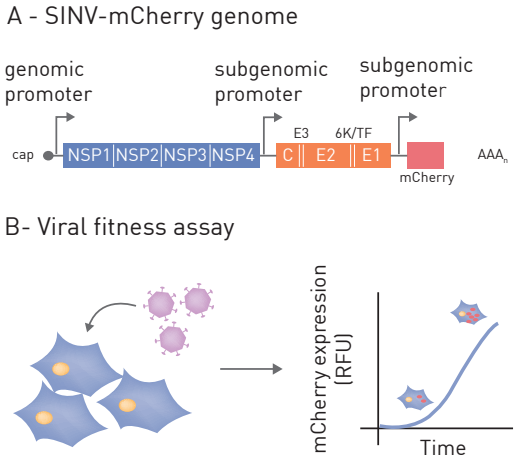

Fig. 1: Schematic representation of (A) SINV-mCherry and (B) the viral fitness assay.

This application note illustrates a fluorescent-based method for monitoring viral gene expression using a BMG LABTECH CLARIOstar *Plus* microplate reader with Atmospheric Control Unit (ACU). As an illustrative example, we employ Sindbis virus (SINV, Alphavirus genus) with engineered genome containing the coding sequence for mCherry fluorescent protein. Expression of mCherry can be used as a proxy for viral gene expression and can be measured at regular intervals throughout the infection. Although exemplified with SINV, this approach has been applied successfully to a broad range of viruses including the human immunodeficiency virus (HIV).

Materials & Methods

- 96-well microplate, flat µclear bottom (Greiner Bio-One). This protocol is, also compatible with 384-well microplates.
- Colourless DMEM medium
- SINV-mCherry virus stock (~5x10^8 pfu/ml)
- BMG LABTECH CLARIOstar *Plus* microplate reader with ACU

Experimental Procedure
HEK293 cells were seeded at a density of 5x10^4 cells in a 96-wells microplate with flat µclear bottom. Infection was performed using SINV-mCherry at 0.1 multiplicity of infection (MOI), and cells were incubated at 37° C with 5% CO_2 in the CLARIOstar *Plus* plate reader for 24 hours. Incubation time should be adapted to the lifecycle duration of each virus (e.g. 72 hours for HIV). The viral genome expression was monitored by the detection of the red fluorescent signal derived from mCherry (excitation: 570 nm, and emission: 620nm), which was measured every 15 minutes. Addition of drugs or other reagents (e.g. interferon) can be performed before or after starting the programme in the plate reader.

Instrument settings

Optical settings	Filters	Excitation 570-15 Dichroic 593.8 Emission 620-20
	Focal height	4.4 mm
	Gain	2900
General settings	Number of flashes	8
	Settling time	0 s
Scan settings	Orbital averaging	
	Scan diameter	4 mm
Kinetic settings	Number of cycles	92
	Cycle time	900 s
Incubation	37° C, 5% CO_2	

Results & Discussion

To assess viral genomic replication we infected WT (wild type), XRN1+/- (partial knockout), and XRN1-/- (complete knockout) cells with SINV-mCherry and the red fluorescence protein was monitored every 15 minutes for 24 hours using the CLARIOstar *Plus* microplate reader. The detection of fluorescent signal every 15 minutes for 24 hours post infection (hpi) was enough to cover both, the early and late phases of SINV infection. In HEK293 WT cells, SINV gene expression was detected at around 8 hpi with a subsequent constant increase (figure 2). In cells completely lacking XRN1, viral gene expression drops to background level. These results reflect that XRN1 is a critical host factor for SINV infection and the lack of it causes cells to become refractory to infection[1] (figure 2).

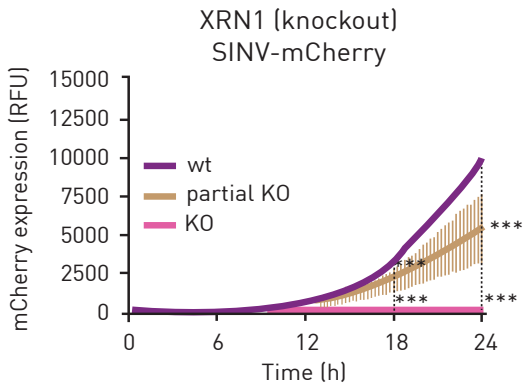

Fig. 2: Detection of mCherry fluorescence signal in the indicated HEK293 cells. Adapted from[1]

The high sensitivity of the assay allows for the profiling of intermediate phenotypes in a very reproducible manner. For example when XRN1 is partially depleted, SINV infection is delayed but not fully supressed[1] (figure 2).

In addition, this assay allows the testing for drug effects on viral fitness. For example, figure 3 shows that cyclosporin A, a known inhibitor of cyclophilin A (PPIA), moderately delays SINV gene expression to a comparable level to that of a PPIA knock out (figure 3, bottom panel). This example goes to highlight the potential of this application for screening the effects of proteins and drugs on viral gene expression.

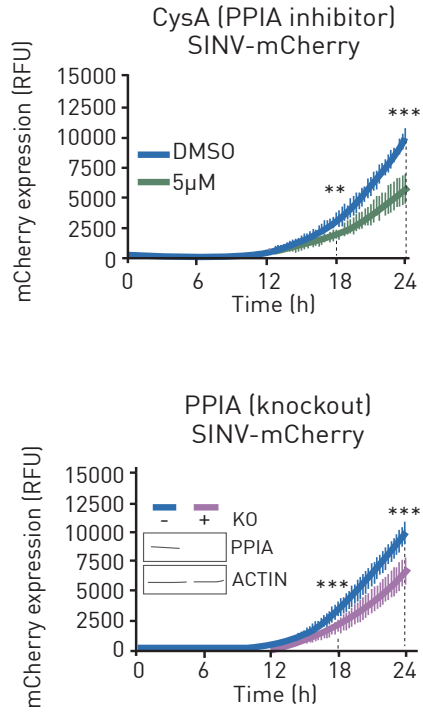

Fig. 3: Detection of mCherry fluorescence signal in the presence of the PPIA inhibitor (top panel) and in the wild type and PPIA knock out HEK293 cells (bottom panel). Adapted from[1]

Conclusion

The viral fitness assay can be successfully performed using the CLARIOstar and CLARIOstar *Plus* fluorescence microplate reader with ACU permitting the study of viral gene expression in multi-well plates in real time for up to 72 hours post infection. This method allows for the simultaneous screening of a broad range of experimental conditions, such as drug libraries and knock out cell lines in virus infection.

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Fluorescence-polarization based RNA synthesis assay

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- **HTS-compatible assay to identify compounds targeting influenza virus polymerase (FluPol)**
- **Suited for enzymatic characterization of FluPol and mechanism of action-studies**
- **Reliable detection is provided by the CLARIOstar's FP detection system**

Introduction

Influenza virus is a major threat to global public health. Essential to virus propagation is the viral RNA-dependent RNA polymerase (FluPol) which amplifies the viral genome and transcribes mRNAs coding for viral proteins. FluPol, a ~260 kDa multifunctional heterotrimeric complex, is an attractive target for anti-influenza drug discovery (1). Here, we present a novel fluorescence polarization assay that directly reads out RNA synthesis by FluPol (2). Moreover, the assay is applicable for measuring RNA synthesis in general and is compatible with high throughput screening.

Assay Principle

The fluorescence polarization (FP) of fluorescently-labelled nucleic acids is sensitive to the conformation of the fluorophore's environment. Specifically, the FP of FAM-labelled single-stranded RNA increases when it becomes double-stranded (Figure 1). Using an 18 nucleotide 5'-end FAM-labelled single-stranded "template" RNA, the highest signal amplitude (ΔFPobs) is observed when adding 18 nt complementary RNA (black), resulting in a perfect 18 bp dsRNA. Increasing the single-stranded environment of the fluorophore by shortening the complementary RNA to 16 nt (yellow) or 14 nt (purple), respectively, reduced ΔFP^{obs} max. Active-site titration of the perfectly complementary RNAs of 18 nt (black) and 16 nt (yellow) was observed, while the interaction of the non-perfectly complementary 14 nt RNA (purple) would correspond to a K_D of ~0.5 µM. Note that changing the RNA sequence or the hybridization strength may affect ΔFP^{obs} max and shift the detection limit where the FP-signal is directly proportional to the amount of product RNA. The 5'-end FAM-labelled 18 nt "template" RNA used here actually corresponds to the influenza virus promoter 3' RNA and this serves as template for RNA synthesis by FluPol in biochemical assays *in vitro* (2). This will produce a complementary 18 nt product RNA at a certain rate, which if it hybridizes to the FAM-labelled template will produce a change in its FP-signal. Before recording the FP-signal of the enzyme-catalyzed RNA synthesis, the reaction needs to be quenched (e.g. by high salt) to dissociate FluPol and RNAs while allowing RNA-RNA-interactions (Figure 2). With this "trick", the contributions to the observed FP-signal of FluPol and the FAM-labelled template RNA interacting are removed and the detected FP-signal is directly proportional to the ratio of product RNA over template RNA (Figure 1).

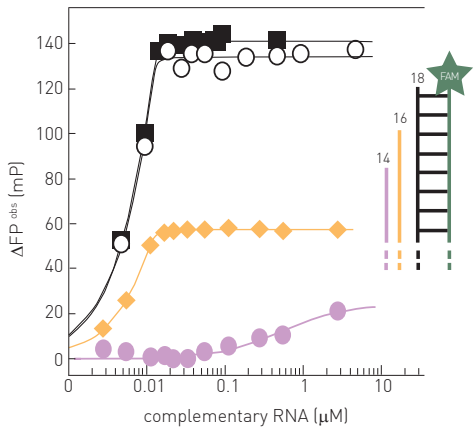

Fig. 1: Assay Principle
Observed Fluorescence Polarization is proportional to and most sensitive for a full-length RNA complementary to a 18 nt FAM-labelled single-stranded "template" RNA. Constant 0.015 µM "template" RNA (5'-(FAM-Ex-5)-UAUACCUCUGCUUCUGCU-3') interacting with 18 nt complementary RNA (black), 16 nt complementary RNA (yellow) or 14 nt non-perfectly complementary RNA (purple; 5'-pAGUAGUAACAAGAG-3'). The FP-signal of synthetic 18 nt complementary RNA and the labelled "template" RNA interacting was not affected by 0.02 µM FluPol (black squares). Modified from (2).

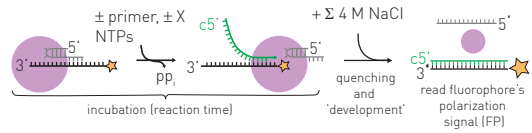

Fig. 2: Enzymatic RNA synthesis assay - Workflow
Influenza polymerase (FluPol, purple sphere) activated by 5' RNA and bound to the 3' template RNA (labelled by FAM-Ex-5 at its 5' end) is incubated with NTPs and optionally a primer or generally a molecule X whose effect on RNA synthesis is to be monitored. The reactions are quenched by addition of 4 M NaCl which perturbs polymerase-RNA-interactions but permits RNA-RNA-interactions. Fluorescence polarization (FP) is directly proportional to the ratio of full-length product RNA over labelled template RNA. Modified from (2).

Materials & Methods

- Oligonucleotides (IBA Lifesciences)
- Primer (5'-(N7MeGppp)AAUCUAUAAUAG-3'), 2'F-2'dNTPs (Trilink Biotechnologies)
- NTPs (Sigma)
- FluPol (influenza B polymerase; Reich *et al.*, 2014)
- 384 well plates (Greiner, #781076)
- CLARIOstar® (BMG LABTECH)

RNA synthesis was performed as described. Briefly, RNA synthesis was initiated by supplementing 0.25 µM FluPol (influenza B polymerase; bound to v5' RNA nts 1-14) with 0.15 µM FAM-labelled template RNA (5'-(FAM-Ex-5) UAUACCUCUGCUUCUGCU-3'), 0.5 µM primer and 25 µM NTPs (each) in assay buffer (50 mM HEPES/NaOH, 150 mM NaCl, 10% (v/v) glycerol, 5 mM $MgCl_2$, 2 mM

TCEP, 1% (v/v) DMSO, pH = 7.4). RNA synthesis proceeded in polypropylene reaction tubes and at indicated times aliquots of 10 µl were transferred to 80 µl of quenching solution (4.5 M NaCl) before the FP-signal was recorded in 384 microplates using the CLARIOstar® (BMG LABTECH). Dr. J. Timmins (IBS Grenoble) is acknowledged for access to the microplate reader.

Instrument Settings

Optic settings	Fluorescence Polarization, endpoint	
	Filters	Ex: 482-16 Dichroic: LP504 Em: 530-40
	Focus and gain	adjusted before measurement
	Target mP	35
	Flashes	50 per well
General settings	Set tling time	0.1 s

Results & Discussion

With the described RNA synthesis assay, infl uenza polymerase can be enzymatically characterized *in vitro*, e.g. regarding promoter RNA requirements, primer effi ciency, K_M (NTPs), turnover numbers, initiation strategies or inhibition (2). Kinetics of RNA synthesis are most informative and report on the amount of product and the rate it is produced. Figure 3 shows complete kinetics of RNA synthesis catalyzed by FluPol (primed with capped RNA) at 25 µM NTPs (black) and the effect of increasing concentrations of the inhibitor 2'F-2'dCTP (X; purple (8 µM) to dark red (8.3 mM)). Progress curves are fi tted double-exponentially according to a pseudo-fi rst order rate law (solid lines) enabling the RNA synthesis rate constants *k* (min^{-1}) to be derived.

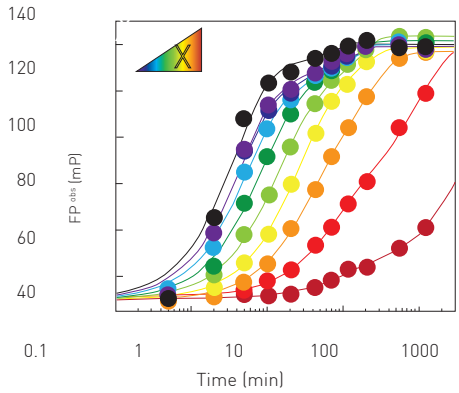

Fig. 3: RNA synthesis kinetics (and inhibition)
RNA synthesis kinetics of FluPol at 25 µM NTPs (each) and 24 °C. The NTP-analogue 2'F-2'dCTP (X; purple blue (8 µM) to dark red (8.3 mM) impeded the uninhibited RNA synthesis reaction (black) in a concentration-dependent manner. Modified from (2).

The rate constants corresponding to the prominent, fast RNA synthesis phase are shown in Figure 4, open black circles and yielded an IC50 (2'F-2'dCTP) of 0.2 mM. When quenching the synthesis reaction at a defined reaction time (within the linear range), the assay becomes compatible with high throughput screening campaigns. Figure 4 shows dose-response curves of NTP-analogues at conditions as in Figure 3 but allowing RNA synthesis to proceed for 5 minutes only and yielded e.g. IC50 (2'F-2'dCTP) = 0.2 mM (Figure 4, black squares), in agreement with the IC50-value determined from complete kinetics (Figure 4, black open circles).

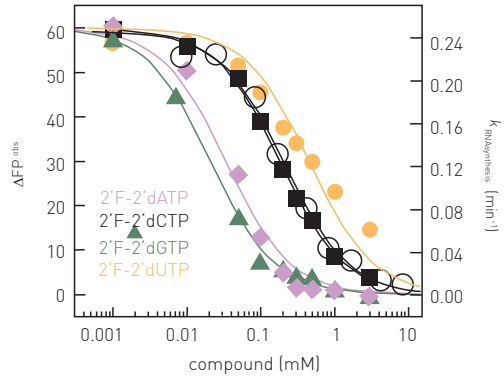

Fig. 4: Inhibition of RNA synthesis in dose-response Characterization of NTP-analogues inhibiting FluPol catalysed RNA synthesis in HTS-compatible mode. Here, FP-signals were recorded after 5 minutes reaction time and yielded IC_{50}-values of 0.03 mM, 0.2 mM, 0.02 mM and 0.45 mM for 2'F-2'dATP (purple diamonds), 2'F-2'dCTP (black squares), 2'F-2'dGTP (green triangles) and 2'F-2'dUTP (yellow circles), respectively. For comparison, a similar IC50-valueof 0.2 mM for 2'F-2'dCTP is determined by recording and fitting complete RNA synthesis kinetics (see Figure 3) and analysing the observed rate constants (black open circles) Modified from (2).

Conclusion

We developed a simple *in vitro* RNA synthesis assay that utilizes fluorescence-polarization changes of FAM-labelled model template RNAs associated with FluPol catalyzed product RNA formation. The assay is high throughput compatible and can easily be performed in 384 well microplates. It reliably reports on compounds inhibiting RNA synthesis, readily can be miniaturized to 0.2 pmole/reaction of recombinant (active) FluPol and might provide an attractive choice for drug discovery campaigns.

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