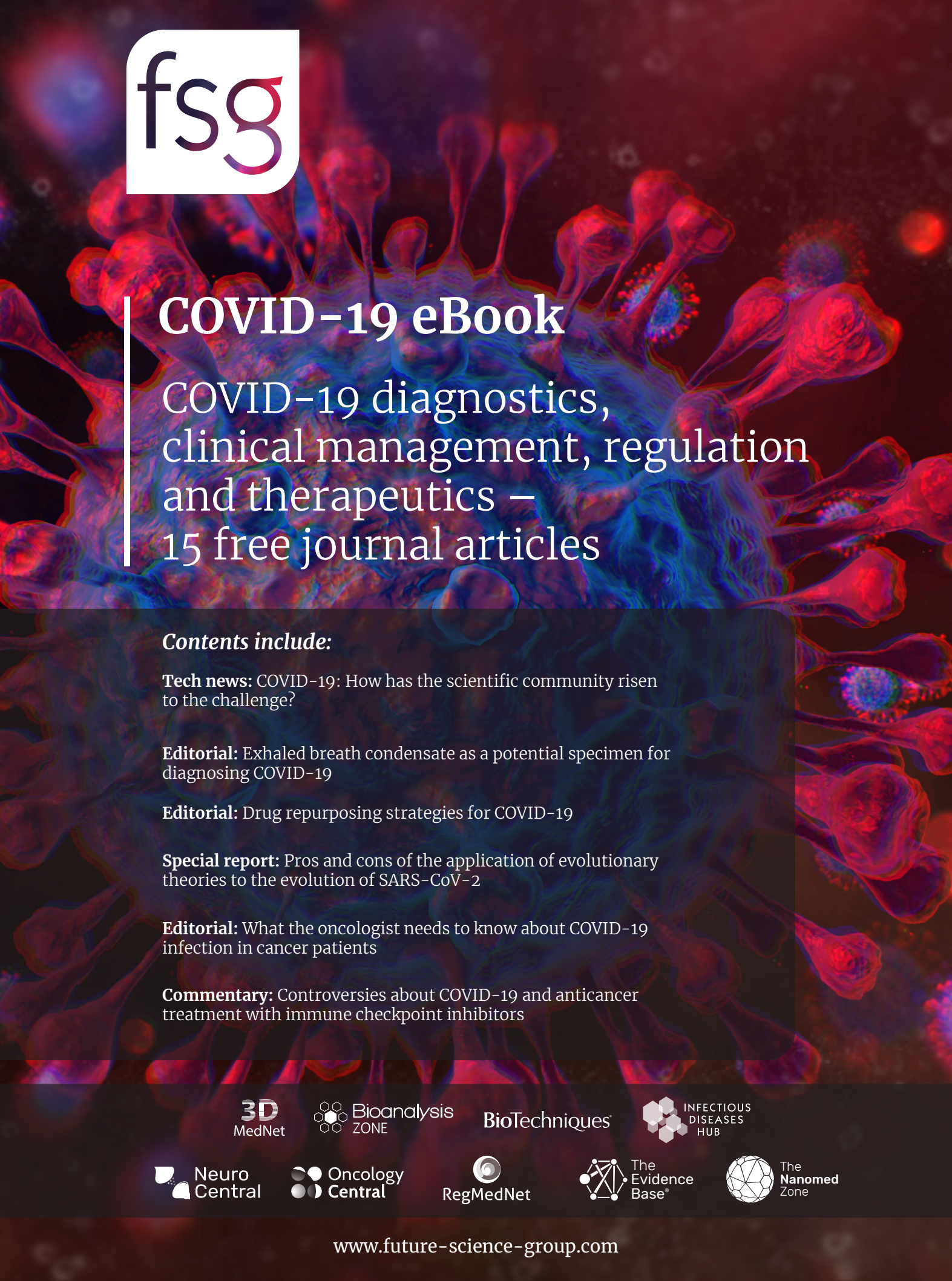

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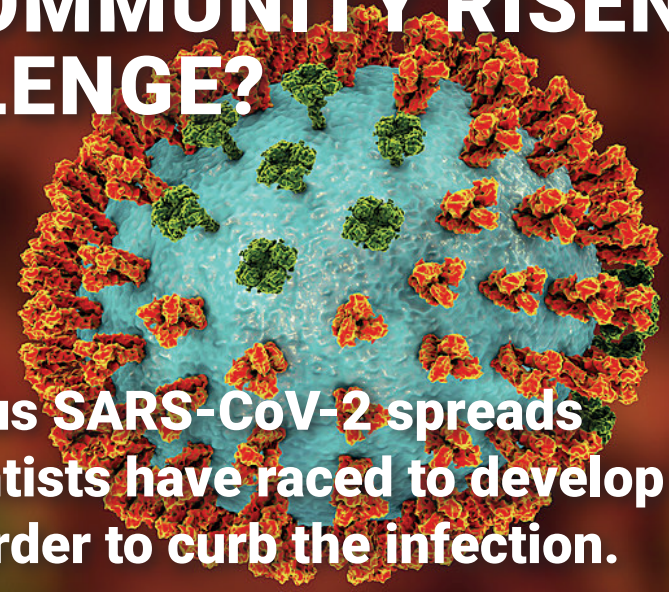
COVID-19: HOW HAS THE SCIENTIFIC COMMUNITY RISEN TO THE CHALLENGE?



As the novel coronavirus SARS-CoV-2 spreads around the world, scientists have raced to develop vaccines and tests in order to curb the infection.

Throughout history, some of the greatest advances in science and medicine have come out of the worst situations. During the Crimean War, Florence Nightingale, among others, revolutionized the practice of nursing; by cracking the Enigma code in the Second World War, Alan Turing brought about the earliest computer; and the Cold War battle of will between America and the USSR resulted in the first man on the moon. Whatever the war, whichever the countries in battle, scientific advancement remained a neutral good that would benefit all involved.

Now, the latest war the world at large has faced is not between countries, but between mankind and a virus. "*Nous sommes en guerre [We are at war],*" announced French president Emmanuel Macron on 16 March 2020; a statement soon to be adopted by other world leaders across the globe as all nations began to acknowledge the severity of the coronavirus situation.

However, despite the delay in political recognition of the virus, the scientific community was already on the frontline. On the same date as Macron's declaration, the first Phase I trial for a vaccine for the novel coronavirus was announced by the biotechnology company Moderna, Inc. (MA, USA) [1]. By the time politicians and the world at large began taking the virus seriously, scientists had already determined the full genome sequence of SARS-CoV-2 [2], isolated it from clinical samples [3], and were on the hunt for drug combinations that could be repurposed against this novel strain. The scientific community had risen to the challenge before many others even acknowledged that it existed.

THE RACE TO DEVELOP A VACCINE

Traditionally, the timeline to create a new vaccine spans over 15–20 years as a product is proven safe, first in lab animals and then in human volunteers. Although not written in law, animal testing is almost always used prior to human trials in order to check safety and negate the risk of adverse effects. Given the urgency to develop the COVID-19 vaccine, the researchers at Moderna, Inc. broke from the usual testing protocol and are planning on conducting nonclinical research in parallel to the clinical trial. "*I don't think proving this in an animal model is on the critical path to getting this to a clinical trial,*" commented Tal Zaks, Moderna, Inc's Chief Medical Officer [4].

The benefit of vaccines has been long proven and, if successful, their ability to stop an infection has been well documented. For many, the potential benefits outweigh the risk of an unproven treatment as stopping the rapidly spreading virus remains priority one. However, not all agree: "*Outbreaks and national emergencies often create pressure to suspend rights, standards and/or normal rules of ethical conduct. Often our decision to do so seems unwise in retrospect,*" noted Jonathan Kimmelman, a Professor in Biomedical Ethics at McGill University (QC, Canada) [4].

Despite the controversy, following Moderna, Inc's announcement more and more companies announced the launch of new therapeutic programs, clinical trials or collaborations, each in an attempt to develop the first viable vaccine to stop the spread of the virus [5].

The speed at which the first vaccine is being developed is, in part, due to the prompt actions of Chinese scientists who quickly sequenced the novel coronavirus, identifying SARS-CoV-2 as the ▶

cause of the disease. The genetic sequence for SARS-CoV-2 was shared in early January and, since then, researchers all over the globe have been able to study the virus and gain a greater understanding of how it enters and infects the human body.

However, it was not only dependent on the work done since the identification of the SARS-CoV-2. For years, vaccinologists have worked on early-stage vaccines for 'prototype' pathogens, getting ready for the next outbreak. The Coronaviridae family of viruses shares large quantities of genetic material with SARS-CoV-2, sharing between 80 and 90% of DNA with the virus that caused the SARS outbreak in 2002, SARS-CoV – hence the name. This means that vaccine developers already had a set of semi-developed vaccines that they could pull out and adapt to the latest member of the family; a method exemplified by vaccine company Novavax (MD, USA) who announced that, utilizing technology developed for the SARS and MERS outbreaks, they are developing a vaccine for COVID-19 that they hope to have in human trials by the end of spring 2020 [6].

Even with the unprecedented speed at which these vaccines have been brought to Phase I trial, it seems unlikely that they will be available for clinical use for many more months, if not years. From then, further challenges will have to be overcome before the vaccine is globally available, with politics and economics a likely barrier to scientific development. "*Getting a vaccine that's proven to be safe and effective in humans takes one at best about a third of the way to what's needed for a global immunisation programme*," Jonathan Quick, a Professor of Global Health at Duke University (NC, USA), stated in an article for The Guardian. "*Virus biology and vaccines technology could be the limiting factors, but politics and economics are far more likely to be the barrier to immunisation*." [7].

DRUG REPURPOSING FOR COVID-19

Given that the time needed to create a new drug or vaccine is significant, even if in vivo animal tests are avoided, many researchers looked to what they already had around them to try and quickly find a drug. Utilizing the strategy of drug repurposing, medicinal chemists take drugs that have been previously approved by licencing bodies, such as the US FDA (MD, USA), and redeploy them to treat novel or otherwise difficult-to-treat diseases.

In a study published in February, researchers from the Wuhan Institute of Virology and Beijing Institute of Pharmacology and Toxicology (both China) evaluated a set of FDA-approved antivirals to see whether they could be utilized against SARS-CoV-2 [8]. They found that remdesivir, initially developed to treat Ebola, and chloroquine, an antimalarial, were both able to inhibit SARS-CoV-2. Having both already been proven safe for human use, following the results of this study clinical trials for their use to treat COVID-19 were soon initiated.

As the virus spread and case numbers increased, so did the number of strategies and potential drug repurposing candidates. In one publication, Andersen et al. reviewed 120 antiviral agents that had been previously proven safe in man, finding 31 that had potential applications for the treatment of COVID-19 [9]. Another strategy included the development of new combination therapies; often broad-spectrum antivirals demonstrate a weak activity when used alone, while combination treatments hope to capitalize on the antiviral activity of multiple drugs at a time [10].

TESTING FOR COVID-19

"*We have a simple message to all countries: test, test, test*," stated World Health Organization (Geneva, Switzerland) Director General, Tedros Adhanom Ghebreyesus, in a press conference on 16 March 2020 [11]. Despite the clear advice, many countries failed to follow a large-scale testing protocol, reserving tests for only those most at risk or with the severest symptoms.

Current testing methods involve taking a nasopharyngeal swab from a potential COVID-19 case, extracting the viral genetic material, converting the RNA to DNA and then ultimately utilizing PCR to amplify the genetic material and detect the presence of tell-tale SARS-CoV-2 genes [12]. The tried-and-tested diagnostic technique is reliable at detecting disease, although can be slow and in some cases take days to get a result. Therefore, alternative methods have been investigated in an attempt to speed up the process, make it more accessible to all and easier to track the spread of the disease.

One method to speed up the process, as well as limit social interaction and potential spread of the disease, is to bring in at-home testing kits, where individuals self-swab and then send their sample to a diagnostics lab for analysis [12]. Another approach is by reducing the size of the testing equipment. Instead of the large, automated PCR machines used by many hospitals, many companies are introducing smaller amplification devices that do the same job in a much shorter timeframe. For example, Cepheid (CA, USA) recently received US FDA approval for the use of Xpert® Xpress, a molecular diagnostic test based on their GeneXpert® system, which can detect SARS-CoV-2 in 45 min [13].

Alternative testing opportunities are being explored and, in perhaps the cutest story to come from coronavirus, the charity Medical Detection Dogs (Milton Keynes, UK) announced that they are investigating whether their specialist sniffer dogs could detect COVID-19 infection. Already trained to identify the scent of malaria, cancer and Parkinson's disease, the dogs could provide a rapid, noninvasive diagnosis of the disease. "*The aim is that dogs will be able to screen anyone, including those who are asymptomatic and tell us whether they need to be tested. This would be fast, effective and non-invasive and make sure the limited NHS testing resources are only used where they are really needed*," explained Claire Guest, CEO and Co-Founder of the charity [14].

As more evidence comes to light that many cases of COVID-19 have passed by undetected, the focus has shifted from a test for whether an individual has the disease, to a test for whether an individual has had the disease. Antibody tests have become a top priority for many as they would allow the full scale of the pandemic to be visualized. Mirroring the race to find a vaccine, companies worldwide are now working to develop large-scale antibody tests that can be distributed for widespread testing. In addition, a team based at the Icahn School of Medicine at Mount Sinai (NY, USA) has posted a preprint, detailing how to replicate their newly developed assay for detecting SARS-CoV-2 seroconversion in the blood [15].

Antibody testing will likely help in understanding the full impact of the disease as well as guide more practical decisions in society, such as when it is safe to end social distancing protocols and return to work. It may also shed light on the long-term immunity gained following infection, as well as elucidate the effect the virus has on children [16].

Unlike wars of the past, the ongoing war against coronavirus has a predetermined victor. Through scientific development of vaccines and advances in testing, humans will gain control of the as-of-now seemingly unstoppable virus. However, in the meantime, the British wartime mantra of 'keep calm and carry on' has never been more relatable, as the world at large avoids the pull of coronavirus-induced hysteria and carries on with life – albeit in the safety of their own homes and with very clean hands.

Written by Jenny Straiton

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Short Communication

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The divergence between SARS-CoV-2 and RaTG13 might be overestimated due to the extensive RNA modification

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Aim: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread throughout the world. There is urgent need to understand the phylogeny, divergence and origin of SARS-CoV-2. **Materials & methods:** A recent study claimed that there was 17% divergence between SARS-CoV-2 and RaTG13 (a SARS-related coronaviruses) on synonymous sites by using sequence alignment. We re-analyzed the sequences of the two coronaviruses with the same methodology. **Results:** We found that 87% of the synonymous substitutions between the two coronaviruses could be potentially explained by the RNA modification system in hosts, with 65% contributed by deamination on cytidines (C-T mismatches) and 22% contributed by deamination on adenosines (A-G mismatches). **Conclusion:** Our results demonstrate that the divergence between SARS-CoV-2 and RaTG13 has been overestimated.

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Keywords: divergence • overestimate • RaTG13 • RNA modification • SARS-CoV-2

The spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) needs to be controlled [1–3], and meanwhile its outbreak provides an opportunity for evolutionary biologists to investigate the viruses from the angle of evolution. The ultimate ambition might be finding out the origin and evolving patterns of SARS-CoV-2.

With or without much knowledge of virology, the evolutionary formula or algorithms could be easily applied to the virus sequences by using software or manual calculation. A previous study focusing on the origin and continuous evolution of SARS-CoV-2 (Tang *et al.* 2020 [4]) has an interesting finding that the synonymous substitution rate (dS) between SARS-CoV-2 and RaTG13 (one of the bat SARS-related coronaviruses) is 17%, which is 14-times the divergence between human and chimpanzee. This divergence as high as 17% is much greater than the estimation of earlier studies. The authors commented that the difference between SARS-CoV-2 and RaTG13 has been underestimated by earlier papers.

The authors' opinion is that only the silent mutations should be used to calculate the divergence between SARS-CoV-2 and RaTG13, because these neutral sites are not affected by selection forces. By using the formula $dS = 2ut$, where dS represents substitution rate and u is the mutation rate, one could estimate the divergent time (t) between the two species.

Despite the terminology 'mutation' widely being used by evolutionary biologists, in many cases 'mutation' has been used in broad-sense, which represents all kinds of mismatches observed in the sequence alignment, no matter these mismatches are caused by natural mutation (such as replication errors) or other factors.

The cellular organisms have multiple RNA modification systems, which could modify any types of RNAs in the cell. Since SARS-CoV-2 and RaTG13 are coronaviruses (RNA viruses), when they infect the human cell, the RNA modification enzymes might act on the viral RNAs as they usually do to the host RNAs. Modified viral RNAs such as the methylated adenosines have been commonly observed [5–7]. Apart from the minor decorations such as methylation, two major deamination enzymes, ADAR [8] and APOBEC [9,10], are responsible for adenosine-to-inosine deamination and cytidine-to-uracil deamination, leading to an observed A-to-G and C-to-T change in the sequencing results. No matter which of SARS-CoV-2 and RaTG13 is modified, it will produce an A-G

Table 1. The length and aligned length of each ORF of SARS-CoV-2.

ORF ID	Length (amino acids)	Aligned length in RaTG13
E	75	75
M	222	222
N	419	419
ORF10	38	38
ORF1AB	7095	7093
ORF3A	275	275
ORF6	61	61
ORF7A	121	121
ORF7B	43	43
ORF8	121	121
S	1273	1273

or C-T mismatch in the alignment between two viruses. In mammals, ADAR is required to fight against the infected hepatitis C virus (HCV) [11–13]. Similar to coronavirus, the HCV is a positive-strand RNA virus, and the case of ADAR acting on HCV means that the deamination on viral RNAs (thus inducing mismatches against the reference sequence) is prevalent. In other invertebrate organisms, the mismatches induced from ADAR deamination is observed in sigma virus, a negative sense RNA virus [14–16]. Evidence shows that the ADAR-modified viral RNAs are not rapidly degraded so that the 'offspring' of the deaminated RNA would permanently carry this mutation [11].

In the dS calculation, any observed mismatches in the sequence alignment are regarded as mutations. Of course, the software would not automatically tell the users whether a mismatch is a natural mutation caused by replication error or an RNA modification site.

However, for DNA organisms like humans, the classic definition of mutation rate should mainly (perhaps not absolutely) refer to the replication error rate of DNA. For SARS-CoV-2, the mutation rate should mainly refer to the RNA replication error rate. Accordingly, the calculation of dS should only include the natural mutations introduced during RNA replication rather than the RNA-to-RNA mismatch sites caused by RNA modification system. The replication error rate should be very low while the occurrence of RNA modification could appear in any virus RNA which is exposed to the host's deamination enzymes. The RNA modification rate could be higher than RNA replication rate for orders of magnitude. The phenomenon that the viral RNAs or even proteins are modified by host cells is not rare at all [13,17] so that this issue should be considered when studying the divergence of RNA viruses.

Our idea is that when checking the sequence alignment between SARS-CoV-2 and RaTG13, if one found that plenty of the synonymous substitutions could be potentially explained by C-to-T deamination or A-to-G deamination then the actual divergence between SARS-CoV-2 and RaTG13 might have been overestimated by many times. We re-emphasize that we only say the C-T and A-G mismatches could be potentially explained by RNA modification but not definitely caused by RNA modification. The aim is to rationally estimate the real divergence between the two RNA viruses.

Materials & methods

We downloaded the sequences of SARS-CoV-2 and RaTG13 from GeneBank and aligned the coding sequences with MUSCLE [13]. The 11 nonredundant ORFs are annotated with names (such as *M*, *N*, *ORF1AB*) so that we put the two ortholog genes into a file and run the sequence alignment. The length of each ORF (number of amino acids) and the aligned length of each ORF are given in Table 1. For example, we put the two sequences of SARS-CoV-2 ORF10 and RaTG13 ORF10 into one file and run MUSCLE with default parameter. Then the output file would give us the aligned sequences of these two ORFs. From Table 1, we could see that the ORFs in two virus species are almost of the same length so that the parameters hardly affect the alignment results. We manually extract each codon in the alignment file using our own python script. The unaligned regions are gaps. As shown in Table 1, only ORF1AB have two triplets (codons) unaligned, and the other regions and other ORFs are well aligned. Next, most of the aligned regions are identical. The nonidentical regions are either missense or synonymous mutations. To help readers understand the process of extracting mutations (mismatches) from the alignment, we listed the first ten missense and synonymous mutations of ORF1AB in Tables 2 & 3, respectively.

Table 2. The first ten missense mutations in ORF1AB.

Position	SARS-CoV-2	RaTG13	Amino acid (SARS-CoV-2)	Amino acid (RaTG13)	Mismatch (nondirectional)
38	GTC	GCT	Val	Ala	C-T
110	CAT	TAT	His	Tyr	C-T
114	ATA	ACA	Ile	Thr	C-T
117	GCT	GTT	Ala	Val	C-T
172	GAA	GAT	Glu	Asp	A-T
280	ATA	ACA	Ile	Thr	C-T
376	TCA	CCA	Ser	Pro	C-T
395	ACC	CCC	Thr	Pro	A-C
417	CAT	TAC	His	Tyr	C-T
424	GTT	ATT	Val	Ile	A-G

Table 3. The first ten synonymous mutations in ORF1AB.

Position	SARS-CoV-2	RaTG13	Amino acid (SARS-CoV-2)	Amino acid (RaTG13)	Mismatch (nondirectional)
20	GTT	GTC	Val	Val	C-T
59	GGC	GGT	Gly	Gly	C-T
74	TCG	TCT	Ser	Ser	G-T
82	GGT	GGC	Gly	Gly	C-T
92	CTC	CTT	Leu	Leu	C-T
97	TAC	TAT	Tyr	Tyr	C-T
104	CTT	CTC	Leu	Leu	C-T
138	GCC	GCT	Ala	Ala	C-T
142	TCA	TCG	Ser	Ser	A-G
169	GTT	GTC	Val	Val	C-T

From Tables 2 & 3, we already see prevalent C-T mismatches.

It is possible that sometimes the mutation may be lethal, producing shortened protein if TAA is produced instead of CAA. We scanned the 11 nonredundant ORFs in SARS-CoV-2 and RaTG13. We did not find any internal stop codons in these ORFs.

For the multiple alignment incorporating other virus species ZXC21, ZC45 and BM48-31, we aligned the ORFs with the same method. Together with SARS-CoV-2 and RaTG13, we put the orthologous ORF of the five species into one file and run MUSCLE. The output alignment file was manually inspected. Each codon located in the ORFs were simply extracted by our own python scripts. The results of aligning SARS-CoV-2 and RaTG13 and the results calculated from aligning five species were compared. The relative alignment and mismatch profiles between SARS-CoV-2 and RaTG13 were found to be identical under two sets of strategies.

The ID of SARS-CoV-2 is NC_045512. The link of SARS-CoV-2 ORF1AB (coding sequence) is: https://www.ncbi.nlm.nih.gov/nuccore/NC_045512.2?from=266&to=21555&report=fasta

The ID of RaTG13 is MN996532. The link of RaTG13 genome is: https://www.ncbi.nlm.nih.gov/nuccore/MN996532.1/?report=fasta

The beginning of SARS-CoV-2 ORF1AB is 'ATG|GAG|AGC|CTT|GTC', the end of SARS-CoV-2 ORF1AB is 'GAT|GTT|CTT|GTT|AAC|AAC|TAA'. By manually searching 'ATGGAGAGCCTTGTC' and 'GATGTTCTTGTTAACAACTAA' in the RaTG13 genome sequence, we can anchor and extract the ORF1AB in the RaTG13 genome. The ORF1AB CDS alignment between SARS-CoV-2 and RaTG13 is provided in Supplementary Table 1. As we can see, most codons are identical. The nonidentical codons mostly have synonymous mutations.

Results

Substitutions between SARS-CoV-2 & RaTG13

We aligned the ORFs of SARS-CoV-2 and RaTG13 and manually extracted the codons in the alignment file (see Materials and methods). The statistics of the alignment results (Table 1) show that most of the ORFs are well

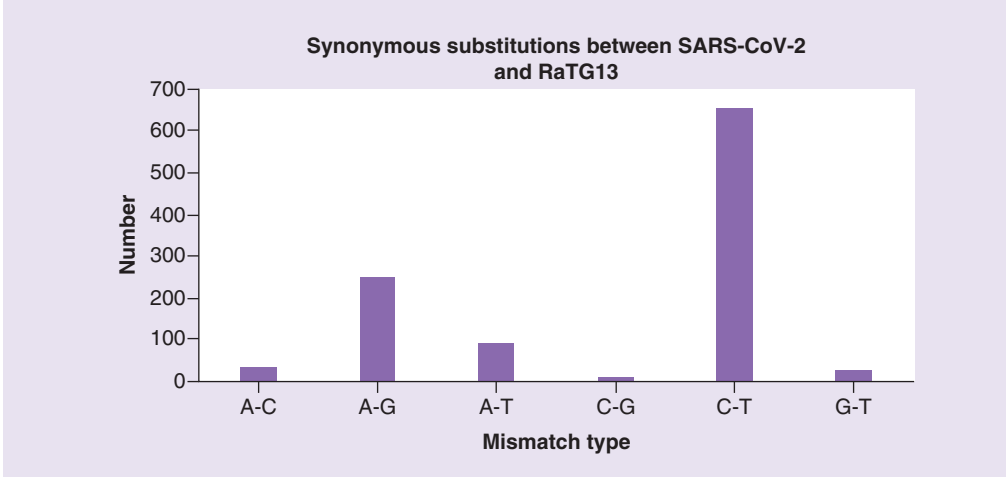

Figure 1. The numbers of mismatch types on synonymous substitution sites between SARS-CoV-2 and RaTG13.

aligned and only ORF1AB has two gaps. From the 9.7 thousand codons in the ORFs, we totally obtained 1076 nonidentical codon positions between SARS-CoV-2 and RaTG13, 931 of which encode the same amino acid (synonymous) and 145 of which encode different amino acids (missense). That is to say, there are 931 synonymous substitutions and 145 missense substitutions between SARS-CoV-2 and RaTG13. The other ORF regions (90%) are identical between SARS-CoV-2 and RaTG13.

Among the 9.7 thousand codons in the 11 nonredundant SARS-CoV-2 ORFs, the content of C and T is 51.2%. However, among the 931 codons with synonymous substitutions, the content of C and T is 56.1%, and the difference is significant using Chi-square test (p = 5.7E-3). It proves that the occurrence of synonymous substitutions is nonrandom and it tends to take place on codons containing C or T.

87% of the synonymous substitutions are C-T or A-G mismatches

We checked the 1076 substitution sites between SARS-CoV-2 and RaTG13, 84.4% of the mutations are A-G or C-T mismatches (61.0% C-T mismatches and 23.4% A-G mismatches). Among the 931 synonymous substitution sites (Figure 1), 86.7% of them are A-G or C-T mismatches (64.9% C-T mismatches and 21.8% A-G mismatches). This mismatch spectrum resembles the enrichment of C-to-T(U) deamination and A-to-G(I) deamination. Nearly 87% of the observed synonymous 'mutations' between SARS-CoV-2 and RaTG13 could be potentially explained by the RNA modification systems in host cells.

To help readers understand how the mismatches were extracted from the alignment file, we listed the first ten missense and synonymous mutations in ORF1AB, respectively (Tables 2 & 3). We have said that 90% of the aligned regions is identical and the nonidentical codons usually differ with a single nucleotide. In Table 2, seven out of the ten missense substitutions were C-T mismatches. In Table 3, eight out of the ten synonymous substitutions were C-T mismatches. Given the high similarity of the SARS-CoV-2 and RaTG13 sequences, these mismatches may not be caused by mis-alignment.

One may also be concerned whether the alignment and mismatch profile is different when using multiple virus species to run the alignment. We downloaded the ORFs of other SARS-related coronaviruses ZXC21, ZC45, and BM48-31 (see Materials and methods). We found that using multiple species does not affect the aligned regions between SARS-CoV-2 and RaTG13. Although additional gaps are introduced in the alignment, the relative position between SARS-CoV-2 and RaTG13 remains the same. So, the mismatches between SARS-CoV-2 and RaTG13 are not affected by different alignment strategies. The prevalence of C-T and A-G mismatches is robust.

Mismatch profile excluding the protease digestion sites in ORF1AB

The ORF1AB (pp1AB) would be cleaved into multiple proteins (nsp1-16) by protease. The cleavage sites are LQS and LQA sequences [18,19]. We could not simply call ORF1AB as one gene, so it is rational to exclude the mutations in digestion sites in the divergence analyses. We checked the mutations in the LQS and LQA regions. We only found one case. Amino acids 4252-4254 is Leu-Gln-Ala, and the Leu codon is CTA in SARS-CoV-2 and TTA in

Table 4. dS values and the fold of overestimation.

ORF	dS (Tang *et al.*)	C-T mismatch	A-G mismatch	Explained by modification (upper bound)	Fold of overestimation of dS (upper bound)
All	0.17	65%	22%	87%	7.7
ORF1AB	0.152	67%	22%	89%	9.2
S	0.321	59%	19%	78%	4.5
Other	Not provided	64%	27%	91%	10.9

RaTG13. This single C-T mismatch in digestion regions does not affect the overall mismatch profile. This also proves that the amino acid sequences of digestion sites might be highly conserved to avoid the loss of protease recognition. Again, our finding of prevalent C-T and A-G mismatches is robust.

ORF1AB & S contribute most of the mismatches

It is necessary to provide the influence of the tested number of genes on the estimated divergence. As seen in Table 1, ORF1AB and S are the longest ORFs. They contribute most of the mismatches if we look at the mismatch profile in all the ORFs. Here we list the dS values calculated by Tang *et al.* [4] and the percent of mismatches potentially explained by RNA modification (Table 4). ORF1AB, S, and the other ORFs are listed separately. Clearly, the choice of tested genes does not severely affect the pattern. In all genes, 87% mutations could be (potentially) explained by modified RNA. In ORF1AB, 89% mutations could be (potentially) explained by RNA modification. In S, 78% mutations could be (potentially) explained by modified RNA. In the remaining ORFs, 91% mutations could be (potentially) explained by modified RNA. Presume that 91% of the mismatches are caused by RNA modification, then the dS value is overestimated for more than tenfolds. The S ORF has a pretty high dS value, so it is especially necessary to question if the modification system contributes to the divergence.

Discussion

One argument is that in the alignment between SARS-CoV-2 and RaTG13 we did not use an outgroup species so that the direction of the mutation is uncertain. Yes, that is true. We do not worry about the ancestral state. SARS-CoV-2 and RaTG13 are RNA viruses. As long as we observe a C-T or A-G mismatch in the sequence alignment between them, we could speculate that the C-to-T or A-to-G deamination might have occurred in one of the two virus species.

Note that we only say 87% of the mutations could be potentially explained by RNA modification, rather than 87% of them are definitely caused by RNA modification. From the sequence alignment alone, it is impossible to know whether the mismatch is a '*de novo*' mutation or an RNA modification site. The software would not tell users what has caused this mismatch since it is technically indistinguishable. Improving the parameters only makes alignment more accurate but does not tell us the origin of the mismatch.

As understood by common researchers, the definition of dS between RNA viruses mainly (but not absolutely) refers to the natural mutations introduced by RNA replication error rather than the RNA modification sites caused by host cells. The RNA modification rate is many times higher than the replication error rate. This fact is consistent with our notion that the divergence between RNA viruses is overestimated.

According to our results, potentially 87% of the synonymous substitutions between SARS-CoV-2 and RaTG13 could be caused by RNA modification system in hosts. The remaining 13% of the substitutions should be genuine interspecific mutations as they could not be explained by known RNA modification types. The claimed dS = 0.17 should have been overestimated. The upper bound of overestimation is 1/0.13 = 7.7-times so that the lower bound of the dS value is 0.17/7.7 = 0.022.

Indeed, if the authors argue that the definition of dS itself already included any mutation types such as those RNA modification sites then the dS value of 17% would be valid. However, this definition of dS is not what we commonly understand, and the authors should have pointed this out in their article. Again, adjusting the parameters of any software only makes the alignment more accurate but is not helpful in determining whether the observed mismatches are modified RNA or the natural mutation introduced during RNA replication. A rational way to avoid a wrong and misleading conclusion is to calculate the upper bound and lower bound of the divergence value. Anyway, the currently proposed divergence (dS = 17%) between SARS-CoV-2 and RaTG13 has been severely

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overestimated. We appeal that when calculating dN and dS between RNA viruses, the RNA modification should be taken into account.

The limitation of our study is that we were currently unable to provide experimental evidence for the modification on viral RNAs although this phenomenon is not new for virologists. At the same time, neither did Tang *et al.* [4] provide evidence to prove that the mismatches in the alignment are not caused by RNA modification. Since both sides lack experimental evidence, it is reasonable to think about this dilemma from the angle of maximum likelihood. That is, if the mismatch sites between SARS-CoV-2 and RaTG13 are really introduced by accumulation of RNA replication errors, they should not exhibit an excessive number of C-T and A-G mismatches (in that case the mutation types should be random).

Another limitation of our work is that we did not give an estimation of the real divergence value. As we have stated, the RNA modifications and normal mutation sites are technically indistinguishable. We only say that the proposed 17% divergence is higher than the real value but we still do not know what the real value is. Promisingly, experts in mutations could estimate the relative abundance of each type of mismatches and give a reasonable value of the divergence between SARS-CoV-2 and RaTG13.

Conclusion

Since we found 87% of the synonymous substitution sites between SARS-CoV-2 and RaTG13 could be potentially explained by RNA modification system in host cells, we are strongly concerned that the previously defined divergence between SARS-CoV-2 and RaTG13 has been overestimated.

Summary points

- The outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused severe damage to the world.
- It is necessary to understand the origin and evolution patterns of SARS-CoV-2.
- A previous study claimed that SARS-CoV-2 and RaTG13 have 17% divergence on synonymous sites.
- We aligned the coding sequences of SARS-CoV-2 and RaTG13, and checked the substitution sites between them.
- The substitution sites are CT-enriched compared with background.
- Potentially 87% of the synonymous substitutions between SARS-CoV-2 and RaTG13 could be explained by RNA modification system in hosts.
- The divergence between SARS-CoV-2 and RaTG13 has been overestimated.
- The calculation of dN or dS between RNA viruses should take the RNA modification into consideration.

Author contributions

The corresponding author designed and supervised this research. All authors contributed to writing this article.

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Special Report

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Pros and cons of the application of evolutionary theories to the evolution of SARS-CoV-2

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The outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused severe damage to the world. With the support of classic evolutionary theories and population genetics principles, many studies on the origin of SARS-CoV-2 have revealed encouraging results but meanwhile some are still under debate. We are concerned with the validity of applying classic evolutionary theories and formula to the evolution of RNA viruses. We have raised several confounding factors like the RNA replication feature and the RNA modification systems of the hosts, which might jeopardize the validity of the application of classic methods to analyze the SARS-CoV-2 data.

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Keywords: application • concerns • evolution and origin • RNA virus • SARS-CoV-2

The outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused severe damage to China, especially the Hubei province [1–3]. Recently, the cases in China are coming under control but the situation in some other countries has become exacerbated. It is urgent to find ways to control its transmission and cure the infected patients.

The emergence of papers on the evolution of SARS-CoV-2 is as fast as the spread of the virus itself. Based on genome sequencing followed by sequence alignment and sequence similarity analysis, researchers have characterized the evolutionary patterns of SARS-CoV-2 and postulated its origins. Theories of bat origin [4–6] and pangolin origin [7,8] emerged and even the snake plays an intermediate role in the transmission of SARS-CoV-2 [9]. Many other similar studies and results are not exhaustively listed here at all. In a word, it seems that the continuous change of theories on the evolution of SARS-CoV-2 even exceeds the evolution rate of the virus itself. It seems that several results appear to be encouraging. For example, a recent paper by Tang *et al.* [10] has taken advantage of RNA-seq data. By traditional mutation calling pipeline, the authors discovered two lineages (L and S) of SARS-CoV-2, and claimed that the L type is more virulent than S type based on their frequency among the population. This might be a typical bioinformatic study that informs us of the scenario of SARS-CoV-2 population and evolution. The 'snake' paper [9] also emphasized the important fact that viruses adapt to the host expression system, so that their codon content should adapt to the host environment. No matter whether the final conclusion is correct or not, the main contribution is that the associated terminologies like relative synonymous codon usage and codon adaptation index are nicely incorporated in the relevant studies, indicating a correlation between the codon content of host and parasite.

As far as we know, the classic theories and principles of evolutionary biology, especially population genetics, are based on the central dogma, where DNA should be first transcribed to RNA and then produce proteins, and meanwhile the DNA replicates itself under a certain error rate. The validity of many formula/software is also based on multiple hidden hypotheses such as random mating, bi-allelic DNA, bi-sexual gender and infinite population size.

However, SARS-CoV-2 is a positive-strand RNA virus. It reproduces its own RNA by RNA replication. We doubt the validity of applying classic evolutionary theories and formula to the evolution of RNA viruses. In this

article, we would raise and discuss several concerns regarding how the confounding factors would jeopardize the validity of the application of classic methods to analyze the SARS-CoV-2 data. We should emphasize that we neither criticize any studies, nor prove any ideas wrong. Instead, we aim to raise some questions and wish that these concerns could be further discussed by the broad community. We hope our concerns would contribute to the accurate identification of the origins of SARS-CoV-2.

The problem of RNA modification system in host cells

We have stated that SARS-CoV-2 is an RNA virus. The classic theories, principles and formula of evolutionary biology are based on the central dogma, which assumes the DNA-to-RNA-to-protein chain. The basic notion is that the mutations on DNA could be inherited rather than the modifications on RNA. For example, despite the numerous types of RNA modifications in higher eukaryotes, these organisms do not suffer from extraordinarily high mutation rates because the modified RNAs could not be transmitted to the next generation. However, for RNA viruses, their RNA is actually their genetic information. Whether the evolutionary principles could be applied to RNA viruses should be seriously debated. The host cells have multiple RNA modification systems/enzymes. The changes in viral RNA (by host cells) would permanently change its genetic information and be transmitted to the next 'generation', which is similar to genetic mutations in higher organisms. Technically, one could not distinguish genetic mutations and RNA modifications from the RNA-sequencing data of RNA viruses. So, what is the point of detecting positive/negative selection based on the mutations in the virus population? These mutations are possibly conferred by the host cell's RNA modification systems. How could the randomly occurring RNA modification events have preference on missense or synonymous sites?

The recent study by Tang *et al.* [10] claimed that the divergence between SARS-CoV-2 and RaTG13 (a bat SARS-related coronavirus) is 14-times larger than the divergence between human and chimpanzee. The authors concluded that only the neutral evolving sites should be considered rather than all different sites. Let us assume that both SARS-CoV-2 and RaTG13 undergo the RNA modification by host cells, and the modified viral RNA is inheritable, then their sequence (SARS-CoV-2 and RaTG13) could become quite different within a short time scale. The divergence time is calculated as $t = dS/2u$. When dS might be largely contributed by the RNA modification system of host cells, this estimation could be inaccurate. In the dN and dS calculation, it is necessary to rule out any mismatch sites that might be produced by RNA modification. They should at least mention why they should or should not consider this factor. And then, the mutation rate 'u', how to define 'u'? Does 'u' include the nucleotide changes conferred by the host's modification enzymes? Therefore, the authors' logic chain is questionable.

More importantly, the single nucleotide polymorphism and modified RNAs are technically indistinguishable. The software and algorithms only align the sequences but do not tell you whether the observed mismatch is a single nucleotide polymorphism or RNA modification site. This is a biological problem rather than technical problem, and could not be solved by adjusting or improving the alignment parameters or filtering criteria.

One may argue that some studies have analyzed RNA modifications by using classic evolutionary theory, but note that the viral RNA is modified by the host cell rather than by the virus itself, and their equivalence (modified by the host or modified by itself) should be formally proven before conducting any analyses.

Indeed, the coronaviruses isolated from the hosts (like human, bat or pangolin) are only compared with SARS-CoV-2. Therefore, RNA virus sequence is compared with the SARS2 RNA, and hence, the used strategy is almost sound. So, these studies compared virus to virus or RNA to RNA and conclude the potential host or carrier based on the highest percent of identity or potential concluded recombination events. However, as we have stated, the sequence similarity could be largely and even randomly skewed by the hosts' RNA modification systems. Therefore, aligning RNA with RNA is fine, but the concern is how to distinguish whether the observed divergence (or mismatch sites) really reflects the phylogeny of the viruses. It could simply be shaped by the arbitrary modification of the host's enzymes.

The next concern caused by RNA modification system is the 'batch effect'. In population genetics, DNA mutations take place randomly among different individuals. But when RNA viruses are modified by host cells, the modification enzymes are likely to modify multiple sites at a time. Since the genes of an RNA virus (like the 12 genes of SARS-CoV-2) are linked, they are prone to be modified 'in a batch'. In contrast, in higher organisms, it is not possible to see numerous individual-specific mutations linked within an entire haplotype unless it is driven by selective sweep. This reality again challenges the application of traditional theories to RNA viruses.

In our opinion, the RNA viruses should obey a different evolutionary theory. So far, the functional experiments are more important and reliable than the pure evolutionary analyses in this case of RNA virus. When traditional evolutionary principles are jeopardized by additional mutation forces, the functional experiments work well as they did in the past.

Problems raised from the RNA replication process

Apart from being modified by the host RNA modification systems, there are other concerns about whether the evolutionary theories could be applied to the RNA viruses like SARS-CoV-2.

First, for cellular organisms, the DNA mutations are majorly introduced during the DNA replication process. The mutation rate is largely connected with the fidelity of DNA replication. The next step is the natural selection force acting on these mutations, after which the deleterious mutations are purged and those beneficial mutations are maintained. However, RNA viruses either undergo the reverse-transcription process (like HIV) or the RNA replication process (like SARS-CoV-2). For RNA viruses, every newly transcribed RNA molecule is a potential offspring of the original virus. The mismatches introduced during reverse transcription or RNA replication would be maintained and kept in the offspring. Before applying the evolutionary formula to RNA viruses, one should state whether RNA replication has similar mismatching rates as DNA replication. Intuitively, DNA–DNA pairing (DNA replication), DNA–RNA pairing (transcription) and RNA–RNA pairing (such as RNA replication) should have different mismatching rates. Thus, when applying theories to SARS-CoV-2, should the authors consider the potentially different mutation rates during the reverse transcription or RNA replication processes? Take the paper by Tang *et al.* [10] for instance, what exactly does mutation rate 'u' refer to? Even the problem of technically indistinguishable RNA modification and the '*de novo*' RNA mutation is not mentioned by the authors at all, let alone the mutations introduced during the RNA replication process. At least, the authors could briefly introduce the reproduction mode of SARS-CoV-2 rather than 'mechanically' apply the formula to an organism which they are not familiar with.

Second, the DNA generated from the reversetranscription is only an intermediate, and could not be packaged into the envelope protein. The same goes for the negative-strand RNA generated by the positive-strand RNA during the RNA replication. So that the DNA/negative-strand RNA and any changes on it could not be directly transferred to the next host cell. However, if a DNA intermediate could transcribe N RNA molecules, and a mutation takes place when $N/2$ RNA molecules have been transcribed, then only part of the offspring would have this mutation. All these random processes could not be predicted by any algorithms. How could theories on DNA mutations be applied to a transiently existing DNA intermediate or negative-strand RNA?

These factors may shed concerns on the accuracy and validity of the results of previous works. However, we do not claim any studies to be wrong since we have neither better methods nor enough evidence. Hopefully, our opinions could be seen and discussed by other researchers and benefit the studies on the evolution and origin of SARS-CoV-2.

Conclusion

We are concerned with the validity of applying classic evolutionary theories and formula to the evolution of RNA viruses. We have raised several confounding factors like the RNA replication process and the RNA modification systems of the hosts, which might jeopardize the validity of the application of classic methods to analyze the SARS-CoV-2 data. However, we neither criticize any studies nor prove any ideas wrong. We hope our concerns could be considered by the broad community and could contribute to the accurate identification of the origins of SARS-CoV-2.

Future perspective

We anticipate and speculate that the field of virus evolution could be incorporated with more novel evolutionary theories that distinguish cellular organisms, DNA viruses and RNA viruses, due to their distinct features of nucleotide components, mutation rates, and reproductive patterns.

Author contributions

The corresponding author designed and supervised this research. All authors contributed to writing this article.

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Executive summary

- The recent evolutionary studies on SARS-CoV-2 have revealed encouraging results as well as debatable questions.
- The problem of RNA modification system in host cells: RNA modification system in the host cells could act on viral RNAs, which jeopardizes the validity of many hidden hypotheses behind the evolutionary theories.
- Problems raised from the RNA replication process: the traditional evolutionary theories did not mention the RNA replication process at all, while SARS-CoV-2 exactly uses this approach to replicate itself.

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Editorial

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Bioanalysis

Exhaled breath condensate as a potential specimen for diagnosing COVID-19

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"Since the virus spreads via respiratory droplets, and the success rate of BLF is better than sputum, nasal and pharyngeal swabs, exhaled breath condensate (EBC) could be considered a more appropriate sample to follow virus NAT using RT-PCR due to its similarities with BLF (i.e., biochemical contents and origin of production)"

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Keywords: clinical specimen • COVID-19 • exhaled breath condensate

Coronavirus disease 2019 (COVID-19) is an emerging condition threatening the biosecurity of all nations on the planet. This pandemic of respiratory disease, caused by a novel β-coronavirus (called severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2]), has high sequence similarity to SARA-CoV, which was responsible for a major outbreak in 2002–2003 [1]. Early diagnosis of suspected cases is a vital task to manage patients and to control the spread of the pathogen, especially as there can be a long period before the clinical symptoms of the infection present [2]. The current COVID-19 pandemic affects a wide range of parameters from the global economy to the personal behavior of human beings. In addition to important concerns to control the spread of the disease, further attention should be paid to the mental healthcare of members of society [3]. At the present time, the laboratory tests to diagnosis SARS-CoV-2 samples take between a number of hours and a number of days to complete, depending on the nature of the test [4]. Employing novel technologies, such as microfluidics, could provide a fast and accurate test for outbreaks [5].

The SARS-CoV-2 virus can be detected in variety specimens such as bronchoalveolar lavage fluid (BLF) [6], sputum [7], saliva [8] or by using throat [9] or nasopharyngeal swabs [8]. Reliable and rapid laboratory diagnosis of a SARS-CoV-2 infection is critical and experiments have revealed that nucleic acid detection by real-time reverse-transcription polymerase chain reaction (RT-PCR) is the clinical standard for diagnosis of patients with COVID-19 [4]. Replication of the specific regions of SARS-CoV-2 genome including coding area of virus S, E or N structural proteins or RNA-dependent RNA polymerase (RdRp) nonstructural protein allowed the development of RT-PCR tests for diagnosis of this novel virus in patients with COVID-19 [10]. Sequencing of the viral RdRp gene is also used for diagnosis, especially when the PCR result is not convincing for one target gene but the clinical/epidemiological suspicion for COVID-19 is high. In addition, whole genome sequencing of the virus genome RNA from positive samples provides more evidence for better understanding the genomic similarities between patients (molecular epidemiology) and possible mutations of the virus genome [1].

A retrospective analysis of different respiratory specimens (including nasal and pharyngeal swabs, BLF and sputum) was carried out to determine the reliability of the test at detecting SARS-CoV-2 in patients with COVID-19 infections [11]. Liu *et al.* concluded that nucleic acid test (NAT) is a rapid, easy to conduct and widely used laboratory diagnostic test for identifying the RNA of SARS-CoV-2. According to their findings, BLF exhibited the highest (100% cases) true positive results where nasal and pharyngeal swabs showed low (61% cases) true positive results for targeting the *ORF1ab* gene of the virus [11]. This is of note because the false negative results could provide misleading information for control and management of COVID-19. In another study, some failures in detecting

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SARS-CoV-2 in a proportion of samples apparently taken using an inappropriate technique were reported [12]. Furthermore, in a recent study [13], biodistribution of SARS-CoV-2 was evaluated in 205 COVID-19 patients using real time RT-PCR targeting *ORF1ab*. The researchers collected different clinical specimens from patients across multiple sites and demonstrated that the type of sample collected can affect the result of virus diagnosis as well as influence accurate identification of patients with COVID-19. According to their results, the BLF specimen showed the highest rate of true positive results (14 of 15; 93%) to identify patients, with the lowest false negative results. However, it should be considered that this cannot be recommended for all cases in the screening stage as this is an invasive sampling procedure and requires highly skilled professionals to collect the BLF samples. BLF is collected by a bronchoscope passed through the mouth or nose into the lung, then a given volume of a sterile solution (usually NaCl 0.9%) is introduced to the lung and the fluid is collected for further biochemical examinations [14].

Since the virus spreads via respiratory droplets, and the success rate of BLF is better than sputum, nasal and pharyngeal swabs, exhaled breath condensate (EBC) could be considered a more appropriate sample to follow virus NAT using RT-PCR due to its similarities with BLF (i.e., biochemical contents and origin of production) [6,11,13]. EBC is a condensed form of small droplets of lung lining fluid [15] which is normally exhaled and contains a variety of components from small ions to proteins and organelles, even viruses, fungi and bacteria [16–18]. In a recent article [19], some technical tips for improving the quality and quantity of extracting NAT from EBC samples were reported. The same procedure with some modifications could be used to detect the genome of SARS-CoV-2 by using RT-PCR. EBC samples could be easily collected using a simple cold trap, commercially available EBC sampling device (such as EcoScreen® or RTube®) or even using a tube passing water–ice mixture. The mechanism of sample collection by these devices is cooling down the temperature of the collection chamber from 0 to -25°C as has been reviewed in recent works [15,20]. Ahmadzai *et al.* [21] compared the efficacy of different collection devices for measuring the variations of biomarkers in EBC samples. Despite BLF, collection of EBC is simple, well tolerated by sample donors and no adverse effects have been reported so far, therefore it could be employed for sampling on a large scale to screen the suspected patients in viral epidemics such as the recent pandemic.

Conclusion

We propose that EBC samples should be tested as a noninvasive sampling method in clinics, since it seems a promising specimen for diagnosis of patients with COVID-19 infections. EBC sampling could be performed as many times as needed in follow-up investigations of patients, which is not possible with BLF sampling. Unfortunately, our team does not currently have access to a safety level 3 laboratory in our research center to test the applicability of this hypothesis. There is also a possibility of developing microfluidics or other single-use technologies to collect and analyze the samples to provide faster screening tools in pandemics such as COVID-19.

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Research Article

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Bioanalysis

Precision multiparameter tracking of inflammation on timescales of hours to years using serial dried blood spots

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Aim: High-frequency longitudinal tracking of inflammation using dried blood microsamples provides a new window for personalized monitoring of infections, chronic inflammatory disease and clinical trials of anti-inflammatory drugs. **Results/methodology:** Using 1662 dried blood spot samples collected by 16 subjects over periods of weeks to years, we studied the behavior of 12 acute phase response and related proteins in inflammation events correlated with infection, vaccination, surgery, intense exercise and Crohn's disease. Proteins were measured using SISCAPA mass spectrometry and normalized to constant plasma volume using low-variance proteins, generating high precision within-person biomarker trajectories with well-characterized personal baselines. **Discussion/conclusion:** The results shed new light on the dynamic regulation of APR responses, offering a new approach to visualization of multidimensional inflammation trajectories.

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Keywords: acute phase response • dried blood spot • inflammation • longitudinal • mass spectrometry • SISCAPA

In this paper, we explore the longitudinal behavior of inflammation-related acute phase response (APR) proteins in a unique collection of dried blood spot (DBS) samples. These included 1522 samples collected longitudinally by eight individuals over periods of up to 9 years (with extended periods of daily sampling) and 140 samples collected by eight elite athletes at multiple time points per day during 1 week of Olympic training using a Sportomics approach [1]. Using peptide-based SISCAPA immunoaffinity-mass spectrometry (SISCAPA-MS) and a novel optimized plasma volume normalization method, we measured the scale and temporal relationships between inflammatory proteins and the frequencies of large and small changes from personal baselines. We also developed novel means for visualizing this rich data.

Inflammation is central to the understanding of human health and disease. Underlying the classical definition (heat, pain, redness and swelling), inflammation involves a complex balance between protective and destructive processes implemented by a varied cast of proteins under active regulatory control. In clinical contexts, these complex processes are usually summarized by measuring only a single blood biomarker: CRP [2], a key component of the innate immune system involved in recognizing and destroying bacterial pathogens. Enormous clinical value has been obtained through quantitative measurement of CRP, as reflected by the fact that there are more US FDA cleared commercial tests for CRP than for any of the >100 other clinically measured blood proteins. CRP levels have well-established clinical significance over a very wide dynamic range, from small (less than twofold) increases associated with increased cardiovascular disease risk [3] to increases of more than 100-fold in major infections [4]. Considered more broadly, increases in CRP are associated with negative developments in a wide range of health situations, including infection [5], arthritis [6], surgery [7], intense exercise [8], sleep apnea [9], depression [10], air pollution [11], welding fume exposure [12], Parkinson's disease [13], pregnancy [14], inflammatory bowel disease [15], and various cancers [16], to name a few examples.

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Many therapeutics that reduce inflammation are in use or under development. These usually act either directly (by targeting inflammation pathway signaling) or indirectly (by treating causes of inflammation). Inflammation signaling is generally targeted by biologics, including a wide and rapidly growing range of molecules such as the anti-TNF alpha antibodies infliximab [17] and adalimumab [18]; TNF inhibitor etanercept [19]; anti-IL-6 mAbs siltuximab [20] and olokizumab [21]); anti-IL-6 receptor tocilizumab [22]; anti-IL-1β mAb canakinumab [23]; and IL-1 receptor antagonist anakinra [24]. ISIS 329993, an antisense oligonucleotide complementary to the coding region of the human CRP messenger RNA, directly targets CRP translation [25]. A similarly wide spectrum of small molecules target causes of inflammation, including antibiotics (such as teicoplanin [26], vancomycin [27] and cefuroxime [28]), various statins [29], the COX/5-LOX inhibitor tenidap [30], methotrexate [31], the Lp-PLA2 inhibitor darapladib [32] and the MAPK inhibitor dilmapimod [33]. All of these drugs reduce CRP levels, and in fact CRP has been used as a pharmacodynamic biomarker in trials of many of them as a means of selecting dose and dosing schedule [18,19,21–23,31]. CRP thus carries a heavy load in measuring inflammation, both in terms of diagnostic evaluation and in the assessment of treatment efficacy.

Can a single biomarker adequately summarize such complex mechanisms and outcomes? The current use of CRP as a biomarker implicitly assumes that inflammation can be considered a fixed response [34] to many disparate triggers, following the same basic script in all individuals. In reality, the inflammatory response exhibits wide variations in scope and timing, as well as major differences between individuals. At the molecular level, it involves significant changes in the concentrations of many proteins besides CRP, including cytokines (short-lived, low-abundance signaling proteins that regulate inflammatory responses [35]) and a broad set of acute phase response (APR) proteins [36] that implement many of the functions required to recognize and deal with inflammatory stimuli. The cytokines, including primary inflammation regulators such as IL-6, are of central importance in research on regulatory mechanisms, but are only occasionally used as clinical diagnostic tests because of their short half-lives, localized pleotropic effects and very low concentrations (low pg/ml). APR proteins like CRP, on the other hand, represent the systemic effects of cytokine regulation and have longer half-lives, a wide range of specific effector functions and higher concentrations. Differences among APR proteins in terms of their regulation, half-lives and functions imply that each has the potential to contribute additional non-redundant diagnostic information, which in turn suggests that a panel of APR proteins would have greater sensitivity and power than CRP alone in analyzing inflammatory responses. In this paper we explore that concept using a set of 12 APR and APR-related proteins in blood, each with distinct roles and behaviors. These include clinically established molecules involved in both innate (SAA, CRP, LPSBP, MBL; see Table 2 for complete list of protein abbreviations) and adaptive (IgM) immune responses, as well as proteins involved in drug transport (Alb, A1AG), iron scavenging and transport (Hp, Hx), the complement cascade (C3), coagulation (FibG) and neutrophil activity (MPO). High precision measurement of protein panels in DBS is achieved by mass spectrometry combined with a sample preparation workflow involving stable isotope standards and capture by anti-peptide antibodies (SISCAPA [37–39]). From an informatics viewpoint, this breadth of analysis increases the functional dimensionality of inflammation measurement, giving a clearer picture of what is happening at any moment in time.

Tracking biomarker changes through time completes the biological picture. Concentrations of various inflammation markers change over timescales ranging from minutes to years, exposing associations with a variety of underlying time-dependent disease mechanisms and contextual variables. For this reason, longitudinal studies of serial blood, plasma, or serum samples provide the clearest path to detailed understanding of the dynamics of inflammatory processes needed to distinguish specific causes, guide therapy, predict outcomes and account for differences between individuals. In medicine, longitudinal sampling allows interpretation of results against personal baselines for each protein, thereby personalizing diagnostic interpretation. Such an approach exposes relationships between biomarkers over time, potentially uncovering novel diagnostic multi-parameter indices. In pharmaceutical trials, longitudinal sampling allows construction of mathematical models relating the changing levels of a drug *in vivo* (pharmacokinetics; PK) to changes in one or more biomarkers of effect (pharmacodynamics [PD]; together referred to as PK/PD models). However, since frequent longitudinal blood sample collection by venipuncture is generally impractical outside a medical environment (and generally discouraged for ethical reasons, including the associated reduction of subject hematocrit [40]), longitudinal sample collections rarely include more than five to ten samples per individual. Thus high-frequency (e.g., daily) sampling is rarely attempted, even in clinical trials.

Longitudinal sample collection can be radically improved by using a less intrusive means of blood collection: fingerprick capillary blood dried on filter paper ('dried blood spots': DBS). DBS have been used as clinical samples since the pioneering studies of Guthrie [41] on inborn errors of metabolism in neonates. As practiced routinely by

Table 1. Set I capillary blood dried blood spot samples.

Subject	Age (years)	Sex	Overall health	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	Total samples	Span (days)
S-01	62	F	Good		7	17				24	17	54	67	186	2791
S-04	71	F	Good						10	15	43	52	130	250	1647
S-07	55	M	Good						18	31				49	393
S-10	37	M	Crohn's							49	52	183		284	852
S-17	32	M	Good					2	28	19	19	19		87	1415
S-18	69	M	Good	14	12	6	52	22	31	50	52	48	124	411	3292
S-20	47	F	Good						21	24				45	390
S-22	72	M	Good						99	29	40	42		210	1224
			Total	14	19	23	52	24	207	241	223	398	321	1522	12,004

diabetic patients, individuals can collect capillary blood by lancet fingerprick several times each day for glucose measurement without injury or undue discomfort, blood that can be collected and stored in the form of DBS by subjects themselves. Numerous efforts are now underway to further improve the user-friendliness and reproducibility of capillary blood collection that will further enable the approach described here.

Here we report results that illustrate the complex and informative behavior of APR proteins (and related proteins) in tracking inflammation due to multiple causes. The data demonstrate the potential for major improvements in diagnostic test interpretation using personalized baseline values and the feasibility of personalized multiparameter biomarker response models useful in clinical trials.

Materials & methods

Samples

A unique set of 1522 long-term longitudinal capillary blood DBS samples (sample set I) was self-collected at home between 2008 and 2017 by 8 participants using lancet finger-pricks (Medlance Plus Extra or Special, HTL Strefa; Medline, Cat. No. HTD7045BX) and dried on Whatman 903 Protein Saver DBS cards (Table 1). The DBS cards were stored at 4°C in the presence of desiccant except for brief periods at room temperature or at -20°C and were barcoded prior to analysis. Transportation of specimens to the laboratory for processing followed the guidelines provided by the US Center for Disease Control (CDC) and Prevention for shipment of DBS specimens. In addition, a set of 140 EDTA-treated venous whole blood samples (sample set II) was collected from eight Brazilian professional athletes during four consecutive days of Olympic-level beach volleyball training in a study organized by Prof LC Cameron and A Bassini-Cameron (Universidade Federal do Estado do Rio de Janeiro, Brazil). Samples were collected before breakfast, before training, after training and 60 min after training) and after 1 or 3 days of recovery, and were spotted and dried on Whatman 903 filter paper.

SISCAPA-LC-MRM protein measurement

A panel of proteins of known clinical significance was measured using SISCAPA-MRM mass spectrometry [38]. These included a broad set of APR and other inflammation-related proteins reported here (Table 2) and other biomarkers to be reported elsewhere. Sample preparation and SISCAPA peptide enrichment were performed using an automated protocol essentially as described [37] with small modifications as described in Supplementary Information. Target protein amounts were expressed as femtomole (fmol) of proteotypic peptide in each sample, calculated by multiplying the observed peak-area ratio (PAR; the ratio of the endogenous target peptide MRM peak area to that of the stable isotope standard (SIS)) by the known amount of added SIS. The proteins reported here constitute a subset of the panel described previously [39], exhibiting similar precision in replicate standards (Supplementary Table 1).

Samples were analyzed in four campaigns at different times, measuring slightly different but largely overlapping sets of measured protein targets (Table 2). These yielded datasets A–C (Group I) comprised of fingerprick (capillary blood) DBS from subjects shown in Table 1, and dataset D (Group II) comprised of venous DBS from eight beach volleyball athletes. In dataset C, the analytes reported here were run in two successive SISCAPA-MRM panels (C1 & C2).

Table 2. Proteotypic peptides and their proteins measured by SISCAPA-MRM.

Protein	Role	Peptide	I				II
			A	B	C1	C2	D
A1AG	APR+	NWGLSVYADKPETTK	1		1		1
Alb	Normalization, APR-	LVNEVTEFAK	1	1	1	1	1
C3	Complement, APR+	IHWESASLLR	1		1		1
CRP	Innate immunity, APR+	ESDTSYVSLK	1	1	1		1
FibG	Coagulation, APR+	YEASILTHDSSIR	1			1	1
Hp	Fe metabolism, APR+	VTSIQDWVQK	1	1	1		1
Hx	Fe metabolism, APR+	NFPSPVDAAFR	1	1	1	1	1
IgM	Immune response, APR±	YAATSQVLLPSK	1	1	1	1	1
LPSBP	Innate immunity, APR+	LAEGFPLPLLK	1	1	1		1
MBL	Innate immunity, APR+	EEAFLGITDEK	1	1	1		1
MPO	Neutrophil count	DYLPLVLGPTAMR	1	1	1		1
SAA	Innate immunity, APR+	GPGGVWAAEAISDAR	1	1	1		1

Data analysis

PAR data were assembled in Tableau Prep Builder (www.tableau.com) and joined with Excel tables defining sample characteristics (e.g., date of collection, subject contextual health notes, SIS concentrations, analytical run structures, etc). Data analysis and visualization were performed in Tableau Desktop (Tableau Software, Inc.).

To minimize the effect of variations in plasma volume between DBS from the same individual (typically ±10–15%), we refined and applied a method developed previously [39] to normalize plasma volume using a sample-specific scale factor computed from an equally weighted combination of three proteins (Alb, Hx and IgM) whose abundances are usually very stable within individuals over time. Consistency of values across datasets was established by including replicate punches of subject samples (i.e., punches from the same DBS cards) in datasets A, B and C (88 samples common to A and B, 19 common between B and C; Supplementary Information). This approach compensates for any drift in the absolute amounts of SIS peptide internal standards that could occur in the time between the different analytical runs. Replicate standard DBS samples were included in each 96-well plate processed for SISCAPA measurement, normalized using the above methods and used to evaluate the precision of each assay in each dataset. The average coefficient of variation (CV) across the assays used in Datasets A, B and C were 6.7, 5.9 and 4.5% respectively, consistent with expectations for clinically useful assays (Supplementary Table 1).

After this subject-specific normalization, subject-specific baselines were computed for each protein as the average level in the half of samples with lowest CRP (i.e., the samples showing least evidence of current inflammation). Fold-changes were computed relative to these baselines and so focus on relative responses in the individual rather than absolute scale of change.

Results

Amounts of selected APR and related proteins were measured using an automated, multiplexed SISCAPA-MS protocol [37,39] and normalized to a consistent plasma volume using a personalized approach based on a combined index of three proteins (Alb, Hx, IgM) whose levels are generally very stable in an individual over time (i.e., they exhibit low within-person variance [39]). The calculated scale factors applied to individual longitudinal DBS spots in this study fell in the range of 0.7–1.3 as expected. This volume normalization substantially reduces longitudinal baseline 'noise' in measurements from an individual: Supplementary Figure 1A shows DBS protein levels before and after normalization in 250 serial samples collected by subject S-04 over almost 5 years.

In addition to maintaining stable levels over time, two of the normalizing proteins (Hx and IgM) exhibit large quantitative baseline differences between individuals. As a result, each subject's sample set, in many cases including hundreds of samples collected over many years, forms a tight cluster on a plot of Hx versus IgM, almost completely separated from the other subjects (Figure 1A). In general, points diverging from the cluster centroids are associated with major inflammation events. Subjects S-04 and S-18 (of opposite sex, contributing 411 and 250 samples respectively) share a living environment, while their sample sets appear as completely separated clusters, indicating that most of this between-subject variation is not determined by environment at the time of collection. Most of the other APR proteins measured showed significant inter-individual differences as well (Supplementary Figure 2).

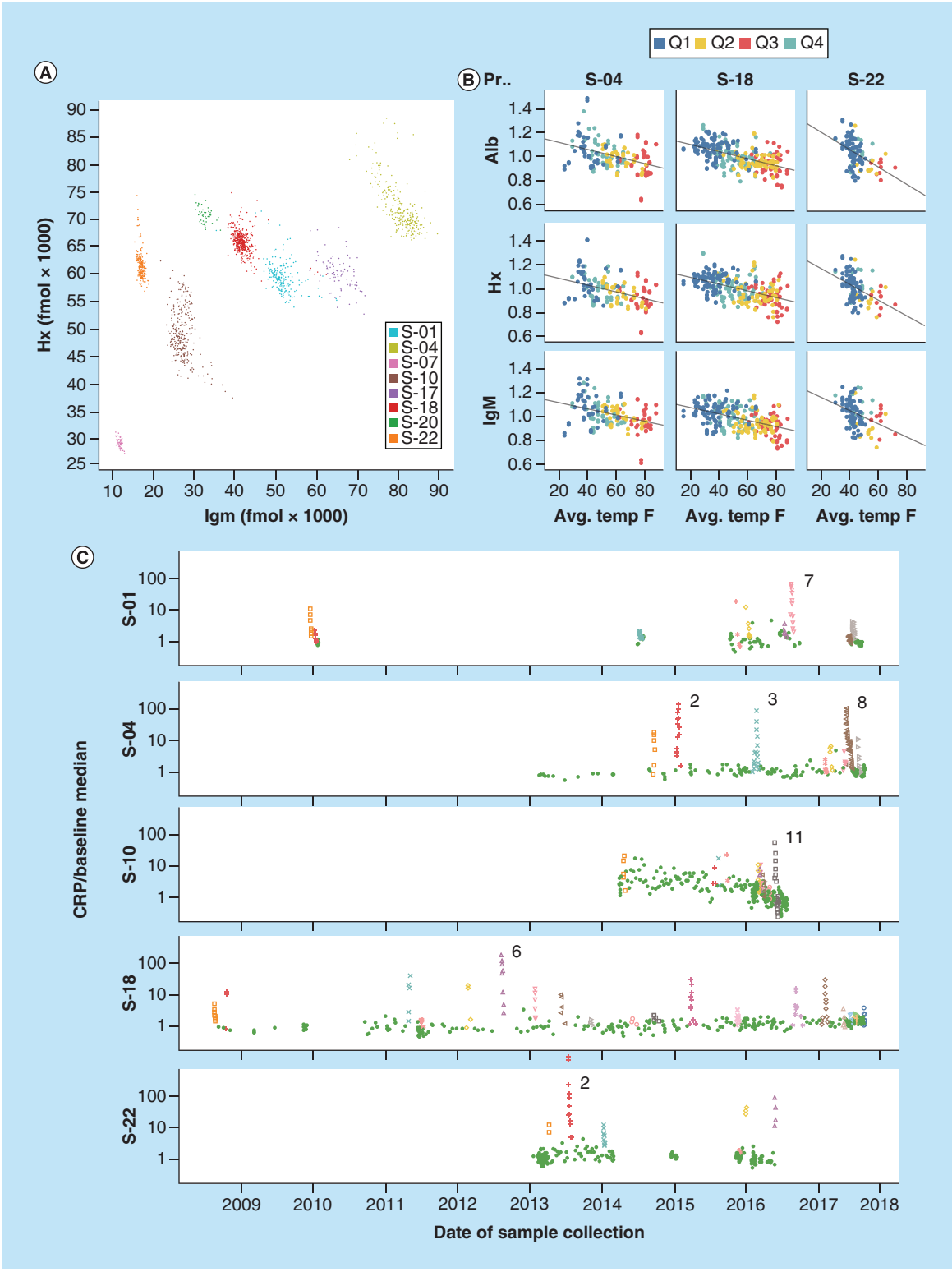

Figure 1. Biomarker baseline stability, and appearance of inflammation events. (A) Plot of fmol IgM *versus* Hx for all 1522 samples in Set I, color coded by subject. **(B)** Abundances of Alb, Hx and IgM (un-normalized fmol divided by personal average value, excluding inflammation event samples) in three subjects *versus* local daily average ambient temperature on the date of collection, colored by calendar quarters. **(C)** Abundance of CRP (normalized fmol divided by personal baseline average) in five subjects over time. Inflammation events are color coded (remaining samples are shown as green dots) and most intense events numbered.

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While the selected normalizing proteins exhibit striking longitudinal stability, we nevertheless observed two significant sources of variation in these proteins within subjects. Figure 1B shows small (± 10%) systematic changes in three proteins in three subjects that correlate with average ambient temperature on the date and location where the samples were collected, an effect previously noted in the form of seasonal variation in some DBS analytes [39]. In addition, as shown in Supplementary Figure 1B, extreme inflammatory events such as major infections affect the concentrations of most plasma proteins to some degree, including those used here for normalization. With the present approach, normalization bias due to inflammation is mitigated due to the fact that while IgM varies little, Alb shows a weak negative APR and Hx shows a weak positive APR, so that the combined index is to a great extent balanced with respect to inflammation.

Inflammation events

The largest perturbations observed in levels of most proteins occurred during inflammatory events that were noted by the study subjects and were coincident with increases in the acute phase reactants CRP (Figure 1C) and SAA. However, many events were observed that appear to be 'sub-clinical': in the five subjects for which extended (>150) longitudinal sample series were available, we identified 57 inflammation events in which CRP was increased significantly above baseline levels in multiple samples collected over a brief interval, allowing identification of a temporal maximum (colored spikes in the CRP time series presented in Figure 1C). Out of these events, approximately half (29 events) included at least one sample in which CRP was increased by more than tenfold above baseline and all events were confirmed by coordinated increases in CRP, SAA and LPSBP. In contrast to the large increases seen in SAA and CRP in these events, the remaining APR proteins (LPSBP, A1AG, Hp, MBL, FibG, C3 and Hx) showed much smaller effects (Supplementary Figure 1B), with fold increases relative to baseline ranging from 0.036 (LPSBP) as great as the fold increase in CRP to 0.002 (Hx).

Given the significant fraction of samples that showed evidence of an inflammatory response, we defined personal 'normal' baseline levels for each biomarker protein based on the subset of samples with CRP below a cutoff equal to the personal median CRP value across all of a subject's samples; in other words, selecting the half of each subject's samples with lowest CRP values. For each protein, the average value in these low-inflammation baseline samples was taken as the subject's personal baseline. The standard deviation in these baseline samples allowed calculation of a personal baseline CV. The average baseline CV calculated over all subjects, samples and proteins was 12.3% (12.4, 17.0 and 10.2% in datasets A, B, and C, respectively) compared with 38.0% (39.2, 68.7 and 31.3% in datasets A, B and C) for combined baseline and nonbaseline (i.e., all) samples (Supplementary Table 3). On average, the ratio of assay CV (from replicate standards) to average subject baseline CV (from subjects' longitudinal samples) was 0.52, satisfying the criterion (<0.6) allowing statistically meaningful analysis of within-subject biological variation [42] at very low levels of inflammation. In subject S-22, CRP and SAA showed maximum increases of up to 497- and 1062-fold (equivalent to 2673 and 3688 personal standard deviations) respectively, above personal baselines in a major kidney infection, illustrating the >1000-fold dynamic range of statistically significant (>2 SD) within-person changes. The proportion of samples in which CRP was more than twofold higher than the personal baseline ranged from 12 to 45% among these five subjects (Supplementary Table 2).

Infection

Infections of various kinds constituted the major driver of inflammation events. Figure 2A shows time course plots for seven significant infections occurring in five subjects, each involving SAA increases >100-fold from personal baseline average values. These events were reported by subjects as: respiratory infection (S-01 E7; i.e., event 7 in subject S-01); respiratory infections (S-04 E2, E3 and E8); food poisoning (S-10 E11); pneumonia confirmed by x-ray (S-18 E6); and kidney infection (S-22 E2). Subject S-18, with the largest sample series, exhibited a number of discrete events (Figure 1C) in addition to S-18 E-6 that were subject-annotated as 'nose colds' and have a remarkably consistent magnitude and structure, likely indicating a reproducible response to similar infectious agents (e.g., rhinoviruses). None of the infections required hospitalization, though in some cases antibiotics were administered.

The ratio of SAA over CRP (each in terms of fold-change from personal baseline average; Figure 2B) shows large changes through the course of each infection. SAA induction exceeds CRP induction at the peak of each event, while it appears that CRP exceeds SAA before and after the peak.

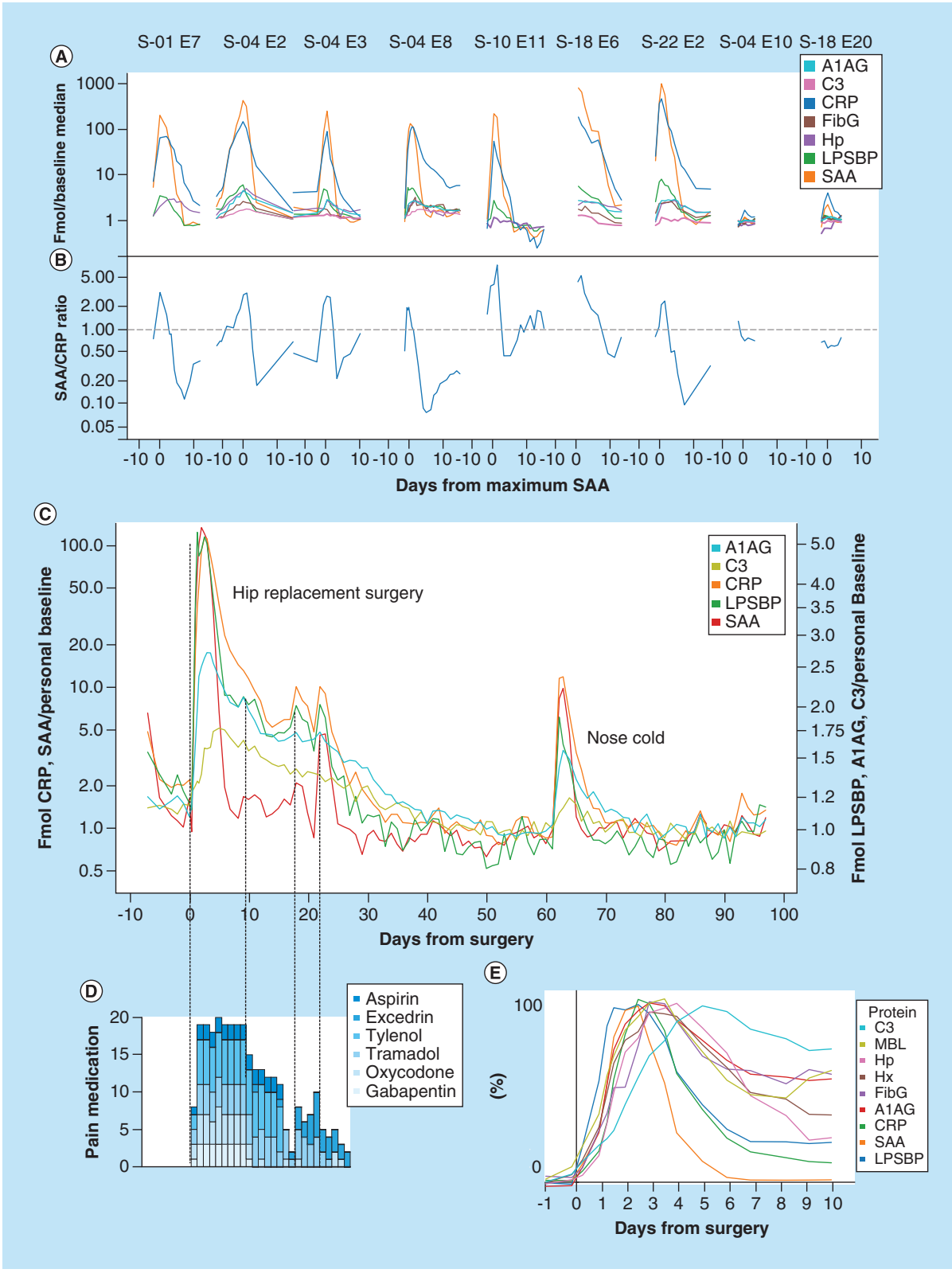

Figure 2. Inflammation profiles related to infection, vaccination and surgery. (A) Amounts of six proteins (normalized fmol divided by personal baseline average, log scale) in seven largest inflammation events and two influenza vaccinations. **(B)** SAA/CRP ratio for the same events shown in **A**. **(C)** Amounts of five inflammation proteins (normalized fmol divided by personal baseline average, two separate log scales) over a 100-day period including a total hip artheroplasty (day 0) and an upper respiratory infection (day 63). **(D)** Requirement for pain medication associated with the surgery. **(E)** Amounts of nine inflammation proteins over 10 days including a total hip artheroplasty, with each protein normalized to approximately the same scale of change in order to illustrate differences in timecourse.

Influenza vaccination

Two subjects collected daily samples in periods that included a vaccination against influenza (Fluzone™ high dose 2017). Inflammatory responses (Figure 2A; S-04 E10 and S-18 E20) in SAA and CRP were measurable (1.3- and 2.2-fold increases in SAA from respective personal baselines), but were ~100× smaller than those associated with major infections. Out of the 57 identified inflammation events, 41 involved SAA increases larger than 2.2-fold (i.e., a greater response than seen with either of these two vaccination events).

Surgery

Subject S-04 underwent elective anterior total hip arthroplasty involving a short (~2 h) surgical procedure and subsequent recovery period of 97 days during which daily (or more frequent) samples were collected (Figure 2C). Following an initial rapid rise after surgery, inflammation markers (here plotted on two log scales, left and right, differing by 20-fold in magnitude), declined at different rates until day 55, at which point the levels approached a new baseline approximately 50% below the presurgery level. CRP and SAA were induced much more strongly than other inflammation indicators (with maximum inductions of 113- and 136-fold, respectively). The magnitudes of the observed responses (in fold-change from personal baselines) were: SAA > CRP >> LPSBP > FibG, Hp, A1AG > MBL, C3 > Hx (136, 113, 5.4, 3.0, 2.9, 2.7, 2.0, 1.8 and 1.2-fold, respectively), overall a ~500-fold range of response magnitudes relative to personal baselines. In the period between days 8 and 30, a series of post-surgical jumps in SAA and CRP (e.g., days 9, 18 and 22) indicated renewed inflammatory activity that generally coincided with increased requirement for pain medication (Figure 2D) and subject reports of strains in the surgical area, followed by smooth declines to baseline. Figure 2C also includes an infection event (an upper respiratory tract infection perceived by the subject as a 'nose cold') on day 62 following surgery. While the overall magnitude of the inflammation response in this infection was roughly 10% as great as the response to surgery, the kinetics of the early response of the five proteins shown were similar.

Figure 2E shows the time course after surgery of nine acute phase proteins normalized to the same peak response (0–100%) and smoothed in order to reveal relative peak times. LPSBP achieved its peak level first (~1.2 days post-surgery), followed by SAA, CRP, A1AG and MBL, Hp, FibG, Hx and C3, with the last peaking approximately 5 days post surgery.

Crohn's disease

A combination of rapid APR protein changes superimposed on a slow declining trajectory were observed in subject S-10, who has Crohn's disease and has diligently explored dietary and other measures to control symptoms. Over a period of ~2.5 years, CRP and SAA levels were substantially elevated on numerous occasions (Figure 3A shows the entire time period consisting of almost 2 years of weekly samples followed by 180 daily samples shown expanded in Figure 3B). Overall, CRP was above two-times the average personal baseline value in 48% of the individual's samples, a significantly higher proportion than exhibited by the other subjects. The subject made careful notes of perceived Crohn's attacks (reported on a scale of 0.5–2 in intensity at times indicated in Figure 3); 'colds' (labeled 'C') and one episode attributed to food poisoning ('FP'). It is noteworthy that three of eight recorded Crohn's attacks occurred at times of lowest CRP (150, 460 and 800 days from the first sample), while the rest coincide with CRP peaks. Conversely, at least eight CRP peaks of magnitude equal to those linked to Crohn's attacks are not noted by the subject as either Crohn's attacks or infections. Over the period of sample collection, S-10 reported success in significantly reducing the frequency and intensity of gut inflammatory events and this was reflected in the significant downward trend in CRP and SAA, reduced intensity of CRP spikes and a progressive decline of almost threefold in Hp, a slowly-responding acute phase protein.

Figure 3C shows results from weekly samples (Thursdays) extracted from the daily sample set shown in Figure 3B. Weekly sampling gives a significantly different picture of inflammation activity than the daily samples in this case, consistent with the short timespan of most Crohn's flares in subject S-10.

Intense exercise

The most rapid APR protein changes observed were the result of intense physical training, in this case using dried venous blood samples from elite Brazilian beach volleyball athletes during a week of Olympic training. Four samples were collected on each training day: Figure 4 shows longitudinal changes in four teams (1–4) of two athletes (A & B) training together over the course of four training sessions and two subsequent recovery samples later in the week. Hp decreased significantly during training sessions, presumably due to complexation with Hb released

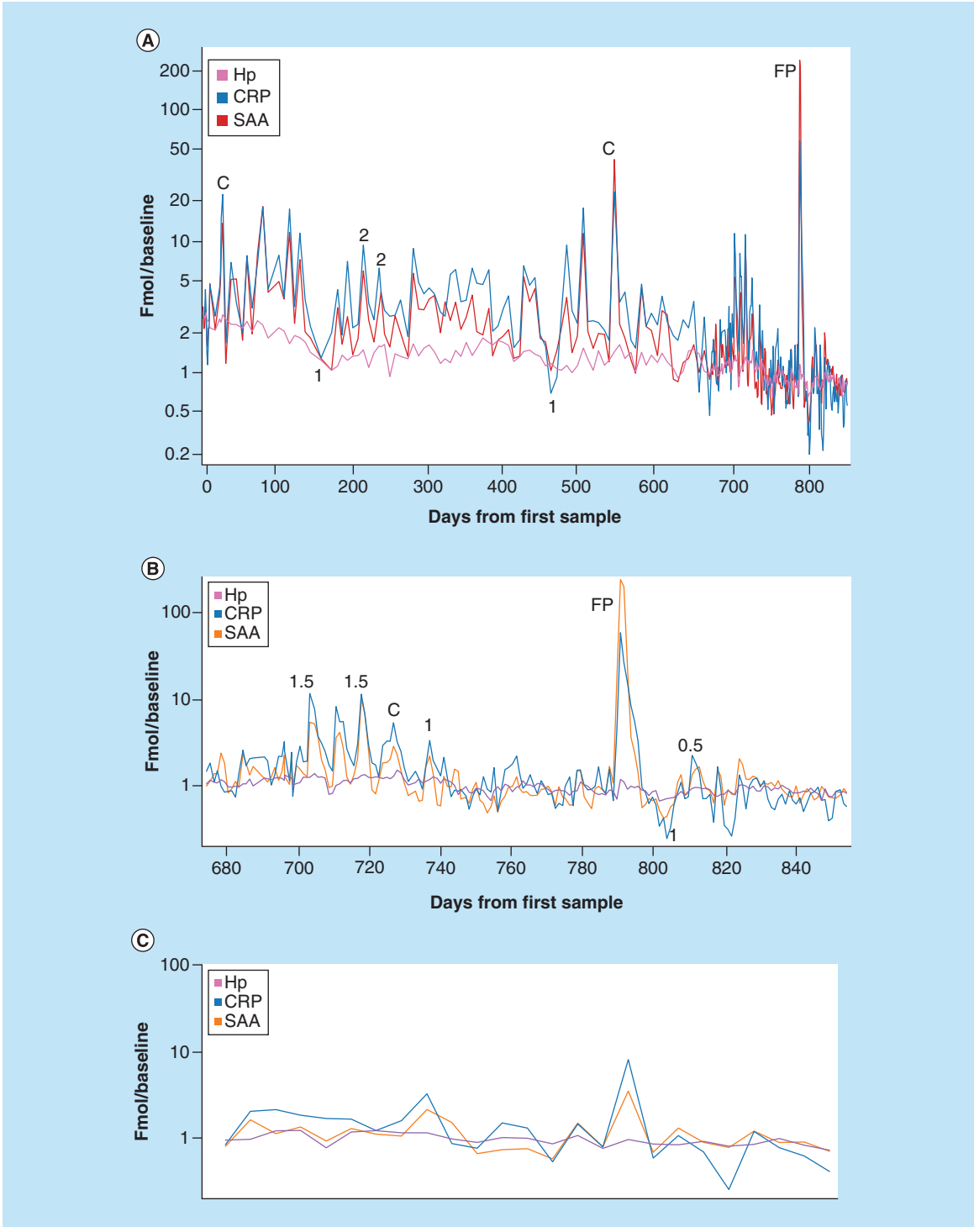

Figure 3. Timecourse of changes in Crohn's disease. (A) Amounts of Hp, CRP and SAA (normalized fmol divided by personal baseline average) in 284 samples from subject S-10 (Crohn's disease) over 852 days (daily sampling from day 675, weekly sampling prior to that). Numbers indicate severity of Crohn's attack (0–2 scale) assessed by subject, 'C' = cold, 'FP' = episode of food poisoning. **(B)** Expanded view of daily samples. **(C)** Weekly subsample (Thursdays) of daily sample data.

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Figure 4. Amounts of haptoglobin, MPO, CRP and serum amyloid A (normalized fmol divided by personal baseline median) in eight elite beach volleyball athletes (4 teams of 2) during a week of Olympic training. Four training sessions are outlined in gray rectangles.

through RBC hemolysis associated with violent muscle contraction [43], declining by a total of ~10–60% among the eight athletes by the end of session 4, and then recovering to normal levels 3 days later. MPO (reflecting the neutrophil count) increased by ~10–100% during training sessions and rapidly returned to baseline [44], exhibiting large differences between athletes. CRP and SAA were not strongly affected during training sessions, though SAA appeared to decrease in most athletes during the 4 days of training. There were, however, several specific events indicating inflammatory responses from other sources: athlete A of team 3 (A3) began the week with CRP and SAA strongly elevated, and both declined according to typical half-lives. Athlete 4B experienced a large increase in neutrophils (MPO) during the first training session, followed by the largest observed CRP increase the following day, suggesting a potential injury (though none was noted by the coaches). While the overall pattern of Hp and MPO responses to intense training was consistent, individual athletes differed significantly in the magnitude of these changes.

Correlations among inflammation biomarkers across the data

Figure 5A shows a protein:protein correlation matrix calculated over all 1522 samples from subjects in sample set I after volume normalization and division by personal baseline average values (to minimize the impact of between-subject differences in protein amount). The positive APR proteins showed significant correlations overall as expected, with the highest pairwise correlations occurring between proteins with similar time signatures (Figure 3E): thus CRP and SAA (both fast APR's) show a pairwise correlation of 0.89; A1AG and Hp (both slow APR's) correlate at 0.84. CRP and Hp (fast vs slow responses) showed a correlation of only 0.49. Alb and IgM showed strong negative correlations with the positive APR proteins, consistent with their known roles as negative APR markers. The three normalizing proteins Alb, Hx and IgM also exhibited strong negative correlation with each other, as expected given their collective balancing role in the volume normalization process.

Nonlinear regulation

Figure 5B explores the correlation of CRP with SAA and LPSBP in more detail using log:log plots in samples that are above (blue) and below (orange) personal average CRP levels for the five subjects with large sample numbers. During inflammatory events, where CRP varies by up to ~100-fold, the SAA response was consistent

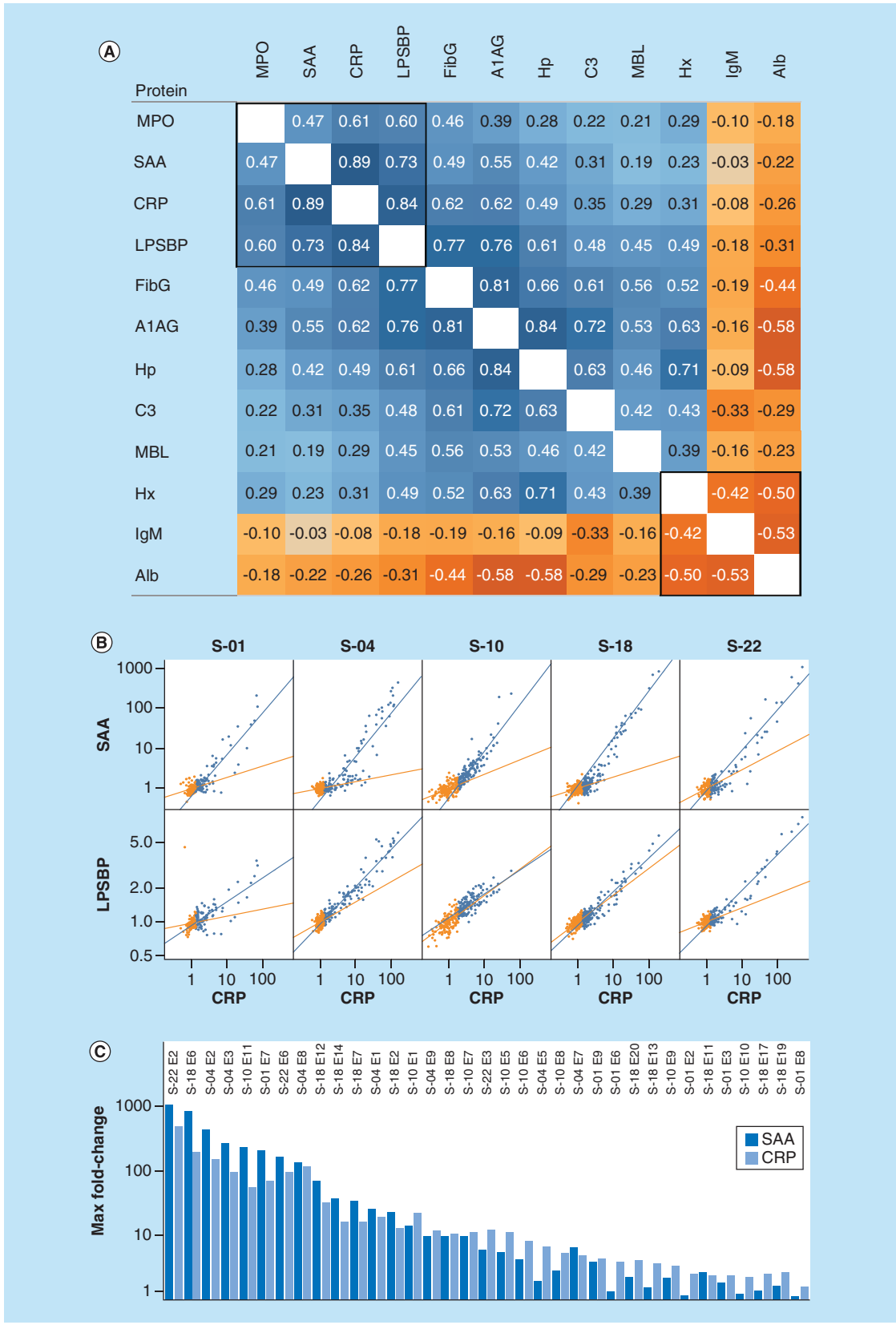

Figure 5. Correlations among inflammation proteins. (A) Protein:protein correlation matrix calculated over all samples from all subjects after sample volume normalization and division by personal baseline average values (to minimize the impact of subject:subject differences in protein amount). **(B)** Scatterplots (normalized fmol divided by personal baseline average, log-log) relating CRP to SAA or LPSBP in the individual samples (dots) contributed by five subjects. Baseline samples (the half with lowest CRP) are shown in **orange**, and the higher half of samples in **blue**. **(C)** Maximum fold-change from baseline in SAA and CRP during inflammation events.

across subjects, increasing by up to 1000-fold over baseline in major infections. LPSBP levels increased in a similar pattern, but the observed increase was less than tenfold at maximum. The strong relationships evident in the blue samples, while appearing linear in log:log format, are, in fact, nonlinear (best-fit here by exponential relationships). This is confirmed in the set of events (Figure 5C) in which a local maximum SAA timepoint was determined (i.e., for which samples were available with lower levels just before and after a maximum), which shows that SAA's maximum induction relative to baseline exceeds CRP's for the highest-level events, but CRP generally achieves higher levels of induction than SAA for the smaller events. This nonlinear relationship can be modeled by an exponential fit in which SAA is related to CRP raised to a power between 1.31 and 1.52, except for subject S-10 for which the power is 1.75.

Figure 5B also reveals significant correlations between CRP and SAA or LPSBP in the half of each subject's samples (orange) with lowest CRP (i.e., in the baseline low-inflammation data). Such persistent correlations among proteins in what could be considered baseline samples demonstrate that small changes in APR proteins represent real biological signal ('microinflammation'), not simply noise in pre-analytic and analytic steps.

Visualization of inflammation responses & recovery

Differences in timing between earlier and later-peaking inflammation markers suggest an improved method for visualizing an inflammatory response trajectory as loops in two dimensions, starting at baseline, through injury and returning to baseline [45]. Figure 6A shows three such event trajectories in one subject (S-04) visualized by plotting SAA (fast response, up to 439-fold above baseline on a log scale) versus Hp (slow response, maximum at 5.3-fold above baseline, also on a log scale) in successive DBS from the respective events. These events include a 'deep cough with earache' (S-10 E2; red), as well as the hip replacement surgery (S-10 E8; green) and 'nose cold' (S-10 E9; blue) events shown in Figure 2C. The largest magnitude event (cough) reaches maximum response at days eight and nine after first increase, while the much smaller response to a cold reaches maximum in 3–4 days. Response to the comparatively short 2-h surgical intervention peaked in 2–3 days, and proceeded through initial healing (day eight), followed by a period of re-inflammation (days 10–27; coincident with increased use of pain medication) and recovery to baseline (day 86).

The same approach can be applied (Figure 6B) to visualize three subjects' complete inflammation trajectories presented in Figure 1C. In these plots, the identified inflammation events are drawn in colors, while the samples constituting baseline are shown in green. Subjects S-04 and S-18 each experienced a number of inflammation loops (all associated with infections, except S-04 E7 (surgery). Subject S-10 (Crohn's) has a different distribution of measurements, with only a single major infection (E11) and a wider range of Hp variation due to the progressive decline of this marker during the 2.5 years of sample collection.

Discussion

Here we present a high-resolution multi-dimensional molecular picture of inflammation events and personal baseline biomarker levels in 16 individuals, combining dense longitudinal sampling *via* DBS, a broad panel of acute phase proteins and precise, multiplexed wide-dynamic-range quantitation using SISCAPA-MS. This picture captures details of inflammation processes on multiple time scales, reveals complex relationships among inflammation-related proteins and extends the measurable dynamic range downward to cover biology-driven fluctuations in APR baselines ('microinflammation'). The results move beyond what can be observed through sporadic measurements of a single marker such as CRP and offer the potential for major improvements in diagnostic medicine and for monitoring subject responses in clinical trials.

While previous studies have examined many of these proteins alone, or occasionally in small groups, this study is unique in applying a broad set of APR markers across many indications at high frequency. Key to this broader coverage is selection of a group of APR proteins that vary widely in response kinetics, magnitudes and function. Each inflammation-associated protein biomarker shows a different characteristic time course of expression during an inflammation event. APR proteins, which are generally produced in the liver, exhibit a protein-specific early phase increase, likely governed by mRNA transcription and expression triggered by a short-lived cytokine signal. Among the APR proteins, LPSBP typically rises most rapidly, followed in order by SAA, CRP, A1AG, FibG, Hx, MBL, Hp and C3. MPO, which in the context of whole blood serves as a surrogate for the neutrophil count, typically shows a spike just before or coincident with LPSBP, reflecting the fact that pre-existing neutrophils can be released from the bone marrow immediately upon cytokine signaling, without a time-lag required for new protein synthesis. APR proteins decline following an order (SAA, CRP, LPSBP, Hp, Hx, FibG, MBL, A1AG,

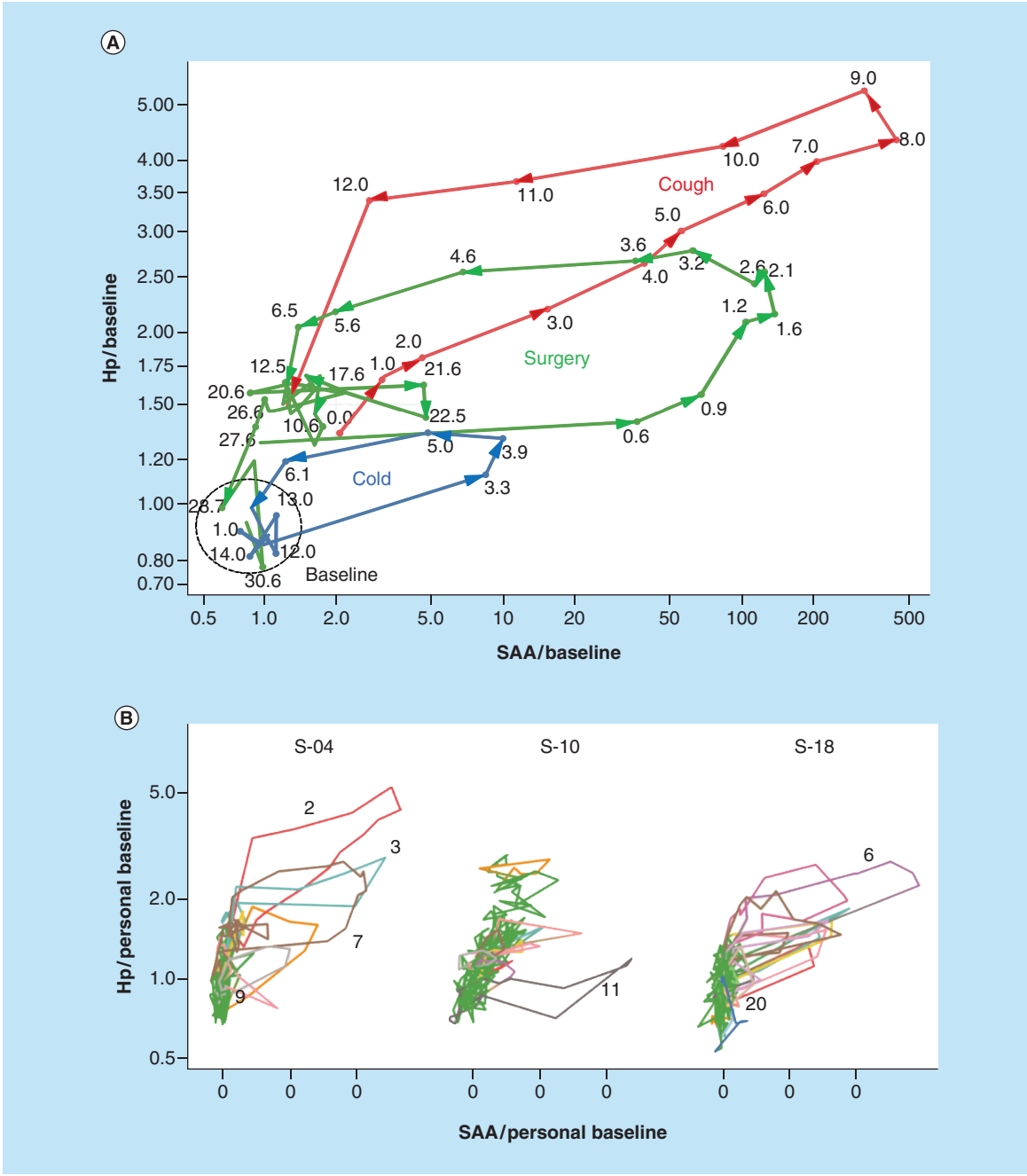

Figure 6. Multiparameter visualization of inflammation event trajectories. (A) SAA versus Hp loop plot (normalized fmol divided by personal baseline average, log-log) for three inflammation events (cold, surgery and cough) in subject S-04, with successive time points linked by lines to form loops. Numbers indicate days from beginning of inflammation event. **(B)** Loop plots showing all samples for three subjects. Inflammation events color-coded, with baseline samples shown in **green**.

C3) that is generally consistent with previously measured half-lives of CRP, LPSBP, A1AG and C3 of 26, 32, 104 and 84 h, respectively [46]. Proteins peaking earlier and later provide complementary information, analogous to the relationship between glucose and HbA1c measurements in diabetes: fast responders provide a measure of inflammatory stimuli in the previous 1–3 days, while the slower responders reflect stimuli that have occurred over much longer periods comprising a slower initial rise and a slower decay. Thus at any given timepoint, the combined set of proteins provides a readout reflecting inflammatory stimuli occurring over the previous 2–4 weeks.

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In addition to different expression times, the APR proteins also differ widely in the amplitude of changes during inflammation events. Proteins exhibiting fast responses typically show very large changes (10- to 1000-fold from personal baseline values) while those showing smaller, though still statistically significant changes (in some cases 1.1- to 1.5-fold changes from personal baselines), typically show slow responses. Changes in Alb and IgM generally involve decreases from baseline during major infections (i.e., they are negative APRs, changing with polarity opposite to APRs like CRP).

The data also demonstrate directly, for the first time, that the quantitative relationships between strongly responding APR proteins such as CRP, SAA and LPSBP are nonlinear; in other words, producing different relative change magnitudes in large *versus* small scale inflammation events. The APR panel used here thus achieves its overall design goal: to include proteins responding differently in time, magnitude, polarity and functional responses to inflammatory signals.

Underlying these observations is an implicit assumption that proteins (or more specifically the proteotypic peptide analytes they contain) are themselves stable in DBS samples over extended time periods (e.g., up to a decade). While the present study was not designed to test stability directly, several results presented here provide compelling evidence of long-term stability: the general constancy of individual subject baselines for most proteins across many years (Figure 1C & Supplementary Figures 1–4); the impressive tight clustering of subjects' samples in Figure 1A; and the consistent detection of seasonal variations over many years shown in Figure 1B.

Distilling and visualizing these complex data is challenging. Simple ratios between two proteins or two timepoints have been explored with limited success. The ratio of SAA to CRP, for example, has been proposed as an indicator of infectious disease severity in children [47]. Our results show that while this ratio does appear to distinguish between large and small infection responses when measured at the peak of response (Figure 5C); nevertheless, the ratio changes so rapidly during an infection (up to 20-fold change over the course of a few days as shown in Figure 2B) that sampling at any point off-peak can generate an incorrect prediction.

An alternative approach explored here incorporates measurements of multiple proteins at multiple time points. As a first step, we have used two APR proteins responding on different timescales (fast-responding SAA and slower-responding Hp) which allows inflammation events to be visualized in two dimensions as shown in Figure 6A. In this representation, similar to visualizations of pharmacodynamic 'hysteresis' loops [48] or personalized health curves [45], inflammatory events unfold over time as counterclockwise loops. Loop size and distance from the subject's baseline samples indicate the magnitude of the response, while any two adjacent (i.e., consecutive) samples on such a loop generate a directional line segment whose position and slope indicate the size of the loop (whose area approximates the intensity and duration of the whole response) and where the subject is on the loop trajectory at that time (i.e., position on the path from insult, through recovery and back to baseline). Given the relatively smooth structure of these loops, it appears that comparison of two samples taken 6 h (0.25 days) apart would provide a useful diagnostic estimate of a subject's position and direction on the response loop, given the assay precision and baseline data obtained here.

Our long-term objective, however, is to create a true multi-dimensional, personalized longitudinal inflammation model including additional APR components and related features such as neutrophil bursts (measured here as released MPO) preceding APR and adaptive immune responses (measured here as IgM) following some infections. Such a model will incorporate the temporal and magnitude relationships among the biomarkers (including the nonlinear regulatory effects first reported here), fitting multi-protein data from observed events to generate important derived parameters. Some of the required methods may be adapted from classical pharmacodynamic (PD) modeling [48], in which biomarkers are used to measure response to a known cause (e.g., a pharmacokinetic [PK] drug dosing model). Simple PD models using CRP as a single biomarker have been developed and used successfully in clinical trials of antibiotics [26–28], various biologics [20,23,24]; antisense to CRP [25] and an MAPK inhibitor [33]. However PD-like models of inflammation have not been adopted in clinical diagnostics despite a small handful of successful examples [49], due in part to the rarity of applicable longitudinal samples. Personalized PD inflammation models, developed using baselines and responses to inflammation events (e.g., vaccinations, colds) in a subject's longitudinal samples, would provide increased precision in tracking inflammatory phenomena and optimal design of sampling protocols. Additional methods for uncovering disease:biomarker relationships, including machine learning approaches developed in 'big data' applications, are also likely to benefit from personalized longitudinal multiplex data described here.

A number of novel observations emerged from examination of our extended longitudinal sample series. First is the unexpectedly large number of identifiable inflammation events, most of which were apparently due to infections in

apparently normal subjects. A surprisingly high proportion (12–45%) of each subject's samples showed indications of inflammation, whether defined as CRP levels >twofold above personal baselines, or inclusion in the 57 discernable APR events. Similar proportions are obtained when considering only those samples collected on a near-daily basis (about 1/3 of the samples), in which the timing of collection should be less susceptible to subjects' selection bias. Such high event frequencies, if confirmed in a larger subject population, suggest that unrecognized short-term "sub-clinical" inflammatory events are likely to occur frequently during investigational studies and drug trials, and perhaps at even higher frequency in unwell individuals (of which the Crohn's disease subject is an example). Such changes may impact behavior of drugs not directly related to inflammation, for example, due to the well-known binding of many small molecules to the APR proteins Alb and A1AG [50].

The frequency of small inflammation events also suggests that randomly-timed single CRP measurements (such as those included in annual checkups) may overestimate a patient's true baseline level if collected during an inflammatory episode (i.e., 12–45% of the time according to our estimate of samples indicating inflammation), and thus bias predictions of cardiovascular disease risk [3,51]. Conversely a single CRP measurement is likely to underestimate the likelihood that a patient has inflammatory episodes when used as a qualification for anti-inflammatory therapy [52]. A clear improvement will be to use personal average baseline APR levels (including CRP) derived from a series of longitudinal samples thus providing a significantly better risk estimate by capturing baseline inflammation separately from fluctuations related to transitory subclinical events. As a practical matter, these results suggest that the four lowest values from eight serial samples be averaged for a baseline magnitude, and CV be estimated from the lowest 8 of 16 samples. As to the timing of these samples, our results suggest an intersample period of 5–10 days for such a baseline measurement of inflammatory responses, given that most of the larger perturbations appear to last 3–10 days. Biomarkers that show different patterns of variation (e.g., those that are not affected significantly by inflammatory events) likely require different baseline definitions based on exploratory studies of variation timescales.

Characterizing personal baseline inflammation proved to be more challenging (and interesting) than anticipated. In the half of each individual's samples with the lowest CRP (presumably the samples showing the lowest levels of inflammation), we observed persistent correlations between CRP, SAA and LPSBP, indicating that a significant amount of the observed baseline variation represents low-level, but real, biological response fluctuations ('microinflammation'), rather than analytical 'noise'. As a result, the spectrum of inflammatory responses measurable using APR biomarkers appears to extend smoothly from ±10% (i.e., 0.9–1.1-fold) to 1000-fold, a total dynamic range of over 10,000. This observation suggests the possibility of correlating detectable subclinical inflammatory triggers with a variety of contextual data including diet, environmental exposures, recurring infections and chronic inflammatory disease.

The 57 discrete inflammation events we cataloged included events driven by a variety of causes, both 'planned' (surgery, vaccination and intense exercise) and 'unplanned' (infections, and episodic inflammation related to Crohn's disease). Given the small number of subjects covered, these observations represent individual case reports and require follow-up in well-designed studies of larger cohorts to confirm the generality of our observations.

Total hip replacement surgery in subject S-04 represented a 'planned' inflammatory intervention, whose known start time and 2-h duration allowed precise timing of samples with respect to the inflammatory stimulus (Figure 2C, D, E & Figure 6A). Post-surgical inflammation events were clearly discernable ~9, 18 and 22 days after surgery, the latter two of which correlate in time with physical 'strains' noted by the patient, causing increased requirement for pain medication (Figure 2D). Between 50 and 60 days post-surgery, the levels of APR proteins reached a new baseline significantly below pre-surgery levels, a decrease in inflammation that provides a quantitative measure of the benefit of replacing an arthritic hip joint. The level of temporal detail observable by this approach provides a much more complete picture of recovery, including interruptions, than shown in previous studies using a single marker (typically CRP) to detect post-surgical infection [53] or to assess surgical damage [54].

A series of infection events generated the largest APR protein changes observed here, seven of which resulted in SAA levels ≥100-fold above personal baselines (Figure 2A). These events, and a series of smaller events usually attributed to common 'nose colds', provides evidence of a nonlinear relationship between maximal CRP and SAA inductions, as well as the lack of synchrony mentioned above which limits use of the SAA/CRP ratio. In addition to acute phase proteins, we also observed increases in IgM and MPO in a subset of infections. While most infections did not cause measurable increases in IgM, three episodes resulted in increases of 30–50% in total IgM 8–10 days after SAA peaked (Supplementary Figure 4), after which the levels subsided to pre-existing baseline levels. The production of such large amounts of IgM after specific infection events is consistent with the expected timeframe

for an adaptive immune response to a pathogen, and may provide an opportunity to identify endogenous human monoclonal antibodies from DBS samples with potential therapeutic value as anti-infectives. In contrast, MPO, which serves in whole blood as a surrogate for neutrophil count, was frequently increased at the earliest stage of infection followed by spikes every 2 or 3 days (Supplementary Figure 3), consistent with periodic release from and regeneration of neutrophil pools in the bone marrow [55].

In a single case of Crohn's disease, we observed a higher frequency of APR events than in the other subjects, including eight subject-identified 'Crohn's attacks', three 'colds' and one instance of food poisoning. Interestingly, three of the Crohn's attacks occurred at times of minimal APR levels, while the others coincided with APR spikes of 2- to tenfold above baseline levels. The brief timecourse of the attacks highlights the necessity for frequent sampling as a basis for selecting an optimal sampling strategy for any given condition: a weekly sampling schedule would have completely missed most of the events (Figure 3B & C). Of practical importance, over a 2- and 5-year period, average CRP, SAA and Hp levels decreased alongside steady improvement in the subject's control of the disease. Since the relationship of Crohn's disease activity to inflammatory biomarkers such as CRP may be useful in a 'treat-to-target' approach to biologic therapy [56], it will be important to further elucidate these relationships in multiple 'subjects.

Intense exercise with accompanying heat stress and strong sun, in this case occurring during Olympic training for beach volleyball, was included here to assess the potential magnitude of very short-term physical effects on APR responses. The most significant changes observed were large decreases in Hp (presumably due to rapid removal of Hp:Hb complexes formed as a result of exercise hemolysis [57]) and increases in MPO (presumably due to release of pre-formed bone marrow neutrophils) during daily training sessions, neither of which depend on expression of new protein.

Taken together, these results demonstrate that DBS collected under field conditions and stored for periods up to 9 years can be analyzed by SISCAPA-MRM to deliver high-precision biomarker measurements spanning a very wide dynamic range.

However, we note several limitations of the present study. While the sample numbers analyzed here exceed those in all but a handful of biomarker studies, the number of subjects is modest, with only five individuals contributing more than 100 longitudinal samples each. Future studies will require larger numbers of subjects in order to generalize conclusions about the frequency, scale and time courses of inflammation events, to link this data with specific causes and outcomes, and to determine optimal sampling frequencies for specific applications. While not strictly necessary in studies such as ours that refer biomarkers to personal baselines, we also look forward to reporting calibration of the individual biomarkers against validated clinical assays, and rigorous comparison of dried capillary blood with liquid venous serum (UNPUBLISHED DATA).

Future perspective

In the future, a number of important opportunities can be addressed using inflammation and other protein panels measured in subject-collected DBS and interpreted against carefully defined personal baselines. Aside from obvious diagnostic needs in infectious and chronic inflammatory diseases and in trials of related therapeutics, APR responses are frequently noted in cancer biomarker discovery studies and are likely to prove useful in monitoring cancer patients during treatment and in detection of recurrence. From a wider perspective, the practicality of collecting large scale longitudinal biomarker data using DBS microsamples establishes a new dimension of dynamic biological 'Big Data', orthogonal and complementary to the established streams of data from genomics, electronic medical records and wearable sensors.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Summary points

Background

- Inflammation is a key clinical feature of many disease states and its reduction is the goal of a rapidly expanding range of biologics and small molecules.
- C-reactive protein is currently used as a single diagnostic indicator of inflammation in patients and is the primary biomarker of efficacy in anti-inflammatory drug trials.
- Here we explore a higher-resolution picture of inflammation, using multiple protein biomarkers and high-frequency sample collection to provide deeper insights into structure of inflammatory events as well as asymptomatic normal baseline.

Experimental

- A unique set of 1662 dried blood samples (DBS) was collected from 16 individuals over periods up to 9 years, including daily collection for extended periods.
- A panel of nine positive and three negative APR proteins was measured by SISCAPA-LC-MRM mass spectrometry.
- Spot-to-spot volume variation in dried blood spots was reduced substantially by normalization of a three-protein panel, providing total workflow assay CVs on replicate DBS samples of 2.5–6%.

Results

- A total of 57 discrete inflammation events were observed related to major infections, vaccination, surgery, extreme exercise and Crohn's disease.
- APR proteins were differentially regulated during these events, with unique nonsynchronous time courses and nonlinear regulatory relationships that require PD-like modeling and interpretation more advanced than currently used ratios.
- CRP, SAA and LPSBP remained correlated in the subset of samples with lowest CRP, indicating that very low level 'microinflammation' phenomena can be measured and extending the dynamic range of measurable APR to >10,000.

Discussion

- Multiplexed measurement of a broad panel of acute phase response (APR) proteins, including CRP, at frequent timepoints using DBS microsamples provides improved insights into the magnitude and dynamics of inflammation processes.
- The approach provides personal baselines for each protein, allowing improved diagnostic performance.
- Clinical trials of anti-inflammatory biologics, antibiotics and cancer therapeutics can benefit from improved multi-marker pharmacodynamic models of inflammation.

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Commentary

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Future Medicinal Chemistry

miRNA nanotherapeutics: potential and challenges in respiratory disorders

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"miRNAs are therefore considered as potential biomarkers and new therapeutic targets for respiratory diseases"

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Chronic respiratory disorders are among the most common causes of severe illness and death worldwide. Globally, approximately around 4 million people die annually prematurely due to chronic respiratory diseases [1]. miRNAs are small noncoding RNA molecules that are used to suppress protein translation in order to modulate gene expression. Alterations in miRNA abundance can be observed both in the lung tissue and in the inflammatory cells, as regulators of the disease [2,3]. miRNAs are therefore considered as potential biomarkers and new therapeutic targets for respiratory diseases namely, asthma, chronic obstructive pulmonary disease, lung cancer and fibrosis. Several miRNAs have been identified, especially in the let-7 family, miR-10, miR-26, miR-30, miR-34, miR-99, miR-122, miR-124, miR-122, miR-140, miR-145, myR-146, miR-190, miR-192, miR-219, miR-222 and miR-223 [4], which downregulate their expressions during respiratory disorders. Nevertheless, there is an emerging need to resolve numerous physicochemical and biological challenges associated with miRNAs, particularly, off-target effects, in order to obtain an effective *in vivo* delivery. This commentary, in particular, addresses the challenges associated with the use of miRNAs and the advantages of nonviral methods of delivery in respiratory diseases.

Therapeutic challenges associated with miRNA

Several biological and physicochemical factors are associated that minimize the delivery of miRNA to the target cells. These include low plasma half-life: in addition to a lack of stability in biological systems and fluids, primarily blood, the short-lived nature of their gene silencing effects is one of the principal obstacles in the use of miRNA. These are enzymatically degraded by cellular nucleases and quickly removed through the kidneys due to their low molecular weights. Studies show that naked miRNA quickly degrades in plasma having half-life of seconds to 30 min *in vivo* [5]. Immunotoxicity: the systemic miRNA delivery, like other types of nuclear acids, stimulates the innate immune system resulting in unintended toxicity and severe adverse effects [6]. Secretion of inflammatory cytokines and type I interferons by Toll-like receptors (TLRs) result from the systemic administration of miRNA duplexes. These TLR sensor molecules (dsRNA) stimulate both the cellular endosomal and lysosomal interferon type I pathways, and subsequently, induce the development of cytokine in terms of its structure, sequence and deliveries. TLRs bound by miRNA may also be neurotoxic [7]. Off-target effects: previously, miRNA therapeutics were considered highly specific in their action, but later on studies demonstrated that they are less specific than

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formerly assumed. They not only bind to complementary sequences but also to similar sequences that lead to show unwanted phenotypes, adverse side effects and sometimes completely negating the therapeutic effect of miRNA. Cell membrane barriers: the naked miRNAs are not free to disperse across the cell membrane. This is because of their comparatively larger molecular weight and polyanionic nature [8].

MiRNA inhibitors & nanotechnology: a symbiotic association

miRNA inhibitors/antagomirs (AMO) are synthetic miRNA antagonists, also known as miRNA silent agents. They are commonly found bound to the miRNA. They mask the target site, which essentially avoids interaction with the miRNA, enabling them to be translated. This approach has the benefit of annulling the likely off-target effects of a wide range of miRNAs [9]. Like AMO, miRNAs are not degraded by this method. Thus, specific functions of the miRNAs remain intact in other genes. Several reported studies have shown a greater potential for AMO such as AMO-106a and AMO-9, in asthma and hyper reactive steroidal airways in respiratory diseases [10].

Nanotechnology has made significant strides in recent years, in both developing new materials and also with their applications. These advancements have led to the development of new DNA and RNA delivery systems to monitor diseases that can be used, instead of viral vectors [11]. Inorganic varieties namely, gold, silver, calcium phosphate, graphene, quantum dots, iron oxide and silica; organic substances namely, chacosanes, fabric, protein/peptides and aptamers; polymerized nanomaterials may be used in the architectural creation of these non-viral vectors. Nanomaterial-based delivery systems have several advantages over viral vector delivery systems, which may include, having less immune response and design versatility to work in low cytotoxicity areas [12]. For example, proteins, antibodies and carbohydrates could be used for combining nanoparticles in carboxy and amino groups. The cellular absorption of miRNA is increased because of its small size and the probability of mixing cell penetration peptides [13]. Despite these potential benefits, the applications of nanobiotechnology in the respiratory area are still in its infancy and have not yet made its mark in comparison to other disorders. Few attempts which are made in the respiratory area have been discussed below.

Evidences on drug delivery approaches of miRNA in respiratory disorders

One of the recent attempts made was to deliver miR146a using polymeric nanoparticles for the treatment of chronic obstructive pulmonary disease. Results showed that miR-146a has maintained its gene and protein functional structure. The high concentration of miR-146a-nanoparticles decreased the *IRAK1* target gene expression to 40% [14]. In addition to that, there is another study which had reported that the presence of miR-660 lipid-nanoparticles decreased lung cancer tumor growth by inducing P53-dependent cell cycle arrest in low doses when compared with the controls [15]. McKiernan *et al.* prepared nanoparticles of miR-126 with cationic polymers and demonstrated their ability to promote the incorporation of miRNA into the CFBE41o cells (the human F508del transmembrane epithelial bronchial regulator for cystic fibrosis), thus significantly decreasing the expression of *TOM1* [16]. Neutral lipid delivery systems have been developed to minimize the adverse impacts and nonspecific interactions of cationic particles. In a non-small-cell lung cancer mouse model for efficacy, a neutral fat emulsion was compared and correlated with synthetic miRNA-34a and let-7 [17]. When compared with cationic lipoplexes, the systemic administration of the neutral lipid particles demonstrated lesser hepatic aggregation, and more uniformly distributed pattern in other tissues such as the lungs. Treatment with the miRNA imitation caused a decrease in the tumor size and an increase in the tissue level of miR-34a [18]. Shi *et al.* developed a codelivery system that comprised of solid lipid nanoparticles enclosing miRNA-34a and paclitaxel (PTX) for synergistic effect to target lung cancer cells. The drugs administered co-operatively and more effectively blocked the metastatic production of BF10-CD44^{+} originating in the lung. In addition this approach also decreased the doses of PTX, thereby, reducing the side effects associated with PTX alone [19]. A similar study was conducted by Song *et al.* in 2020, which also encapsulated myricetin and siRNA in mesoporous silica nanoparticles and showed its synergistic effect to suppress tumor growth in lung cancer cells with lesser side effects [20].

Conclusion

The expanded and in-depth research in understanding the roles of miRNA opens up new possibilities for pulmonary disorders. The blend of miRNA with nanotechnology paves the way to achieve enhanced cellular uptake, endosomal escape and improved bioavailability. However, there are several challenges related with the unknown structure and stoichiometry of polymers along with the accumulation of nanoparticles in normal organs. Other key issues awaiting

further exploration include effective gene silencing effect with absolute safety and the challenge to increase the delivery of the target substances to the organ of interest or even the cell of interest alone.

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Future Drug Discovery

Editorial

Drug repurposing strategies for COVID-19

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"drug repurposing will have to compete with structure-based design of preventative/therapeutic vaccines and small molecules on efficacy and off-target toxicity turfs"

COVID-19 has now been declared a pandemic and new treatments are urgently needed as we enter a phase beyond containment. Developing new drugs from scratch is a lengthy process, thus impractical to face the immediate global challenge. Drug repurposing is an emerging strategy where existing medicines, having already been tested safe in humans, are redeployed to combat difficult-to-treat diseases. While using such repurposed drugs individually may ultimately not yield a significant clinical benefit, carefully combined cocktails could be very effective, as was for HIV in the 1990s; the urgent question now being which combination.

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Keywords: broad spectrum antiviral agents (BSAAs) • COVID-19 • drug repurposing

It is no secret that COVID-19 has caught the global public health community by surprise, with the number of laboratory-confirmed human cases surpassing the 200,000 mark as of late March 2020, leading to the WHO declaring it a pandemic. While all efforts should be made toward prevention and/or containment of the 2019/2020 outbreak, it is evident that contingency measures with experimental therapeutics must be urgently sought. With the average cost of *de novo* drug development reportedly over $1 billion USD, what are viable strategies to discover potential candidate drugs to combat severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)? Here we take a snapshot look at the strategy of drug repurposing – also known as drug reprofiling or repositioning – that promises to identify antiviral agents for the novel coronavirus disease in a time-critical fashion. We also offer a perspective that antiviral combinations with a 'double hit effect' may offer the best chance of success and clinical translatability.

Repurposing of existing antivirals

Broad-spectrum antiviral agents (BSAAs) that have been deemed 'safe-in-man' through testing on early phase clinical trials have been touted as good drug repurposing candidates [1]. Andersen *et al.* have recently summarized 31 potential candidates for COVID-19 in a highly accessible database of 120 experimental, investigational and approved agents [2]. Conceptually, BSAAs take advantage of the promiscuity of viral replicative mechanisms and host interactions to target two or more viral families [3]. Following the COVID-19 outbreak in December 2019, a few existing BSAAs have been rapidly introduced into clinical trials, spanning Phases II though IV. Umifenovir is a membrane fusion inhibitor targeting viral entry and lopinavir/ritonavir is a drug combination targeting viral protease, both approved for the indications of Influenza and HIV. They are currently being considered in different combinations in a Phase IV clinical trial for pneumonia associated with COVID-19 (ClinicalTrials.gov ID: NCT04255017) [4].

At the Phase III level, remdesivir, a viral RNA-dependent RNA polymerase inhibitor, is under investigation for mild and moderate SARS-CoV-2 (ClinicalTrials.gov Identifier: NCT04252664) [5]. Remdesivir has activity in preclinical studies against the species of *coronaviridae* implicated in SARS-CoV and Middle East respiratory syndrome (MERS-CoV) [6]. Notably, it has already been studied in a randomized, controlled trial for Ebola

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virus disease, demonstrating an antiviral effect [7]. Other Phase III agents being evaluated in combination therapy for viral pneumonia interestingly include the antimalarial hydroxychloroquine, based on promising *in vitro* data (ClinicalTrials.gov Identifier: NCT04261517) [8]. Chloroquine, in addition to its immunomodulating properties, has been shown to have antiviral activity at entry and post-entry stages of the SARS-CoV-2 infection. It can enhance the antiviral activity of remdesivir and potentially serve as a synergizer of BSAAs [9].

At more early stages, the viral RNA polymerase inhibitor favipiravir in combination is also on a Phase II clinical trial for novel coronavirus-associated pneumonia (Chinese Clinical Trial Registry Identifier: ChiCTR2000029544) [10]. Finally, preclinical studies of ribavirin (ribonucleic analog) has shown *in vitro* activity against SARS-CoV-2 [9].

BSAA combination therapy

One limitation of phenotypic screens is the low potency of hit compounds as single agents, as their maximal tolerated dose is often subtherapeutic for the new indications being sought [11]. One way to circumvent this issue is to evaluate two or more drugs acting on different cellular signaling pathways involving viral replication with minimal redundancy. Another strategy is high-throughput screening of compound libraries for synergistic combinations at the host–virus interactome level for emerging and re-emerging infectious diseases, which may allow researchers to narrow down the spectrum of individual antimicrobials [3,12,13]. These strategies promise to address the often-weak activity of BSAAs by improving efficacy while potentiating dose reductions, reducing duration, cost of the drug development pipeline, lowering toxicity and minimizing emergence of secondary resistance.

Future perspective

A notion that will determine the effectiveness of this drug repurposing strategy is whether such agents will compare favorably with virus-specific vaccines or small molecules, both options traditionally considered gold standards of modern drug development. If one were to extrapolate the experience from precision oncology, where targeted cancer therapies and immunotherapeutics have made major gains against cancer, a broad-spectrum strategy will have fundamental flaws. Importantly, the lack of specificity of BSAAs may become implicated in the emergence of drug resistance and more virulent strains.

For example, the nonstructural protein nsp14-ExoN, together with its cofactor nsp10, of *coronaviridae* has been found to repair nucleotide mismatches caused by nucleoside analogs such as ribavirin, thus potentially negating the antiviral effect of BSAAs [14]. As elegantly demonstrated by Ferron *et al.*, the nsp14-ExoN of SARS-CoV forms part of a highly flexible complex with multienzymatic properties, likely facilitating compensated low fidelity of replication. What are viable strategies to circumvent this remarkable innate ability of *coronaviridae* to proofread their RNA and maintain genomic integrity, while allowing some degree of evolutionary freedom to mutate? Again high-throughput screening can come to the rescue here. Possibilities include compounds with affinity toward the nsp14-ExoN catalytic subunit or those with allosteric effects at critical sites with conserved residues causing conformational change of the entire viral RNA repair complex. Therefore, the hunt should now be on to identify an inhibitor of this viral nuclease.

Further, drug repurposing will have to compete with structure-based design of preventative/therapeutic vaccines and small molecules on efficacy and off-target toxicity turfs. The CoV spike glycoprotein used by SARS-CoV-2 at the atomic resolution and human ACE2 enzyme as its port of cellular entry have now been determined. These discoveries are hoped to spur rapid efforts to develop vaccines and antibodies. However, such processes typically take up to a decade and can be hindered by the potential for blunted antigenicity of epitopes due to genetic drift of the virus [15–17].

Another important dimension in this quest for repurposed drugs involves patent protection issues under current national and/or international regulations [18]. A global health emergency of this magnitude calls for a bold, international response at the governmental and political levels. Therefore, the regulatory community must act fast to minimize any financial hurdles implicating private industry and update guidelines for drug licensure through repurposing if necessary. It should not escape us that this is a vital behind-the-scene act while efforts are underway to seek new indications for existing compounds.

The urgently launched clinical trials worldwide on investigational medicinal products for the current COVID-19 outbreak should read out within weeks to months. We can anticipate the notion of drug repurposing for emerging viral diseases to be scrutinized based on these results. At a deeper level, this is a battle not only against COVID-19

but for the very soul concept of new antimicrobials and their clinical indications: 'one drug, one virus versus one drug, multiple viruses or multiple drugs, one virus are the contenders' [19,20].

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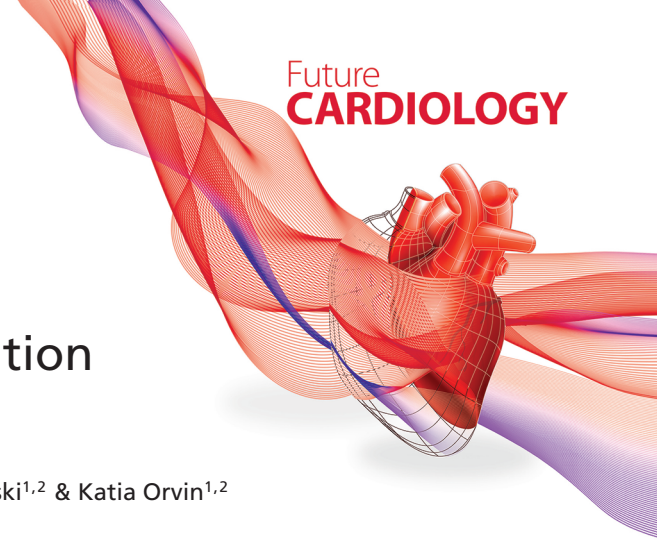

Future CARDIOLOGY

Extracorporeal membrane oxygenation therapy in the COVID-19 pandemic

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"ECMO has developed from being used as a 'rescue therapy' to become an accepted treatment option for patients with severe ARDS. Providing complex therapies such as ECMO during outbreaks of emerging infectious diseases has unique challenges."

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Keywords: COVID-19 • ECMO • extracorporeal membrane oxygenation therapy

The coronavirus disease 2019 (COVID-19) pandemic is upon us. We are learning and experiencing this viral tsunami with limited published data regarding management guidelines and patient outcomes. COVID-19 can cause severe disease requiring intensive care, extracorporeal membrane oxygenation (ECMO) could be of use in those unresponsive to conventional care. Providing complex therapies such as ECMO during outbreaks of emerging infectious diseases has unique challenges – we aim to review ECMO as a mechanical support option in COVID-19 patients with critical disease.

Severe acute respiratory syndrome coronavirus-2 is the virus that causes COVID-19 disease. The spectrum of disease severity is wide, ranging from asymptomatic patients to those in whom the most serious manifestations of the disease are pneumonia, acute respiratory distress syndrome (ARDS) and an acute cardiomyopathy. Those that develop severe disease are critically ill, requiring intensive care management and often mechanical ventilation. Despite these measures, initial case series have reported a high mortality rate in those with severe disease [1]. ECMO has developed from being used as a 'rescue therapy' to become an accepted treatment option for patients with severe ARDS. Providing complex therapies such as ECMO during outbreaks of emerging infectious diseases has unique challenges. We aim to review ECMO as a mechanical support option in COVID-19 patients with critical disease.

The rational for ECMO

ECMO is a form of mechanical cardiopulmonary support that can provide prolonged extracorporeal life support in patients who are unresponsive to conventional management. There are two forms of ECMO: veno-venous (VV) typically used in cases of severe respiratory failure, and veno-arterial (VA) or veno-venous-arterial (VVA) for patients with cardiogenic shock.

In VV ECMO, blood is extracted from the vena cava or right atrium and returned to the right atrium. This form of ECMO provides respiratory support, but not hemodynamic support, and is used for patients with acute respiratory failure. By replacing the gas exchange function of the lungs, ECMO facilitates protective mechanical ventilation as oxygenation and carbon dioxide clearance are provided by the extracorporeal circuit. This decreases the magnitude of ventilator-induced lung injury secondary to volutrauma, barotrauma and oxygen toxicity.

VA ECMO or VVA ECMO provides both respiratory and hemodynamic support and is used in patients with cardiogenic shock or cardiac arrest. In VA ECMO, blood is extracted from the right atrium and returned to the arterial system, thus bypassing the heart and lungs.

ECMO therapy is associated with potentially severe side effects. These include bleeding and vascular complications, an increased risk of nosocomial infections and poor neurological outcomes. VA ECMO, in particular,

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provides hemodynamic support but does not reduce the left ventricular afterload and can exacerbated pulmonary congestion.

Some patients exhibit a systemic inflammatory response syndrome due to the exposure of the patient's blood to the nonendothelialized surface of the ECMO circuit. A potential concern is that the systemic inflammatory response syndrome from ECMO can be detrimental to the recovery process of patients with COVID-19 as lymphopenia and raised IL-6 have been reported to be poor prognostic indicators in these patients. It has been suggested that monitoring the lymphocyte count and IL-6 as prognostic markers could be useful in those with COVID-19 who are treated with ECMO [2].

ECMO therapy is used as rescue therapy in the most critical patients. Therefore, there is a selection bias in that it is used in patients who, from the outset, have significant morbidity and mortality. Patient selection for ECMO should be judicious to optimize patient outcome.

VV ECMO in ARDS

Reassuring results of the CESAR trial (conventional ventilatory support vs extracorporeal membrane oxygenation for severe adult respiratory failure) [3] and the successful use of ECMO for severe ARDS cases during the influenza A (H1N1) pandemic in 2009 [4] have led to an exponential use of VV ECMO for acute respiratory failure in the last decade. ECMO provides full blood oxygenation and carbon dioxide elimination and allows 'lung rest' by enabling low-volume, low-pressure ventilation [5]. It is now considered as a reasonable therapeutic option to support patients with severe acute lung injury refractory to conventional measures. This approach is supported by the Berlin consensus document on ARDS [6].

VA-ECMO in cardiogenic shock

Short-term mechanical circulatory assist devices such as VA ECMO can be used as a bridging therapy to recovery in those with cardiogenic shock by providing both respiratory and cardiac support, improving end-organ perfusion, reducing filling volumes and augmenting coronary perfusion. However, the outcomes of those on VA-ECMO for refractory cardiogenic shock are poor with an overall reported survival rate of about 40% [7,8] but considerably better prognosis in case of fulminant myocarditis (65–71%) [9]. Prediction tools such as the Survival After Veno-arterial ECMO score have been developed to assist in predicting which patients with cardiogenic shock may have a higher chance of survival with ECMO therapy [10].

Use of ECMO in previous outbreaks of viral respiratory syndromes

The Extracorporeal Life Support Organisation Registry is an ongoing registry on patients who have been on ECMO support. From these data, 926 adult patients with viral pneumonia of any cause showed an overall survival of 65% with an average duration on ECMO use of 13.5 days [9].

During the 2009 H1N1 influenza A pandemic, almost a third of patients admitted to intensive care units (ICUs) were supported by ECMO therapy [11,12]. Various case series reported a trend toward a decrease in mortality in those who received ECMO therapy [11–13]. An Italian cohort reported a 68% survival to hospital discharge in 61 patients receiving ECMO during the H1N1 influenza A pandemic [11].

Avian influenza A (H7N9) is a viral pneumonia that manifests with varying degrees of dyspnea with most patients developing ARDS and with a subsequent high mortality rate of about 30% [14]. In a review of 35 patients in which ECMO was used for ARDS in patients with Avian influenza A, the ventilator parameters of patients with ECMO, including Fi02 and tidal volume were significantly decreased and physiological parameters, such as pH, were significantly improved. The in-hospital mortality in this cohort was 63% [15].

The Middle East respiratory syndrome (MERS), caused by a coronavirus (MERS-CoV), is characterized by respiratory failure and is associated with a high mortality rate of over 35% [16]. This disease was primarily seen in the Middle East with its' peak in 2015. In a retrospective analysis on 35 MERS-CoV patients in ICUs with refractory respiratory failure, those who were treated with ECMO had significantly lower in-hospital mortality (65 vs 100%; $p = 0.02$), compared with those who received conventional therapy. This was also associated with a longer ICU stay (25 vs 8 days; $p = 0.001$). Of the 21 patients who died, five (14%) died due to uncontrolled hemorrhage and nine (26%) of septic shock [17]. These highlight the potentially fatal complications of ECMO therapy.

Clinical reports of COVID-19 & the use of ECMO

Reports have recently been published describing the clinical characteristics of this severe disease. A recent report from Wuhan, China, published in *The Lancet* reported that in this cohort six patients (11.5% of COVID-19 cases) in the ICU received ECMO of which only one patient survived [1]. In another report from Wuhan, China, published in *JAMA*, of 36 patients with COVID-19 admitted to the ICU, four patients (11.1%) were treated with ECMO [18]. In a letter in *JAMA*, describing the characteristics and outcomes of 21 critically ill patients with COVID-19 in Washington State, USA, an acute cardiomyopathy was reported in seven patients (33% of the cohort) [19]. It is still unknown if this is due to an acute viral myocarditis or due to overwhelming illness.

The data so far report high rates of potentially fatal complication of COVID-19. However, the clinical courses and outcomes of these patients have not yet been reported and the benefit of ECMO cannot yet be determined.

Current guidelines in COVID-19 pandemic

The Society of Critical Care Medicine has released tentative guidelines for the management of COVID-19 patients. In these guidelines, they recommend VV ECMO in the management of ARDS in those in whom refractory hypoxemia persist despite optimized ventilation, the use of rescue therapies (such as the use of inhaled pulmonary vasodilators) and proning.

They provide an algorithm to help guide decision-making with the following criteria promoting a recommendation for early ECMO usage:

paO_2:Fi02 <80 mmHg for >6 h
paO_2:Fi02 <50 mmHg for >3 h
pH <7.25 with $PaCO_2$>/60 mmHg for >6 h

This is currently a weak recommendation with a low-quality evidence [20].

However, this comes with some important caveats. ECMO is a resource-intensive therapy. In the current pandemic and wave of new COVID-19 cases, ECMO should be considered only in those with a good prognosis; those without significant co-morbidities, less than 7 days on mechanical ventilation, and should not be considered in the critically ill elderly due to poor outcomes regardless of ECMO support. Furthermore, ECMO should only be used in resource-stable centers and in settings where personal protective equipment for the staff is not limited.

Moving forward & ongoing research

In the wake of COVID-19, the Extracorporeal Membrane Oxygenation for 2019 novel Coronavirus Acute Respiratory Disease study is being conducted. Hopefully, this extremely important registry will give us further insights into the role of ECMO in this disease.

The Lancet has published a document regarding planning for ECMO preparedness and provision for the COVID-19 pandemic [14]. The high transmission rate of the severe acute respiratory syndrome coronavirus-2 and the ARDS-related mortality are sweeping the world. The number of patients who may need this specialized support is currently unknown. Also unknown is the ability of national healthcare systems to provide this specialized therapy during this resource-straining pandemic.

In conclusion, ECMO is a resource-intensive form of life support that can be used as a rescue therapy in critical patients. ECMO could be of use in COVID-19 patients with severe ARDS or cardiomyopathy in which conventional therapy has failed. The number of patients who might require this level of support is currently unknown and further data are needed.

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Future VIROLOGY

Editorial

Is regulation preventing the development of therapeutics that may prevent future coronavirus pandemics?

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> "Given the massive amount of morbidity and mortality associated with EIDs over the past 30 years, the balance between public health risk and inaction is clear."

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In the last century, hundreds of new emerging infectious diseases (EIDs) have arisen in human populations most of which originate from wild animals as zoonoses [1]. The recent surge of zoonotic EIDs in human populations is driven by a constellation of socioeconomic factors including human population growth, eroding public health infrastructures, changes in land use and agriculture and ease of global travel. HIV, Ebola virus, avian influenza (H5N1, H7N9, etc.), severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) are but a few recent examples of highly virulent, zoonotic viral EIDs that have catastrophically affected global economies and public health [2]. Geopolitical flux since the 1990s and the potential for weaponizing EIDs provoked the creation of myriad policies aimed at protecting the USA from bioterrorist threats and the accidental release of potential pandemic pathogens from laboratories. Are these policies effective? Are they impacting countermeasure development for current and future EIDs? How are they shaping the direction of individual research programs, the recruitment of new investigators and the stability of impacted fields? Below, we discuss our experiences in developing therapeutics against SARS-, MERS- and zoonotic CoV in an ever changing regulatory environment.

Gain of function (GOF) research is critical for microbiological research in determining causal relationships between a mutation and its phenotype. In 2012, two studies describing GOF mutations facilitating the transmission of highly pathogenic avian influenza in ferrets set off a firestorm of debate ultimately resulting in a 'pause' of active GOF research and its future funding [3]. While influenza research was primarily targeted, GOF policies were also extended to studies that could enhance the pathogenicity and/or transmissibility of SARS- and MERS-CoV in mammals, requiring regulatory approval of each experiment by NIH or its equivalent. Since the types of mammals were not specified and the assessment of mutant virus in humans or all the other approximately 5400 mammals was obviously not possible, the GOF policies by nature of their vagueness could never be adequately satisfied. During the 'pause,' a risk and benefit analysis conducted by the National Science Advisory Board for Biosecurity (NSABB) helped guide the creation of 'Recommended Policy Guidance for Departmental Development of Review Mechanisms for Potential Pandemic Pathogen Care and Oversight (P3CO)' released in January 2017 [4,5]. Almost a year later, US Department of Health and Human Services (HHS) lifted the pause and released policy intended to guide funding decisions of new grant applications involving potential pandemic pathogens that have been 'enhanced' for pathogenicity or transmissibility [6]. While the policies for new grant applications are now clear and in place, the triage process of amendments to currently funded grant applications remains unknown, as oftentimes a new recombinant must be made to address an evolving research question.

For CoV researchers, regulatory complexity increased in 2012 when SARS-CoV was designated as a 'select agent' by the HHS and the Centers for Disease Control and Prevention (CDC) [7]. The Federal Select Agent Program (FSAP) requires those working with pathogens or toxins that pose a severe threat to public health to register and meet certain safety and security standards. Many of the 66 pathogens and toxins accompanying SARS-CoV on the select agent list are infamous including Ebola virus, smallpox and anthrax. As such, the FSAP has many positive

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consequences including the registration of pathogens/toxins with the government, increased oversight on biosafety and biosecurity, increased standardization of high containment facilities and procedures, and research funding prioritization. For SARS-CoV, both the virus and its RNA genome became select agents as the genome itself is 'infectious' and can produce live virus if injected into mammalian cells under highly defined conditions. But what is SARS-CoV? Is it the epidemic strain that spread across the globe? Is it a bat virus that is 99.9% similar to SARS-CoV at the amino acid level, yet has never circulated among humans? What about chimeric viruses containing pieces of SARS-CoV? These questions illuminate the complexities in defining the FSAP policy, as biology is not binary but an ever-evolving continuum. For FSAP compliance, many US institutions spent tens to hundreds of thousands of dollars renovating BSL3 facilities. In addition to BSL3 renovations at UNC, an FSAP compliant BSL2 laboratory was created to process and analyze (i.e., qRT-PCR, sequencing, microarray, RNA-seq, etc.) samples containing SARS-CoV RNA since they could no longer be processed by core facilities or contracted out. Upkeep of select agent paperwork and annual inventory requires periodic review by a team of UNC Environmental Health and Safety Officers and their work is supported by 5–10% effort of each BSL3 worker and 35% effort of a facility manager. Initially, the CDC estimated the cost for FSAP compliance would be less than US$20,000/lab, but this is a gross under-estimate especially when personnel time is included. New policies and paperwork also evolve like the FSAP's policy for "validated inactivation procedures." All told, select agent work is serious business and the University's commitment to retaining a SARS-CoV research program has not been without sacrifice, approaching US$500,000 in facility renovation/maintenance costs over the years. Thus, quantitative data detailing the financial impacts of these policies and their effect on the current and future research programs are desperately needed, as the true impact is unknown and likely under-estimated.

The CoV family has a proclivity for emergence. This paradigm was established with the emergence of SARS-CoV in 2002 and was solidified with the identification of MERS-CoV in 2012 [8]. While epidemic SARS-CoV is no longer a threat, similar 'prepandemic' viruses (i.e., Bat CoV WIV1 and SHC014) have been found in bats in China that can readily infect human cells without adaptation and are thus poised for human emergence [9,10]. To maximize the benefit of therapeutics targeting virus families prone to emerge, they should be broadly active against both human and zoonotic viruses, which will likely seed future EID. To this end, we have constructed recombinant virus from infectious cDNA clones for multiple human and zoonotic CoV [9,11–15]. Mutation and manipulation of the cDNA clone is essential to identify determinants of pathogenesis and to gauge zoonotic virus pandemic potential. Unfortunately, the study of zoonotic and human CoV outside of their natural host often times requires genetic manipulation and GOF to be useful. For example, the epidemic strain of SARS-CoV, SARS Urbani, replicates to low levels in laboratory mice and does not cause clinical disease. Since mouse models mimicking human disease more powerfully test therapeutic benefit than those with virus replication only, SARS Urbani was passaged in mice producing a mouse-adapted strain (SARS-MA) with pathogenesis paralleling human disease [16]. SARS-MA, used across the globe, has been an invaluable tool for the rigorous assessment of therapeutics and also helped critically identify inactivated vaccine associated immune pathology leading to a refocusing of vaccine efforts into safer technologies [17]. Similarly and prior to the 'pause', we reconstructed SARS-like (i.e., Bat CoV HKU3) and MERS-like (Bat CoV HKU5) bat CoV that could replicate in mammalian cell lines but could not propagate suggesting receptor and virus spike glycoprotein incompatibilities [15,18]. To facilitate sustained replication in cell culture and mice, portions of the Bat CoV HKU3 and HKU5 spike glycoproteins were exchanged with those of SARS-CoV. As defined by the 2014 GOF policy, the creation of SARS MA15, HKU3 and HKU5 adapted viruses would have been prohibited, seriously eroding the development of key systems for therapeutic evaluation. In fact, the 'pause' halted all US efforts to adapt MERS-CoV in small animals. This moratorium was lifted months later for a few laboratories leading to the development of several animal models that replicate many aspects of human disease. Given that there are no US FDA approved therapies for any CoV, these mouse models have been essential for the preclinical development of multiple MERS-CoV therapeutics.

We recently reported a nucleoside analog (GS-5734) capable of preventing SARS-CoV disease in mice and could inhibit replication of multiple CoV in cell culture [19]. Interestingly, GS-5734 is also efficacious against Ebola virus and respiratory syncytial virus [20]. Phase I clinical trial for GS-5734 has been completed and a feasibility assessment for conducting a Phase II trial for MERS-CoV is underway. Importantly, we determined GS-5734 had broad-spectrum efficacy against the CoV family through challenge with our diverse panel of human and zoonotic bat CoV discussed above. This analysis would not have been possible without passage of CoV to increase pathogenicity, the shifting of zoonotic CoV host range to infect human cells, and the reconstruction of 'prepandemic' SARS-like bat CoV poised for emergence (i.e., WIV1, SCH014). Since several branches of the CoV family tree have not

yet been evaluated with GS-5734, additional zoonotic CoV must be constructed, which will likely require genetic manipulation, adaptation or a host range shift to be tractable for use in the laboratory. The generation of genetically diverse panels of emerging virus is absolutely essential to rigorously evaluate therapeutics and proactively gauge their efficacy against pre-epidemic zoonotic viruses. Given the massive amount of morbidity and mortality associated with EIDs over the past 30 years, the balance between public health risk and inaction is clear. For EIDs, the myopic 'one bug, one drug' approach will forever be regressive as the therapy specifically designed for the past epidemic strain (e.g., SARS-CoV vaccine) will likely fail against future emergence (i.e., MERS-CoV). Therefore, we must change the paradigm to 'one drug, many bugs' and prospectively develop broadly active countermeasures within and across virus families. Regulation must adequately protect public safety but should also be fluid enough to evolve in step with EIDs. Despite the best intentions, one of the greatest risks of biosafety policy is unintended over-reach that limits our understanding of EID biology and prevents countermeasure development that could limit or prevent future pandemics.

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Editorial

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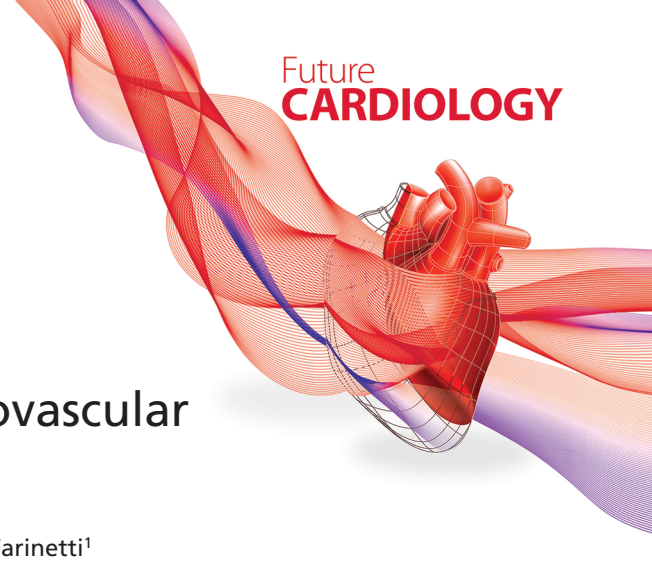

COVID-19 outbreak: impact of the quarantine-induced stress on cardiovascular disease risk burden

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"emotional eating and reduction of physical activity lead to obesity and metabolic syndrome; both risk factors have a pivotal role in cardiovascular risk. Obesity is also associated with an increased risk of Type 2 diabetes"

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Keywords: COVID-19 • lifestyle • physical activity • quarantine • stress

The novel Coronavirus, CoV-19/SARS-CoV-2 is causing a global pandemic with a rising number of deaths [1,2]. Although pandemic preparation plans have been developed, little attention has been paid to the cardiovascular burden of such an event [2]. Cardiovascular risk factors are strongly influenced by quarantine, an effective measure that minimizes the impact of infectious disease outbreaks [3]. These restrictions will strongly influence lifestyles leading to an increased burden of cardiovascular disease [4].

Previous research has revealed a profound and wide range of psychosocial impacts on people at the individual, community and international levels during outbreaks of infection [5]. Psychological distress is an important side effect of quarantine [6]. Mass quarantine, self-quarantine and isolation are associated with depression, anger, and chronic stress. During this period, additional stress is caused by longer quarantine duration, frustration, boredom, inadequate supplies, inadequate information, financial loss and stigma. In addition, during outbreak, people are fearful of falling sick or dying themselves. These negative feelings are associated with systemic inflammation and endothelial dysfunction as well as tendency to adopt an unhealthy lifestyle [7]. Both acute and sub-acute stress activate the adrenergic system and increase the inflammatory response and endothelial dysfunction, leading to an increase in atherosclerotic plaques [5,8,9]. Some types of stress preceded a substantial percentage of myocardial infarctions. This was reported after the occurrence of earthquakes [10]. The reason has been associated with changes in neurohormonal, hemodynamic and coagulation systems that cause rupture of a vulnerable atherosclerotic plaque, platelet activation and coronary vasoconstriction. Events such as pandemics may also prompt the emergence of collective stress among the population.

The sympathetic system activation influences the cardiovascular system in several ways: increase in heart rate and ventricular contractility, increase in blood pressure, increase in systemic and coronary resistance, promote thrombus formation and increase in the risk of arrhythmias. The hypothalamic–pituitary–adrenocortical axis releases plasmatic cortisol that increases blood pressure and plasma glucose levels. Moreover, cortisol alters the platelet function and the systemic inflammatory response. CRH produced by the hypothalamic-pituitary-adrenocortical axis increases the inflammatory response, macrophage activation, adhesion of monocyte to endothelial cell and endothelin-1 release.

Previous studies found that mental stress induced paradoxical vasoconstriction at the level of coronary artery stenosis and the degree of vasoconstriction is correlated with the degree of atherosclerosis [11]. In addition acute or chronic stress negatively influences nutritional behaviors such as alcohol consumption, smoking and diet [12,13]. Some individuals respond to stress by eating more and selecting foods high in sugar and fat [12,13]. This emotional

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eating may contribute to excess energy intake and weight gain [12–14]. Torres and Nowson identified that people cope with stress by eating and drinking in an attempt to feel better ('stress-related eating') [14]. These stress driven eaters and drinkers were more likely to consume unhealthy foods and alcohol. Moreover, the lack of emotional support from friends and relatives increases stress driven eating and drinking behaviors [14].

Quarantine is associated with a diet poor in fresh fruit and vegetables; however it is known that higher vegetable intake is correlated with lower anxiety and fear severity. A previous study found that higher nonrefined grain consumption is significantly related to lower depression and anxiety compared with controls and these relationships persisted after adjustment for other food groups [15]. The Mediterranean diet is a healthy diet rich in vegetables, fruits and nonrefined grains. It is interesting that higher consumption of nonrefined grains, vegetables, fruits, potatoes, fish and olive oil were inversely related to depression or anxiety severity, while higher consumption of poultry and high fat dairy products was positively associated with greater anxiety symptoms. During the quarantine, changes in diet contribute to increasing the stress and depression associated with isolation. In addition, emotional eating and reduction of physical activity lead to obesity and metabolic syndrome; both risk factors have a pivotal role in cardiovascular risk. Obesity is also associated with an increased risk of Type 2 diabetes [8,9].

Technologies could be useful; a high number of health and nutrition applications are available on Google Play and the Apple app store. Apps may help the control of diet and to maintain healthy weight. The social support improves the use of these tools for adopting a healthy lifestyle. Physical and relaxing activities could be useful in reducing stress during quarantine. However, the limitations imposed by government restrictions on outdoor activities affects the vast majority of physical activities. The main consequence of quarantine has been the reduction of physical activity. Physical activity motivation is strongly related to social aspects, such as indoor gym groups and team competitions. With these activities restricted during quarantine conditions, it is expected that physical activity will be reduced. In order to reduce the negative effects of quarantine on health the WHO has released guidance to "*Stay physically active during self-quarantine*" [16]. These indications are intended for people in self-isolation without any symptoms or diagnosis of acute respiratory illness and contain practical advice on how to stay active and reduce sedentary behavior while at home. To acheive these objectives, new technologies and internet solutions could be useful; online exercise classes and video- or app-guided aerobics training at home can be a simple and economic tool for performing physical activity. Therefore, to maintian the healthy lifestyle habits and active behavior at home is very important for the health of the overall population but, especially, for subjects with cardiovascular and metabolic risk factors and for older people [8]. There are several options for exercising and training at home; aerobic exercises like walking inside the house and dancing can be easily done. Resistance training can be carried out by going up and down a step or the home stairs, sitting and getting up from a chair and transporting items with light and moderate weights. Exercise and physical activity play a pivotal role in prevention of cardiovascular disease [17]. Limited physical activity, sedentary behavior and sitting time are associated with increased risk of cardiovascular disease and with several metabolic and mental effects that could also contribute to increase the cardiovascular risk [8,9,17]. There are several mechanisms through which exercise training reduces chronic inflammation, including improvement of endothelial function and the capacity to regenerate endothelium after injury [9].

WHO suggest that meditation and deep breaths can help to remain calm and reduce stress. A review analyzed the relationship between yogic practice and decline in anxiety and stress and concluded that scientific studies do not report significant reduction. However, due to the self-reported beneficial outcomes for the use of yoga as an intervention for stress and anxiety, yoga may be considered as a possible adjunctive therapy for those experiencing stress during quarantine. The word yoga, meaning 'union', is a mind-body-spirit practice that can include meditation, breath awareness, asanas or postures and relaxation. It is thought to alter nervous system regulation, physiology, psychological well-being and physical fitness. Due to the difficulties in performing exercise in the right way, the practice could be useful in people familiar with this technique [18].

Conclusion

There is a strong relationship between the cardiovascular system and SARS-CoV-2. The virus could affect the cardiovascular system by several mechanisms: direct myocardial injury, systemic inflammation, altered myocardial demand-supply ratio, plaque rupture and coronary thrombosis, adverse effects of various therapies (i.e., prolonged QT interval) and electrolyte imbalances [19]. Patients with previous cardiovascular disease appear to be more inclined to develop COVID-19 and have more severe clinical disease with a worse outcome [19]. Previous cardiovascular disease is associated with a threefold greater risk of severe COVID-19 disease requiring, in many cases, admission to intensive care units. Similarly, several cardiovascular risk factors (i.e., diabetes and hypertension) adversely affect

prognosis of these patients. We cannot exclude that quarantine stress could lead to Takotsubo Syndrome due to hyper-activation of the sympathetic nervous system. A case of myocarditis, as a possible late phenomenon of the COVID-19 respiratory infection, has recently been reported from a group in Brescia hospital [20]. Quarantine for containing the COVID-19 outbreaks affects cardiovascular risk factors, leading to an increase of cardiovascular risk burden.

Author contributions

AV Mattioli, M Nasi, C Cocchi & A Farinetti conceived of the idea at the basis of the article, AV Mattioli, M Nasi developed the different parts of the manuscript, AV Mattioli, M Nasi, C Cocchi & A Farinetti performed the final supervision. All authors contributed to and approved the final manuscript.

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Editorial

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What the oncologist needs to know about COVID-19 infection in cancer patients

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> “In this paper, we provide the oncologists with the available evidence concerning COVID-19 in cancer patients to better guide management decisions.”

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Keywords: chemotherapy • coronavirus • COVID-19 • immune checkpoint inhibitors • oncologist • oncology • review

The outbreak of Coronavirus Disease 2019 (COVID-19) started back in December 2019 with a cluster of pneumonia cases in Wuhan, a city within the central part of China, and has rapidly evolved into a global pandemic [1]. The putative pathogen is a novel coronavirus that presents a close resemblance to a known bat coronavirus termed BatCoV RaTG13, thus favoring a bat-to-human transmission hypothesis before eventually identifying human-to-human transmission [2,3]. It presents a high transmission rate with one new infected case producing an average of 2.9 new secondary cases and a fatality rate of 2.3% [4,5]. As a result, thousands of severe cases have died every day worldwide due to the pressure on the healthcare system and lack of specific treatments. Not surprisingly, cancer patients, compared with the general population, are regarded as a highly vulnerable group because of their immunosuppressive state due to malignancy, chemotherapy and comorbidities. Thus, oncologists are obliged to reconsider anticancer treatments while taking into consideration the risk of complication and cancer progression [6]. An updated WHO report demonstrates a mortality of 7.6% among patients with cancer [7]. Subsequently, several societies have issued conservative guidelines inviting oncologists to consider, on a case-by-case basis, the possibility of delaying treatment administration [8–12]. In this paper, we provide the oncologists with the available evidence concerning COVID-19 in cancer patients to better guide management decisions.

COVID-19 infections in cancer patients

As China was the first epicenter for the pandemic, the Chinese epidemiology data constitute the bulk of the published literature reporting on COVID-19 infections. The early case series including a total of 300 COVID-19 patients identified 2 cancer patients only [4,13]. Later case series by Liang *et al.* (18 cases), Zhang *et al.* (28 cases) and Zhang *et al.* (67 cases) reported a higher prevalence of cancer patients with COVID-19 infections compared with the overall population (1 vs 0.29%) [14], a higher median age at diagnosis (63–66 vs 49 years) [14–16] and a male predominance (61%) [15,16]. Lung cancers (22–25%), gastrointestinal cancers (14–16%) and breast cancers (11%) were the most commonly encountered tumors [15,16]. The clinical features included fever (80–82%), dry cough (75–81%) and dyspnea (50–66%) [15,16]. Dyspnea was more frequently noted at admission in severe cases (56.3 vs 11.4%) and in nonsurvivors (66.7 vs 20.4%) whereas the other symptoms were similar between mild and severe cases [16]. Laboratory tests showed hypoproteinemia (89%), lymphopenia (82%), increased level of CRP (82%) and anemia (75%) [15]. In comparison with patients without cancer, cancer patients had a higher risk of adverse events (39 vs 8%; $p = 0.0003$) and deteriorated more rapidly (13 vs 43 days, HR = 3.56; 95% CI: 1.65–7.69) [14]. Severe events were reported in 48–54% of cases (versus 16% in the overall population), notably among patients receiving anticancer treatment within the previous 2 weeks (OR = 4.079; 95% CI: 1.086–15.322) [14–16]. Compared with the mild illness group, patients in the severe illness group were older (69 vs 64 years; $p < 0.001$) and had more comorbidities (72 vs 37%; $p = 0.004$) [16]. Serious complications included acute respiratory distress

syndrome (20.9 vs 3.4% in the overall population), heart failure (16.4%) and acute renal injury (3 vs 0.5% in the overall population) [16,17]. Empirical antibiotics, antiviral agents, glucocorticoids and intravenous immunoglobulins were administered in 82, 71–85, 45 and 20–26%, respectively [15,16]. Oxygen therapy, noninvasive ventilation and invasive mechanical intubation were required in 73, 30 and 12–36%, respectively [14–16]. Cancer patients had a higher case-fatality rate (5.6–29 vs 1% in the overall population) [14–16]. The median duration to recovery and death was 31 and 20 days, respectively [16].

Diagnosis of COVID-19 infections

The diagnosis of COVID-19 seems obvious but is not straightforward in clinical practice. Patients may be very symptomatic at presentation showing fever and respiratory symptoms, which are very commonly encountered in daily practice. The COVID-19 diagnosis adds to a long list of differential diagnoses including bacterial, fungal or other viral infections. Patients may also present with very subtle symptoms that may not be clinically relevant. For example, the earliest reports from Wuhan described two patients presenting ground-glass opacities in their lungs, a characteristic radiological finding in COVID-19 patients, who had undergone lobectomies to remove early-stage lung cancers but ended up having a COVID-19 infection. Both patients eventually became severely ill, and one of them died of respiratory failure [18]. COVID-19 also adds to the etiologies of pneumonitis following cytotoxic chemotherapies, immune checkpoint inhibitors and radiotherapy. In such instances, steroids are the mainstay of any treatment plan however its use during COVID-19 infection is controversial as it slows the elimination of the virus. The confirmation of a COVID-19 infection is currently largely based on reverse-transcriptase polymerase chaine reaction (RT-PCR). This technique requires a deep nasopharyngeal swab sampling and is available broadly. However, RT-PCR testing seems to present low accuracy especially in places that perform large numbers of tests. In one case series of 1014 patients, 75% of patients with negative RT-PCR had positive chest computed tomography findings of COVID-19 infections (48% highly likely cases and 33% probable cases) and were attributed to faulty design of some PCR kits and inadequate sampling [19].

Anticancer treatment during COVID-19 infections

Most patients with cancer were recommended to withdraw or delay cancer treatment during the pandemic as almost 30% of cancer patients' infection was suspected to be hospital-associated transmission [15]. However, the risks of cancer progression make this issue controversial. In contrast to chemotherapy which is immunosuppressive, immune checkpoint inhibitors may be a safer option as one case series of cancer patients with COVID-19 infection did not report any case receiving immunotherapy [14]. Thus, patients may be less prone to severe infections but are at a theoretical risk of a cytokine release syndrome that would exacerbate a COVID-19 infection [20–22]. The biologic findings including lymphopenia, neutrophilia, elevated D-dimer and LDH very frequently encountered in cancer patients seem to increase the risk of severe COVID-19 infections [23]. A case report of a patient with EGFR (L858R, T790M) mutant metastatic lung adenocarcinoma and diagnosed with COVID-19 infection maintained his daily osimertinib concomitantly with broad-spectrum antibiotics and antiviral treatment with lopinavir plus ritonavir uneventfully [24]. Concerning clinical trials inclusions, the US FDA and the EMA have issued special guidance for the conduction of clinical trials during the COVID-19 pandemic [25,26].

Cancer patients with suspected or confirmed COVID-19 should be discussed with an infectious disease specialist. Based on the data suggesting patients with cancer are at high risk of respiratory complications related to COVID-19 infection, many societies favor delaying treatments on a case-by-case basis [8–12]. The treatment of COVID-19 has been a matter of controversy with one single-arm trial showing the potential efficacy of the azithromycin-hydroxychloroquine combination. Unfortunately, this study had major methodology issues and was not adopted by the medical society [27]. In the absence of solid evidence for effective antiviral therapy, the research activity has never been this active. The number of ongoing trials registered increased from 84 trials on 24 March (at the conception of the paper) to 306 on 4 April 2020 (at the time of submission). Several therapies varying from classical antiviral drugs such as lopinavir-ritonavir (NCT04330690 and NCT04307693 currently recruiting, NCT04321993 active but not yet recruiting) and remdesivir to unconventional treatments such as chloroquine and hydroxychloroquine (NCT04328272 and NCT04307693 currently recruiting, NCT04321993 active but not yet recruiting) are undergoing evaluation in randomized clinical trials. The role of immune therapies is also being explored in patients with severe infections including, tocilizumab an anticytokine therapy which binds IL-6 receptors (NCT04317092 currently recruiting), hyperimmune plasma (NCT04321421 active but not yet recruiting). The

eagerly awaited study is the Phase III trial (DisCoVeRy, NCT04315948) randomizing 3100 patients to remdesivir, lopinavir-ritonavir, IFNβ-1A, hydroxychloroquine and standard of care.

Conclusion & perspective

At present, there is a global pandemic of COVID-19 that has infected more than 1 million cases and killed more than 60,000 cases [28]. In comparison with the overall population, cancer patients are at a higher risk of severe events in 48–54% of cases (vs 16% in the overall population) and death in 5.6–29% (vs 3.4% in the overall population on 3 March 2020 vs 2% in the overall population on 10 February 2020) [28]. The current evidence remains insufficient to explain a conclusive association between cancer and COVID-19. The majority of the position papers and guidelines were based on the epidemiology data of Liang *et al.* published on 1 March 2020 [8–12,14]. However, 12 of the 18 cancer patients reported by Liang *et al.* were older than the general population, had no active cancer and were long-term cancer survivors [14]. The other case series do not circumvent this issue as Zhang *et al.* reported a concomitant chronic disease in 64% of cancer patients and higher fatality rate among patients in the active treatment phase in comparison with those at the follow-up phase (39 vs 21%) [16]. The relatively small sample size, limited clinical information and heterogeneity of the disease course between patients limit robust conclusions. At last, the higher rate of cancer patients with COVID-19 could be biased and related to the closer medical follow-up of these patients and the higher mortality to delayed hospitalization while coping with the rapid influx of severe cases. Several questions remain unanswered notably the risks of waiting for the COVID-19 epidemic to subside before treating cancer patients or the risks of exposure to this virus during admission for cancer treatment. This risk should be particularly assessed in patients that may be cured by oncologic treatments. Moreover, the risk of patients receiving hormonal therapy, immune checkpoint inhibitors and targeted therapies should be assessed. Today, abiding by the old *primum non nocere* concept, clinicians may have to balance the risks of developing a COVID-19 infection against the risks of tumor progression, while taking into consideration the prevailing state of the healthcare system.

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Editorial

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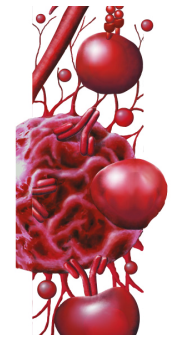
Immunotherapy



Do checkpoint inhibitors compromise the cancer patients' immunity and increase the vulnerability to COVID-19 infection?

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"Since we are not able to consider ICIs treatment as highly immunosuppressive, avoiding it in cancer patients to reduce coronavirus infections could deprive these patients from a highly active class of drugs."

The severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) has been declared a pandemic by the WHO that claimed the lives of thousands of people within a few months. Cancer patients represent a vulnerable population due to the acquired immunodeficiency associated with anti-cancer therapy. Immune checkpoint inhibitors have largely impacted the prognosis of a multitude of malignancies with significant improvement in survival outcomes and a different, tolerable toxicity profile. In this paper, we assess the safety of ICI administration in cancer patients during the coronavirus pandemic in order to guide the usage of these highly efficacious agents.

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Keywords: cancer • checkpoints inhibitors • coronavirus • COVID-19 • immune therapy • immunosuppression

Since it was first reported in the Wuhan region in China, the severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) has been declared a pandemic by the WHO with severe social and economic implications [1]. Until 25 March 2020, COVID-19 has affected more than 400,000 people around the globe and has claimed the lives of more than 18,000 persons [2]. While China, the origin site of the coronavirus spread, has succeeded in reducing the number of infected people, other countries have failed to halt the transmission of the virus with the struggle of their healthcare systems [3]. Generally, the clinical presentation of the COVID-19 infected patients is commonly encountered in cancer patients on a daily basis with a high incidence of fever, fatigue, dry cough and dyspnea while most common laboratory abnormalities include lymphopenia, prolonged prothrombin time and increased lactate dehydrogenase (LDH). So far, no vaccine or therapeutic strategy is yet available with the initial management of these patients involving optimal supportive care and oxygen supplementation for their respiratory failure symptoms [1].

Worldwide, cancer remains a heavy burden with more than 18 million cases diagnosed in 2018 according to the Globocan reports while the prevalence of cancer is largely beyond 43 million people [4]. Cancer patients might be more susceptible to a higher risk of infections and the COVID-19-related complications due to systemic immunodeficiency, mainly due to the effects of anticancer therapy [5–7]. Growing evidence supports the fact that avoiding immune destruction or immunoevasion is a new aspect of the hallmarks of cancer [8]. This conclusion is based on the theoretical idea that cells are constantly monitored by an active immune system that is responsible for the recognition and elimination of cancer cells and nascent tumors [8]. It is also thought that the chronic inflammation associated with cancer can help the development of an immunosuppressive tumor microenvironment, thus helping the tumor escape from immune surveillance [9]. However, not all cancer patients should be considered equally immunocompromised but particularly patients on active chemotherapy or those with hematological tumors such as leukemia and lymphomas.

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COVID-19 & cancer

In a report from China on 72,314 Covid-19-positive patients, the crude-fatality rate (CFR) was 2.3% among infected patients with higher mortality rates among those aged 70 years and older. Among cancer patients, the CFR was 5.6% [10]. On the other hand, the CFR in the Italian population was higher reaching 7.2%, which was attributed to the older age distribution and the different and selective testing strategy, mainly for symptomatic patients. In a small sample of Italian patients, 20.3% had an active cancer as a common comorbidity [11]. From another report from the Chinese population, 18 out of 1590 COVID-19 cases had history of active cancer. In these patients, there was a higher proportion of serious events occurrences (defined as the percentage of intensive care admissions with invasive ventilation or death) in comparison to those without cancer while older age represented the only risk factor for serious events [7]. Several strategies have been proposed by the authors to closely monitor cancer patients, including postponing chemotherapy or surgery in stable patients and a closer surveillance strategy in the elderly patients and those with multiple comorbidities [7]. However, these data should be cautiously interpreted due to relatively small sample size. Also, history of smoking should be considered as a predisposing factor in these patients with increased susceptibility to infection with COVID-19 since tobacco increases the expression of angiotensin-converting enzyme 2, a binding receptor for the SARS-Cov-2 [12]. Additionally, cancer is associated with an overexpression of immunosuppressive cytokines, reduced proinflammatory danger signals and enhanced functional immunosuppressive leukocyte population, which may induce a blunted immune system and increase the possibility of infectious complications [13].

COVID-19 & immune checkpoint inhibitors

Role of ICIs in cancer

On 17 March 2020, the National Health Service (NHS) published a report on the management of cancer patients during the coronavirus pandemic in which a large proportion of cancer patients were considered to be at utmost risk of infection by COVID-19. These patients were those receiving active chemotherapy or radiation therapy, those with hematological cancers or bone marrow transplants, as well as those receiving targeted therapy (protein kinase or PARP inhibitors) or immune therapy [14]. Interestingly, patients treated with immune checkpoint inhibitors (ICIs) were considered highly vulnerable if they were to be tested positive for the coronavirus infection. Since their introduction into the therapeutic arsenal against cancer, ICIs have revolutionized the treatment sequencing in the cancer management [15]. These monoclonal antibodies targeting immune checkpoints (PD1 and PD-L1), by their role in restoring the antitumor immunity through the reversal of immune escape or evasion, have led to a significant antitumor activity with confirmed impact on the duration of tumor response and prolonged overall survival. They have earned fast approvals across multiple cancer subtypes including melanoma, lung cancer, urological tumors, breast cancer and other solid tumors [16–20]. More importantly, these agents have earned several indications as monotherapy without association to chemotherapeutic agents, thus predisposing cancer patients to a different and more tolerable toxicity profile [16,17,19,21]. These agents have led to the occurrence of a new spectrum of immune-related adverse events (irAEs) ranging from moderate to severe and life-threatening ones. These side effects include a wide variety of adverse events such as endocrine abnormalities (thyroid dysfunction, adrenal insufficiency and hypophysitis), gastrointestinal events (colitis, hepatitis), and dermatological or respiratory events (interstitial pneumonitis), which merit a high index of suspicion with clear therapeutic strategies in order to prevent irreversible outcomes [22].

Immunosuppression & ICIs

During the pandemic phase of the coronavirus there should be careful monitoring of cancer patients undergoing active therapy to prevent irreversible and life-threatening outcomes. In fact, cancer patients receiving cytotoxic chemotherapy are at high risk of developing infectious complications, mainly due to their impact on the myeloproliferative cells in the bone marrow but also on the rapidly dividing cells including the gut mucosa cells leading to the disruption of the protective barrier [23]. Therefore, patients receiving active chemotherapy will often develop neutropenia and up to 5–30% of patients might develop febrile neutropenia [23]. Also, patients undergoing hematopoietic stem cell transplantation or receiving chemotherapy for hematologic cancers are at the highest risk of developing prolonged neutropenia or febrile neutropenia episodes [23–25]. On the other hand, data on the hematological irAEs secondary to ICIs seems to be more comforting. Their occurrence is uncommon with different forms of adverse events including autoimmune thrombocytopenia and neutropenia, antibody-mediated hemolytic anemia and thrombotic thrombocytopenic purpura [22]. These adverse events highlight the crosstalk between the

humoral and cellular immunity and the Treg-mediated self-tolerance [22]. In fact, ICIs might play a role in boosting pathogen-specific immune response in contrast to other immune checkpoints agonists such as Abatacept [26]. Their impact on the immune system may be beneficial in countering the immunosuppressive microenvironment and does not cause immunosuppression by itself [26]. Few cases have reported on severe ICI-related neutropenia, mostly secondary to anti-CTLA4 agent Ipilimumab but the overall rate of grade 3–5 rate of neutropenia remains low around 0.94% (overall occurrence around 0.3–1.07%) while the incidence of febrile neutropenia was 0.54% [27–31]. A small series by Finkel *et al.* analyzed the characteristics of 32 patients with immune-related neutropenia (irN). In these patients, the median time to onset of irN after the introduction of ICIs was 60 days with an incidence of febrile neutropenia of 50%. IR neutropenia resolved in 84% of cases after a combination of therapeutic agents including oral or intravenous steroids, granulocyte colony-stimulating factors and intravenous immunoglobulin [32]. That said, the incidence of severe neutropenia secondary to ICIs is very rare and can be controlled with an adapted therapeutic strategy if it occurs. Moreover, the instauration of prolonged immunosuppressive agents such as corticosteroids in a subset of patients with IrAEs might predispose to higher risk of infection and should also be closely monitored during the pandemic phase [33]. Few cases of infections secondary to the treatment of ICI-related side effects have been reported, since up to 10 and 50% of patients treated with ICI as monotherapy or combination therapy might develop irAEs [26,34]. There are also very few nonsolid data in the literature describing viral infections or reactivations as a complication to ICIs usage regardless of the development of irAEs such as varicella zoster infection, JC virus, hepatitis B, cytomegalovirus or Epstein–Barr virus [34,35].

Conclusion

With the increasing risk of the COVID-19 pandemic, the management and selection of anti-cancer therapy in oncology patients including ICIs must be carefully balanced on a case-by-case scenario with the potential increase in the risk of complications or death from the coronavirus infection. Data on the immunosuppressive impact of ICIs in cancer patients are inconclusive but they seem to be more tolerable and reassuring than the heavy burden of hematological toxicities associated with other chemotherapeutic agents. Since we are not able to consider ICIs treatment as highly immunosuppressive, avoiding it in cancer patients to reduce coronavirus infections could deprive these patients from a highly active class of drugs. A special consideration should be given to patients treated for irAEs who are exposed to prolonged duration of immunosuppressive agents. While the world is preoccupied by the fight against coronavirus, adapted strategies and recommendations by the cancer community should be implemented to optimize the management of oncology patients. This could be achieved by a careful selection of the most efficacious anti-tumor weaponry with the lower risk of weaning the patients' immune system against a potential infection by the COVID-19. Real-world data from the COVID-19 affected areas are eagerly needed to assess the outcomes of oncology patients in order to enhance their therapeutic strategies.

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Commentary

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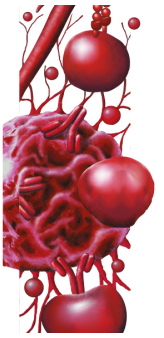

Controversies about COVID-19 and anticancer treatment with immune checkpoint inhibitors

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"Since ICI can restore the immune-competence, if on one hand it can be paradoxically needed to develop the cytokine storm characterizing the acute respiratory distress syndrome (ARDS) phase, on the other hand the epidemiological features of SARS-CoV-2 infection lay for a lower probability to affect these patients compared with their chemo-treated immune-suppressed counterpart."

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Corona virus disease-19 pandemic & cancer patients

On 11 March, the WHO formally declared the corona virus disease-19 (COVID-19) outbreak a pandemic [1]. After the first cluster of cases emerged from Wuhan, in China, at the end of 2019, up today almost 287000 cases of infections from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have been diagnosed across all five continents in the last few months [2,3].

COVID-19 morbidity and mortality have been linked to elderly age and comorbidities, leading to a poorer outcome to the viral infection for frail patients and more often resulting in hospitalization, intensive care unit admittance and need for invasive tracheal intubation [4]. Among such individuals, cancer patients represent a large subgroup at high risk of developing coronavirus infection and its severe complications. A recent nationwide analysis in China demonstrated that, of 1590 COVID-19 cases from 575 hospitals, 18 had a history of cancer (1 vs 0.29% of cancer incidence in the overall Chinese population, respectively), with lung cancer as the most frequent diagnosis [5]. Patients with cancer were observed to have a higher risk of severe events compared with patients without cancer (39 vs 8%; p = 0.0003). Moreover, cancer patients who underwent recent chemotherapy or surgery had a higher risk of clinically severe events than did those not receiving treatment. With the limit of a small sample size, the authors concluded that patients with cancer might have a higher risk of COVID-19, and poorer outcomes, than individuals without cancer. As a consequence, they recommended to consider an intentional postponing of adjuvant chemotherapy or elective surgery for stable cancer in endemic areas [5].

Nevertheless, as subsequently highlighted by other authors, the true incidence of COVID-19 in patients with cancer would be more informative in assessing whether such patients have an increased risk (and morbidity) from this viral illness [6]. Furthermore, the limited cancer patient population described in this first report from the literature, was curiously characterized by the lack of individuals receiving anticancer immunotherapy. Indeed, only chemotherapy and surgery were cited among treatments received by patients in the month prior to developing COVID-19. Maybe, this could simply be due to the casualty of a small sample, or otherwise, it could suggest that cancer patients receiving immunotherapy are less prone to develop COVID-19 or to be admitted in hospital due to severe coronavirus symptoms. Currently, we are aware of the probably higher incidence of misdiagnosed coronavirus infections compared with that reported and updated every day; it is likely that a great portion of healthy and young population develop COVID-19 with mild symptoms, not requiring hospital admittance and thus escaping the laboratory confirmation of the disease [7]. Cancer patients undergoing treatment with anti-PD-1/PD-L1 or anti-

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CTLA-4 immune checkpoint inhibitors (ICI) currently used in everyday practice to treat solid tumors such as melanoma, lung cancer, renal carcinoma, urothelial cancers and head and neck carcinoma constitute a growing oncological population [8]. Their specific susceptibility to bacterial or viral infections has not been investigated. Considering that immunotherapy with ICI is able to restore the cellular immunocompetence, as we previously suggested in the context of influenza infection, the patient undergoing immune checkpoint blockade could be more immunocompetent than cancer patients undergoing chemotherapy [9,10].

Potential interference between COVID-19 pathogenesis & immune checkpoint blockade

In the recent weeks, in the countries heavily interested by the COVID-19 outbreak, such as Italy, the scientific associations recommended the prudential postponing of active cancer treatments, especially for stable patients not needing urgent interventions [11]. On one hand, this recommendation could be reasonable for advanced cancer patients receiving chemotherapy, with the risk of hematological toxicity and of worsening an immunosuppressed status, thus favoring COVID-19 morbidity [5]. On the other hand, some oncologists are even currently wondering about the risk of administering ICI in the middle of the COVID-19 outbreak, essentially due to two major concerns.

The first seems to be represented by the potential overlap between the coronavirus-related interstitial pneumonia and the possible pneumological toxicity from anti-PD-1/PD-L1 agents. Even if lung toxicity is not the most frequent adverse event of ICI, it can be life threatening. The overall incidence rate of ICI-related pneumonitis ranges from 2.5–5% with anti-PD-1/PD-L1 monotherapy to 7–10% with anti-CTLA-4/anti-PD-1 combination therapy [12]. The dominant radiological pattern of lung immune-related adverse events (irAEs) is organizing pneumonia, but ICI-related pneumonitis could exhibit a variety of patterns, also including nonspecific interstitial pneumonitis [13]. Despite being rarer than other irAEs, pneumonitis is the most fatal AE associated with PD-1/PD-L1 inhibitor therapy, accounting for 35% of treatment-related toxic deaths [14]. Considering that underlying lung disease, particularly including interstitial pneumopathy, is considered a risk factor for ICI-related pneumonitis, it could be reasonable taking into account the risk of treating patients while they are developing an initial form of COVID-19. The synergy between the two lung injuries, despite only hypothetical, cannot be surely ruled out. Nevertheless, such an epidemiological coincidence should not prevent the oncologist from offering a potentially effective and often well-tolerated treatment even in the middle of the COVID-19 outbreak, since the duration of the pandemic is still currently unpredictable. This is true in particular considering the potentially curative aim of ICI treatment in the context of highly responsive diseases, such as melanoma and renal cell carcinoma and in the adjuvant setting even more than in the advanced disease.

The second concern seems to be represented by a possible negative interference of ICI in the pathogenesis of COVID-19. Cytokine-release syndrome (CRS) is a phenomenon of immune hyperactivation typically described in the setting of T cell-engaging immunotherapy, including CAR-T cell therapy but also anti-PD-1 agents [15]. CRS is characterized by elevated levels of IL-6, IFN- γ and other cytokines, provoking consequences and symptoms related to immune activation, ranging from fever, malaise and myalgias to severe organ toxicity, lung failure and death. In parallel, one of the most important mechanism underlying the deterioration of disease in COVID-19 is represented by the cytokine storm, leading to acute respiratory distress syndrome or even multiple organ failure [16]. The cytometric analyses of COVID-19 patients showed reduced counts of peripheral CD4 and CD8 T cells, while their status was hyperactivated. In addition, an increased concentration of highly proinflammatory CCR6+ Th17 in CD4 T cells has been reported, and CD8 T cells were found to harbor high concentrations of cytotoxic granules, suggesting that overactivation of T cells tends to contribute to the severe immune injury of the disease [17]. Moreover, the pathological findings associated with acute respiratory distress syndrome in COVID-19 showed abundant interstitial mononuclear inflammatory infiltrate in the lungs, dominated by lymphocytes, once again implying that the immune hyperactivation mechanisms are at least partially accountable for COVID-19 severity [17]. Considering these aspects, the hypothesis of a synergy between ICI mechanisms and COVID-19 pathogenesis, both contributing to a counter-producing immune hyperactivation, cannot be excluded.

In spite of this fascinating rationale, we should remember that ICI-induced CRS is a quite rare phenomenon as well as that the cytokine storm is not an early event in the COVID-19 pathogenesis, indeed characterizing the late phase of its most severe manifestation, occurring in a minority of patients. It is not likely that cancer patients are still receiving ICI during this phase of the viral illness. Obviously, in the current pandemic scenario, careful attention should be dedicated in delaying treatment for those patients presenting flu-like symptoms at the time of the intended ICI treatment.

Therapeutic implications: tocilizumab & the risk of hasty conclusions

Since its first outbreak in China, COVID-19 was empirically treated with antiviral therapy, first employing agents already used in prior severe acute respiratory syndrome epidemics [18]. Then, several randomized clinical trials were initiated in China and more recently in Italy, investigating different treatment options, varying from classical antiviral drugs as lopinavir/ritonavir, to newer antiviral as remdesivir, to unconventional agents such as chloroquine and hydroxychloroquine [19]. The latest treatment frontier against COVID-19 seems to be represented by a recombinant humanized monoclonal antibody, named tocilizumab, which binds the human IL-6 receptor, inhibiting its signal transduction [20]. Tocilizumab is currently used for rheumatoid arthritis, but its efficacy has been demonstrated also against ICI-induced irAEs, starting from the rationale of an ICI-induced systemic inflammatory response syndrome similar to CRS [21]. Moreover, along with the improvement in symptoms related to systemic inflammatory response syndrome, some authors reported a clinical improvement in other irAEs with tocilizumab used in cancer patients with immune-related toxicity from anti-PD-1 agents [21,22].

With these premises, the risk of hasty conclusions is around the corner. In fact, one can argue that the alleged tocilizumab efficacy both for treating COVID-19 and irAEs might suggest a potentially increased danger from SARS-CoV-2 infection for ICI-treated patients, maybe hypothesizing a synergy in the promotion of the viral morbidity. Nevertheless, this is probably a thoughtless deduction.

First, it can be a matter of time. The time at which the COVID-19 patient develops the pathologic hyperactivation of the immune response, eventually contributing to the final injury, is probably in the late phase of the disease manifestation, occurring together with the respiratory distress [17]. Furthermore, the time matters also in the case of ICI therapy, since the majority of patients develop irAEs within the first 6 months from the first administration [12]. Thus, a certain caution for ICI administration during the pandemics could be applied mostly for those patients needing therapy initiation or in their first months of treatment.

Second, it is probably a matter of patient. Patients more prone to developing immune hyperactivation are probably those more likely to respond to ICI [23]. There is a possibility that such patients would be also more prone to fall in the cytokine storm in the case of SARS-CoV-2 infection. Nevertheless, these patients do not correspond to the average advanced cancer patient, who is supposed to be immunosuppressed, with a blunted immune status [6]. The epidemiology of the COVID-19 observed up today suggests that SARS-CoV-2 tends to infect more frequently the frail patient populations, such as the elderly and cancer patients [4,5]. Cancer is usually associated with overexpression of immunosuppressive cytokines, suppression of proinflammatory danger signals, impaired dendritic cell maturation, and enhanced immunosuppressive leukocyte populations [6]. Since ICI can restore the immune-competence, if on one hand it can be paradoxically needed to develop the cytokine storm characterizing the acute respiratory distress syndrome (ARDS) phase, on the other hand the epidemiological features of SARS-CoV-2 infection lay for a lower probability to affect these patients compared with their chemo-treated immune-suppressed counterpart.

Third, the efficacy of tocilizumab for COVID-19 is still under investigation, with still unexplored backstage and with uncomfortable upstream evidence coming from the setting of influenza infection. Despite clinical studies associating IL-6 with high disease severity in influenza-infected patients and its levels correlated directly with symptom occurrence in human influenza virus infection, the role of this cytokine is still ambiguous [24]. It was demonstrated in mice models that IL-6 is essential for preventing virus-induced neutrophil cell death and H1N1-associated mortality, limiting influenza-induced cytokine storm and protecting against fatal lung pathology [25]. Furthermore, IL-6 is crucial in secondary infections to recall virus-specific memory CD4 T cells, favoring virus clearance and host survival, as supported by the inability of IL-6 deficient mice to control influenza viral titers in the lung [25]. Such preclinical evidence suggests that, despite probably being harmful in the ARDS phase, IL-6 role could be crucial, in the early phase of the viral infection, to defuse the pathogenesis of severe and lethal forms of influenza. Thus, hoping for positive results from tocilizumab randomized clinical trial on COVID-19 patients, we could only argue about the evident diversity of this viral infection from previous SARS outbreaks and even more from influenza epidemics, probably both in terms of clinical features and of pathogenetic implications.

Conclusion

Clinical decisions about cancer patients deserving immunotherapy in the current context of the COVID-19 pandemic should be characterized by separated reflections, avoiding generalizations and remembering their deeply different immunological status compared with that of cancer patients undergoing chemotherapy or targeted agents. In the end, beyond any charming scientific speculations, it is unfortunately likely that in this COVID-19 pandemic,

the greatest risk for cancer patients is the unavailability of the usually high-level medical services, since all our hospital resources, in terms of structures, tools and healthcare professionals, are currently strongly dedicated to the outbreak management.

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Commentary

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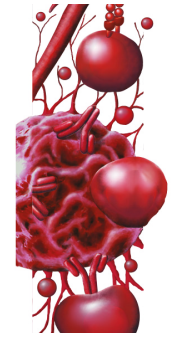

COVID-19: the use of immunotherapy in metastatic lung cancer

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"Significant gains have been made in lung cancer morbidity and mortality since the introduction of PD-1 therapy. These benefits still exist in the ongoing COVID-19 pandemic, however, ongoing treatment may place a subset of patients at increased risk"

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Lung cancer remains among the most lethal malignancies with a high mortality rate, which is in part due to the metastatic nature at the time of diagnosis. Untreated, the prognosis of metastatic non-small-cell lung cancer (NSCLC) is poor with a median overall survival of 7 months [1]. The introduction of drugs that target the axis between programmed death-1 (PD-1) and its ligand (PD-L1) have rapidly changed the treatment and prognosis of NSCLC over the past decade. Use of immunotherapy as monotherapy for the second-line setting [2] or in the first-line setting for PD-L1-high tumors [3] has resulted in significant improvements in overall survival. Newer first-line protocols looking at combination immunotherapy with chemotherapy have seen benefits across the board, including both squamous and nonsquamous histology, EGFR mutant and ALK rearrangements [4–6]. The emergence of the novel coronavirus severe acute respiratory syndrome ([SARS]-CoV-2 or COVID-19) pandemic poses significant challenges for the treatment of all cancer patients, but in particular lung cancer patients where an increase in mortality has already been reported [7,8]. Here, we re-evaluate the risks, benefits and delivery of immunotherapy for NSCLC patients during the COVID-19 pandemic.

The clinical manifestations and severity of COVID-19 are broad but typically manifest as fever, cough, dyspnoea and myalgia [9]. Initial reports suggest that 81% of cases are mild and the remainder are classified as either severe or critical [10]. In this subset of patients, cough and fever can be present for approximately 7–10 days prior to the development of acute respiratory distress syndrome (ARDS), acute cardiac injury and acute kidney injury [11]. Patients with severe COVID-19 admitted to the intensive care unit were more likely to have proinflammatory cytokines such as IFN-γ, IP-10, MCP-1, IL-1β, IL-4 and IL-10 [9]. Initial pathological examination of ARDS in COVID-19 implies overactivation of T cells which may account for the severe immune mediated injury seen in patients [12]. While myocardial dysfunction and renal impairment are found in severe COVID-19, the cause and its relation to excessive inflammation is less clear. Currently, no vaccine or validated disease-modifying agents are available, however, treatment with high-dose glucocorticoids and medications that inhibit IL-6 such as tocilizumab have been reported [13]. No benefit of immunosuppression has yet been empirically demonstrated.

The immune-related adverse events (irAEs) of anti-PD-1 or anti-PD-L1 agents are typically inflammation caused by the immune system directed against organ-specific targets. While the exact pathophysiology of irAEs is not known, patients receiving anti-PD-1 or PD-L1 therapy can develop complications not limited to pneumonitis, myocarditis, nephritis, hepatitis, colitis, thyroiditis, hypophysitis, dermatitis, arthritis and encephalitis. No clear effect of microbial coinfection has been demonstrated in the type or frequency of irAEs. Specifically, there was no increased incidence of hepatitis in patients with chronic viral hepatitis nor increased risk of immune reconstitution in patients with HIV infection receiving treatment with immunotherapy [14,15]. However, there have been cases of PD-1 therapy causing a reactivation of latent tuberculosis through an increase in the immune response [16]. Case reports of fatal PD-1 induced encephalitis or myocarditis found Epstein Barr Virus positive lymphocytes in the

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affected histological region, suggesting some role of the infection in this idiosyncratic irAE [17]. Ultimately, the interaction between the immune system and microbes is complex and an area of emerging research. The impact of COVID-19 and whether it has any impact of irAE in lung cancer irAE will be an area of ongoing interest.

While PD-1 therapy was not in use during recent viral outbreaks, we can look at their impact on lung cancer patients to gain insight into the specific challenges that COVID-19 will bring. A total of 79 NSCLC were prospectively followed during the SARS outbreak in 2003 [18]. While there was considerable concern and anxiety regarding contracting SARS, there was minimal delay or interruption to treatment. The Middle East Respiratory Syndrome outbreak in 2015 demonstrated a mortality rate of 84% in cancer patients, which was twice as high when compared with nononcology patients [19]. Lung cancer patients comprised of 15.8% of these patients. The H1N1 influenza pandemic had considerably lower mortality but affected more people than the two previously mentioned coronavirus outbreaks. Hospital admission data during the H1N1 outbreak suggests that cancer within the last 12 months was one of the highest risk factors for death. During the H1N1 outbreak in 2009, hospital mortality for oncology patients admitted with H1N1 was up to 18.5% in some studies [20]. While the H1N1 outbreak was global and prolonged, it has not had the same societal impact as the COVID-19 outbreak, nor does it appear to be as lethal.

While the data for the impact of COVID-19 are limited and emerging, there are early indications that suggest significant impact on the oncology patient population, in particular, lung cancer. A retrospective case study across three hospitals in Wuhan identified 28 cancer patients suffering from COVID-19 [7]. Lung cancer was the most common cancer type in that group (25%) with one patient receiving immunotherapy. Lung cancer patients were more likely to have earlier symptoms of dyspnoea, to develop anoxia and more rapid progression of COVID-19 symptoms. Anticancer therapy administered within 14 days of presentation were more likely to have severe clinical outcomes such as admission to ICU, need for mechanical ventilation or death. A prospective cohort of laboratory confirmed COVID-19 cases in China identified 18 patients with a history of cancer [8]. Lung cancer was the most common cancer type (28%) with most patients being older (mean age 63.1 years) and in routine follow-up following treatment from cancer (75%). Patients with cancer were at higher risk of severe events compared with noncancer patients (39 vs 8%) and recent anticancer treatment was an independent risk factor for severe events. While evidence is limited currently, emerging reports suggest higher risk of adverse outcomes in cancer patients who received recent anticancer therapy. It is difficult to confirm that lung cancer patients were at higher risk based on this limited data without knowing the specific lung cancer incidence rate for this region of China. Nethertheless for lung cancer patients for whom treatment may be continued for up to 2 years, this may represent a significant risk.

Another challenge for lung cancer patients receiving immunotherapy and the current COVID-19 pandemic is a diagnostic one. The current WHO definition of a confirmed case of COVID-19 is based on laboratory confirmation typically via PCR. There is an evolving role for computed tomography imaging in the diagnosis of COVID-19, with advantages including rapid results and the ability to reflect the severity of ARDS [21]. There is a broad range of radiological features found in patients infected with COVID-19. Similarly, PD-1 inhibitor-induced pneumonitis can present with a broad range of radiological findings [22]. Most commonly, this presents as cryptogenic organizing pneumonia but in its more severe grade, is consistent with ARDS. Given the diagnostic overlap radiologically and the common clinical characteristics of cough and hypoxia, this can present a diagnostic challenge to the physician. The differential diagnosis of PD-1-induced pneumonitis may complicate the management of patients receiving PD-1 therapy and suspected of being infected with COVID-19.

Models of care associated with the delivery of immunotherapy in lung cancer will also have to be reconsidered. Currently, patients continue on immunotherapy for 2 years or longer in some instances should they continue to derive benefit. This can represent a significant period of time during which patients are attending clinic appointments, visiting pathology, radiology diagnostic centers and spending time in infusion centers. With no vaccine or disease modifying intervention currently available for COVID-19, the only strategy to reduce mortality associated with the outbreak is social distancing [23]. For patients stable on flat dosed immunotherapy, home-based treatment in selected patients may be utilized more frequently to minimize social interaction. Telehealth will undoubtedly replace a large proportion of face-to-face consultation and treatment protocols involving less chair time will likely be preferentially used.

The duration of treatment should also be considered when reducing direct patient interaction with the healthcare system. The duration of treatment for patients who are responding to immunotherapy has been a point of contention in lung cancer immunotherapy. Depending on the drug used and the line of therapy, some clinical trials have opted

for 2 years of drug treatment or until progression. The Checkmate 153 study randomized patients who had completed 1 year of nivolumab to either treatment discontinuation with the option to resume nivolumab at time of progression and continuing nivolumab until progression. Importantly, this study reported superior progression free survival in patients who continued nivolumab [24]. 2 years of therapy has been standard in pembrolizumab-containing protocols. In patients who continued to respond after 2 years of therapy with pembrolizumab in the Keynote-10 trial, 75 of 79 patients had ongoing response [25]. With concerns about recent treatment for cancer increasing the risk of serious events with COVID-19, keeping the duration of treatment to an appropriate time would be important. Therefore, it may be reasonable to discuss stopping treatments in patients who have achieved a complete response or prolonged response for more than 2 years during this COVID-19 pandemic.

While recent cancer therapy appears to be a risk factor for serious events with COVID-19 infection, it is not clear whether the treatment modality mediates this risk. In a retrospective case series of cancer patients from three hospitals in Wuhan, six patients had received anticancer therapy in the preceding 14 days from COVID-19 diagnosis; two with cytotoxic chemotherapy, two with targeted therapy, one with radiotherapy and one with combination chemoimmunotherapy. Although it is difficult to draw a conclusion from this series due to small numbers, it is reasonable to make the assumption that cytotoxic chemotherapy would be more immunosuppressive than immunotherapy, and hence more harmful in patients with COVID19 coinfection. While there is a clear benefit of single-agent pembrolizumab versus conventional chemotherapy in the PD-L1 >50% group, there has been no comparison of single-agent immunotherapy versus chemoimmunotherapy in a clinical trial. Therefore, in the current climate, clinicians may be inclined to use protocols with single-agent immunotherapy versus combination chemoimmunotherapy, especially in patients with PD-L1 >50%. This has to be balanced with the inferior response rate seen in Keynote 024 of single-agent pembrolizumab (44%) versus combination chemoimmunotherapy (61.4% for PD-L1 >50%) in Keynote 189 [3,4].

The current end point to the COVID-19 pandemic likely involves reaching heard immunity or the mass availability of an effective vaccine. The time frame, delivery and toxicity of any potential vaccine is beyond the scope of this commentary. However, it is important to reiterate the safety of inactivated influenza vaccination for cancer patients receiving immunotherapy [26]. In the period preceding any potential COVID-19 vaccine, it is important that lung cancer patients continue to receive their yearly vaccinations and any potential exposure or hospitalization from seasonal influenza is avoided.

The impact of COVID-19 and the social distancing required to combat this pandemic has had a devastating impact on our society, healthcare and culture. We have highlighted the challenges within thoracic oncology, where significant number of patients whose life expectancy and quality of life have been vastly improved with novel anticancer therapy such as immunotherapy. Initial data from China's experience with COVID-19 highlighting that these patients are at higher risk for serious events from COVID-19 should be taken into serious consideration when making decisions regarding patient selection for therapy, duration of therapy and the decision to combine immunotherapy with cytotoxic chemotherapy. Significant gains have been made in lung cancer morbidity and mortality since the introduction of PD-1 therapy. These benefits still exist in the ongoing COVID-19 pandemic, however, ongoing treatment may place a subset of patients at increased risk. As the COVID-19 crisis impacts upon healthcare systems across the world, we hope this commentary provides some guidance to thoracic oncologists as they deal with this emerging problem.

Author contributions

SC Kao devised the project with AP Davis being primarily responsible for writing and drafting the commentary. SC Kao had ongoing supervision with SC Ka providing further conceptual ideas and refining of the commentary. JH Lee and M Boyer provided feedback and guidance to further develop the manuscript.

Financial & competing interests disclosure

M Boyer is part of the advisory board for BMS, AstraZeneca & Janssen and receives honorarium from AstraZeneca, BMS and Boehringer Ingelheim. JH Lee receives honoraria from AstraZeneca and BMS. SC Kao receives research funding from AstraZeneca and honorarium (paid to his institution) from MSD, AstraZeneca, Roche, BMS, Boehringer Ingelheim, Pfizer, Takeda, Amgen. SC Kao also has travel expenses to conferences paid by Roche, BMS, Boehringer & AstraZeneca. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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White Paper

Colorectal Cancer

Colorectal cancer care in the age of coronavirus: strategies to reduce risk and maintain benefit

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We are in unprecedented times requiring us to approach our medical obligations in unprecedented ways. Cancer care is not elective and therefore must be maintained throughout the COVID-19 pandemic [1]. However, we recognize that there is early evidence based on small studies from China [2] and Italy [3] that cancer patients are at increased risk of severe illness if they are to develop a COVID-19 infection and there is even some evidence to suggest that healthcare providers are at increased risk, possibly due to exposure to larger viral loads and repeated exposure [4,5].

Behind the scenes there are ongoing, organic collaborations among medical providers and scientists around the world working to help us endure the pandemic, but more importantly, helping us to fight back through development of treatments, prognostic and predictive markers and minimizing infections. Unpublished Data and metrics assessing the outbreak permeate across social media. New data is emerging so quickly that it is often difficult to determine our best course.

Should we continue to treat our patients using standard guidelines or should we modify our treatments to mitigate risk? In so doing, are we trading off short-term risk for ultimate worse long-term outcomes? We have already seen many instances in our own practice where we have felt forced to modify standard treatments to optimize the balance between the effective delivery of cancer care and minimize the risk to our patients and frankly to ourselves.

Many disease groups, including those representing breast cancer and melanoma, have published treatment guidelines attempting to standardize treatment modifications for the months ahead [6–8]. Individual cancer centers have developed internal guidelines for colon cancer management, but no major national group has published any recommendations to date. In partnership with the Colorectal Cancer Alliance (DC, USA) and the Otto J Ruesch Center for the cure of gastrointestinal cancers at the Georgetown University Lombardi Comprehensive Cancer Center (DC, USA), we are presenting a practical set of guidelines and recommendations for the management of colorectal cancer during the COVID-19 pandemic.

It is virtually impossible to detail every possible scenario that clinicians and patients could face over the coming months, so instead we are providing a broader outline of the basic principles we are incorporating into decision making, specific ideas of how to modify common treatment regimens and a table highlighting strategic guidance to consider when making multidisciplinary recommendations. We assume our readers will incorporate important clinical variables such as MSI-High, right-versus left-sided disease, *RAS*/*BRAF* and *HER 2* into individual patient decisions.

Basic principles:

- Avoid clinic and hospital exposure

While this may be highly variable depending on the nature of an individual healthcare practice, we know that there will continue to be an increasing number of COVID-19-positive individuals in and around hospitals. It is increasingly likely that our medical staff will become infected and, despite masks, gloves and other personal protective equipment, given the high rate of asymptomatic carriers, we want to minimize the chances of our patients becoming infected. In our way of thinking, every trip to the clinic or hospital carries some risk. We must do everything we can to reduce this controllable variable;

We are all rapidly evolving to replace face-to-face encounters with remote, 'telemedicine' visits. Although embraced positively by both our staff and our patients, we recognize that we lose an important element of the human touch and confirmatory diagnostic abilities that come from a directed physical examination. But for the vast majority of our patient encounters, this technology has proven to be an effective bridge. Indeed, it is likely that these visits will permanently replace in-person visits in some circumstances, particularly long-term follow-up appointments;

However, we have not replaced the need for in-person visits for those patients who are on intravenous therapies. Somewhat unique to colorectal cancer, we prescribe home intravenous 5-fluorouracil (5-FU) as a routine, but other agents such as oxaliplatin, irinotecan, bevacizumab, anti-EGFR agents and others, are still administered in an infusion unit by skilled nurses and supported by expert pharmacists. These agents are important to improve outcomes for our patients. Therefore, we must weigh the importance of these drugs versus the risk of infection;

- Maintain optimal clinical outcomes, especially in the curative setting.

  Our current guidelines are based on a series of clinical trials and practice patterns, which are brought together as recipes for optimized clinical outcomes. It is important to recognize that while our current guidelines do reflect the current standards, there are significant modifications that can be made, which are unlikely to result in any major significant negative impact on an individual patient;

  However, there are key moments in the treatment of colorectal cancer where we are delivering curative therapy. Certainly, in the adjuvant setting for stage II and stage III patients, chemotherapy has a significant curative impact. We would suggest that curative therapies can still be modified without compromising long-term survival. In the metastatic setting, our treatment is primarily palliative and while we cannot simply put this treatment on hold for the next few months, we can make significant modifications that are unlikely to have a major negative impact on patient outcomes;
- Reduce myelosuppression.

  In colorectal cancer, we are increasingly recommending regimens that generate regular myelosuppression: FOLFOXIRI;
- Trifluridine-tipiracil, and other regimens are associated with high rates of grade 3–4 neutropenia and anemia [9,10].

  While there is a little hard evidence that being myelosuppressed when exposed to COVID-19 will increase one's risk of infection, we feel it is prudent to avoid low counts if at all possible. Unlike influenza, secondary bacterial pneumonias do not appear to be as commonly associated with COVID-19 but are still seen in a minority of patients [11]. Lymphopenia is frequently seen in COVID-19, but this likely reflects active viral infection, more than vulnerability to becoming infected. Nevertheless, we recommend modifying regimens to reduce myelosuppression where possible, particularly in the palliative setting;
- Avoid grade 3–4 toxicity that would require emergency room (ER) visits or hospitalizations

  Many of our treatments induce nausea, vomiting, diarrhea, mucositis and febrile neutropenia, among others, which result in emergency room evaluations and often admissions. While many of these toxicities can be predicted, most are not. Careful monitoring of kidney and liver function can help predict risk. In addition, pharmacogenetic tests of germline mutations in *DPYD*, *UGT1A1* and others, can identify rare patients at risk for significant toxicities, but these are unlikely to significantly reduce unexpected adverse events and are not recommended in standard guidelines [12];

  We do know that by lowering doses we can reduce the frequency of grade 3–4 toxicities and, fortunately in colorectal cancer, there is little evidence that dose intensity carries a survival advantage. Therefore, it would be our recommendation for those patients that are being continued on more intensive regimens that dose modifications by as much as 25% be made proactively, particularly in the first few cycles, to ensure that grade 3/4 toxicities do not emerge. Moreover, we would favor adding prophylactic growth factor support in patients with borderline neutrophil counts at baseline. We would caution using growth factor in patients with confirmed COVID-19 given the risk of capillary leak syndrome;
- Planning for 2–3 months, not a few weeks.

While there is no specific agreement as to the timeline ahead, it is likely that the impact of the COVID-19 pandemic will be felt for the next several months. Therefore, as you are making plans with your individual patients, we recommend that you consider that the current situation will be in place for several months.

Modification ideas:

- Drop the bolus 5-FU.
  There is little consistent evidence that the bolus 5-FU portion of a FOLFOX or FOLFIRI regimen adds significant benefit. There is clear evidence that it adds toxicity in the form of myelosuppression, mucositis, and diarrhea [13]. There is controversy as to whether the leucovorin should be maintained if the bolus is dropped. To reduce the time in the infusion unit and possibly minimize toxicity further, we also recommend dropping the leucovorin;
- Change intravenous 5-FU to oral capecitabine.
  In virtually every clinical scenario, from adjuvant therapy to metastatic treatment to concurrent chemoradiation, capecitabine has proven to be equal or superior to iv. 5-FU [14–16]. To simplify dosing and schedules, we recommend continuous dosing of 1000–1500 mg by mouth twice daily, Monday through Friday. Other modifications of standard dosing include a 7-day on, 7-day off regimen. Capecitabine given at full doses, either alone or in combination with intravenous chemotherapy, is associated with more mucositis and diarrhea and should therefore, be avoided;
- Skip cycles of treatment.
  As outlined above, we are planning for several months of modified treatments. Therefore, skipping a single cycle is unlikely to have a major impact apart from the patient avoiding a trip to the hospital. Certain therapies such as bevacizumab when given as maintenance or pembrolizumab when given as chronic therapy due to their long half-lives could be skipped for a month or more. Delaying a treatment from every 2–3 weeks could have an impact over a few months and therefore should be considered when possible;
- Drop the iv. portion of a regimen and just maintain with capecitabine or other oral medications.
  We must remember that the addition of oxaliplatin to adjuvant therapy adds only a relatively small incremental improvement over 5-FU/LV or capecitabine [17]. Certainly, patients initiating adjuvant chemotherapy for high risk disease should be offered doublet chemotherapy, but one could justify starting with single agent oral therapy and adding oxaliplatin later, depending on the impact of the pandemic. While the impulse to delay adjuvant chemotherapy is enticing, data demonstrates that delaying initiation of adjuvant chemotherapy leads to inferior survival outcomes [18]. In the metastatic setting, maintenance therapy has been firmly established and can be initiated as soon as after 2–3 months of induction therapy. Standard maintenance therapy includes capecitabine with or without bevacizumab and, single-agent capecitabine should be considered. Treatment holidays or oral therapies could be used as a bridge to surgeries that are planned;
- Manage orals using telemedicine visits and outside labs.
  Supported with regular nursing education and remote visits, it is appropriate to manage patients on most oral regimens without in-person clinic visits. Most regimens do require regular laboratory testing and we would recommend referring patient's to neighborhood providers such as LabCorp (NC, USA) and Quest (NJ, USA) for these labs instead of visits to the clinic or hospital;
- Spread out mediport flushes to 6–8 weeks.
  Many of our patients are used to the routine of having their Mediport flushed every month. If there is no other reason for the patient to come to the hospital, we recommend extending this to 6–8 weeks;
- Short-course radiation when possible.
  In the USA, the standard approach for preoperative radiation for rectal cancer is a treatment that is delivered daily over 5–6 weeks. When appropriate, we would recommend that short-course radiation be used for neoadjuvant treatment of rectal cancer [19,20]. Newer technologies including stereotactic radiosurgery have enabled much shorter treatment schedules and we would recommend using these techniques for palliative radiation where available;
- Consider ctDNA for adjuvant decision making.
  Circulating tumor DNA technologies have burst onto the colorectal scene in the last year. Primary surgery for colorectal cancer will continue over the course of the next few months and patients will be looking to us for adjuvant therapy decisions. If available, we recommend ctDNA testing to assist in adjuvant therapy decision

Table 1. Treatment modification strategies for CRC during the COVID-19 pandemic.

Treatment setting/modality	Treatment that should be commenced if possible	Treatment should not be commenced without justification	Treatment should not be stopped without justification	Treatment can potentially be stopped or delayed after careful consideration
Adjuvant	– Rectal cancer, neo-adjuvant chemo – 5FU/Oxalipatin stage III, 4–8 weeks post op	Oxaliplatin for stage II crc, cape OK	Adjuvant 5FU/cape for stage III (month 1–3)	– Oxaliplatin in adjuvant – Adjuvant after 3 months – Routine follow-up labs and scans – Routine colonoscopy
Metastatic	Front line met CRC		Induction chemo for met resection	– Maintenance therapy – Palliative chemo – Follow-up scans if stable
Surgery	– Primary resections – Obstruction, severe bleeding	Elective liver or other met resections		– Elective liver or other met resections – Rectal resections after major neo-adjuvant response
Radiation	Palliative RT	Rectal chemo/RT	Rectal chemo/RT	– Post Op RT – Palliative RT if pain controlled

CRC: Colorectal cancer; RT: Radiotherapy.

making, by detecting minimally residual disease. Some companies providing this technology are offering in-home sample collection in support of this process. While not definitive or established, a positive tumor DNA test would compel us to initiate chemotherapy even during the pandemic. A negative test will be more difficult to apply but could justify delaying treatment or being less aggressive. Similarly, Immunoscore® quantifies immune cell infiltration in resected colorectal cancer and if a patient had a high-risk stage II colorectal cancer but with high Immunoscore, adjuvant chemotherapy has a lower likelihood of benefit and should be avoided [21];

- Delay surgeries when appropriate (may be upwards of 2–3 months).

  Over the course of the next few months, many of our patients will come up on the time when their surgery was planned as part of an overall multidisciplinary strategy. This could be surgery to remove their primary tumor, a metastasectomy or the long awaited re-anastomosis. Decisions to delay surgery must be based on a multidisciplinary discussion, but could be justified based on hospital and ICU resources and patient risk. Some rectal cancer studies indicate that a watch-and-wait approach for patients with a clinical complete response to neoadjuvant chemoradiation is safe and leads to similar outcomes compared with patients who undergo surgery; however, other studies highlight an increased rate of distant metastases in those forgoing surgery [22,23]. Regardless, a watch-and-wait approach may be appropriate in order to avoid surgery and its resulting hospitalization during the pandemic. Likewise, patients scheduled for resection of metastases could be maintained on oral chemotherapy or even treatment holiday until such time that it is safe to have surgery. These delays in surgery are unlikely to have a major negative impact on a given patient apart from the anxiety of waiting, which should not be minimized. In fact, any change in therapy which is recommended solely due to COVID-19 will cause some level of anxiety among our patients. Extra time will be required to explain the rationale for the recommendation and the establishment of a revised plan that is mutually agreed upon. During this period, more will be expected of our patients and patient selection, patient resources and caregiver support are critical to optimized outcomes.

The following table was developed not as absolute guidelines but more as a framework for thinking through an individual patient's treatment during the COVID-19 pandemic. Emphasis should continue to be placed on multidisciplinary discussions balancing the patient's individual benefit from a given therapy against potential exposures within the hospital setting and limited resources within many hospitals dealing with a surge in COVID-19 patients.

As stated, treatment for colorectal cancer is not elective and therefore cannot simply be canceled for the next few months. We must continue to support our patients so that they receive optimized treatment for their colorectal cancer, while at the same time minimizing their individual risk of infection. We hope this review will prove useful to you as you are making individual patient decisions.

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or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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