On Covid-19 for WSU Emergency Medicine Residents:
Killing the virus & Calming the storm

From Blaine White, M.D. (updated March 10, 2021)

This is to provide continuing update on adult Covid-19. My views and opinions here do not represent Wayne State University or the Detroit Medical Center. They are, however, informed by study and many years of substantial experience in both patient care and basic science research. This is continuing development of a **6th** version with newest material is VIOLET. This document provides a comprehensive disease model, examination of hypercoagulability, information on risks in the adaptive immune response, enlarged focus for "Long Haulers" and vaccines, and basic science and patient data with HCQ, cyclosporine, ivermectin, niclosamide, colchicine, aspirin, dipyridamole, anti-coagulants, famotidine, steroids, and monoclonal antibodies. I am grateful to Drs. Brian O'Neil and James Paxton for helpful thoughts, and also grateful to Drs. Scott Freeman and Jonathon Sullivan for overcoming my ineptitude with web-posting. Curiosity and constant knowledge expansion are what research is really about; we just keep adding to our files throughout our career.

I hope this continues to expand our shared understanding by putting together in one place pieces regarding (1) the molecular biology of the virus, (2) the process by which it gets into our cells, (3) our immune system and its battle with Covid-19, and (4) potential strategies to combat the disease with available drugs focused on attacks on the molecular biology of the virus and some adjustments to our immune response. There are now ~200 references at the bottom of pages for your access. In more general terms we're entering an era of being able to treat viral diseases based on the growing scientific understanding of their molecular biology, and these principles are worth our learning as Emergency Physicians.

Molecular biology of the virus

The Covid-19 virus has a single-stranded RNA genome that contains 29,891 nucleotides, encoding for 9,860 amino acids[1]. Nobody knows enough to design this, and nobody did; although it could have escaped from a lab for bat viruses. Bats carry a number of coronaviruses, and Covid-19 shares **96%** nucleotide sequence identity with a coronavirus carried by a common Asian bat - *Rhinolophus affinis*[2]. It also shares up to 80% of its sequence with the coronaviruses that were responsible last decade for the SARS (Severe Acute Respiratory Syndrome) and MERS (Middle-East-RS) diseases. Although the CoVs' genomes are the largest of the known RNA viruses, they are less than double the size of our small mitochondrial genome.

The Covid-19 genome does not contain catalytic RNA (a ribozyme) but rather is a positive-sense RNA ready for immediate translation into proteins by a host cell's ribosomes to first produce **Nsp1** (more later) nearest its 5' end[3] and then its replication-transcription-complex containing its RNA-dependent RNA polymerase (**RdRp**)[1]. That complex works by synthesizing both (1) full-

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1 Cascella M *et al.* Features, Evaluation and Treatment of Coronavirus (COVID-19). StatPearls https://www.ncbi.nlm.nih.gov/books/NBK554776/.

2 Perico L, *et al.* Should COVID-19 Concern Nephrologists? Why and to What Extent? The Emerging Impasse of Angiotensin Blockade. Nephron 2020; 23:1-9. doi: 10.1159/000507305.

3 **Remember:** DNA and RNA are formed from a backbone of sugar (ribose) – phosphate – sugar – phosphate …. The 5-carbon chain of the sugar is circularized and by convention the carbons are numbered. Except for the ends of the DNA or RNA, each phosphate is attached to a 3-carbon on one ribose and a 5-carbon on the next ribose. We describe the ends of the DNA or RNA as 5' or 3'.

length negative-sense RNA as a template for viral replication and (2) subgenomic negative-sense RNAs as templates for mRNAs for the viral proteins. The negative-sense templates with the positive-sense viral genome or mRNAs are **double-strand RNA** (dsRNA) that our cellular antiviral defenses can recognize. During translation by our ribosomes, reading frame shifts on viral mRNAs and the post-translational activity of viral-encoded proteases result in production from the viral genome of 29 proteins that include structural proteins (**S**pike, **M**embrane, **E**nvelope, and **N**ucleocapsid proteins), 16 non-structural proteins (*i.e* Nsp1 to Nsp16), and 9 accessory proteins.

For the full-length viral genomic RNA, ribosomes initially walk along the RNA in the 5' → 3' direction to synthesize the viral polyprotein by translating the RNA; in contrast, the RdRp will operate in the 3' → 5' direction. Thus after early rounds of translation there must be a molecular mechanism for clearing ribosomes off the viral genomic RNA[4] so negative-sense RNA transcripts can be produced. The viral **N**ucleocapsid protein (AKA "**N**" protein) appears to be an important actor in the switch from translation to transcription of the viral genome[4]. The negative-sense RNA transcripts are a perfect hybridization match (C to G and A to U) for the positive-sense genomic RNA, thus dsRNA. And those dsRNAs must be separated in order to use the negative-sense strands as templates for production of both the full-length positive-sense viral genome and the subgenomic viral mRNAs. That strand separation requires a viral dsRNA helicase, since we don't have dsRNA and don't require such a helicase. The reason for including this discussion is that both the **N** protein and the viral helicase are targets for approved drugs with newly appreciated antiviral activity.

Another crucial issue is interaction of viral proteins with our proteins. This has been approached with yeast-2-hybrid studies[5] using human and viral gene transcripts together with artificial intelligence modeling of these interactions[6]. Of particular importance,

1. the **S**pike protein binds strongly with cell-surface angiotensin-converting enzyme-2 (ACE2) as a receptor[5,6]; it also binds the CD4 surface protein on helper T-lymphocytes[7]. **S**pike binding to the cell surface is followed by viral infection and eventual death of the cell.
2. the **N**-protein contains amino acid sequences for both nuclear translocation and also binding poly-A on the 3'-end of mRNAs; it thus interacts with both the "importins" of the nuclear pores and all mRNAs with 3'-poly-A tails.
3. **Nsp1** interferes with ribosomal translation of cellular proteins and attracts a RNAse for selective degradation of cellular mRNAs. Nsp1 also binds strongly with several important cellular proteins including immunophilins, that have long been known to interact with the drugs tacrolimus (FK-506) and cyclosporine A.

In rodent experiments vector-directed over-expression of Nsp1 by itself inhibited immune response and caused prolonged cytokine dysregulation[5], as has been seen clinically in both SARS and Covid-19 cases. Active Nsp1 is essential for CoV replication, and counter-intuitively CoV replication is blocked through immunophilin binding by both tacrolimus and cyclosporine A[8], which are commonly used as immunosuppressants to inhibit transplant rejection.

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4 Tsai TL, *et al*. Interplay between the poly(A) tail, poly(A)-binding protein, and Coronavirus Nucleocapsid protein regulates gene expression of Coronavirus and the host cell. J Virol 2018; https://doi.org/10.1128/JVI.01162-18.

5 Pfefferle S, *et al*. The SARS-Coronavirus-host interactome: Identification of cyclophilins as target for pan-Coronavirus inhibitors. PLoS Pathog 2011; 7(10): e1002331. doi:10.1371/journal.ppat.1002331.

6 Srinivasen S, *et al*. Structural genomics of SARS-CoV-2 indicates evolutionary conserved functional regions of viral proteins. Viruses 2020, 12, 360. doi:10.3390/v12040360.

7 Davanzo G *et al*. SARS-CoV-2 Uses CD4 to infect T helper lymphocytes. MedRxiv 2020; https://doi.org/10.1101/2020.09.25.20200329.

8 Carbajo-Lozoya J, *et al*. Replication of human coronaviruses SARS-CoV, HCoV-NL63 and HCoV-229E is inhibited by the drug FK506. Virus Res. 2012; 165:112-7. doi: 10.1016/j.virusres.2012.02.002.

Other than vaccine production against the **S**pike and other Covid-19 proteins, 4 potential therapeutic targets emerge from these molecular insights. They are

1. the interaction of the **S**pike protein with ACE2 and CD4 as receptors,
2. the viral RNA polymerase (Nsp-12) and helicase,
3. the interactions of viral proteins with our macromolecules, and
4. the proteases produced by the virus.

We will see that obstructing any of these targets could stop the virus.

I note that Covid-19 is inactivated by ultraviolet light, heat, lipid solvents including soaps, 50% ethanol, chlorine-containing disinfectants, and peracetic acid, but not chlorohexidine.

The process by which Covid-19 gets into our cells

In general all pathological viruses enter our cells by a specific interaction with the cell surface. This interaction is highly conserved in families of viruses and reflects a long period of coevolution of viruses with hosts - in this case coronaviruses with mammals. So for example, influenza viruses bind to cell-surface sialic acids. Covid-19 in particular and the coronaviruses in general use their **S**pike protein to bind to ACE2 as their receptor on the surface of many cells. Hosts often make antibodies against the binding site on a viral surface. Viruses in turn mutate their binding site to escape antibodies, and this appears to have occurred with the Covid-19 **S**pike protein as compared with that of the SARS and MERS coronaviruses[6]. However, these viral mutations do not suddenly leap to bind an alternative host surface receptor; the spike proteins of SARS, MERS, and Covid-19 all dock with the cell-surface ACE2. We will see later that this is an important aspect of some approaches to therapy and passive-immunization.

ACE2 is highly expressed in the mouth and tongue, facilitating viral entry in the host[2]. It is also expressed in the gut and kidneys, which may explain some of the clinical involvement of these organs. In our lower lungs, ACE2 is expressed on type I and II alveolar epithelial cells, which normally make surfactant protecting alveoli from collapse. After lung infection, Covid-19 entry starts by binding of the **S**pike protein to ACE2 on the alveolar surface. This can stimulate fusion at the cell membrane and clathrin-dependent endocytosis of the whole Covid-19-ACE2 complex in endosomes. This is facilitated by endosomal cysteine proteases that are dependent on a low pH, and raising endosomal pH can inhibit the endocytosis of the virus. VeroE6 cells (from African green monkeys) are often used to culture and study viruses, and Covid-19 readily infects these cells. However, VeroE6 cells have lost their genes for Trans-Membrane Protease Serine -2 (TMPRSS-2). When VeroE6 cells were transformed to express TMPRSS-2 at 10-fold the level of human lung cells, their production of Covid-19 virus was increased 100-fold[9]. This indicates TMPRSS-2 proteolytic processing of the **S**pike protein enhances endocytosis, and this is important for therapeutic approaches seeking to inhibit viral endocytosis. Once inside the cells, Covid-19 exploits the endogenous translational machinery to replicate.

Molecular biology of our response as an unwilling viral host

1. Introduction and translation control

It is essential to use some of the basic science alphabet soup that follows, and in fact it is much worse than what is here. Actually, I'm careful about cytokines because they seem to multiply more rapidly than rabbits and have an unclear literature that often claims each of them is a "master

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[9] Matsuyama S *et al*. Enhanced isolation of SARS-CoV-2 by TMPRSS2-expressing cells. Proc Natl Acad Sci (USA) 2020; 117:7001-7003.

switch." Clinical studies that report 20 statistically elevated cytokines drive me crazy – who can tell what's fundamental in that? In a kinder reflection, some of this surely results from the complexities of a 3.5-billion year old biological conflict between cells and viruses. What I've tried to do here is find a central causally-connected path. Of course in choosing to ignore a myriad of finer detail, that approach could include errors. Nevertheless, we'll start with a mountaintop view.

There are two connected levels of our struggle with any virus, the **innate immune response** of local cells and the **adaptive immune response** of the whole organism's immune system. At the level of our individual cells the struggle with the virus boils down to who controls the ribosomes. The ribosome is the oldest part of our cells and reflects an RNA-world in which catalysis by RNA was central[10]. In fact ribosomal RNAs (not proteins!!) are the catalytic molecules (ribozymes) that produce the peptide bond between amino acids to generate all our proteins, and of course the mRNA-triplicate codons are what code the sequence of amino acids assembled in our proteins. The virus has to use our ribosomes to produce Nsp1 and its first replication-transcription-complex and then to translate its mRNAs for all the viral proteins. At the level of the whole organism, we have to stop that or we will surely die. The whole organism adaptive immune response is supposed both to eliminate infected cells and also to produce an immunologic memory – immunity.

To understand our antiviral host-defenses that begin at infected cells, we need to briefly review the initiation of protein synthesis by our ribosomes[11]. A ribosome has a small and large subunit, and translation initiation begins with two crucial steps involving interactions of eukaryotic-initiation-factors (eIFs) with the small ribosomal subunit before it is joined by the large subunit for active translation.

1. The eIF-4F complex delivers the mRNA to be translated to the small ribosomal subunit. Almost all our mRNAs have a 5'-GTP cap and a 3'-poly-A tail. The eIF-4F complex recognizes and binds to the 5'-GTP cap and also binds to the 3'-poly-A tail. This circularizes the mRNA, which the eIF4F complex delivers to small ribosomal subunits. This circular structure facilitates repetitive initiation by multiple ribosomes, so picture the electron microscope photos of a circularized mRNA with multiple ribosomes making nascent proteins as they walk around the mRNA. This is the normal mechanism for delivering mRNA to the ribosomes, but the eIF-4F complex can be inactivated by proteolysis (as by the polio virus or during brain ischemia[11]), and special non-cap-dependent mRNAs with "internal ribosome entry sites" (IRES) can directly associate with the small ribosomal subunit for translation. None of the Covid-19 mRNAs use IRES. The reason for telling you about this is that some viruses produce mRNAs without the 5'-GTP cap or the 3'-poly-A tail, and our cellular defenses can recognize that. But coronavirus genomic RNA itself has both the cap and tail (so ready for immediate efficient translation). Furthermore, all other mRNAs subsequently made from the coronavirus also have both the 5'-cap and 3'-tail, although unlike cellular mRNAs, production of coronavirus mRNAs involves dsRNA intermediates.
2. The eIF2 complex delivers the first amino acid of the new protein - always Methionine. This event is also subject to regulation, and phosphorylation of serine-51 on the α-subunit of eIF2 produces an inhibitor [eIF2(αP)] of the methionine delivery process[11]. This can produce a nearly complete shutdown of protein

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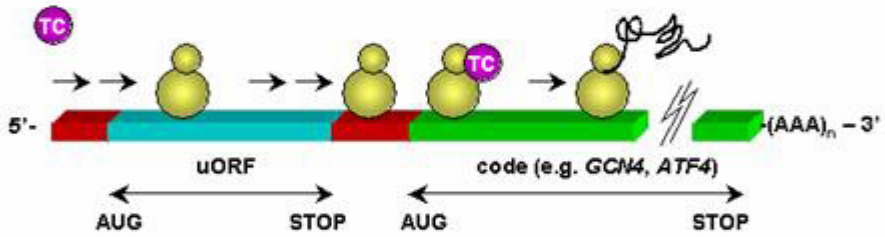
[10] Noller HF. Evolution of protein synthesis from an RNA world. Cold Spring Harb Perspect Biol. 2012; 4:a003681.

[11] DeGracia, DJ *et al*. Molecular pathways of inhibited translation during brain reperfusion: Implications for neuronal survival or death. J Cereb Blood Flow Metab 2002;22:127-141.

synthesis. This process will be important when we discuss cyclosporine as a therapeutic approach to Covid-19 below. Our genome codes for 4 serine-51 phosphorylating eIF2α kinases. The oldest is GCN2 (General Control of Nutrition-2) present already in yeast, and we will see that it together with PKR and PERK are involved. The 4th is HRI, which regulates hemoglobin production.

There are some select mRNAs that undergo preferential translation when eIF2(αP) is increased. They have a long 5'-leader (5'-untranslated region = UTR) before the genuine methionine start codon (AUG) for the protein, and that leader contains an AUG with a short reading frame (an "upstream open reading frame" or uORF). This results in the ribosome making a short junk peptide and falling off the mRNA most of the time. However, increased eIF2(αP) causes the ribosome to not initiate translation at the upstream AUG but rather continue more prolonged scanning and find one of the last good methionine-eIF2 complexes before it reaches the genuine start codon. In amino-acid-starved yeast, GCN2 brings about translation of GCN4 and activation of de-novo amino acid synthesis by this process. This is ribosomal "bypass scanning," illustrated in the figure below from DeGracia *et al*[11].



Ribosome Bypass Scanning on a mRNA: "TC" is the methionine-tRNA-eIF2 "ternary complex." The ribosome scans the mRNA for the AUG start codon, but at the first one in the "junk" upstream open reading frame (uORF) there are few TCs because of inhibition by increased eIF2(αP). Thus the ribosome scans past the uORF without initiating translation. By the time the ribosome reaches the genuine coding sequence, it has found a TC and initiates translation there. Examples of stress-responsive proteins produced by this process include GCN4 in amino-acid starved yeast. Of greater interest in our present discussion, ATF4 is one of our transcription factors for resolution of endoplasmic reticulum stress by unfolded proteins, and SOCS1[12] and SOCS3 are **S**uppressor(s) **o**f **C**ytokine **S**ignalling important in macrophages.

Covid-19 genomic RNA and all its derived subgenomic mRNAs share an identical 70-nucleotide 5'-UTR before the first AUG start codon. That 5'-UTR does not have an uORF but has an unusual 2° structure involving 4-self-hybridization hairpins (C to G and A to U) that allow CoV mRNAs to be recognized by Nsp1 for translation and protection from its RNAse[13].

So part of our cells' response to Covid-19 could involve proteolysis of eIF4F and use of IRES mRNAs with scanning-bypass 5'-leaders, and that is in fact a basic response to more general stress including what brain neurons experience during and following cardiac arrest[11]. The problem is that this type mRNA selection for translation can take cells down the path to molecular suicide – that is, apoptosis. Indeed, high eIF2(αP) and proteolysis of eIF4F are present in apoptotic cells and part of how badly infected cells can choose both to block synthesis of viral proteins and to die.

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12 Gregorieff A *et al.* Regulation of SOCS-1 expression by translational repression. J Biol Chem 2000; 275: 21596–21604.

13 Lokugamage K *et al*. Middle East Respiratory Syndrome Coronavirus Nsp1 inhibits host gene expression by selectively targeting mRNAs transcribed in the nucleus while sparing mRNAs of cytoplasmic origin. J Virol 2015; 89:10970-10981.

2. Innate immune response

In the above first overview we can already see some of the fundamental ways a virus versus cell war is fought. The cellular machinery is going to try to detect and destroy the virus, deny it use of ribosomes, and use its ribosomes to produce both warnings for nearby cells and screams for help (cytokine proteins). The virus is going to try to escape detection, hide its genome, take over ribosomes, and suppress or redirect warnings and screams for help to the advantage of the virus. This is an ugly-dog fight, but in the long run of evolution there are the limits of Mutual Assured Destruction. The virus can't be too lethal or it loses its hosts, and our anti-viral defenses must not themselves kill us all.

Kikkert[14] has written a terrific review of this battle that includes specific information about coronaviruses. The innate immune response signaling cascade against RNA viruses in the lungs starts with recognition of pathogen molecules by **P**attern **R**ecognition **R**eceptors (PRRs). These sensor proteins include RIG-1 and MDA5, which recognize viral forms of RNA (*e.g.*, 5′-uncapped RNA and dsRNA). Coronavirus transcription does produce dsRNA[15], but its RNAs are 5'-capped. Recognition of coronavirus dsRNA by PRRs leads to induction of nuclear DNA transcription to express cellular mRNA for type-I interferons (IFNs), which are the conductors of the early cellular innate immune response against viruses[16]. IFN-β (one of two type-I interferons - IFN-α and IFN-β) is produced by almost all virus-infected cells, and large amounts can be secreted by lymphocytes. The binding of type-I IFNs to a specific cell-surface Interferon Receptor (IFNAR) triggers intracellular signaling pathways that result in activation of or enhanced transcriptional expression for antiviral proteins. Thus, type-I IFN can serve as a warning to nearby cells. The IFN-up-regulated genes encode proteins such as MxA, dsRNA-activated PKR, and RNase L, and shift to this sort of "proteome" in neighboring cells can create marked viral resistance. Although coronaviruses limit production of type-I IFN (below), genetic knockout of the IFNAR cell-surface receptor allows explosive coronavirus replication and organ destruction with early death in mice[17]. In coronavirus infections production of type-I IFN may be very limited, but it is vital.

An obvious viral strategy would be to shield its RNA intermediates and their recognizable features from the innate immune sensors roaming the cell[14]. Indeed, coronaviruses' Nsp3 and Nsp4 modify intracellular membranes to form "replication organelles" (ROs – lots of little bags) that hide viral RNA and serve as headquarters for its replication. In addition, coronavirus Nsps 14 & 16 facilitate addition of a 5'-cap structure to viral mRNAs. Cells' MxA-protein induced by type-I IFN can interfere with viral ROs.

The dsRNA-activated PKR can recognize viral dsRNAs and could block their translation by phosphorylating eIF2α. PERK is a related eIF2α kinase that also produces eIF2(αP) in response to unfolded proteins in the endoplasmic reticulum (ER)[11], which do accumulate during rapid synthesis of coronavirus proteins. In fact, for SARS-CoV (and surely Covid-19), activated PKR, PERK, and eIF2(αP) are present in virus-infected cells[18]. A virus-derived protein binds viral dsRNAs in a way that obstructs their recognition by PKR, and experimental knock-down of PKR does not reduce

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14 Kikkert M. Innate Immune Evasion by Human Respiratory RNA Viruses. J Innate Immun 2020; 12:4–20. DOI: 10.1159/000503030.

15 De Wilde A *et al*. Cyclosporin A inhibits the replication of diverse coronaviruses. J Gen Virol 2011; 92:2542–2548.

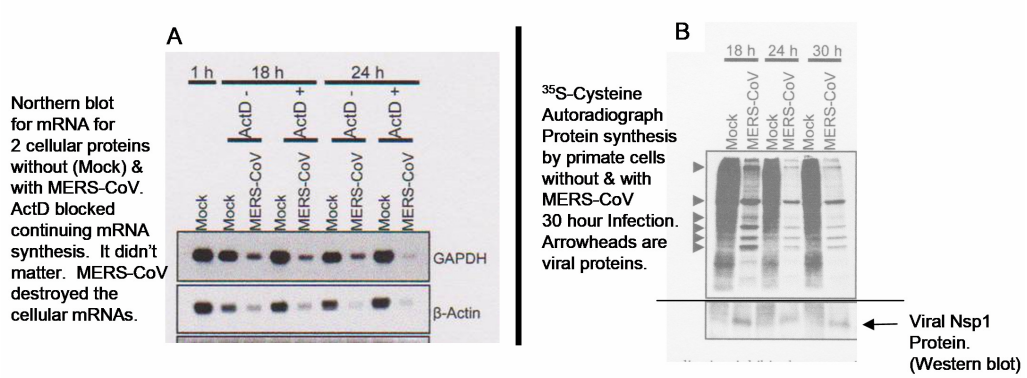
16 Frese M *et al*. Interferon-γ inhibits replication of subgenomic and genomic hepatitis C virus RNAs. Hepatology 2002; 35:694-703.

17 Cervantes-Barragan L *et al*. Control of coronavirus infection through plasmacytoid dendritic-cell–derived type I interferon. Blood 2007; 109:1131-1137.

18 Krähling V *et al*. Severe acute respiratory syndrome coronavirus triggers apoptosis via PKR but is resistant to its antiviral activity. J. Virol. 2009; 83: 2298–2309. doi: 10.1128/JVI.01245-08.

SARS-CoV-induced eIF2αP. Thus coronavirus-induced eIF2(αP) and some translation inhibition is a result of ER stress (PERK) rather than antiviral activation of PKR.

However, in this infection the host isn't the only player trying to shut down translation of the opponent's mRNAs. SARS-CoV, MERS-CoV, and Covid-19 all work to shut off translation of host proteins. Nsp1 attracts a host RNAse that degrades host mRNA, but Nsp1 also recognizes the hairpin-structured 5'-UTR of coronavirus mRNAs and spares them[13,14].



The above data is from the work of Lokugamage *et al*[13] with MERS-CoV. Although copious mRNAs for cellular proteins including interferon are produced by cells early in the course of coronavirus infection, mRNAs for all cellular proteins including interferon are degraded (**A** above) with the result that CoV proteins are selectively produced (**B** above). Covid-19 Nsp1 also mediates general degradation of host mRNA and selective translation of viral transcripts[19]. This destruction of host-cell mRNA is so extensive that by 8-hours post-infection, 88% of cytoplasmic mRNA is viral[20]. The low level of IFN-β produced in CoV infections distorts the pattern and effectiveness of the innate and adaptive immune responses.

Another way to reduce recognition of viral RNA by intracellular defenses is for a viral RNAse enzyme to destroy viral RNAs that have served their purpose[14]. Coronaviruses encode in another of their Nsps an RNAse that helps avoid recognition of viral dsRNA by the sensor MDA5 and cellular RNAse[14]. It may be counter-intuitive for a RNA virus to express an RNAse, but the virus apparently destroys its own RNA products at certain locations or in certain stages of the infection to avoid triggering cellular dsRNA sensing and virus-destroying machinery.

Another aspect of the cellular innate immune response is cell-surface antigen presentation by the Major Histocompatability Complex-I (MHC *aka* HLA) system[21]. Some of the translation products from the virus get recognized as "Defective Ribosomal Proteins" (DRiPs – really!) and are labeled with the small peptide ubiquitin for proteolytic degradation to ~10-amino-acid fragments in proteasomes. The protein fragments are then joined to the MHC-I heavy and light chains to form a moleculer triamer that is moved to the infected cell membrane as a MHC-1 antigen epitope for presentation to and recognition by Natural Killer cells (NKs - large granular lymphocytes present in the normal circulation). This MHC-1 antigen presentation can occur as early as 90-minutes after a cell is infected[21].

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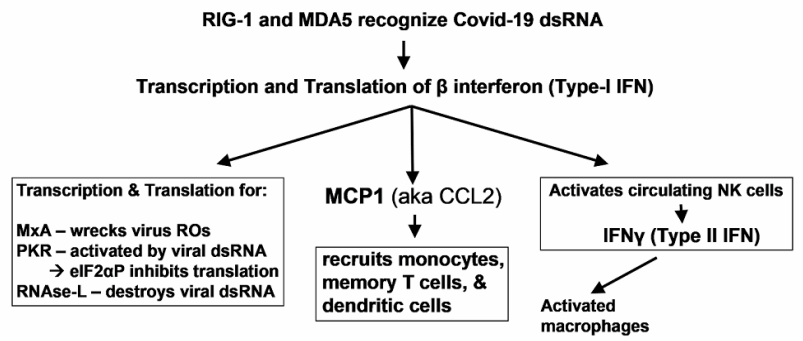
19 Rao S *et al*. Genes with 5′ terminal oligopyrimidine tracts preferentially escape global suppression of translation by the SARS-CoV-2 NSP1 protein. BioRxiv 2020; https://doi.org/10.1101/2020.09.13.295493.

20 Finkel Y *et al*. SARS-CoV-2 utilizes a multipronged strategy to suppress host protein synthesis. BioRxiv 2020; https://doi.org/10.1101/2020.11.25.398578.

21 Goldberg A and Rizzo L. MHC structure and function – antigen presentation. Part 2. Einstein (Brazil) 2015; 13:157-62. doi: 10.1590/S1679-45082015RB3123.



The above figure summarizes the central elements of innate immune response signaling. Inadequate production of IFN-β by failed translation of its missing mRNA greatly weakens the initial innate immune response of infected cells and their neighbors.

Our immune escalation from the **innate immune response** of infected cells, their immediate neighbors, and local NK cells to the systemic **adaptive immune response** also utilizes type-I interferon[22] to induce production of the chemokine Monocyte Chemoattractant Protein (MCP1 *aka* CCL2) to recruit monocytes, memory T cells, and dendritic cells to the infection. NK cells begin to recognize MHC-1 presentation of viral antigens, use their cytotoxic granules to kill infected cells, and respond to Type-I interferon binding their IFNAR by producing Type II IFN, *aka* IFNγ, that is a key link to the adaptive immune response. IFNγ is a homodimer, and it binds its cell-surface receptors (IFNGRs) in a 1 IFNγ to 2 IFNGRs complex[23]. This binding of an IFNγ to the 2 IFNGRs brings the receptors together in a configuration that activates their intracellular signaling to nuclear transcription promoters. This IFNγ signaling up-regulates proteosomes and stimulates macrophages, which can "eat" virus-infected cells and activate cytotoxic T-lymphocytes. Together NK cells, dendritic cells, and macrophages can all act as "Antigen-Presenting Cells" (APCs) to the adaptive immune response. APCs present antigens on MHC-**2** complexes, and this antigen presentation is recognized by lymphocytes[25]. APCs are our only cells that express the MHC-2 complex.

3. Host-driven viral-codon changes

Although not usually viewed in the context of innate immune response, this is an ancient antiviral response that occurs in infected cells. Our cells have evolved some ability to "foul-up" viral nucleotides and so force changes in codons and the amino acids they code for in viral proteins. If this doesn't disable the virus, it can also drive mutations that may allow escape from some of the antibodies and T-cells of the adaptive immune response. SARS-CoV-2 variants are a worry for vaccination, and examination of such variants has highlighted the role of two of our antiviral enzyme systems in their development.

Azgari *et al.*[24] retrieved 66,938 SARS-CoV-2 genome sequences (Global Initiative on Sharing All Influenza Data database). Rather than simply count mutations in the RNA, they constructed a mutational heredity tree. This allowed them to see relative abundance for the nucleotide changes. There are 4 nucleotides, each of which could be randomly changed to any of the other 3, so 12 potential mutations, each with a random probability of 8.3%. In fact the distribution of SARS-CoV-2 mutations is not random with two of the most common being C→U =

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22 Lee A and Ashkar A. The dual nature of Type I and Type II Interferons. Front Immunol 2018; 9:2061. doi: 10.3389/fimmu.2018.02061.

23 Alspach E *et al.* Interferon γ and its important roles in promoting and inhibiting spontaneous and therapeutic cancer immunity. Cold Spring Harb Perspect Biol 2019; doi: 10.1101/cshperspect.a028480.

24 Azgari C *et al.* The mutation profile of SARS-CoV-2 is primarily shaped by the host antiviral defense. BioRxiv 2021; https://doi.org/10.1101/2021.02.02.429486.

33.8% and U→C = 13.7%. Mammalian APOBEC enzyme [apolipoprotein-B RNA-editing catalytic - (I hate that name)] has an established antiviral effect and drives the C→U mutation. Similarly, our ADAR enzyme (adenosine deaminase acting on RNA) drives U→C mutation. The effect on amino acid incorporation in a protein of course depends on the position of a mutation in a triplicate codon. But the fact that almost ½ of the observed CoV nucleotide mutations can be assigned to 2 mammalian enzymes that act against RNA viruses is a compelling argument against only random copying errors as the cause of the viral mutations. Both the long evolutionary course of the cells vs viruses wars and the consequences in this infection are entangled and complex.

4. Adaptive immune response

There are two types of adaptive immune responses[25]. The **cell-mediated immune response** utilizes T-lymphocytes (T-cells, named for the Thymus where they were first recognized) to kill cells that have been infected with viruses. Normally T-cells are ~80% of circulating lymphocytes, and there are three types of T cells: helper, cytotoxic, and suppressor (T-regs) T cells. Helper T-cells play a part in activating both cell-mediated and the antibody immune responses. Cytotoxic T-cells destroy virus-infected cells. Suppressor T-cells deactivate T-cells and B-cells when needed, and thus prevent the immune response from becoming too intense. The **humoral immune response** (antibody production) is produced by activated B-lymphocytes (B-cells).

Both T-cells and B-cells originate from a "common lymphoid progenitor" (see next Figure from[25]) and are antigen-naïve until they are activated by recognizing an antigenic epitope. Naïve T-cells can express either CD4 or CD8 on their surface. Naïve CD4+ T-cells bind APCs via MHC-2 antigen complexes and become activated helper T-cells **(T_H)**. There are CD4+ **T_H** subsets known as Th1 and Th2 cells that are characterized by production of different cytokines. Th1-dominated responses (interferon-γ, IL-2, and TNF-β) support eradication of infectious agents, while Th2 responses (IL-4, IL-5, IL-10, and IL-13) support protection against parasites and limit potentially harmful Th1 responses, such as those associated with multiple sclerosis and thyroid autoimmunity.

Naïve $CD8_+$ T-cells bind MHC-1 antigen complexes and become activated cytotoxic T-lymphocytes **(CTLs)**. CTLs directly kill infected cells and destroy the virus using granules containing both perforin (which puts holes in infected cells) and serine proteases that activate in infected cells many proteases and nucleases that destroy both cellular and viral proteins and nucleotides. A CTL can kill an infected cell in as little as 5 minutes. Each activated T-cell (both T_H and CTL) has a T-cell-receptor specific to the activating antigenic epitope and then clones many copies of itself with that antigen receptor. So we develop an enormous diversity of activated T-cells, and an antigen matching to a T-cell receptor amounts to an instruction to expand that line of T-cells – so we have memory T-cells for the adaptive immune system. CTLs are particularly important in viral infections because they recognize and eliminate infected cells and the viruses they contain.

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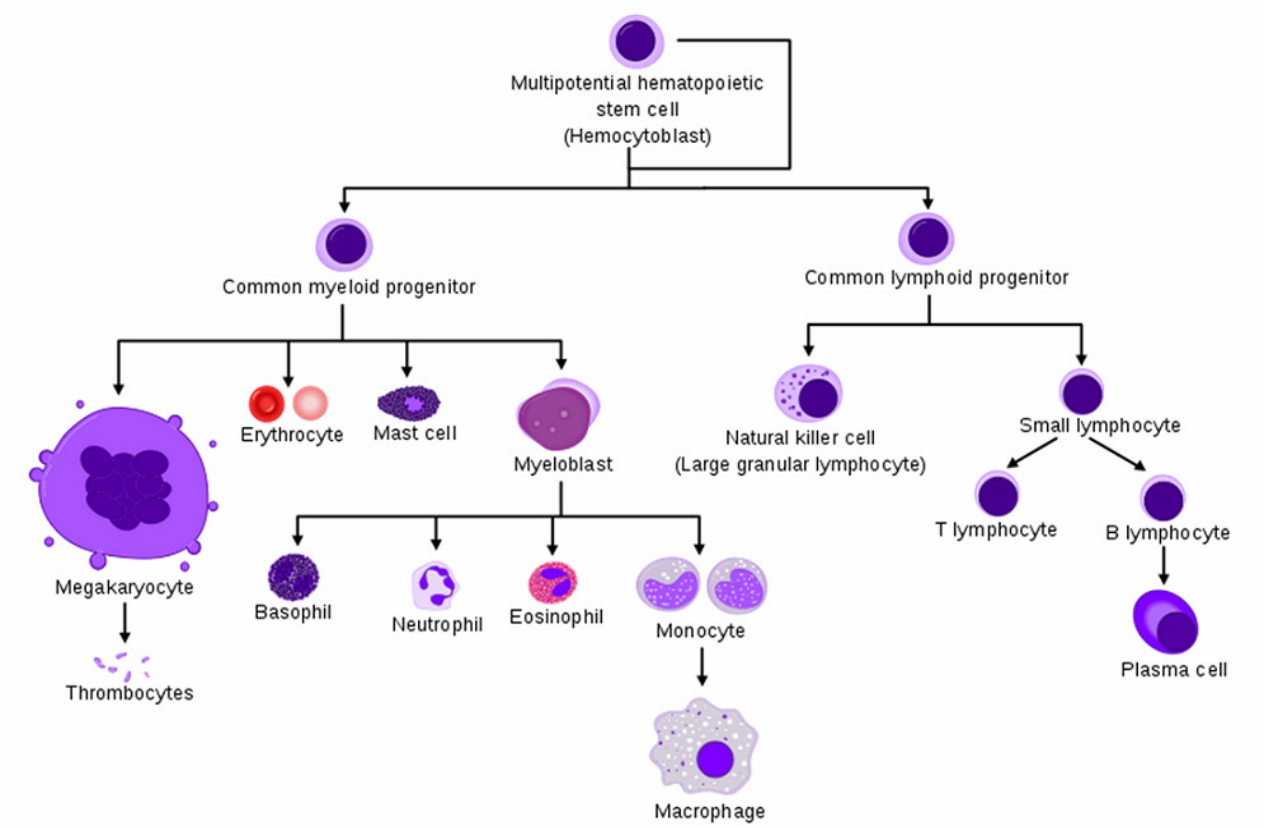
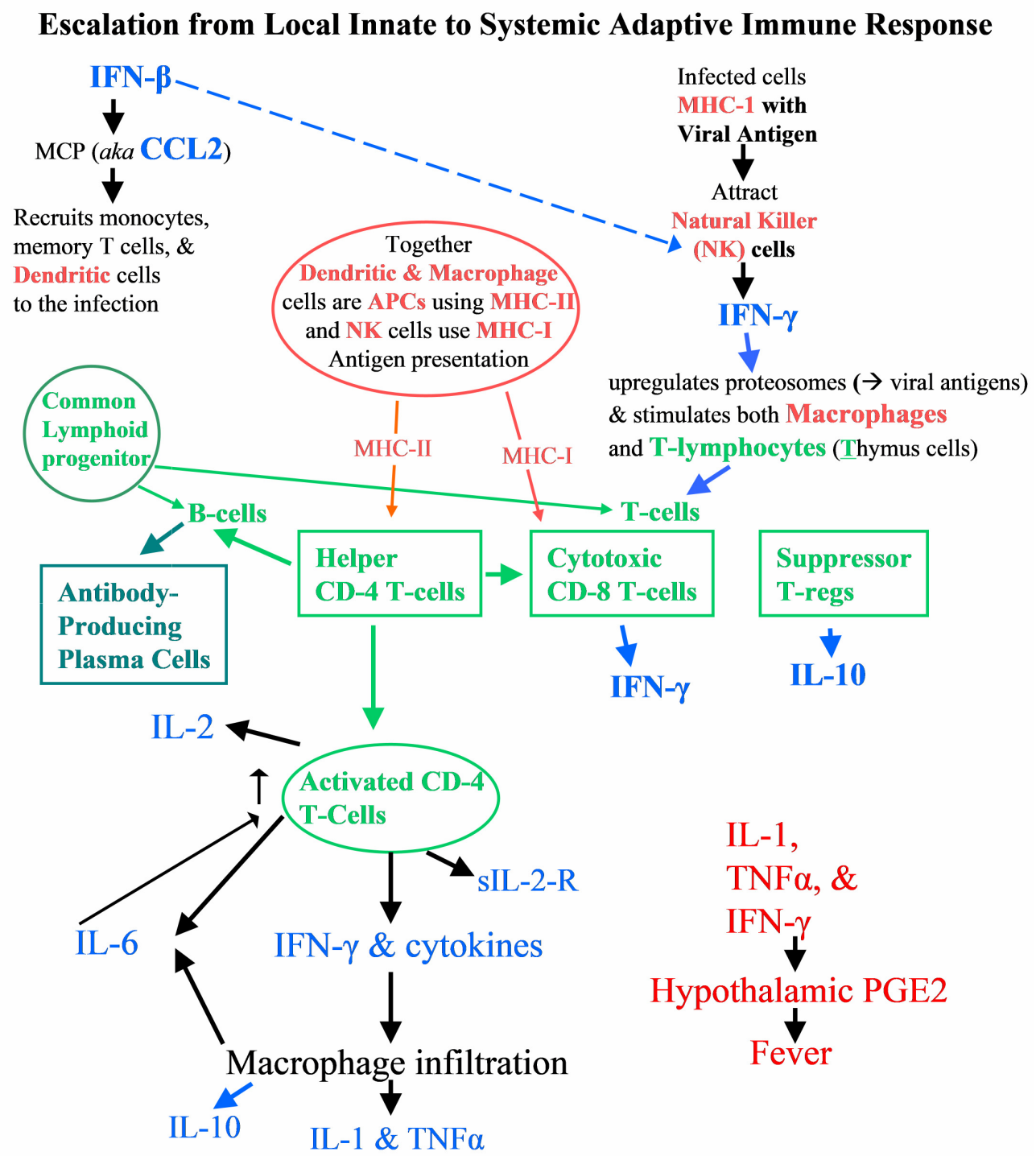
[25] Molnar C and Gair J. Adaptive Immune Response. Concepts of Biology - 1st Canadian Edition. British Columbia Ministry of Advanced Education, 2019.



TH cells can both stimulate CD8+ T-cells to become CTLs and also stimulate naïve B-lymphocytes to differentiate into antibody-producing plasma cells. Zillions of naïve B-lymphocytes are covered with zillions of different IgGs (one type IgG per naïve B-lymphocyte). The B-lymphocyte IgG binds places on full-length undigested antigens because they will produce antibodies for direct recognition of the pathogen outside cells rather than small pieces after it has been inside. When a TH cell recognizes that a previously naïve B-lymphocyte has bound an antigen, the TH cell secretes cytokines causing that B-lymphocyte to differentiate into a plasma cell and clone lots of copies of itself that secrete the antigen-binding IgG as antibodies. Memory B-cells preserve humoral memory for the adaptive immune system. Antibodies do not penetrate our cells even if they contain viruses, but instead attack pathogens when they are extracellular.

The following is a Power-Point figure using the above text and references to help track our way through the escalation to the adaptive immune response. A very central question is what happens if we don't have enough IFN-β from infected cells because Nsp1 has removed its mRNA? Remember, losing Type-1 IFN receptors is disastrous[17]; too little too late IFN-β could be too.



See also Fujiwara F *et al*. Hypercytokinemia in hemophagocytic syndrome. J Ped Hematol/Oncol 1993; 15:92-98.

Post-infection adaptive immunity is persistent but does not result in our permanently maintaining high levels of specific antibodies. If we did that for a lifetime of infections, our blood would be Jello! Instead, Memory-B-cells and Sentinel-T-cells are maintained in lymph nodes. This means that blood concentrations of antibodies against SARS-CoV-2 will drop post infection.

However, we now know that a broad based immunity[26] with memory B-cells and sentinel T-cells (both CD4 and CD8) against SARS-CoV-2 persists at least 8-months after infection[27]. In convalescent patients interrogation of Memory-B-cells reveals that[26], "More than half of all of the antibodies generated were directed at non-S viral proteins, including structural nucleocapsid (N) and membrane (M) proteins, as well as auxiliary open reading frame-encoded proteins" – including ORF3a (ion channel and viral release), ORF6 (antagonist of interferons), ORF7a (membrane-type protein interferes with restrictive tethering of virus to plasmalema), ORF8 (lumenal ER protein inhibits MHC1 expression), and ORF10 (small 38 aa sequence may be non-functional)." Convalescent antibodies have a much broader target set than just **S**pike. As with many other infectious diseases, such a post-infection broad based immunity makes reinfection (even with viral variants) RARE (<1 in 1,000 recovered patients)[28].

The immunologic response in patients and progression to serious Covid-19 lung trouble

1. Interferons from innate immune response

In a study of innate immune response in 50 Covid-19 patients, Hadjadj *et al*.[29] reported IFN-β was undetectable at all stages, and in 24 Covid-19 patients, Blanco-Melo *et al*. reported[30], "serum samples consistently tested negative for both IFN-β and the IFN-λ" (IFN-λ is a type III interferon). This patient data shows that Covid-19 causes a failure of the innate immune interferon response from the cells that are primarily infected, and there is evidence this is caused by Nsp1. In lab studies, wild-type SARS-CoV virus efficiently suppresses production of Type-1 interferons, but a mutant SARS-CoV virus lacking Nsp1 fails to suppress production of Type-1 interferons[31,32].

2. Cellular adaptive immune response

With respect to the cellular adaptive immune response, Diao *et al*.[33] reported that in 522 Covid-19 patients compared to 40 controls, there was marked cytopoenia (lymphocyte deficiency) of CD4+ and CD8+ T-cells. As patients deteriorated this was associated with remarkably increased levels of IL-6, TNFα, and IL-10 (IL-10 is produced mainly by T-regs and macrophages to down-regulate the cellular immune response). In the final stages of patient deterioration, the result was interpreted as "T-cell exhaustion" with increasing cytopoenia and circulating markers of T-cell apoptosis[34]. This was initially thought of as a consequence of defective immunologic signaling, but the Covid-19 virus now appears to be more directly involved. This damn virus is directly infecting

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26 DiMuzio J *et al*. Unbiased interrogation of memory B cells from convalescent COVID-19 patients reveals a broad antiviral humoral response targeting SARS-CoV-2 antigens beyond the spike protein. BioRxiv 2021; https://doi.org/10.1101/2021.01.27.428534.

27 Dan J *et al*. Immunological memory to SARS-CoV-2 assessed for up to 8 months after infection. Science 2021; doi 10.1126/science.abf4063.

28 Abu-Raddad L *et al*. SARS-CoV-2 reinfection in a cohort of 43,000 antibody-positive individuals followed for up to 35 weeks. MedRxiv 2021; doi.org/10.1101/2021.01.15.21249731.

29 Hadjadj J *et al*. Impaired type I interferon activity and exacerbated inflammatory responses in severe Covid-19 patients. 2020; doi.org/10.1101/2020.04.19.20068015.

30 Blanco-Melo D *et al*. Imbalanced host response to SARS-CoV-2 drives development of COVID-19. J Cell 2020; DOI: 10.1016/j.cell.2020.04.026.

31 Narayanan K *et al*. Severe Acute Respiratory Syndrome Coronavirus Nsp1 suppresses host gene expression, including that of Type I Interferon, in infected cells. J Virology 2008; 82: 4471–4479.

32 Wathelet M *et al*. Severe Acute Respiratory Syndrome Coronavirus evades antiviral signaling: Role of Np1 and rational design of an attenuated strain. J Virology 2007; 81: 11,620–11,633. doi:10.1128/JVI.00702-07.

33 Diao B *et al*. Reduction and functional exhaustion of T Cells in patients with Coronavirus disease 2019 (Covid-19). 2020; doi: 10.1101/2020.02.18.20024364.

34 Chiappelli F *et al*. Covid-19 immunopathology & immunotherapy. Bioinformation 2020; 16:219-222. DOI**:** 10.6026/97320630016222.

and probably killing lymphocytes needed for effective adaptive immunity. Here's the Pontelli *et al*. abstract[35].

> Although SARS-CoV-2 severe infection is associated with a hyperinflammatory state, lymphopenia is an immunological hallmark, and correlates with poor prognosis in COVID-19. However, it remains unknown if circulating human lymphocytes and monocytes are susceptible to SARS-CoV-2 infection. In this study, SARS-CoV-2 infection of human peripheral blood mononuclear cells (PBMCs) was investigated both *in vitro* and *in vivo*. We found that *in vitro* infection of whole PBMCs from healthy donors was productive of virus progeny. Results revealed that monocytes, as well as B and T lymphocytes, are susceptible to SARS-CoV-2 active infection, and viral replication was indicated by detection of double-stranded RNA. Moreover, flow cytometry and immunofluorescence analysis revealed that SARS-CoV-2 was frequently detected in monocytes and B lymphocytes from COVID-19 patients, and less frequently in CD4+T lymphocytes. The rates of SARS-CoV-2-infected monocytes in PBMCs from COVID-19 patients increased over time from symptom onset. Additionally, SARS-CoV-2-positive monocytes and B and CD4+T lymphocytes were detected by immunohistochemistry in post mortem lung tissue. SARS-CoV-2 infection of blood circulating leukocytes in COVID-19 patients may have important implications for disease pathogenesis, immune dysfunction, and virus spread within the host.

It looks like when Covid-19 infection is overwhelming, it crushes both the innate immune response (no measurable interferons) and the adaptive immune response (infects lymphocytes and → lymphopenia). That leaves NK cells responding to MHC-1 antigen presentation → macrophages with a macrophage cytokine release storm (with production of IL-6, TNFα, and IL-10), ARDS, and hypercoagulability. We'll see more about "macrophage activation syndrome" (MAS) below.

3. Antibodies from the adaptive immune response

With respect to antibodies, very recent seminal evidence from Hoepel *et al.*[36] at the University of Amsterdam suggests the adaptive immunity onset of IgG production can make a significant contribution to macrophage hyperactivity and emergence of pulmonary pathology in advanced Covid-19 SARS. This work also suggests causal unification of some Covid-19 disease phenomena, including respiratory distress and hypercoagulopathy, and it may raise significant issues for prospective vaccines focused only on producing anti-**S**pike IgG. It begins with observation that severe pulmonary disease occurs ~10 days after initial symptom onset, coinciding with production of IgG after the initially suppressed interferon response. The authors cite Liu *et al.*[37], who passively immunized rhesus monkeys with anti-**S**pike IgG and showed that, although viral titers were reduced in subsequent SARS-CoV lung infection, **the anti–Spike IgG caused severe lung injury by skewing the inflammatory response of macrophages**. Liu reported inhibition of lung injury by blocking macrophage Fc receptors (FcR).

We need to digress a moment to be reminded about what the Fc is in the antibody structure of IgG. Please don't be insulted if you know this in your sleep. IgG is a tetramer protein (2 "heavy chains" and 2 "light chains") shaped like a **Y**, with the antigen-binding sites of both the heavy and light chains at the ends of the 2 upper arms. Because even 50 years ago we knew that the complement system bound to the bottom arm (of the 2 "heavy chains") and that arm could be removed by a protease, the proteolytic fragment constituted by the bottom arm got named the "Fc-

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35 Pontelli M *et al*. Infection of human lymphomononuclear 1 cells by SARS-CoV-2. BioRxiv 2020; https://www.biorxiv.org/content/10.1101/2020.07.28.225912v1.

36 Hoepel W *et al.* Anti-SARS-CoV-2 IgG from severely ill Covid-19 patients promotes macrophage hyper-inflammatory responses. BioRxiv Preprint 2020; doi: https://doi.org/10.1101/2020.07.13.190140.

37 Liu *et al.* Anti–spike IgG causes severe acute lung injury by skewing macrophage responses during acute SARS-CoV infection. JCI Insight 2019; 4: e123158. https://doi.org/10.1172/jci.insight.123158.

fragment" for "fragment complement-binding." The 2 upper arms of the **Y** freed from the Fc by proteolysis are called FAB for "fragments antigen-binding." FABs can be used clinically (like snake venom antidotes) to avoid consequences of Fc binding its receptors. In IgG-antigen immune complexes, Fc functions are more complex that just complement binding – and include binding to Fc-receptors (FcRs) on **macrophages,** histamine-containing **mast cells,** and **platelets**.

M2 macrophages should secrete anti-inflammatory cytokines and so reduce inflammation and promote healing. Hoepel *et al.* assessed the effect on human M2 macrophages of **immune-complexes** of the Covid-19 **S**pike protein with anti-**S**pike IgG antibodies from critically ill Covid-19 patients. **S**pike-IgG immune complexes elicited dose-dependent production of inflammatory cytokines by human macrophages. In a further *in vitro* model with human pulmonary artery endothelial cells, **S**pike-IgG immune complexes and macrophages induced long-lasting endothelial disruption (fluid leak). In addition, upon platelet perfusion of this system, there was an increase in both platelet adhesion to the endothelial cells and also in release of von Willebrand Factor (see discussion of hypercoagulopathy below).

Hoepel *et al.* had developed an anti-**S**pike **recombinant** IgG from plasma cells of Covid-19 patients. They were surprised that immune complexes of recombinant IgG-**S**pike-protein elicited much less pro-inflamatory macrophage response. The anti-**S**pike IgG from severe Covid-19 cases was intrinsically more pro-inflammatory than recombinant anti-**S**pike-IgG, arguing for important post-translational modification of the IgG in severe Covid-19 patients. A key characteristic that determines IgG pathogenicity is glycosylation of the IgG Fc tail[38]. Hoepel *et al.* found increased galactosylation of anti-**S**pike IgG compared to total IgG in sick Covid-19 patients. Furthermore, when they modified their recombinant anti-**S**pike IgG by glycosylation, **S**pike-IgG immune complexes again drove the macrophage inflammatory responses. Fc-receptor specific antibodies that blocked the macrophage Fcγ2 receptor (Fcγ2R) most strongly inhibited the inflammatory response to **S**pike-IgG immune complexes. FcγRs signal through the Syk kinase, which can be blocked using fostamatinib (FDA-approved for immune thrombocytopenia; as yet no animal or human evidence with Covid-19). Fostamatinib inhibited macrophage pro-inflammatory cytokine production induced by **S**pike-IgG immune complexes from severe Covid-19 patients. The authors went on to analyze macrophage gene expression and found fostamatinib down-regulated mRNA expression of genes for both inflammatory cytokines and also a set of platelet activation genes. In January 2021 DOD committed a $16.5-million award to Rigel for a phase-3 study of fostamatinib treatment of Covid-19[39].

Below we will see clinical evidence for reduction of Covid-19 mortality by famotidine, an over-the-counter inhibitor of the histamine H2-receptor. Although we classically think of mast-cell degranulation with histamine release as an allergic response to IgE, IgG immune complexes can also cause mast-cell degranulation. The effects of pro-inflamatory anti-**S**pike IgG immune complexes probably involve mast-cells, which also have FcγRs[40]; mutant mice without IgG FcγRs lack mast-cell degranulation[41].

4. Hyper-Coagulopathy – aka Covid-19 Associated Coagulopathy (CAC)

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38 Jennewein M and Alter G. The immunoregulatory roles of antibody glycosylation. Trends Immunol 2017; 38:358-372.

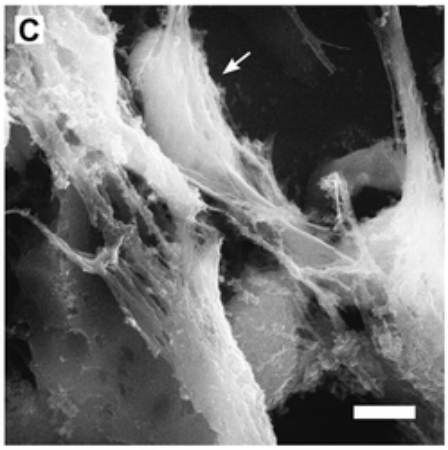
39 https://globalbiodefense.com/2021/01/30/rigel-awarded-16-5-million-from-dod-for-phase-3-trial-of-fostamatinib-in-covid-19-patients/

40 Sylvestre D and Ravetch J. A dominant role for mast cell Fc receptors in the Arthus reaction. Immunity 1996 5:387-90. doi: 10.1016/s1074-7613(00)80264-2.

41 Hazenbos W *et al*. Impaired IgG-dependent anaphylaxis and Arthus reaction in Fc gamma RIII (CD16) deficient mice. Immunity 1996 5:181-8. doi: 10.1016/s1074-7613(00)80494-x.

It starts with platelets. Although the study from Hoepel *et al.*[36] is an online preprint for now, it is vitally important and has received online reviews from both MIT and Oxford. This report of molecular evidence for macrophage-induced platelet activation is consistent with additional molecular evidence of platelet hyperactivity and a general hypercoagulable state in severe Covid-19 patients[42]. Platelets express ACE2, and during SARS-CoV-2 viremia, viral **S**pike can itself activate platelets by binding ACE2[43], although viral RNA does not appear to enter platelets[44]. Platelets also have Fcγ2 receptors, and IgG-**S**pike immune complexes from Covid-19 patients can directly activate platelets via this receptor[45].

Our understanding of platelet function has changed in important ways in the past 20 years. In particular it is now clear that there are important interactions between platelets, neutrophils (PMNs), and coagulation[46]. Intravascular PMNs are drawn to infections and can undergo a process of "NETosis" in which both nuclear and plasma membranes rupture to release decondensed chromatin (including the histones DNA is wrapped around). The resulting DNA fibers form a network which can entrap and kill extracellular pathogens; this structure is called a **Neutrophil Extracellular Trap** or a NET. In fact, it has recently been shown that plasma from Covid-19 patients induces PMN NETosis, and this effect is blocked by the SYK inhibitor fostamatinib[47].



Electron microscope photograph of DNA fibers of a **NET** engulfing fungi that have infected a mouse lung. (https://en.wikipedia.org/wiki/Neutrophil_extracellular_traps)

NETs can also entrap platelets, RBCs, and other PMNs. Moreover, locally activated platelets serve as "landing pads" for PMNs that will produce NETS[46], and a NET-platelet complex is a focus for coagulation and formation of microthrombi. Such NET-platelet-fibrin microthrombi are found in lungs at autopsy of severe Covid-19 patients who have died.

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42 Manne B *et al*. Platelet gene expression and function in patients with COVID-19. Blood 2020;136:1317-1329.

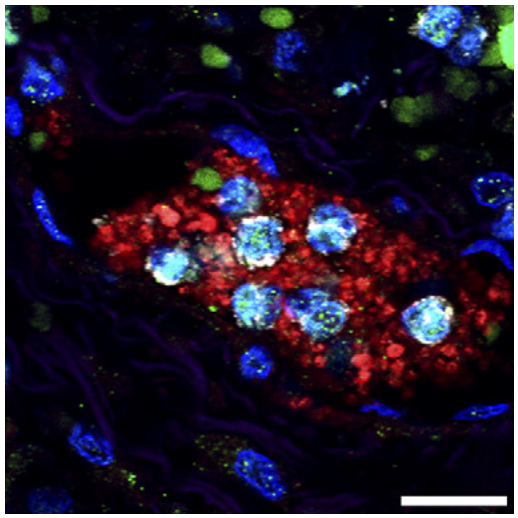
43 Zhang S *et al*. SARS-CoV-2 binds platelet ACE2 to enhance thrombosis in COVID-19. J Hematology & Oncology 2020; 13:120. https://doi.org/10.1186/s13045-020-00954-7.

44 Bury L *et al*. Search for SARS-CoV-2 RNA in platelets from COVID-19 patients. Taylor-Francis Online 2020; https://doi.org/10.1080/09537104.2020.1859104.

45 Zlamal J *et al*. cAMP prevents antibody-mediated thrombus formation in COVID-19. MedRxiv 2020; https://doi.org/10.1101/2020.12.15.20247775.

46 Zucoloto A and Jenne C. Platelet-Neutrophil interplay: Insights into Neutrophil Extracellular Trap (NET)-driven coagulation in infection. Frontiers Cardiovasc Med 2019; doi: 10.3389/fcvm.2019.00085.

47 Strich J *et al*. Fostamatinib inhibits neutrophils extracellular traps induced by Covid-19 patient plasma: A potential therapeutic. J Infectious Diseases 2020; jiaa789, https://doi.org/10.1093/infdis/jiaa789.



Fluorescent photomicrograph of a microthrombus from the lungs of a patient who died from severe Covid-19. The tissue was prepared with various primary and then secondary fluorescent antibodies to differentially identify components in the thrombus. Neutrophils (gray) are imbedded in the matrix of a platelet-rich clot (red) containing NETs revealed by extracellular histones (green). From Middleton *et al*[48].

When this platelet-NET-fibrin business gets going in Covid-19, hypercoagulation can spread beyond the lungs. Studies of the coagulation system in severe Covid-19 show evidence of a hypercoagulable state that is distinct from Diffuse Intravascular Coagulation (DIC)[49]. The major clinical finding in Covid-19 is thrombosis, whereas the major finding in DIC is bleeding. The hypercoagulable state of severe Covid-19 is characterized by markedly increased von Willebrand Factor and D-dimer and more modest increases in fibrinogen and coagulation Factor VIII (with Factor IX forms "Intrinsic Tenase" to activate Factor X at the top of the final common pathway of coagulation). Platelet count is often moderately reduced.

Deep venous thrombosis has been frequently found in patients with severe Covid-19, and arterial thrombosis may also occur. A large study of hospitalized Covid-19 patients (3,334 with 829 in ICU) reported stroke in 1.6 percent and myocardial infarction in 8.9 percent[50]. However, the most lethal coagulation problem appears to affect the lungs. Carsana *et al.*[51] and Middleton *et al.*[48] have published autopsy histopathology identifying platelet-fibrin microthrombi in the lungs, and Ackerman *et al.*[52] have extended these post-mortem observations by showing that platelet-fibrin microthrombi are found at autopsy of Covid-19 lungs at **9-fold** the rate seen with influenza.

5. An integrated theory of Acute Covid-19 pathology

The work of Hoepel *et al.*[36] also suggests pushing the causal reasoning further. The question arises as to what might drive glycosylation of IgG Fc? The glycosylated protein we are most familiar with is HbA1c, which is used as a marker of chronically elevated blood glucose. It is

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48 Middleton E *et al*. Neutrophil extracellular traps contribute to immunothrombosis in COVID-19 acute respiratory distress syndrome. Blood 2020; 136:1169-1179.

49 Cuker A and Peyvandi F. Coronavirus disease 2019 (COVID-19): Hypercoagulability. UpToDate 2020; https://www.uptodate.com/contents/coronavirus-disease-2019-covid-19-hypercoagulability.

50 Bilaloglu S *et al*. Thrombosis in hospitalized patients with COVID-19 in a New York City Health System. JAMA 2020; 324:799.

51 Carsana L *et al*. Pulmonary post-mortem findings in a large series of COVID-19 cases from Northern Italy. Lancet Infect Dis 2020; S1473-3099(20)30434-5. doi: 10.1016/S1473-3099(20)30434-5.

52 Ackermann M *et al.* Pulmonary vascular endothelialitis, thrombosis, and angiogenesis in Covid-19. N Engl J Med. 2020; 383:120-128.

known that our HbA1c increases as we age[53], even in non-diabetics, and HbA1c is an important prognostic indicator in Covid-19[54]. Furthermore, elevated HbA1c is associated with proinflamatory glycosylation of IgG Fc in both Type 1 and Type 2 diabetics[55,56]. Acute stress-induced hyperglycemia also occurs frequently in hospitalized non-diabetic Covid-19 patients and predicts a difficult course and increased mortality[57]. As discussed by Bermingham *et al.*[55], hyperglycemia can drive the hexose-amine biosynthetic pathway to produce diphosphate-N-acetylglucosamine, a key substrate for Fc glycosylation on IgG.

We have now reviewed enough evidence to construct for Covid-19 pathology an integrated theory that embraces the combined basic science and general clinical experience. Covid-19 infection suppresses both innate immunity by obstructing interferon production and also adaptive immunity by infecting lymphocytes, inducing their apoptosis and lymphopenia, and markedly limiting T-cell response. This results in an immune response involving predominantly MHC1 antigenic epitopes, NK cell activity, and macrophage activation. B-cells do eventually respond with IgG antibody. But for older patients, diabetics, and those with stress-induced hyperglycemia, anti-**S**pike Fc-glycosylated-IgG combines with numerous viruses to produce IgG-**S** immune complexes that bind to FcγRs and activate SYK, thereby locally activating platelets, stimulating NETosis, and exacerbating the macrophage inflammatory response into the pathology of full blown macrophage activation syndrome (MAS). This results in platelet-NET-mediated hypercoagulability with lung microthrombi and more general compromised microperfusion together with pulmonary endothelial fluid leakage. The Fc-glycosylated immune complexes may also stimulate local mast-cell degranulation with histamine release. The cumulative result is Severe Adult Respiratory Syndrome with progressive hypoxia leading to death. In addition to testing the cell counts, coagulation profile, and inflammatory markers of MAS, immediate determination of patients' HbA1c is important.

6. The emerging Covid-19 problem of "Long Haulers"

Before we move on from molecular biology and pathophysiology to vaccines and treatment approaches, we need to begin to think about basic mechanisms and this disease process in a longer time frame than we associate with most acute infectious diseases. It is beginning to look as though both the SARS-CoV-2 virus and human hosts can induce longer-term effects on each other.

We've already noted the evidence from Azgari *et al.*[24] that some of the viral nucleotide mutations are not random but rather more directly driven by ancient components of our cellular anti-viral defenses. It now also appears in work by a Harvard-MIT team that some sub-genomic viral RNAs are being reverse transcribed and more-or-less randomly stitched into the DNA of some infected cells[58]. About 17% of our genome is comprised of LINE-1 retrotransposons (~500,000 copies) that contain code for a RNA-dependent-DNA-polymerase – that is a reverse transcriptase.

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53 Pani, LN *et al*. Effect of aging on A1C levels in individuals without diabetes: Evidence from the Framingham Offspring Study and the National Health and Nutrition Examination Survey 2001-2004. Diabetes Care 2008; 31:1991-1996.

54 Wang Z *et al*. Glycosylated hemoglobin is associated with systemic inflammation, hypercoagulability, and prognosis of COVID-19 patients. Diabetes Res Clin Pract 2020; 164: doi: 10.1016/j.diabres.2020.108214.

55 Bermingham M *et al*. N-Glycan profile and kidney disease in Type 1 diabetes. Diabetes Care 2018;41:79–87.l https://doi.org/10.2337/dc17-1042.

56 Liu J *et al*. Glycomics for Type 2 diabetes biomarker discovery: Promise of immunoglobulin G subclass-specific fragment crystallizable N-glycosylation in the Uyghur population. OMICS 2019; 23: doi: 10.1089/omi.2019.0052.

57 Mamtani M *et al*. Association of hyperglycemia with hospital mortality in nondiabetic Covid-19 patients: A cohort study. MedRxiv 2020; doi.org/10.1101/2020.08.31.20185157;

58 Zhang L *et al*. SARS-CoV-2 RNA reverse-transcribed and integrated into the human genome. BioRxiv 2021; https://doi.org/10.1101/2020.12.12.422516.

On average ~100 of our LINE-1 retrotransposons are expressed, with great variability among individuals in which of the numerous LINE-1 sequences are expressed. These authors report that,

> "Human endogenous LINE-1 expression was induced upon SARS-CoV-2 infection or by cytokine exposure in cultured cells, suggesting a molecular mechanism for SARS-CoV-2 retro-integration in patients."

In laboratory infections of human cells, they were able to recover chimeric sequences from DNA by PCR utilizing primers from both the viral **N** sequence and human sequences. Furthermore, they found in some patients' cells (from bronchoalveolar lavage fluid) chimeric RNA (probably post-transcription) containing both a SARS-CoV-2 sequence (most commonly for the **N** protein) and human sequences. The chimeric abundance of **N**-sequence integration into cellular DNA occurs without evidence of whole-viral integration and likely reflects reverse transcription of viral subgenomic RNA. In-situ hybridization indicated the genomic-integrated **N**-sequences up to the full-length protein can be transcribed by our cells. This study did not explore whether those transcripts can be translated into a protein, although simple chance (1 in 3 for an integrated codon) argues that some of these genomic integrations will occur in a correct ribosomal reading frame for the chimeric protein. The authors note that,

> "From an evolutionarily perspective, retro-integration of viral RNA by LINE-1 could be an adaptive response by the host to provide sustaining antigen expression possibly enhancing protective immunity. Conversely, retro-integration of viral RNAs could be detrimental and cause a more severe immune response in patients such as a "cytokine storm" or auto-immune reactions."

This mechanism is an unconvincing etiology for "cytokine-storm," but this seminal study has the potential to explain both prolonged detection of viral nucleotides and "Long Hauler" syndromes.

There is now evidence that both viral nucleotide sequences and proteins can be found in some patients several **months** after the acute infection. Gaebler *et al*.[59] studied the trajectory of post-infection antibodies and then went on to look for persistent viral antigen. They report,

> "IgM, and IgG anti-SARS-CoV-2 **S**pike protein receptor binding domain (RBD) antibody titres decrease significantly with IgA being less affected. Concurrently, neutralizing activity in plasma decreases by fivefold in pseudotype virus assays. In contrast, the number of RBD-specific Memory B cells is unchanged. Memory B cells display clonal turnover after 6.2 months, and the antibodies they express have greater somatic hypermutation, increased potency and resistance to RBD mutations, indicative of continued evolution of the humoral response. Analysis of intestinal biopsies obtained from asymptomatic individuals 4 months after the onset of coronavirus disease-2019 (COVID-19), using immunofluorescence, or polymerase chain reaction, revealed persistence of SARS-CoV-2 nucleic acids and immunoreactivity in the small bowel of 7 out of 14 volunteers."

Things to be sure we notice here are (1) plasma antibody levels go down, BUT MEMORY-B CELLS DON'T, (2) in fact the antibodies from the Memory-B cells get better, and (3) 4 MONTHS POST-ACUTE-INFECTION immunofluorescence for SARS-CoV-2 proteins and RT-PCR products from viral nucleotides were found in small bowel biopsies from ½ of studied subjects. Now those persistent molecules could be from viral sequences reverse transcribed into some of our cell's genomes. Or, the damn virus could be hanging around. I don't like either possibility; they reinforce my desire to use early effective outpatient anti-viral treatment. The sooner we kill this damn thing – the better.

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[59] Gaebler C. *et al*. Evolution of antibody immunity to SARS-CoV-2. Nature 2021; doi. 10.1038/s41586-021-03207-w (2021).

So that molecular evidence just made "psychological stress" a very unsatisfactory explanation for persistent symptoms in post-Covid-19 "Long Haulers." In fact, patients discharged after Covid-19 hospitalization have substantially increased risk. Ayoubkhani D *et al.*[60] examined in the UK-NHS 47,780 hospitalized Covid-19 patients who were discharged alive by August 31, 2020, matched to controls. Per 1,000 person-years, post-Covid-19 readmissions (766) and deaths (320) were 3.5- and 7.7-fold greater than seen in matched controls. Readmissions and deaths commonly involved respiratory, metabolic, and/or cardiovascular events. We are going to need to watch for and study the literature that is sure to emerge regarding diagnostic and treatment approaches we will need for what will surely be millions of "Covid Long-Haulers."

Vaccines

1. Covid-19 molecular targets of our immunological response to infection

I begin by emphasizing the caution advised by Liu *et al.*[37], who noted, "Multiple vaccine platforms *(followed by)* viral infection induces SARS-CoV–specific immune memory that enhanced lung inflammation… in mice and African green monkeys…. Recent studies suggested that T cells play a crucial role in protection of mice against lethal SARS-CoV infection. Enhanced pulmonary immunopathology in vaccinated and challenged animals reflects an inadequate T-cell response." Both anti-Spike Fc glycosylation and T-cell response seem critical parameters for an effective anti-**S**pike vaccine and probably also for convalescent plasma (below).

So we should begin thinking about vaccines by asking about the breadth of the physiologic response to Covid-19 infection by antibodies and T-Cells. With the exception of the Covid-19 vaccines made from whole inactivated virus (in China SinoPharm and Sinovac and in India Covaxin), all of the current vaccine candidates use only the **S**pike protein as the immunizing antigen. That can only produce a more limited immune response than that seen after SARS-CoV-2 infection.

The importance of T-cell adaptive immunity is widely recognized, and a fundamental fact is that **T-cell immunity and humoral antibody immunity arise from different molecular targets**. T-cell immunity arises from 10-15 amino acid pathogen pieces produced by intracellular proteolysis and presented on the surface of the infected cell by MHC complexes. Antibody immunity arises from B-cells random surface immunoglobulins binding an intact antigen outside the cell. So during and following infection, antibodies and T-cells can be expected to engage quite different repertoires of pathogen molecules, and that has been found true in Covid-19.

Schrock *et al.*[61] have reported an extensive and elegant set of experiments to identify Covid-19 proteins recognized by patients' antibodies. They created oligonucleotides encoding 56-amino acid epitopes that stepped every 28 amino acids and also oligonucleotides encoding 20-amino acid epitopes that stepped every 5 amino acids throughout the entire SARS-CoV-2 proteome. These nucleotides were incorporated into engineered phage that expressed the encoded peptide on their surface, and the phage particles were then immunoprecipitated using patient sera. The phage precipitated by the sera then underwent PCR amplification of their nucleotides followed by nucleotide sequencing to identify the viral epitope that had been recognized by the patient's antibodies. These investigators also had already utilized this technology to produce epitope libraries for ~400 other known human viral pathogens, and they used these libraries to examine patients' sera for pre-existing antibodies against "common cold CoVs" and other common pathogens.

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[60] Ayoubkhani D *et al*. Epidemiology of post-COVID syndrome following hospitalisation with coronavirus: A retrospective cohort study. MedRxiv 2021; doi.org/10.1101/2021.01.15.21249885.

[61] Shrock E *et al*. Viral epitope profiling of COVID-19 patients reveals cross-reactivity and correlates of severity. Science 2020; 370: https://doi.org/10.1126/science.abd4250.

They utilized this "Virscan" system to study sera from 232 Covid-19 patients and also sera obtained from 190 volunteers before the Covid-19 outbreak. These investigators report that,

> "Analysis of SARS-CoV-2 proteins targeted by Covid-19 patient antibodies revealed that the primary responses to SARS-CoV-2 are reactive with peptides derived from **S**pike protein and **N**ucleoprotein…. Third-most frequently recognized is the replicase polyprotein ORF1 *(Open Reading Frame 1)*, but unlike **S** and **N**, ORF1 is recognized to a similar extent by sera from Covid-19 patients and pre-COVID-19 era controls."

Antibody response to Covid-19 **S** and **N** proteins was seen ~10 days after symptom onset. Pre-Covid-19-era sera did show some modest cross-reactivity (~20%) with some **S** and **N** epitopes, suggesting residual "common cold CoV" immunity, although there was little evidence this cross-reactivity affected the course of Covid-19. The experimental setup allowed the investigators to identify across patients the protein regions most frequently recognized by antibodies in the amino acid sequences of both **S** (4 regions) and **N** (4 regions). When they grouped the Covid-19 patients into those who required Hospitalization (n = 101) and those who did not (n = 131), they found that the Hospitalized group exhibited stronger and broader antibody responses to **S** and **N** peptides. This is consistent with other reports of greater antibody response seen in sicker patients. Getting into serious trouble with Covid-19 does not reflect a failed antibody response.

Grifoni *et al*.[62] have shown that T-cells target epitopes from a substantially broader array of Covid-19 proteins. These investigators utilized 474 predicted MHC II CD4 T-cell epitopes and 628 MHC I CD8 epitopes, both covering all proteins in the viral genome. Then cytokine production by patients' T-cells was measured in response to epitope stimulation. In samples from convalescent Covid-19 patients, these investigators found CD4 T-Cells ("helpers") targeting **S**, **N**, **M**, and

NSP3 (contains an important viral protease)[63],
NSP4 (host cell membrane rearrangement),
NSP12 (**RdRp**)
ORF3a (an ion channel protein),
and ORF8 (binds interferon regulatory factor to inactivate interferon signaling).

They also found CD8 T-cells (**CTLs**) targeting **S**, **N**, **M**, and

NSP6 (host cell membrane rearrangement),
ORF3a,
ORF8,
and amino acid sequences from another 4 Covid-19 RNA open reading frames.

So we see how much broader is the array of T-cell target protein antigenic epitopes (8 identified for CD4 T-cells and 10 for CD8 T-cells) than the antibody targets. These authors note,

> "The **S**pike protein was a target of human SARS-CoV-2 CD8+ T cell responses, but it is not dominant. SARS-CoV-2 **M** was just as strongly recognized, and significant reactivity was noted for other antigens, mostly NSP6, ORF3a, and **N**, which comprised nearly 50% of the total CD8+ T cell response, on average. Thus, these data indicate that candidate COVID-19 vaccines endeavoring to elicit CD8+ T cell responses against the **S**pike protein will be eliciting a relatively narrow CD8+ T cell response."

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62 Grifoni A *et al*. Targets of T-Cell responses to SARS-CoV-2 coronavirus in humans with Covid-19 disease and unexposed individuals. Cell 2020; 181: 1489–1501. https://doi.org/10.1016/j.cell.2020.05.015.

63 For the functions of these Covid-19 proteins see Yoshimoto F. The proteins of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS CoV-2 or n-COV19), the cause of Covid-19. Protein J 2020; 39, 198–216. https://doi.org/10.1007/s10930-020-09901-4.

That narrow base of T-cell immunity is a concern for the anti-**S**pike vaccines.

2. Vaccine Testing

Vaccines go through 3 phases of human testing. Phase 1 tests for **safety**; does this injure human volunteers? Phase 2 tests **marker efficacy**; does this stuff cause a measurable immune response? Phase 3 tests **clinical effectiveness**; do vaccinated people still get Covid-19 illness? Realistic Phase 3 testing requires giving the vaccine to a large number (tens of thousands) of volunteers in an environment where they will encounter the virus in order to see what then happens.

It is now clear that the **efficacy** target for most of the vaccines is to induce production of IgG antibody against the Covid-19 **S**pike protein. Phase 3 vaccine testing in older subjects and diabetes patients must be done with substantial caution and patience, and a strong T-cell response is likely to be critical. A really good vaccine takes some time.

With this in mind, the following Figure is a July summary (from Derek Lowe at AAAS Science "In the Pipeline") of Phase 3 primate trial results for several vaccines in the summer of 2020[64]. Note that the SinoVac (and the SinoPharm) vaccine utilized inactivated Covid-19 virus. The Johnson & Johnson and Oxford/AstraZeneca vaccines utilized the **S**pike-nucleotide sequence engineered into adenovirus expression vectors. The Moderna (RNA) and Inovio (DNA) vaccines utilized directed **S**pike expression from nucleotide sequences without viral expression vectors.

	SinoVac (PiCoVacc)	Moderna (mRNA-1273)	J&J (Ad26.COV2.S)	Oxford-AZ (ChAdOx1)	Inovio (INO-4800)
***n* (monkeys dosed)**	4 (each dose)	8 (each dose)	6	6 (each group)	5
Vaccine dosage	Either 3 µg or 6 µg, at days 0, 7, and 14 (i.e. two boosters)	Either 10 µg or 100 µg at Day 0 and again at Day 28	10^{11} viral particles, one dose	2.5 x 10^{10} viral particles, optionally again at 4 weeks	1mg at Day 0 + 1mg at Day 28
Neutralizing antibody response	Could be still rising at week 3	Peak at week 6, stronger with 100 µg	Response at week 2 Higher by week 4	2x to 4x higher in the two-dose group	Low at week 4, peak at week 6, slow decline
T cell response	No change compared to controls in either dosing group	8 week data show almost entirely CD4+ Th1 response, no Th2 and no CD8+	Peak at week 2, but generally low	Detected, but no difference between the two dosing groups	Peak at 6 weeks, memory out to at least 15 weeks. No CD4+/CD8+ data
Time until challenge	3 weeks	8 weeks	6 weeks	4 weeks/8 weeks	17 weeks
Viral challenge ***(Results in rows below are post-challenge)***	7.6x10^5 PFU inoculate, intra-tracheal only	7.6x10^5 PFU inoculate, intranasal/intra-tracheal	1.1x10^4 PFU inoculate, intranasal/intra-tracheal	2x10^6 PFU inoculate, intranasal/intra-tracheal	1.1x10^4 PFU inoculate, intranasal/intra-tracheal
Antibody results	Dose-responsive	Equivalent higher levels to pre-challenge	---	---	>20x increase in 7 days
T cell results	---	---	Basically none (no infection)	Low Th1 response, no Th2	Higher than earlier peak
Nasal PCR (sg mRNA)	Not measured (only full genomic RNA and not in nose)	Day 4: only detectable in 2/8 at 10 µg, 1/8 at 100 µg	One animal low at days 1, 2, others 0	No real difference compared to controls (!)	Day 7: 10^7 in controls, $10^{4.5}$ treated
Lung PCR (sg mRNA)	Not measured (only full genomic RNA). High dose showed 0 at Day 7,	Day 2: BAL only detectable in 1/8 animals in each dosing group	BAL: 0 at all time points	BAL Day 5: none left in either group (controls were 5/6)	BAL Day 2 peak: 10^5 in controls, $10^{3.5}$

Screenshot

Targets: SinoVac — Inactivated Virus
Moderna — RNA-coded Spike
Johnson & Johnson — Adeno-vector Spike
Oxford-AstraZeneca — Adeno-vector Spike
Inovio — DNA-coded Spike

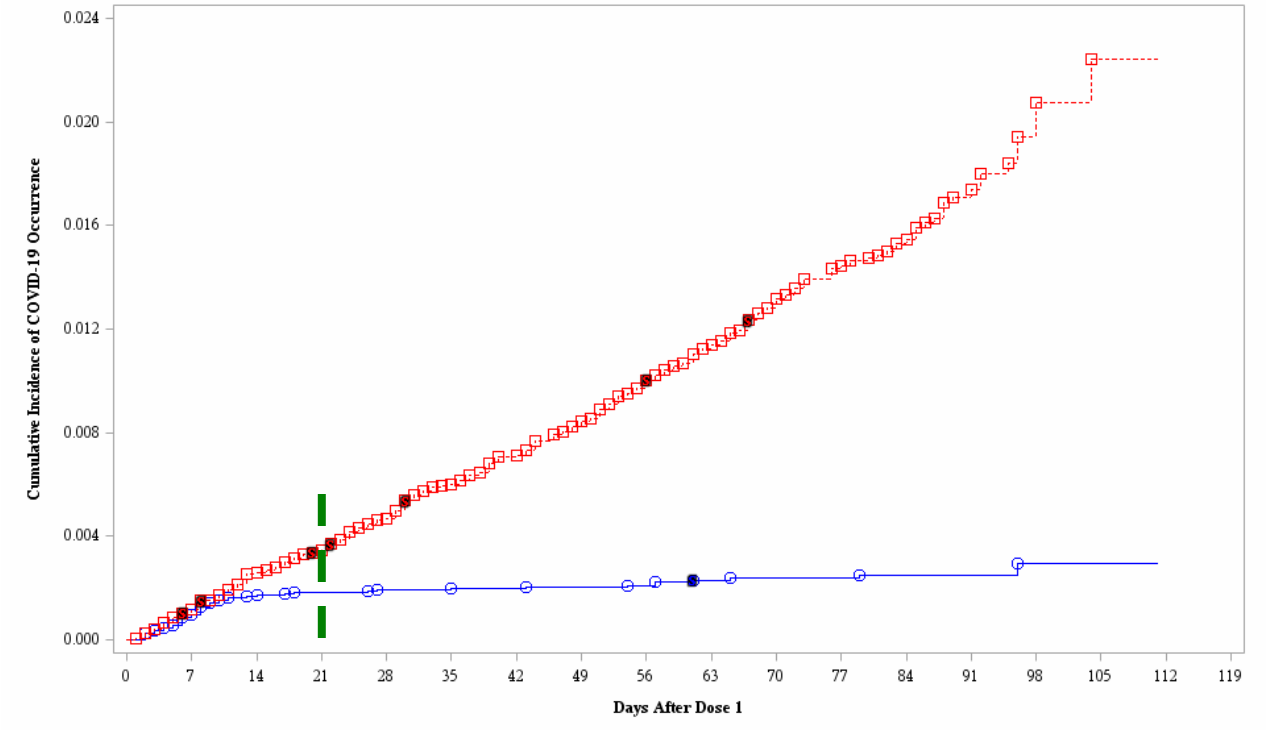
Note: **PFU** = Plaque-Forming Units (measures virus dose), **BAL** = Broncho-alveolar lavage, **J&J** vaccine only needed ONE dose, **Inovio** vaccine did not control Covid viral challenge.

The above is a fairly large amount of data, and we now have more data released from phase-3 human trials. However, there were some specific things to notice. Although the above pre-clinical study of the Inovio DNA-based vaccine showed both some antibody production and T-cell

64 from https://blogs.sciencemag.org/pipeline/archives/2020/07/30/coronavirus-challenges-in-primates-compared

commitment, at a challenge viral dose only 10% of that used with SinoVac, it did not completely control the virus in primate lungs versus unvaccinated controls. The Moderna vaccine also had 1 of 8 animals show persistent lung virus after challenge 8 weeks following vaccination, and it did not report T-cell response. Both the J&J **one shot** vaccine and the Oxford/AstraZeneca vaccine pre-clinical studies showed complete elimination of the virus from the lungs following challenge, although the viral challenge dose for the Oxford/AstraZeneca vaccine was 100-fold greater than that used by J&J. The Oxford/AstraZeneca challenge also produced a recognized T-cell response.

We now have the FDA Phase-3 evaluation (https://www.fda.gov/media/144245/download) of the Pfizer mRNA vaccine showing 95% effectiveness. Here's an impressive part of these results.



Above is Figure 2 from the FDA analysis of the Pfizer vaccine Phase-3 trial (available at https://www.fda.gov/media/144245/download). It shows the incidence of Covid-19 infections in >40,000 trial subjects enrolled 1:1 in the placebo group (red squares) versus the vaccinated group (blue squares) following the first dose at day 0. I have added the dashed green line at day 21 when the second vaccine dose was given. By that point the infection incidence between the 2 groups had already clearly diverged, indicating notable immunity by day 21 after the first dose of vaccine.

Both the Pfizer and Moderna vaccines are now approved for wide clinical use. The Oxford-AstraZeneca adenovirus vaccine is also in UK clinical use and has reported 70% efficacy.

The more recent Novavax vaccine (NVX-CoV2373) utilizes an engineered **S**pike protein produced by recombinant technology in insect cells. Tian *et al.*[65] have reported on the structural properties and stability of the NVX-CoV2373 vaccine and where and why molecular engineering was applied to the **S**pike protein. This paper also shows efficacy of the vaccine (including adjuvant) for inducing antibodies, CD4+ and CD8+ T-cells, and immunity to Covid-19 in both mice and baboons. Phase-3 trial in 15,000 UK subjects showed 90% efficacy against the original virus and little loss of effectiveness against the UK B1.1.7 variant but only 60% efficacy against the South

[65] Tian *et al*. SARS-CoV-2 spike glycoprotein vaccine candidate NVX-CoV2373 immunogenicity in baboons and protection in mice. Nat Commun 2021; 12: doi.org/10.1038/s41467-020-20653-8.

Africa B1.351 variant[66]. Advantages of the NVX-CoV2373 vaccine over the mRNA vaccines include a clearly known antigen dose (amount of **S**pike made from mRNA varies in individuals) and storage stability at ordinary refrigerator temperatures.

With the exception of China's SinoVac and SinoPharm and India's Covaxin, all of these potential vaccines target only the **S**pike protein. All of the Spike-only vaccines produce a narrower immunologic response than infection or vaccination with inactivated virus, and it is reasonable to predict C19 variants will more easily become immune-resistant to Spike-only vaccines.The SinoVac, SinoPharm, and Covaxin vaccines instead utilize inactivated whole SARS-CoV-2 virus, and as expected they produce a more diversified antibody and T-cell immune response against a wider range of antigenic epitopes[67]. In the Sinovac and Covaxin[68] primate trials, when the animals were challenged with Covid-19 three weeks after the booster, no virus was found in the lungs 7 days after the infectious challenge. The human trial data is also promising for both. For SinoVac[69]; immune response, including both antibodies against multiple viral proteins and CTL T-cells, was present 28-days post-vaccination.

Bharat is a major vaccine producer in India and has formulated Covaxin with an adjuvant specifically developed to stimulate CD4+ TH1 (cytotoxic) response. In its Phase-1 human trial Covaxin demonstrated (1) safety, (2) induction of antibodies recognizing both **S**pike and **N**ucleocapsid proteins and neutralizing 3 Covid-19 variants, (3) induction of a predominant CD4+ TH1 response, and (4) induction of CD8+ memory CTLs[70]. A Phase-2 trial of this vaccine demonstrated similar results, and samples again taken from Phase-1 participants 104 days post vaccination found continuing high levels of neutralizing antibodies[71]. Covaxin has been given an EUA for general use in India while it continues to be studied in a Phase-3 clinical trial.

There is also more information on the SinoPharm vaccine. I had to look up to remember how antibody titers are determined and both how and why "antibody geometric mean titers" are calculated in order to obtain from many patients a mean humoral immunologic response to an infection or vaccination. To determine an antibody titer on a single blood sample, the serum is diluted in serial ratios (1:2, 1:4, 1:8, 1:16....), and each dilution is tested for a detectable level of antibody. The titer value is the last dilution in which the antibody was detected. For instance, if the antibody was detected in each of the above serum dilutions but was not detected in a 1:32 dilution, then the titer is 16. The available titer values are neither on a continuous number line nor are they normally distributed. Thus a simple arithmetic mean is inappropriate, and a "geometric mean" is calculated instead. The geometric mean is obtained by multiplying all the titers (A x B x C x …) and then taking the N^{th} root of the product, where N is the total number of patients' serum samples. So for a group N of patients' antibody titers, $\sqrt[N]{A_x B_x C_x \ldots}$ is the "antibody geometric mean titer," and this will be a central number of the distribution of titers – but probably not exactly one of the titer

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66 Taylor N. Novavax COVID-19 vaccine 90% efficacious in phase 3, but protection plummets against one variant. Fierce Biotech 2021; https://www.fiercebiotech.com/biotech/novavax-covid-19-vaccine-90-efficacious-phase-3-but-protection-plummets-against-one-variant.

67 Ganneru B *et al*. Evaluation of safety and immunogenicity of an adjuvanted, TH-1 skewed, whole virion inactivatedSARS-CoV-2 vaccine - BBV152. BioRxiv 2020; https://doi.org/10.1101/2020.09.09.285445.

68 Yadav P *et al*. Remarkable immunogenicity and protective efficacy of BBV152, an inactivated SARS-CoV-2 vaccine in rhesus macaques. ResearchSquare 2020; https://doi.org/10.21203/rs.3.rs-65715/v1.

69 Pu J *et al*. An in-depth investigation of the safety and immunogenicity of an inactivated SARS-CoV-2 vaccine. MedRxiv 2020; https://doi.org/10.1101/2020.09.27.20189548.

70 Ella R *et al*. Safety and immunogenicity of an inactivated SARS-CoV-2 vaccine, BBV152: a double-blind, randomised, phase 1 trial. Lancet Infect Dis 2021; https://doi.org/10.1016/S1473-3099(20)30942-7.

71 Ella R *et al*. Safety and immunogenicity clinical trial of an inactivated SARS-CoV-2 vaccine, BBV152 (a phase 2, double-blind, randomised controlled trial) and the persistence of immune responses from a phase 1 follow-up report. MedRxiv 2020; https://doi.org/10.1101/2020.12.21.20248643.

dilutions. I don't even want to think about the mathematical gyrations to calculate a p-value for significant difference between "antibody geometric mean titers," but rather will just trust the published p-values.

BBIBP-CorV is the name of the SinoPharm vaccine that contains inactivated whole SARS-CoV-2 virus. Phase 1 & 2 trial results were published on 10/15/2020 by Xia *et al.*[72] A particularly important aspect of this work is that it focused some attention on patients more than 60 years old. The phase 1 trial enrolled 1,192 patients aged 18-80 years old; they received vaccine injections of (a) placebo, or (b) 2 μg, or (c) 4 μg, or (d) 8 μg on both day 1 and day 28. There were no serious adverse reactions. All serum samples after placebo-treatment remained negative for neutralizing antibody. Neutralizing antibody titers in serum samples were determined for the SARS-CoV-2 virus growing in cultured primate cells. For the following data, **NAGMT** = **N**eutralizing **A**ntibody **G**eometric **M**ean **T**iter. The 2 μg vaccine dose produced a weaker and slower immunologic response with neutralizing antibody only consistently present in the 60-80 years old group after 42 days following vaccination, and this dose was dropped from the Phase 2 trial. The 4 and 8 μg doses generated a 100% neutralizing antibody response in 60-80 year old subjects by 28 days post vaccination.

Age	2 μg vaccine 42 day **NAGMT**	4 μg vaccine 42 day **NAGMT**	8 μg vaccine 42 day **NAGMT**
18-59 years	87.7	211.2	228.7
60-80 years	80.7	131.5	170.9

I note that because the underlying data for the above NAGMT determinations is actually the last virus-neutralizing serum dilution, for both age groups that dilution is 1:64 at the 2 μg dose and 1:128 for both age groups at the 4 and 8 μg doses. The authors note that at 42 days a virus-neutralizing antibody response was present in 100% of all vaccinated Phase-1 participants,

The phase 2 trial enrolled 2,448 subjects to further examine safety and immunologic response to giving a single vaccine dose of 8 μg or 2 doses of 4 μg with the second dose on either day 14, 21, or 28. On day 28 post-vaccine, the best response (NAGMT = 282.7 - thus basic titer 1:256) was seen with two 4 μg vaccine doses given 21 days apart. There were no serious adverse reactions.

Phase-3 trials with BBIBP-CorV are complete in Brazil, Bahrain, and the United Arab Emirates. UAE and Bahrain have announced it has 86% efficacy and approved it for general use. UAE Prime Minister Sheikh Mohammed bin Rashid Al Maktoum was vaccinated on November 3. SinoPharm's chairman Liu announced (https://www.globaltimes.cn/content/1206008.shtml) November 14 that this vaccine has been administered to over 1-million people in China on an Emergency Use basis, without serious adverse events. I'd still like to see the T-cell responses against the epitopes identified in post-infection by Grifoni et al[62]. But this vaccine looks to be safe and effective and engage a much wider range of viral molecular targets. That should make it very difficult for the virus to escape the induced immunity.

I am concerned that none of the animal or human phase-3 vaccine reports have examined glycosylation of the induced antibodies in diabetic or older patients. The reports we have for Phase-3 efficacy of anti-Spike vaccines, including the RNA vaccines from Pfizer and Moderna and the vector-directed AstraZeneca vaccine all are encouraging and suggest wide use will break the cycle of pandemic infection. However, all these show in the vaccine groups a few subjects who

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[72] Xia S *et al*. Safety and immunogenicity of an inactivated SARS-CoV-2 vaccine, BBIBP-CorV: A randomised, double-blind, placebo-controlled, phase 1/2 trial. Lancet 2020; https://doi.org/10.1016/S1473-3099(20)30831-8.

subsequently develop SARS-CoV-2 infections, and we won't know how that turns out until we see those infections in older patients at risk for hyperinflamatory IgG glycosylation.

Before we move on from vaccines, I want to review another approach suggested by MIT[73] and being pursued in France by Gauttier *et al*[74] that specifically targets a robust, diversified, and long-lasting memory CD8 T-cell response and could be very promising. These French authors bring to this effort their experience developing multi-epitope immunotherapy targeting lung cancer. They utilized an established vaccine platform for optimization of HLA-restricted peptides and 60 **conserved** (among circulating Covid-19 strains) small peptide sequences derived from 11 viral proteins (including **S**, **M**, and **N**). They confirmed binding of these immunization complexes to HLA expressing human cells and then used these complexes to test-immunize transgenic human-HLA-expressing mice with a single subcutaneous injection. This generated immune-positive memory CD8 T-cells (CTLs) against antigenic epitopes. They went on to utilize their antigenic epitopes to challenge mononuclear white cells from recovered Covid-19 patients (in comparison to cells from uninfected controls) for production of IFNγ. This identified in recovered Covid-19 patients the presence of 25 CD8 T cell immunodominant epitopes against 3 structural proteins (**S**, **M**, **N**) and 8 other Covid-19 proteins. From these 25 antigens they selected 12 CD8 T cell antigenic epitopes that cover 11 viral proteins and are predicted to bind efficiently to different human populations' HLA-I alleles (A, B, C) with high genetic coverage in all geographical regions of the world. These 12 T-cell antigenic epitopes combined in a peptide vaccine should induce at least 4 to 5 memory CD8 T-cell responses in each geographical region of the world so as to achieve a long-lasting worldwide 60-70% 'herd immunity' with tissue sentinel T-cells. This vaccine (CoVepiT) is now in Phase-1 human trials.

Diagnostic aspects

This area has been added because I've begun to encounter papers dealing with it. In this first approach to this we're going to look at 2 papers, the first dealing with false-negative RT-PCR (use of Reverse-Transcriptase to make a cDNA from viral RNA followed by Polymerase Chain Reaction to amplify any cDNA from the virus into enough copies for detection). The second paper is about a clinical flowchart developed in Holland to try to standardize and improve diagnosis and accuracy in the ED.

With respect to the diagnostic reliability of RT-PCR, Levine-Tiefenbrun *et al*.[75] presented a paper from Israel, which goes to an important clinical issue I had not considered. To be sure we all have some idea of what RT-PCR is: The test involves using **R**everse **T**ranscriptase (in a common kit) to make a copy-DNA (cDNA) from the viral RNA. Kit manufacturers know the cDNA sequence and include two ~15 nucleotide DNA "primers" that will base-match and hybridize onto the 3' ends of what will be both the primary cDNA and its 1st full-length primed transcript. The cDNA, primers, lots of all 4 nucleotides, and taq-polymerase go into a small (~ ¼ ml) test tube for the **P**olymerase **C**hain **R**eactions (PCR).

> The taq (*Thermos aquaticus*) polymerase is a fun story. A hippy grad student found this heat-resistant enzyme in this bug living in Yellowstone's hot springs and had the imagination to realize one could infinitely copy DNA with this enzyme. They got a PhD, won the Nobel Prize (more than a million $$), made G-d only knows how much off the polymerase, and by rumor devoted their life to surfing!

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73 Liu G *et al*. Predicted cellular immunity population coverage 1: Gaps for SARS-CoV-2 subunit vaccines and their augmentation by compact joint sets. BioRxiv 2020; doi: https://doi.org/10.1101/2020.08.04.200691.

74 Gauttier V *et al*. Tissue-resident memory CD8 T-cell responses elicited by a single injection of a multi-target COVID-19 vaccine. BioRxiv 2020; doi: https://doi.org/10.1101/2020.08.14.240093.

75 Levine-Tiefenbrun M *et al*. Association of COVID-19 RT-qPCR test false-negative rate with patient age, sex and time since diagnosis. MedRxiv 2020; https://doi.org/10.1101/2020.10.30.20222935.

The lab will put our little test tube into an automatic cycling thermal block that will (1) heat it up to 95°C for a few minutes to get all the DNA double-strands to shake apart, (2) cool it off to 55°C for a few minutes so our primer sequences can base-match-hybridize onto the 3' ends of the DNA strands, (3) run it up to the optimum temp for taq-pol at 75°C for a few minutes for taq to extend the hybridized primers into full length double-strand DNAs, and then go back to (1) up to 95° and so on. In theory, only if the cDNA matches our primers, we double our DNA every cycle, and if we run that ~30 cycles in about 5 hours, we get ~2^{30} copies for each strand of cDNA we started with. That's so much it's easy to see by any one of several assays, and it has to be the right length between the two primers. I note from the literature that the number of PCR cycles is being specified (30, 32, 35, etc) to accept negative results because the last few cycles can produce a large majority of the amplified product.

In my mind the beauty of this test is that either that viral RNA is there and makes the right sequence and length cDNA that gets "amplified" - OR NOT. There shouldn't be any false positives, and the specificity is very high; if you have to tell a lover you've got a STD diagnosis from PCR, you've really got it. But the sensitivity is lower, reflecting how essential it is to harvest the viral RNA on the patient's swab; false-negatives can occur, and their rate affects tracing and quarantine strategies.

In the present paper[75], the authors used results of 843,917 RT-PCR tests from 521,696 patients. The overall false-negative rate was 22.8%, but was only 10.7% in the first 5 days when screening to determine contact quarantine. The false-negative rate was higher for women (OR 1.74) and at 20 years old compared to 50 years old (OR 2.54). From this we learn that nasal-pharyngeal viral shedding is high early, and negative tests (especially in young women) require a cautious approach with retesting when they are from symptomatic patients or traced contacts.

In the second paper focused on diagnosis[76], the authors sought a standardized approach to reliable Emergency Department diagnosis of Covid-19 before RT-PCR results were reported in 6 Dutch hospitals. They use a "Corona-Score" plus a chest CT-derived "CO-RADS" score. The "Corona Score" assigns points for **Age** (0 to 2), **Sex** (0 to 1), **CRP** (0 to 3), **Ferritin** (-1 to 3), **LDH** (0 to 3), **Lymphocytes** (0 for ≥1,200 or 1 for <1,200), and **PMNs** (range 0 for <5,100 to minus 4 for >10,400). The CO-RADS score assigns from 0-5 points based on chest CT interpretation on a scale from Normal to Typical Covid-19. The diagnostic Gold Standard remained RT-PCR. They display graphical results obtained from 1,904 patients with 611 positive by RT-PCR and derive cutoff scores and values for sensitivity and specificity.

I was initially quite interested in this report; however, I confess my enthusiasm became inversely related to the amount of time I spent looking at it. Something "feels" wrong. I note the absence of vital signs and O_2 saturation in the evaluation. And I suspect I won't know more from this complicated scoring system than I'll know in a massive pandemic when I see a sick-appearing hypoxic patient with a cough, not really normal chest exam, and lab set suggesting MAS. And marginal calls in the ED nearly always declare themselves with a bit of time – and the result from a RT-PCR. But you still might want to download this paper and have a look as you work diagnoses.

Potential present clinical strategies against Covid-19

1. *Obstruct the virus from entering cells*
   a. Hydroxychloroquine

(Blue is my editorial rant) Donald Trump makes my BS detector scream, and use of this drug has become crazy politicized; however, hydroxychloroquine (HCQ) is not automatic garbage

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[76] Kurstjens *et al*. A chest-CT and clinical chemistry based flowchart for rapid COVID-19 triage at emergency departments – A multicenter study. MedRxiv 2020; https://doi.org/10.1101/2020.10.29.20218743.

just because Trump advocated it. I also want to argue against simply utilizing FDA's or CDC's positions as an automatic intellectual final authority; they have had a large role in the U.S. Covid-19 debacle. That started with rejecting internationally validated Covid-19 tests while we fiddled with developing an "official" USA version - before it had to be withdrawn and replaced, delaying effective testing. Then FDA issued a EUA for HCQ and then withdrew it following what is now a clearly fraudulent negative report[77]. Now FDA has refused Henry Ford's request for authorization to continue using HCQ based on Ford's and others' published reports of effectiveness in clinical studies of over 27,000 patients (below). Furthermore, although U Penn is one of the U.S. top 3 medical research centers, FDA delayed authorization for a cyclosporine study by a U Penn team led by one of the world's top immunotherapists with experience with macrophage activation syndrome (Dr. Carl June, an elected NAS-NAM member). The messes with remdesivir and convalescent plasma looks like further FDA debacles. Dr. Ron Krome, the founding leader of the DRH-WSU Emergency Medicine programs, kept on his desk an engraving that said, "For G-d so loved the world that She didn't send a committee." Even Lister's experience with aseptic surgery and the Royal Society cautions us to remember that big bureaucracies find it difficult to replicate the good that more basic science, clinical experience, and peer-review have done in medicine.

So, beyond my brief rant, quinine was already used in the 17th century to treat malaria, and derivatives such as quinidine, chloroquine, and hydroxychloroquiune (HCQ) were developed in the 19th and 20th centuries. Because of the very prolonged development and multiple applications (antimalarial, antiarhythmic, anti-inflamatory, and anti-viral), these drugs did not go through the rigorous basic or clinical testing associated with modern drug development. Their immuno-modulation was accidentally discovered during anti-malaria prophylaxis of tens-of thousands of troops during WWII, and molecular mechanisms of action remain poorly worked out[78].

Oral HCQ is well absorbed with ~75% bioavailability, a ½-life of ~50 days, and a very large "volume of distribution" indicating extensive cellular absorbtion[78]. The lungs are included among the organs in which HCQ accumulates at ~2-orders-of-magnitude higher concentration than seen in plasma[81]. In cells HCQ accumulates in acidic subcellular compartments including both endosomes and lysosomes, is rapidly protonated, and raises the pH of these compartments[78]. This inhibits virus-endosome fusion, and HCQ has broad spectrum *in vitro* antiviral activity[79], including against Covid-19 replication in Vero cells[80,81]. However, that HCQ inhibition of Covid-19 is largely overcome in Vero cells expressing high levels of TMPRSS-2[82].

HCQ also quiets inappropriate autoimmunity (like Lupus) in part by suppressing autophagocytosis, for which lysosomes are a major component[83]. Autophagosomes can engulf

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77 Mehra MR, Desai SS, Ruschitzka F, and Patel AN. Hydroxychloroquine or chloroquine with or without a macrolide for treatment of COVID-19: A multinational registry analysis. Lancet 2020; S0140-6736(20)31180-6. doi: 10.1016/S0140-6736(20)31180-6.

78 Schrezenmeier E and Dörner T. Mechanisms of action of hydroxychloroquine and chloroquine: Implications for rheumatology. Nature RevIews – Rheumatology 2020; 16:155-166.

79 Devaux CA, *et al.* New insights on the antiviral effects of chloroquine against coronavirus: What to expect for Covid-19? Internat J Antimicrobial Agents 2020, doi.org/10.1016/j.ijantimicag.2020.105938.

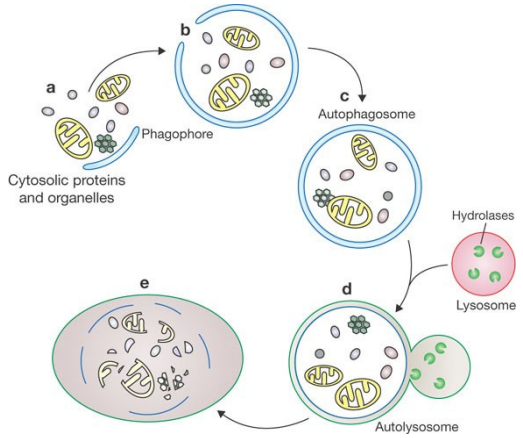
80 Yao X, *et al. In Vitro* antiviral activity and projection of optimized dosing design of hydroxychloroquine for the treatment of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Clin Infect Dis. 2020 Mar 9. pii: ciaa237. doi: 10.1093/cid/ciaa237.

81 Liu J, *et al.* Hydroxychloroquine, a less toxic derivative of chloroquine, is effective in inhibiting SARS-CoV-2 infection *in vitro*. Cell Discovery 2020; 6:16 doi.org/10.1038/s41421-020-0156-0.

82 Hoffmann M *et al.* Chloroquine does not inhibit infection of human lung cells with SARS-CoV-2. Nature 2020; 585:588–590.

83 Eskelinen E *et al.* Seeing is believing: The impact of electron microscopy on autophagy research. Autophagy, 2011; 7: 935-956.

mitochondria, endoplasmic reticulum, and viruses and normally recycle cellular organelles and molecules. The normally acidic lysosome joins the autophagosome and releases proteins that degrade its contents. Increased lysosomal pH caused by HCQ inhibits the joining of lysosomes to autophagosomes[84].



(from Xie and Klionsky[85]) The double-membrane autophagosome encloses a small volume of cytosolic content and is joined by an acidic lysosome releasing proteins that degrade its contents. This process is conserved from yeast up, and starvation is a classic mechanism of activation. Autoimmune disorders such as Lupus demonstrate enhanced autophagocytosis, quieted by HCQ.

HCQ also inhibits the intracellular signaling pathway from the FcγR to reduce induced cytokine production[78], and thus could help down-regulate macrophage activation syndrome.

Although HCQ appears to have no direct action against the virus itself, it does offer both some host-mediated potential antiviral and anti-inflammatory effects. However, the balance of these effects remains unclear, and the very long HCQ ½ life raises unresolved dosing and schedule questions. As I've looked back at the poorly understood details of the drug mechanisms, it is not surprising that reports of clinical results have been widely divergent.

Many scientists and physicians hoped HCQ would be effective, and this was consistent with an early clinical report[86] of more rapid reduction of RT-PCR detectable Covid-19 in 60 patients treated with HCQ. Chen *et al.*[87] also published an early clinical trial involving 62 patients in China with ½ receiving 400 mg HCQ daily and reported better time to clinical recovery and radiologic recovery in the treated group. Subsequently the Henry Ford Hospital system published[88] (with WSU collaborators) an observational study of all 2,541 Covid-19 hospital admissions between March 10 and May 2, 2020. Mortality 28 days following admission was 16.6% for all 1,985 HCQ-treated patients (includes 783 who also got azithromycin) versus 25.4% mortality for 556 patients (includes 147 only azithromycin) without HCQ ($p < 0.001$). This was a 35% reduction of mortality

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84 Mauthe M *et al*. Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. Autophagy 2018, 14:1435–1455.

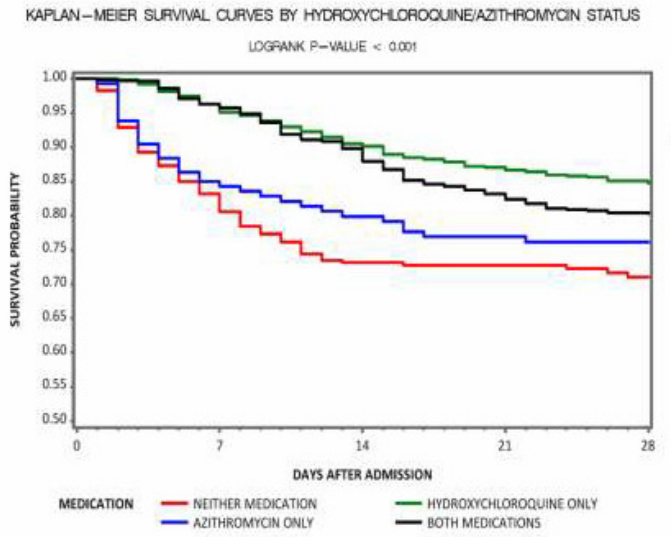
85 Xie Z and Klionsky D. Autophagosome formation: core machinery and adaptations. Nat Cell Biol 2007; 9:1102–1109.

86 Gautret *et al*. Hydroxychloroquine and azithromycin as a treatment of COVID-19: Results of an open□label non□randomized clinical trial. International J Antimicrobial Agents 2020; doi:10.1016/j.ijantimicag.2020.105949.

87 Chen Z et al. Efficacy of hydroxychloroquine in patients with COVID-19: results of a randomized clinical trial. MedRxiv 2020; https://www.medrxiv.org/content/10.1101/2020.03.22.20040758v2.

88 Arshad S *et al.* Treatment with hydroxychloroquine, azithromycin, and combination in patients hospitalized with Covid-19. Internat J Infectious Diseases 2020; https://doi.org/10.1016/j.ijid.2020.06.099.

associated with HCQ. EKG QTc was carefully monitored, and there were no lethal tachycardias. Here are the Kaplan-Meier survival curves from their paper.



The same result favoring HCQ was obtained when risk propensity matching was utilized to construct Kaplan-Meier curves to directly compare HCQ versus no HCQ. The Henry Ford report also noted

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

This large study found early use of HCQ in carefully monitored patients requiring hospital admission to be clinically effective in significantly reducing mortality with no adverse effects of EKG QT prolongation.

A similar result has been published from the New York Icahn SOM/Mt. Sinai Hospital system review of 6,493 Covid-19 patients; these patients had a reduction of Hazard Ratio for death to 0.53 ($p<0.001$) when HCQ was used[89]. An even larger study of 8,075 patients (4,542 with HCQ monotherapy and 3,533 with no HCQ) in Belgium found that HCQ reduced mortality 32%[90]. Another large study of 3,451 Covid-19 patients in Italy has recently also reported a 30% reduction in risk of death in patients treated with HCQ[91]. A prospective cohort study[92] from 238 ambulatory fever clinics in Saudi Arabia has reported results from 5,541 confirmed Covid-19 patients including 1,817 HCQ-treated vs 3,724 supportive care patients, all with 28-day followup. Early HCQ treated patients had lower hospital admission (9.4% vs 16.6%, $p <0.001$) and a 54% reduction ($p = .001$) in a composite outcome of ICU admission and/or death. Still more similar evidence of HCQ real-world

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89 Mikami T *et al*. Risk factors for mortality in patients with Covid-19 in New York City. J Gen Intern Med 2020; DOI: 10.1007/s11606-020-05983-z.

90 Catteau, L et al. Low-dose hydroxychloroquine therapy and mortality in hospitalized patients with COVID-19: A nationwide observational study of 8,075 participants. Internat J Antimicrobial Agents 2020; https://doi.org/10.1016/j.ijantimicag.2020.106144.

91 Castelnuovo A *et al.* Use of hydroxychloroquine in hospitalised COVID-19 patients is associated with reduced mortality: Findings from the observational multicentre Italian CORIST study. European J Internal Medicine 2020; https://doi.org/10.1016/j.ejim.2020.08.019.

92 Sulaiman T *et al*. The effect of early hydroxychloroquine therapy in Covid-19 patients in ambulatory care settings: A nationwide prospective cohort study. MedRxiv 2020; https://doi.org/10.1101/2020.09.09.20184143.

effectiveness has been reported from another large study of 1,645 patients admitted with Covid-19 in Madrid, Spain[93].

The above 6 reports from (1) Detroit - Henry Ford with 2,541 patients, (2) New York with 6,493 patients, (3) Belgium with 8,075 patients, (4) Italy with 3,451 patients, (5) Saudi Arabia with 5,541 patients, and (6) Madrid with 1,645 patients present a total of 27,746 patients from clinical studies reporting improved outcome with HCQ and emphasizing that it should be used early in the course of Covid-19 infection. Although each of the above reports can be criticized, it is not credible to ignore real-world experience on that many patients by calling it only "anecdotal evidence." And Risch[94] has recently reviewed extensive data on HCQ safety for outpatients.

Still we need to take the negative reports on HCQ treatment into consideration. I've already dismissed the study from Mehra *et al*[77] for what is now a widely recognized fraudulent database. Those same authors also retracted another article published by NEJM.

Substantial public attention was garnered by the announcement to news media on June 5 of results indicating no HCQ efficacy in the Oxford RECOVERY RCT with no significant difference in 28-day mortality in 1,542 HCQ patients compared with 3,132 similar patients without HCQ. That data didn't appear in a preprint[95] until July 15, and the study has been criticized on the preprint server for (1) an excess of patients over 70 in the HCQ group, and (2) their use of unusually high doses of HCQ that might be acutely immunosuppressive. This paper (with different group numbers from the preprint) was finally published by NEJM in October and is somewhat unconvincing. However, the World Health Organization SOLIDARITY randomized trial of HCQ, remdesivir, interferon-β, and Lopinavir in 11,266 patients (including 4,088 no-drug controls) found that **none of the study drugs** reduced either requirement for ventilation or mortality[96]. Moreover treatment with HCQ late in the course of advanced Covid-19 is ineffective[97], and a double-blind, placebo-controlled RCT (821 subjects) from Minneapolis found no evidence that HCQ prevented development of infection in subjects with exposure to Covid-19[98].

Million *et al*.[99] (same group as ref[86]) have published a (weak) meta-analysis suggesting HCQ efficacy. They also used public databases of non-HCQ pharmaceutical companies' support (more than 50,000-Euros) of authors of the negative studies to identify potential conflicts of interest. So this is a mess, and this area of research appears to have been significantly contaminated by both political orientation and massive business interests. A look at the clinical experience through the lens of what is known about the drug's mechanisms of action suggests it is ineffective as an antiviral but might blunt development of MAS.

So we've seen the disparity between negative RCTs and the observations of ~30% reduction of mortality by HCQ in often much larger "real world" cohort studies. Very recently Castelnuovo *et*

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93 Bernaola N *et al*. Observational study of the efficiency of treatments in patients hospitalized with Covid-19 in Madrid. MedRxiv 2020; https://doi.org/10.1101/2020.07.17.20155960.

94 Risch HA. Early outpatient treatment of symptomatic, high-risk Covid-19 patients that should be ramped-up immediately as key to the pandemic crisis. Am J Epidemiol 2020; doi: 10.1093/aje/kwaa093.

95 Horby P *et al*. Effect of hydroxychloroquine in hospitalized patients with COVID-19: Preliminary results from a multi-centre, randomized, controlled trial. MedRxiv 2020; doi: https://doi.org/10.1101/2020.07.15.20151852.

96 Pan H *et al*. Repurposed antiviral drugs for COVID-19 – interim WHO SOLIDARITY trial results. MedRxiv 2020; https://doi.org/10.1101/2020.10.15.20209817.

97 Geleris J et al. Observational study of hydroxychloroquine in hospitalized patients with Covid-19. NEJM 2020; DOI: 10.1056/NEJMoa2012410.

98 Boulware D *et al*. A randomized trial of hydroxychloroquine as postexposure prophylaxis for Covid-19. NEJM 2020; DOI: 10.1056/NEJMoa2016638.

99 Million M *et al*. Clinical efficacy of chloroquine derivatives in Covid-19 infection: Comparative metaanalysis between the Big data and the real world. New Microbes and New Infections 2020; https://doi.org/10.1016/j.nmni.2020.100709.

al.[100] have utilized an impressive meta-analysis to engage this head-on using data on 44,521 patients from 26 published studies, including 7,324 patients from 4 RCTs. Overall HCQ used for no more than 5 days at a dose ≤ 400 mg/day with a total dose ≤ 4,400 mg was associated with a 21% reduction of mortality. The authors recognize this as a "low dose" approach with HCQ, and the improvement in mortality vanished above these dosing limits. HCQ use was not associated with either increased or decreased mortality in COVID-19 patients when only the 4 RCTs were evaluated; **however, none of the 4 RCTs were in the low dose category**. In particular the RECOVERY and the SOLIDARITY RCTs used 800 mg/day for 9 or 10 days with a total dose of 9,200 or 10,000 mg of HCQ.

Through our review of mechanisms, we have tentatively concluded that HCQ is ineffective as an antiviral in normal cells expressing the TMPRSS2 protease, but may act by its well-known immunomodulation effects against the onset of MAS. With a ½ life of ~50 days, the present meta-analysis suggests use of high-dose HCQ and HCQ treatment longer than 5 days is ill-advised in Covid-19 patients. Its use in late stage infections in critical patients with full blown MAS is not helpful. Until the 5th revision I kept 5-days of low-dose HCQ in the suggested early outpatient protocol at the end. However, a recent multicenter randomized control trial by Elgazzar *et al.*[101] reported much better results in ivermectin groups than with HCQ. That and the mixed results with HCQ have convinced me we don't need it. We'll look at those results in the ivermectin section.

b. ACE2-Fc

As discussed above, the slow course of coevolution has coronaviruses including Covid-19 committed to and stuck with their spike docking with ACE2 as their receptor on cells. This has led to the idea of administering a recombinant docking peptide derived from ACE2 to saturate the coronavirus spike and thereby prevent it from docking on cells[102]. Monteil *et al.*[103] recently reported from the prestigious Karolinska Institute that recombinant ACE2 can block Covid-19 infection in Vero cells and also in human vascular and kidney cells. There has also already been a Phase II clinical trial of recombinant ACE2 for ARDS[104] not involving CoV infections; the recombinant peptide was well tolerated but did not improve oxygenation. So we have the recombinant ACE2 peptide and evidence it can block Covid-19 infection, and it is well tolerated upon systemic administration. Furthermore, the affinity of recombinant ACE2 for the spike protein is ~1 nM (10^{-9} Molar), which is similar to the affinity of monoclonal antibodies[102]. Apeiron Biologics used unmodified ACE2 in a phase 2 clinical trial that completed recruiting but has not reported by 3/1/2021 (https://www.pharmiweb.com/press-release/2020-12-04/apeiron-biologics-patient-recruitment-completed-in-phase-ii-covid-19-clinical-trial-of-apn01).

Our bodies know not to destroy Fc-containing antibody molecules quickly, and fusing an Fc to the recombinant ACE2 substantially extends its circulating ½ life; in mice this fusion extends the ½ life from ~2 hours to over 1 week. An ACE2-Fc fusion still neutralizes SARS-CoV *in vitro* with an affinity in the 1 nM range[102]. Furthermore, the effector functions of the Fc domain can be retained in this chimerical molecule, allowing recruitment against viral particles of dendritic cells, macrophages, and natural killer cells through the CD16 receptor on Fc. We know the fusion

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100 Castelnuovo A *et al*. Low dose hydroxychloroquine is associated with lower mortality in COVID-19: A meta-analysis of 26 studies and 44,521 patients. MedRxiv 2020; https://doi.org/10.1101/2020.11.01.20223958.

101 Elgazzar A *et al*. Efficacy and safety of ivermectin for treatment and prophylaxis of Covid-19 Pandemic. ResearchSquare 2020; https://doi.org/10.21203/rs.3.rs-100956/v2.

102 Kruse R. Therapeutic strategies in an outbreak scenario to treat the novel coronavirus originating in Wuhan, China. F1000Res. 2020; 9:72. doi: 10.12688/f1000research.22211.2.

103 Monteil V *et al*. Inhibition of SARS-CoV-2 infections in engineered human tissues using clinical-grade soluble human ACE2. DOI: 10.1016/j.cell.2020.04.004.

104 Khan A *et al*. A pilot clinical trial of recombinant human angiotensin-converting enzyme 2 in acute respiratory distress syndrome. Crit Care 2017; 21, 234. doi: 10.1186/s13054-017-1823-x.

chimera doesn't kill mice from the ½ life study. The recombinant ACE2-Fc fusion uses all human proteins, so there's no reason it should elicit an immune response itself. The virus can't just mutate the spike and escape because it's committed to using ACE2 as a receptor. U-Texas-Galveston has worked on this approach with a biotech company, but again at a year into the pandemic this approach has not produced reported clinical results.

2. Target the viral RNA-dependent-RNA-polymerase (RdRP)

Among animals, only primitive eukaryotic nematodes utilize RdRp (for regulatory RNA silencing)[105]; other animals appear to have discarded the genes for RdRp enzymes. Thus, the coronavirus RdRp is a tempting target with no human analog to worry about. This approach has been historically difficult with limited clinical effectiveness against HIV and Ebola, but the pharmaceutical firm Gilead hopes this time might be different.

Remdesivir was developed in the Ebola epidemic and interferes with viral RdRps. It has shown efficacy against the MERS coronavirus in mouse models[102]. Remdesivir is an RNA nucleotide analog with *in vitro* antiviral activity against a diverse panel of RNA viruses such as Ebola, Marburg, MERS-CoV, SARS-CoV, respiratory syncytial virus, and Hendra virus. The mechanism of remdesivir action is competition with the **A**denosine nucleotide for incorporation into RNA transcripts produced from the viral genome by the RdRp[106]. The incorporation of **R**emdesivir in nascent viral RNA instead of an **A** makes garbage and causes premature termination of viral RNA transcripts. Computer molecular modeling predicts that remdesivir is also likely to interact not only with the RdRp but also to form a stable interaction in an active site groove of Nsp1[107]. This suggests patients treated with remdesivir should be studied for possible recovery of interferon levels.

Remdesivir was most effective against the aggressive coronaviruses in animal experiments when it was given early well before viral titers reached their peak[108]. Although remdesivir showed laboratory promise against Ebola, it failed to improve mortality in a clinical trial. Similarly, a Phase 2 Chinese clinical trial during their Covid-19 epidemic appears to have been terminated because other treatments were more effective[108].

Nevertheless, Remdesivir was again tried against Covid-19. Initially encouraging results were published from an open uncontrolled compassionate-trial in Covid-19 ICU patients[109]. Among 53 critically sick patients, 30 were on ventilators and another 4 were even beyond that on extracorporeal membrane oxygenators (ECMO). Based on other contemporary reports, such as from Seattle[110], we would expect at least ~70% mortality in this group of patients; New York has had >80% mortality in such patients. Instead at the time of the report after treatment with Remdesivir, only 7 patients (13%) had died, 57% of the ventilator patients had been successfully

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105 Pinzón N *et al*. Functional lability of RNA-dependent RNA polymerases in animals. PLOS Genetics 2020; doi: 10.1371/journal.pgen.1007915,

106 Zhang L, and Zhou R. Binding Mechanism of Remdesivir to SARS-CoV-2 RNA Dependent RNA Polymerase. Preprints **2020**, 2020030267. doi: 10.20944/preprints202003.0267.v1.

107 Sharma A *et al*. Computational search for potential covid-19 drugs from FDA-approved drugs and small molecules of natural origin identifies several anti-virals and plant products. ChemRxiv 2020; doi.org/10.26434/chemrxiv.12091356.v1.

108 Cao Y *et al.* Remdesivir for severe acute respiratory syndrome coronavirus 2 causing COVID-19: An evaluation of the evidence. Travel Med Infectious Dis https://doi.org/10.1016/j.tmaid.2020.101647.

109 Grein J *et al.* Compassionate Use of Remdesivir for Patients with Severe Covid-19. NEJM 2020; DOI: 10.1056/NEJMoa2007016.

110 Bhatraju PK *et al*. Covid-19 in critically ill patients in the Seattle region — Case Series. NEJM 2020; DOI: 10.1056/NEJMoa2004500.

extubated, and 25 patients had so far been discharged. Beigel *et al.*[111] reported much more modest results from a prospective, placebo-controlled, double-blind RCT of remdesivir in 1,059 hospitalized Covid-19 patients. At 14 days after treatment initiation, mortality with placebo was 11.9% versus 7.1 % with remdesivir (not statistically significant). Furthermore, of the total of 469 SICK patients (246 placebo and 223 remdesivir) requiring either hi-flow oxygen, non-invasive mechanical ventilation, invasive ventilation, or ECMO, ~50% in both treatment groups had recovered within 29 days after remdesivir initiation, and the recovery curves over the 29 days are essentially identical. In this large RCT, remdesivir showed no significant effect on overall mortality or on the rate of recovery in critically ill Covid-19 patients. Last August, Spinner *et al.*[112] reported another RCT of remdesivir in a total of 596 patients with no evidence of an effect on mortality, and most recently this October WHO reported RCT results in which remdesivir had no effect on mortality or disease progression to requiring ventilation[96]. None of the above clinical reports included studies of viral load, so there is no established clinical evidence of antiviral activity by remdesivir. In spite of FDA approval of remdesivir to treat Covid-19, 3 large clinical trials show it to be INEFFECTIVE, and WHO has withdrawn recommendation for its use.

Another important potential suggested by computed molecular modeling is that ivermectin may also obstruct the Covid-19 RdRp[113] with a very strong ΔG[114] of **-**135.2 kJ/mol for ivermectin binding in the RdRp active site. Because ivermectin may interact with at least 3 components involved in Covid-19 infection, we will discuss it separately below.

3. Altering interaction of viral proteins with our proteins: Nsp1, our immunophilins, transplant immunosuppressants, and steroids

a. Cyclosporine and the Macrophage Activation Syndrome (MAS)

In this review I was initially surprised to learn that Nsp1 interaction with our protein cyclophilins is necessary for efficient replication of Covid-19[5,8,15,31]. A brief story behind that is that in 1997 our research group was working to understand failure of protein synthesis in vulnerable neurons during brain reperfusion after resuscitation from cardiac arrest. As part of that we presented 2 key papers[115,116] in Copenhagen at the 1997 meeting of the Society for Cerebral Blood Flow and Metabolism. Those papers showed (1) the very early activation of the calpain-1 protease and (2) identified and immunohistochemically mapped the molecular alteration [eIF2(αP)] in translation initiation that obstructed reperfused neurons' protein synthesis. At that meeting another group presented evidence that treatment with FK-506 during reperfusion facilitated restoration of neuronal protein synthesis. That led us to go literature digging, and we learned that there are a group of proteins that bind FK-506 (*aka* tacrolimus); inevitably they were called FKBPs and are now known to be part of the group of immunophilins (including cyclophilins that are bound by

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111 Beigel J *et al*. Remdesivir for the Treatment of Covid-19 — Preliminary Report. NEJM 2020; DOI: 10.1056/NEJMoa2007764.

112 Spinner C *et al*. Effect of Remdesivir vs standard care on clinical status at 11 days in patients with moderate Covid-19: A randomized clinical trial. JAMA. 2020; doi:10.1001/jama.2020.16349.

113 Sen-Gupta PS *et al*. Binding mechanism and structural insights into the identified protein target of Covid-19 with *In-Vitro* effective drug ivermectin. ChemRxiv 2020; doi.org/10.26434/chemrxiv.12463946.v1.

114 Briefly, ΔG (Gibbs Constant) is a measurement of entropy in a chemical reaction. The 2nd law of thermodynamics says all chemical reactions are driven by an increase in entropy. This large negative ΔG for the interaction of ivermectin with the RdRp predicts it is an avid interaction with a tight binding affinity.

115 Neumar R *et al*. Eukaryotic initiation factor 4G is degraded by calpain during complete global brain ischemia. J Cereb Blood Flow Metab 1997; 17 (Suppl 1):S48.

116 DeGracia D *et al*. Ser-51 phosphorylation of eukaryotic initiation factor 2α during early post-ischemic brain reperfusion. J Cereb Blood Flow Metab 1997; 17 (Suppl 1):S49.

cyclosporine-A). FKBP-12 interacted with tacrolimus to inhibit loss of the normal high calcium concentration in the endoplasmic reticulum (ER). By 2001 we had discovered that it was persistent ER calcium loss into neuronal cytoplasm that caused PERK activation[117] producing eIF2αP that shut off normal protein synthesis. So we had met the FKBPs and cyclophilins.

Now it's been discovered that Nsp1 from coronavirus (1) binds to cyclophilins, (2) obstructs interferon production, and that (3) binding of Nsp1 to cyclophilins is essential for efficient Covid-19 replication[5-8,31]. Here we've seen the cytokine and lymphocyte data from SICK Covid-19 patients can be linked to a failed interferon response for innate and adaptive immunity. Furthermore, that loss of interferon response appears linked to the Nsp1 RNAse destroying cellular mRNAs[13,31]. That line of evidence makes this old DRH ER-doc want to "Fix-Up" Nsp1. Here's a list of relevant facts:

1. Covid-19 infection suppresses production of IFNs in patients[29,30].
2. CoVs' Nsp1 has RNAse activity that selectively destroys cellular mRNAs[13].
3. CoVs' Nsp1 blocks synthesis of cellular but not viral proteins[13].
4. CoVs' Nsp1 binds cyclophilin-A[5,6],and both cyclophilin-A[118] and Nsp1[31] are essential for CoVs replication.
5. SARS-CoV with deleted Nsp1 induces accumulation of IFN-β mRNA and production of IFN-β protein[31].
6. Cyclosporine binds cyclophilin-A as a competitive inhibitor[119].
7. Cyclosporine inhibits coronavirus replication[5,8,31].
8. Cyclosporine in CoV-infected human alveolar epithelial cells and the lungs of infected mice induces IFN response and inhibits viral replication[120].
9. CoV (SARS, MERS, and Covid-19) deaths appear suppressed in transplant patients on cyclosporine or FK506[121].

This looks like cyclosporine can knock Nsp1 off cyclophilin-A and thereby restore Type-1 IFN synthesis, inhibit viral replication, and stop the Severe-Adult-Respiratory-Syndrome.

Of course the main use of cyclosporine-A has been to suppress transplant rejection through its interactions with the immunophilins. But it's now also clear that cyclosporine-A can obstruct, "the replication of CoVs of all genera[8]." A recent helpful review[122] from Zurich, Switzerland summarizes preclinical studies demonstrating anti-coronavirus activity of cyclosporine and (less studied) tacrolimus and point out that both these drugs inhibit transplant rejection by inhibiting calcineurin and its subsequent transcriptional signaling pathways. However, as they note, suppression of coronaviruses by cyclosporine does not involve calcineurin[123]. At the time of writing these authors were not aware of the cyclosporine clinical trials in Madrid or the effects of cyclosporine on cellular eIF2α(P) (below). Nevertheless, the references here provide convenient

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117 Kumar R *et al*. Brain ischemia and reperfusion activates the eukaryotic initiation factor 2α kinase, PERK. J Neurochem 2001; 77:1418-1421.

118 Von Brunn A *et al*. Genetic deficiency and polymorphisms of cyclophilin A reveal its essential role for Human Coronavirus 229E replication. Current Opinion Virol 2015, 14:56–61. doi.org/10.1016/j.coviro.2015.08.004.

119 Ke H *et al*. Crystal structures of cyclophilin A complexed with cyclosporin A and N-methyl-4-[(E)-2-butenyl]-4,4-dimethylthreonine cyclosporin A. Structure 1994, 2:33-44.

120 Sauerhering L *et al*. Cyclophilin inhibitors restrict middle east respiratory syndrome coronavirus via interferon λ *in vitro* and in mice. Eur Respir J 2020; in press (https://doi.org/10.1183/13993003.01826-2019).

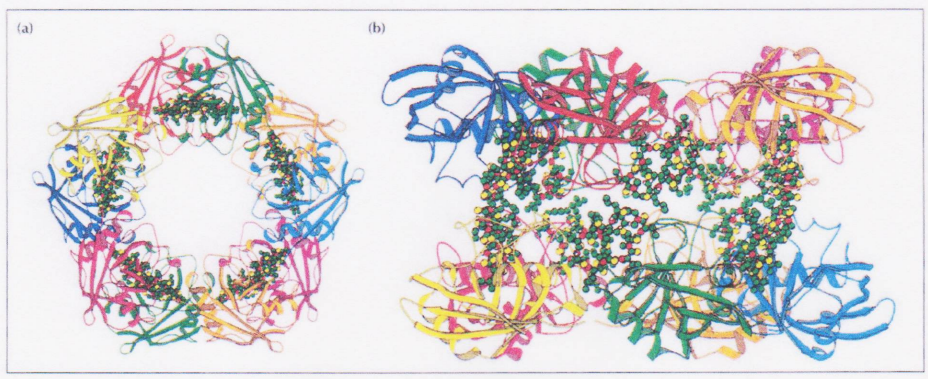
121 D'Antiga L. Coronaviruses and immunosuppressed patients. The facts during the third epidemic. Liver Transpl. 2020; doi:10.1002/LT.25756.

122 Hage R *et al*. Transplant drugs against SARS, MERS and COVID-19. Transplantology 2020, 1:71–84. doi:10.3390/transplantology1020007.

123 Tanaka Y *et al.* Suppression of coronavirus replication by cyclophilin inhibitors. Viruses 2013; 5:1250-1260. doi:10.3390/v5051250.

access to the published evidence for cyclosporine inhibition of both coronaviruses and the cytokine storm of MAS.

The idea that cyclosporine-A is a competitive inhibitor of Nsp1 binding to cyclophilin-A is important to this approach, and the X-ray crystallography of cyclosporine-A bound to cyclophilin-A[119] supports this idea. Cyclosporine-A is an 11-nitrogen peptide ring, and it sits right in the middle of cyclophilin-A, binding with an affinity tighter than 1 nM. The crystallographic images follow.



X-ray crystallography of the non-covalent bonding of cyclosporine-A to cyclophilin-A. The ribbon structure is cyclophilin-A, and the colored balls are the individual atoms of cyclosporine-A sitting in the middle of the cyclophilin protein. The left-hand view is down the central axis of the two non-covalently bonded molecules, and the right-hand view is planar. We can see why this very tight competitive binding of cyclosporine-A to the cyclophilin will exclude Nsp1.

With respect to the antiviral effect of cyclosporine, D'Antiga published (from the Lombardy epicenter of Italy's Covid-19 pandemic) a review of historical information regarding transplant immunosuppression and coronavirus infections in the SARS, MERS, and current Covid-19 epidemics. He wrote[121],

> "Coronaviruses have not shown to cause a more severe disease in immunosuppressed patients. For this family of viruses the host adaptive immune response appears the main driver of lung tissue damage during infection. More importantly, reviewing the mortality and morbidity reports published on Coronaviruses outbreaks such as Severe Acute Respiratory Syndrome (SARS) that emerged in 2002, Middle East Respiratory Syndrome (MERS, still ongoing) and more recently COVID-19, no fatality was reported in patients undergoing transplantation, chemotherapy or other immunosuppressive treatments."

Although cyclosporine now looks promising in Covid-19, recent experience does not fully support the above historical review of its effect on mortality. Tacrolimus is not prophylactic against Covid-19 infection in patients[124]. Cyclosporine isn't either, although an early Covid-19 case series in Spanish transplant patients observed the first suggestion of its effect on reducing mortality[125]. Among 29 kidney transplant recipients with Covid-19, there was a 50% mortality in 6 who had their immunosuppressant (tacrolimus or cyclosporine) reduced, but only 12.5% mortality in 23 patients continuing the usual dose of cyclosporine.

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124 Banerjee D *et al.* COVID-19 infection in kidney transplant recipients. Kidney Internat 2020; https://doi.org/10.1016/j.kint.2020.03.018.

125 Montagud-Marrahi E *et al*. Preliminary data on outcomes of SARS-CoV-2 infection in a Spanish single centre cohort of kidney recipients. Am J Transplant . 2020; 26:1-3. doi:10.1111/ajt.15970.

In addition to its antiviral activity, cyclosporine-A likely also has an important role to play in sick Covid-19 patients with respect to the "cytokine storm" from Macrophage Activation Syndrome (MAS). Hematologists already have experience with an essentially identical clinical disaster that has been recognized in some adults after other virus infections (*i.e*. Epstein-Barr, herpes simplex, cytomegalovirus, and avian influenza). It is called "Secondary Hemophagocytic Lymphohistiocytosis" or sHLH[126].

The syndrome sHLH[126] "is a rare, life-threatening, hematologic disorder with clinical findings of extreme inflammation and unregulated immune activation." It is characterized by[126,127]:

Fevers	Cytopenias	Elevated liver enzymes
Hypofibrinogenemia	Hyperferritinemia	Elevated IL-2
Elevated IL-6	Elevated TNF	Elevated LDH
Elevated D-dimer	ARDS	

Given their role in reticuloendothelial iron signaling, CD163-expressing macrophages are implicated as the source of ferritin[128]. All together the above Table for sHLH is pretty much a description of Covid-19 with MAS. Without intervention this is lethal.

Hematologists have had enough experience with MAS in sHLH that they have worked out a consensus treatment protocol[127] of corticosteroids, cyclosporine-A, and anakinra[129] (a recombinant peptide interleukin 1 receptor antagonist). Indeed, the approach using corticosteroids, cyclosporine A, and anakinra in adult patients with sHLH can produce up to 88% survival[129]. Huet *et al*.[130] have reported encouraging results in a cohort study of anakinra for hospitalized Covid-19 patients with reduced oxygenation, and it is in ongoing clinical trials.

So we've seen a case for cyclosporine as an antiviral inhibiting CoV Nsp1 and its effects. But what is cyclosporine doing to macrophages and MAS? It's been known for some time that macrophages and other cells of the mononuclear phagocyte system bind more cyclosporine than lymphocytes do, and post-transcriptional macrophage production of the cytokines IL6, IL-1β, and TNF-α is reduced by cyclosporine[131]. Very recently it's been discovered that cyclosporine induces activation of both PERK and GCN2 resulting in increase of eIF2(αP)[132]. Ribosomal bypass-scanning of an uORF induced by increased eIF2(αP) is known to increase production of ATF4 to help resolve the problem of unfolded proteins in the endoplasmic reticulum[11]. Furthermore, SOCS1 (**S**uppressor of **C**ytokine **S**ignalling) mRNA level is relatively stable, but its translation is generally suppressed; it has a 5'-leader with 2 uORFs – suggesting production of SOCS1 by ribosomal bypass-scanning in an environment of increased eIF2(αP)[12]. Indeed, just such a mechanism

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126 Kleynberg R, and Schiller G. Secondary Hemophagocytic Lymphohistiocytosis in Adults: An Update on Diagnosis and Therapy. Clin Adv Hematol Onc 2012; 10:726-732.

127 Rose´e P *et al.* Recommendations for the management of hemophagocytic lymphohistiocytosis in adults. Blood 2019; 133:2465-2477.

128 Moore J and June C. Cytokine release syndrome in severe COVID-19. Science 2020; 368:473-4.

129 Kumar B *et al*. A personalized diagnostic and treatment approach for macrophage activation syndrome and secondary Hemophagocytic Lymphohistiocytosis in adults. J Clin Immunol. 2017; 37:638-643. doi: 10.1007/s10875-017-0439-x.

130 Huet T *et al*. Anakinra for severe forms of COVID-19: a cohort study. Lancet Rheumatol 2020; 2: e393–400. https://doi.org/10.1016/ S2665-9913(20)30164-8.

131 Losa-Garcia J *et al*. Cyclosporin A decreases human macrophage interleukin-6 synthesis at post-transcriptional level. Mediators of Inflammation 1999; 8:253–259.

132 Fedele A *et al.* Cyclosporin A but not FK506 activates the integrated stress response in human cells. J Biol Chem 2020; in press, doi/10.1074/jbc.RA120.014531.

upregulates translation of a stable isoform of SOCS3 in an environment of increased eIF2(αP)[133]. These observations suggest that cyclosporine is causing in macrophages an increase of eIF2(αP) with an associated inhibition of cytokine translation at the same time that synthesis of SOCS1 and SOCS3 is increased by ribosomal bypass-scanning. This is a fairly comprehensive set of anti-inflammatory molecular events.

Our usual in-the-box thinking about immunosuppression meant it took some *kahones* to use cyclosporine-A in Covid-19. But the basic science, experience with transplant patients, and experience with sHLH are compelling. The transplant evidence[121,125] that cyclosporine induced resistance to coronaviruses has been joined by evidence from Heili-Frades *et al.*[134], who studied 2,739 hospitalized patients in Spain, of whom 548 died.

	Number of Patients	Number of Deaths	Percent Mortality
Cyclosporine Rx	1,053	165	15.7%
No Cyclosporine	1,686	383	22.7%

Overall, treatment of admitted Covid-19 patients with cyclosporine was associated with a 31% reduction of mortality ($p = 0.0004$). Furthermore, in the 976 hospitalized 51-70 y.o. patients, cyclosporine cut the odds of death in half. This Spanish group is now conducting a cyclosporine RCT (NCT04392531). In October 2020 a different group in Madrid reported data from 607 severe Covid-19 patients[135] with a median age of 69 years and an overall 23.2% mortality. In 253 cyclosporine-treated patients mortality was reduced to 14.2% ($p < 0.001$) versus 29.7% mortality in the 354 patients without cyclosporine. This cyclosporine-associated 52% reduction of mortality is consistent with the results of Heili-Frades *et al.* in 51-70 y.o. patients.

Another recent report compared treatment of moderate/severe Covid-19 using cyclosporine plus low-dose steroids versus steroids only[136]. This study included 209 PCR-confirmed Covid-19 patients who were 18 to 85 years old with O_2 saturation <90% on room air, respiratory frequency ≥ 30 per minute, and $Pa0_2/FiO_2$ less than 400. Cyclosporine plus low-dose steroids were used to treat 105 patients versus only low-dose steroids in 104 patients. Mortality was 22% with cyclosporine and 35% without ($p<0.001$).

The above 3 cohort studies have now reported on 3,555 moderate/severe Covid-19 patients among whom cyclosporine treatment is consistently associated with a near 50% reduction of mortality. A U.S. cyclosporine RCT (NCT04412785) led by Dr. Carl June (a member of the National Academy of Medicine and the Richard W. Vague Professor in Immunotherapy at the University of Pennsylvania) was reportedly delayed by FDA, but now appears to have started recruiting patients on October 23. Moreover, Novartis is now funding a clinical trial (led by Dr. Bryan Burt, chief of thoracic surgery in the Michael E. DeBakey Department of Surgery at Baylor)

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133 Sasaki A *et al*. The N-terminal truncated isoform of SOCS3 translated from an alternative initiation AUG codon under stress conditions is stable due to the lack of a major ubiquitination site, Lys-6. J Biol Chem 2003; 278: 2432–2436.

134 Heili-Frades S *et al*. COVID-19 outcomes in 4712 consecutively confirmed SARS-CoV2 cases in the city of Madrid. MedRxiv 2020; doi.org/10.1101/2020.05.22.20109850.

135 Guisado-Vasco P *et al*. Clinical characteristics and outcomes among hospitalized adults with severe COVID-19 admitted to a tertiary medical center and receiving antiviral, antimalarials, glucocorticoids, or immunomodulation with tocilizumab or cyclosporine: A retrospective observational study (COQUIMA cohort). EClinicalMedicine 2020; https://doi.org/10.1016/j.eclinm.2020.100591.

136 Galvez-Romero J *et al*. Cyclosporine A plus low-dose steroid treatment in COVID-19 improves clinical outcomes in patients with moderate to severe disease. A pilot study. J Intern Med 2020; doi: 10.1111/JOIM.13223.

of cyclosporine to prevent disease progression in hospitalized (not ICU) Covid-19 patients at Baylor U (St. Luke's) and Harvard (Brigham and Women's)[137].

b. Steroids

Clinical reports also indicate steroids may also have an important role in reducing mortality from Covid-19. Salton *et al.*[138] reported their Italian experience using an 8-day course of methylprednisolone (MP) in Covid-19 patients with bilateral lung infiltrates, ARDS, and PAO_2/FIO_2 less than 250 (for example 90/0.5 = 180 for 50% inspired O_2). They studied 83 patients in the MP-treated group and 90 standard-care control patients. At 28-days after study entry, mortality was 7.2% with MP treatment versus 23.3% in the control group ($p<.005$). The duration of viral shedding assayed by RT-PCR was not different between the groups. Similarly, the UK RECOVERY Collaborative Group[139] reported a randomized, controlled, open-label trial of 10-day treatment with 6 mg dexamethasone daily in 2,104 patients versus 4,321 control standard care patients. The overall 28-day mortality between the treated (21.6%) and control (24.6%) groups was not significantly different. However, in patients requiring mechanical ventilation, dexamethasone reduced 28-day mortality from 40.7% in controls to 29% in treated patients ($p<.001$). Taken together these studies indicate that use of steroids is warranted in Covid-19 patients who reach a stage of substantial difficulty in oxygenation. However, the data from the UK RECOVERY study argues that steroid use in lower acuity stages of the disease does not confer any survival benefit.

4. Surprise molecules: Gotta love your grapes and Pepcid!

Ivermectin and niclosamide can both be obtained from grapes by methanol extraction[140], but they are structurally dissimilar and appear to have very different mechanisms of antiviral activity.

a. Ivermectin

Ivermectin is what people use to worm horses, and it has similar human clinical uses against parasites and fungi. So I was initially surprised when a spring 2020 Google coronavirus search turned up a report from Australia that ivermectin efficiently kills Covid-19 in cultured Vero cells[141]!!

In the late 1960s, Satoshi Ōmura (Tokyo's Kitasako Institute) was hunting for new antibacterial compounds in thousands of soil samples from around Japan. He cultured bacteria from the samples and sent them to William Campbell at Merck's New Jersey research labs, who tested their effect against parasitic worms affecting livestock. One culture from a golf course southwest of Tokyo contained a new species, *Streptomyces avermictilis*, that was remarkably effective against parasitic worms. Merck modified avermectin, the active molecule, to ivermectin to increase its activity and safety. In 1981 ivermectin was marketed and became a top-selling veterinary drug. Campbell urged his colleagues to study ivermectin as a potential treatment for onchocerciasis (river blindness) caused by fly-transmitted worms that left millions of Africans blind. Since ivermectin was approved for human use in 1987, more than 3.7 billion doses have been used in successful campaigns against onchocerciasis and lymphatic filariasis (and head lice). Ōmura and Campbell won the Nobel Prize for physiology and medicine in 2015[142].

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137 https://www.bcm.edu/news/study-to-determine-cyclosporines-role-in-treating-hospitalized-covid-19-patients

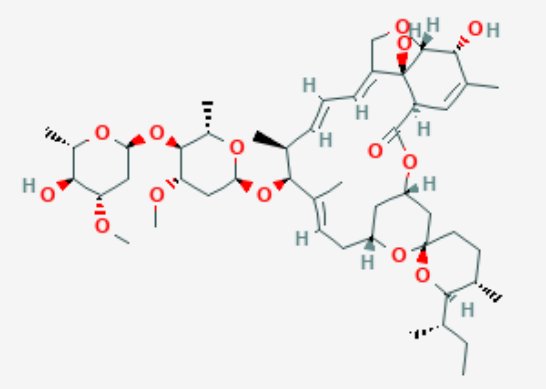
138 Salton F *et al*. Prolonged low-dose methylprednisolone in patients with severe COVID-19 pneumonia. MedRxiv 2020; doi.org/10.1101/2020.06.17.20134031.

139 RECOVERY Collaborative Group. Effect of dexamethasone in hospitalized patients with Covid-19 – Preliminary Report. MedRxiv 2020; doi.org/10.1101/2020.06.22.20137273.

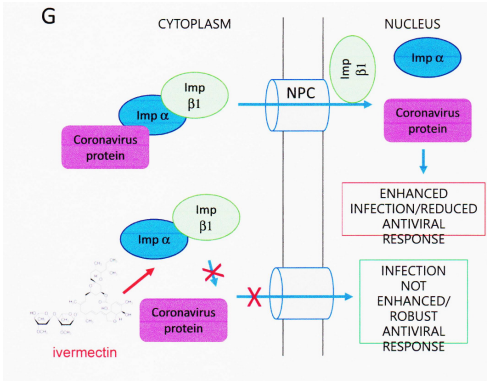
140 Gholami-Ahangaran M *et al*. *In-vitro* anti-leech effects of *Vitis vinifera*, Niclosamide and Ivermectin on mature and immature forms of leech *Limnatis nilotica*. Global Veterinaria 2012; 8: 229-232.

141 Caly L *et al*. The FDA-approved Drug Ivermectin inhibits the replication of SARS-CoV-2 *in vitro*. Antiviral Research 2020; https://doi.org/10.1016/j.antiviral.2020.104787.

142 Wikipedia.



The structure of ivermectin (above) is relatively complex, and it is very difficult to synthesize *de novo*. Thus most ivermectin still starts out as avermectin made by *S avermictilis*. Ivermectin appears to bind at least 3 targets related to activity of the Covid-19 virus. Even at sub-nanoMolar concentrations (1) it binds and inhibits viral dsRNA-helicases[113,143]. Furthermore, (2) as previously noted[113], molecular modeling predicts ivermectin binds in the active site of the Covid-19 RdRp with a large negative ΔG that again suggests affinity in the nanoMolar range. Finally, (3) ivermectin dissociates our importin α/β1 heterodimer (IMPα/β1)[144]. The Covid-19 nucleocapsid protein (**N**) contains nuclear transport signals that would require the IMPα/β1 heterodimer in the nuclear pore complex (NPC) of our nuclear membrane as a shuttle between our cytoplasm and the nucleus[145]. The unimpeded nucleo-cytoplasmic transport of **N** by our IMPα/β1 could reduce the ability of our nuclei to deliver appropriate transcripts to ribosomes from our DNA for antiviral defenses. Here's a picture from Caly *et al*[141].



Further insight into these antiviral features of ivermectin may arise from consideration of the molecule's biological evolution. In other words, why on earth did *S avermictilis* develop avermectin? Production of avermectin by *S avermitilis* is a complex biochemical process involving 4 major steps and a cluster of several genes[146], so avermectin reflects a complex evolutionary development. ***S avermitilis* appears to use avermectin to compete with nematodes for food resources**[147]. It was a couple of weeks after learning this "factoid" that I remembered that the nematodes are the only

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143 Mastrangelo E *et al.* Ivermectin is a potent inhibitor of flavivirus replication specifically targeting NS3 helicase activity: new prospects for an old drug. J Antimicrob Chemother 2012; 67: 1884–1894.

144 Yang S *et al*. The broad spectrum antiviral ivermectin targets the host nuclear transport importin α/β1 heterodimer. Antiviral Res 2020; 2:104760. doi: 10.1016/j.antiviral.2020.104760.

145 Wahyu W *et al*. Nucleocytoplasmic transport of nucleocapsid proteins of enveloped RNA viruses. Frontiers in Microbiology 2015; 6: doi: 10.3389/fmicb.2015.00553.

146 Chemistry & Biology 2001; 8:681-700.

147 https://microbewiki.kenyon.edu/index.php/Streptomyces_avermitilis.

remaining animals that express an RNA-dependent-RNA-Polymerase (RdRp)[105]. Could it be that this molecule developed in nature to poison a general protein configuration involved with dsRNA synthesis and processing – like RdRp and dsRNA helicase? Even the IMPα/β1 dimer is frequently involved in transport of chaperoned RNA complexes across the nuclear membrane. This speculative argument suggests ivermectin could be to RNA viruses what penicillin is to bacterial cell walls. Indeed, Heidary and Gharebaghi published a substantial review this May of the broad-spectrum antiviral activity of ivermectin[148].

Caly *et al.*[141] showed ivermectin at a concentration of 2-5 μM (2-5 x 10^{-6} M) given 2 hours after Covid-19 infection of Vero cells caused a 5,000-fold reduction of Covid-19 replication after 48 hours. This is a large effect, and the nanoMolar (10^{-9}) binding affinities for the drug's molecular targets suggest still important inhibition of the virus may occur at lower ivermectin concentrations. Nevertheless, some critics have suggested it's impossible to reach an effective ivermectin concentration in the lung with an oral dose of ivermectin. Lung concentration of ivermectin in animals is about triple the plasma concentration[149,150], so a usual oral dose of 0.2 mg/kg → ~0.25 μM in lung with a lung ½-life of ~72 hours. In fact FDA human safety trials found a much larger dose of ~1.5 mg/kg p.o. was without adverse effect[151]. But the theoretical argument suggesting such a large dose might be the required *in vivo* to kill coronavirus now appears moot. Arévalo *et al.*[152] have produced direct experimental evidence that an ivermectin dose of 0.5 mg/kg kills coronavirus *in vivo*. Moreover, Jans and Wagstaff[153] (who developed hard-science evidence for ivermectin inhibiting IMPα/β1) have recently reviewed both the mechanism of action and *in vivo* evidence of ivermectin antiviral effectiveness at doses between 0.2 and 0.4 mg/kg. They argue it is "naive to believe that it is necessary to achieve μM concentrations of ivermectin in a patient for maximum clinical benefit." Those observations strengthens confidence in the following clinical reports of ivermectin efficacy in doses between 0.2 and 0.4 mg/kg.

There are now reports from several clinical trials of ivermectin for Covid-19, and more are underway. A group of pulmonology intensivists from the multi-hospital Broward Health System have reported an IRB-approved cohort study[154] of 280 consecutive hospitalized Covid-19 patients, of which 173 were treated with ivermectin (0.2 mg/kg) and 107 were usual care. Severe pulmonary status at study entry was characterized as need for either $FIO_2 \geq 50\%$, or noninvasive or invasive mechanical ventilation. At the time of analysis, all patients in the study cohort had met the endpoints of death, discharge alive, or awaiting transfer to a skilled facility. Results were

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148 Heidary F and Gharebaghi R. Ivermectin: a systematic review from antiviral effects to COVID-19 complementary regimen. Springer Nature J Antibiotics 2020; https://doi.org/10.1038/s41429-020-0336-z.

149 Chiu SH and Lu AY. Metabolism and tissue residues. Ivermectin and Abamectin. (Campbell WC, ed.) 1989; New York, NY: Springer-Verlag, 131–143.

150 Lespine A, *et al*. Influence of the route of administration on efficacy and tissue distribution of ivermectin in goat. Vet Parasitol 2005; 128: 251–260.

151 Guzzo C *et al*. Safety, tolerability, and pharmacokinetics of escalating high doses of ivermectin in healthy adult subjects. J Clin Pharmacol 2002; 42:1122–1133.

152 Arévalo A *et al.* Ivermectin reduces coronavirus infection *in vivo*: a mouse experimental model. BioRxiv 2020; https://doi.org/10.1101/2020.11.02.363242.

153 Jans D and Wagstaff K. The broad spectrum host-directed agent ivermectin as an antiviral for SARS-CoV-2? Biochem Biophys Research Comm 2020; doi: https://doi.org/10.1016/j.bbrc.2020.10.042.

154 Rajter J *et al*. ICON (Ivermectin in Covid Nineteen) study: Use of ivermectin is associated with lower mortality in hospitalized patients with Covid19. Chest 2020; MedRxiv preprint manuscript at doi.org/10.1101/2020.06.06.20124461.

Study Parameter	Ivermectin	Usual Care	P value
1. Overall mortality	15 %	25.2 %	p = 0.03
2. Propensity-matched mortality	12.4%	25.8%	P = 0.02
3. Severe pulmonary mortality	38.8 %	80.7 %	p = 0.001
4. Successful extubation	36.1 %	15.4 %	p = 0.07

Another clinical study from Kahn *et al*.[155] reported 248 hospitalized PCR-confirmed adult Covid-19 patients, of whom 115 were treated at admission with 12 mg ivermectin and 133 received otherwise standard care. Mean initial O_2 saturation was normal in both groups, and about 50% of patients in both groups had lymphopenia, elevated D-dimer (coagulopathy), and C-reactive protein (inflammation). Among the standard care patients 9 died versus one death among the ivermectin patients ($p< 0.05$). During hospitalization 45.9% of standard care patients required supplemental oxygen versus 9.6% of those treated with ivermectin ($p < 0.001$). Median time to negative PCR was 4 days with ivermectin versus 15 days with only standard care ($p < 0.001$).

In November 2020 Niaee *et al*.[156] reported a double-blind RCT of 4 different ivermectin dosing protocols versus standard-care only and standard-care plus placebo for inpatients with Covid-19 confirmed by RT-PCR and CT. The 4 ivermectin dosing protocols were (1) single dose 200 micro-gm/kg, (2) 200 micro-g/kg on days 1, 3, & 5, (3) single dose 400 micro-gm/kg, and (4) 400 micro-gm/kg day 1, and 200 micro-gm/kg days 3 & 5. Admission O_2 saturation was similar in all 6 groups (mean 89%). All the ivermectin dosing schedules were well tolerated. With 45 days follow-up, mortality in the standard-care and standard-care with placebo groups combined (60 patients) was 18.3%. Mortality in all 120 ivermectin-treated patients was 3.3%, with little difference between the dosage schedules. In this RCT a large (~80%) mortality reduction ($p < 0.001$) was seen with ivermectin treatment.

That is consistent with the results reported this December by Elgazzar A *et al.*[101] from a multicenter RCT of either ivermectin or HCQ for treatment of RT-PCR-confirmed Covid-19 classified as either mild/moderate or severe. Severe Covid-19 was defined by patients who met any of 5 criteria including (1) Respiratory rate more than 30/min, (2) Oxygen saturation of less than 93%, (3) PaO2/FiO2 less than 200, (4) Lung infiltrates >50% of lung fields, or (5) Requirement for high flow oxygen or noninvasive or invasive mechanical ventilation. All patients received a Standard Care protocol of Azithromycin 500mg OD for 6 days, Paracetamol 500mg PRN, vitamin C 1gm QD, Zinc 50 mg QD, Lactoferrin 100mg BID, Acetylcystein 200mg QD, and therapeutic anticoagulation if D-dimer > 1000.

Two hundred patients were enrolled in each of the 2 severity classifications and randomized 1:1 to either (A) 4 days treatment with **ivermectin 0.4 mg/kg daily** or (B) **HCQ 400 mg every 12 hours for one day followed by 200 mg every 12 hours for 5 days**. This produced 4 treatment groups of 100 patients each, and the groups were well matched for age, sex, and comorbidities. Significant improvements in clinical evaluation, laboratory parameters, duration of hospitalization, and **mortality (p<0.001)** were seen with ivermectin treatment.

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155 Kahn M *et al*. Ivermectin treatment may improve the prognosis of patients with Covid-19. Arch Bronconeumologia 2020; https://doi.org/10.1016/j.arbres.2020.08.007.

156 Niaee MS *et al*. Ivermectin as an adjunct treatment for hospitalized adult COVID-19 patients: A randomized multi-center clinical trial. Research Square 2020; https://doi.org/10.21203/rs.3.rs-109670/v1.

Covid-19	Ivermectin Mortality	HCQ Mortality
Mild/Moderate	0	4%
Severe	2%	20%

Morgenstern *et al*.[157] have reported on 2,706 Covid-19 patients treated as outpatients with ivermectin in the Dominican Republic. Among these 2,706, there was only 1 death, one other ICU admission, and 16 inpatient admissions without ICU need. There is no placebo control group here, but this 99.3% effectiveness (2,688/2,706) of ivermectin outpatient treatment without hospital admission in a developing nation suggests a sort of "between the eyes" validity.

Many more ivermectin clinical studies have recently been reviewed by Kory *et al*.[158] A British meta-analysis has been done[159] with a finding of an 83% mortality reduction produced by early outpatient treatment of Covid-19; the analyst concluded the overall data was so strong that further RCTs were unethical and should be suspended. On January 19, 2021 Hill *et al*.[160] reported another meta-analysis (supported by WHO) of 18 international RCTs of ivermectin treatment of Covid-19. The study was cautious in its conclusions and suggested further trials, but reported that

> "This systematic review of 18 RCTs (n=2,282) showed ivermectin treatment reduces inflammatory markers, achieves viral clearance more quickly, and improves survival compared with SOC" *(standard-of-care controls).*

This meta-analysis reported a 75% reduction of mortality (p = 0.0002) with ivermectin treatment. It also noted apparent dosing effects and recommended 0.4 mg/kg daily for 3-5 days. Harvard's Dr. Paul Sax (Infectious Disease) has also commented in NEJM JWATCH on the large reduction of mortality[161], and after a discussion with Hill, Sax noted, "The clinical trials data for ivermectin look stronger than they ever did for hydroxychloroquine."

Two additional studies on clinicaltrials.gov are also reporting good results for ivermectin prophylaxis, although neither is yet available as a manuscript. Results of the Egyptian study "Prophylactic Ivermectin in COVID-19 Contacts" were posted 8/27/2020 to https://clinicaltrials.gov/ct2/show/results/NCT04422561. In 304 subjects with close family contact to known Covid-19 patients, ivermectin prophylaxis was given to 203 on day 1 and day 3, and an additional 101 served as controls. Primary outcome was development of symptoms (fever ,cough, sore throat, myalgia, diarrhea, shortness of breath). During 14 days, symptoms occurred in 7.4% of the ivermectin prophylaxis group and in 58.4% of the control group ($p<0.001$)[162], although RT-PCR results have not been posted. There were no deaths in either group. Similar results have now been posted for the IVERCAR study[163] (NCT04425850) from Argentina, which tested daily oral

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157 Morgenstern J *et al*. Observational study on the use of ivermectin in COVID-19 infections. MedRxiv 2020; https://doi.org/10.1101/2020.10.29.20222505.

158 Kory P *et al*. Review of the emerging evidence demonstrating the efficacy of Ivermectin in the prophylaxis and treatment of Covid-19. 2021; https://covid19criticalcare.com/wp-content/uploads/2020/11/FLCCC-Ivermectin-in-the-prophylaxis-and-treatment-of-COVID-19.pdf.

159 Lawrie T. Ivermectin reduces the risk of death from COVID-19 – a rapid review and meta-analysis…. Evidence-Based Media Consultancy Ltd 2021.

160 Hill A *et al*. Preliminary meta-analysis of randomized trials of ivermectin to treat SARS-CoV-2 infection. ResearchSquare 2021; DOI: 10.21203/rs.3.rs-148845/v1.

161 Sax P. Ivermectin for Covid-19 — Breakthrough treatment or hydroxychloroquine redux?. NEJM 2021; https://www.jwatch.org/fw117381/2021/01/04/paul-sax-ivermectin-covid-19-breakthrough-treatment-or.

162 https://www.trialsitenews.com/zagazig-university-randomized-controlled-ivermectin-study-results-confirms-pi-hypothesis-drug-effective-against-covid-19/.

163 https://clinicaltrials.gov/ct2/show/results/NCT04425850.

ivermectin drops and a carrageenan nasal spray for prophylaxis in medical personnel caring for Covid-19 patients in public hospitals. The investigators recruited 131 treated subjects and another 98 serving as controls, and they reported that within 28 days 11 of the controls became infected (RT-PCR) versus zero in the treated group (chi-squared p <0.0001).

Thus considerable evidence of ivermectin effectiveness has accumulated, and ivermectin appears to give substantially better clinical results than remdesivir. In addition to the Broward Health System, intensivists at Eastern Virginia Medical School (Norfolk) have also added ivermectin to their protocol for managing Covid-19 in both outpatients and inpatients[164], as has the Baylor University team. On 1/14/2021 NIH withdrew recommendation against ivermectin use, and there are reports that ivermectin is now being widely used for outpatient treatment in Texas.

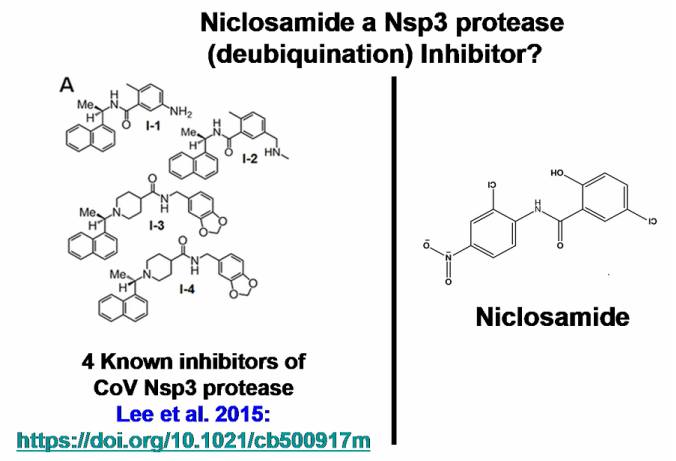
b. Niclosamide

According to Dr. Brian O'Neil, Detroit Receiving Hospital is part of a clinical trial of niclosamide to treat Covid-19. Niclosamide is an oral medication used to treat tapeworm infestations, including diphyllobothriasis, hymenolepiasis, and taeniasis; it is not effective against other worms such as pinworms or roundworms[165].

According to Chen *et al.*[166], niclosamide was discovered in the Bayer chemotherapy research laboratories in 1953 and originally developed to kill the snail host of schistosomiasis (liver flukes). In 1960, scientists at Bayer discovered it was effective against human tapeworm infection, and it was approved by the FDA for use in humans in 1982 and has been used to safely treat millions of patients. However, niclosamide was withdrawn by Bayer from the U.S. market in 1996 because of environmental toxicity.

For such a widely-used drug, niclosamide's mechanism of action is poorly understood. It is thought its anti-helminth actions are produced by uncoupling Ox-Phos in the parasites. In the past several years, mounting evidence has accumulated that niclosamide is a multifunctional drug able to regulate multiple biological processes, and it is again being studied for application as chemotherapy against a number of malignancies.

In vitro niclosamide has been effective against coronavirus at a 1 μM concentration[167]. It is structurally unrelated to ivermectin but resembles other known CoV protease inhibitors.



164 https://www.evms.edu/media/evms_public/departments/internal_medicine/EVMS_Critical_Care_COVID-19_Protocol.pdf.

165 Stuart MC, Kouimtzi M, Hill SR (eds.). World Health Organization Model Formulary 2008; pp. 81, 87, 591.

166 Chen W *et al.* Niclosamide: Beyond an antihelminthic drug. Cell Signal 2018; 41: 89–96.

167 Gassen N *et al.* SKP2 attenuates autophagy through Beclin1-ubiquitination and its inhibition reduces MERS-Coronavirus infection. Nat Commun 2019; 10:5770.

I've flipped the niclosamide structure to help us see the homology around the central nitrogen linkage between the rings, so forgive the upside-down and backward chlorides in the molecule.

Nsp3 of CoVs is a large (~200 kDa) multifunctional protein with 8 conserved domains, including the "Papain-like protease" (= PL^{PRO})[168]. The PL^{PRO} activity of Nsp3 actually releases Nsp3 from the initial polyprotein produced by translation of the full-length Covid-19 RNA genome. Another crucial activity of Nsp3-PL^{PRO} is the removal of ubiquitin[168,169], which, as discussed already, is used by infected cells to mark viral proteins (remember DRiPS?) for proteosome degradation and MHC1 presentation of viral protein fragments. PL^{PRO} mutations that remove its ability to bind ubiquitin without inhibiting its proteolytic release of Nsp CoV proteins from the initial polyprotein restore activity of the IFN-β promoter[170]. Thus completely blocking this protease would inhibit processing of the viral polyprotein, facilitate ubiquination of viral proteins, and help recover production of IFN-β.

Applying 1.6 μM niclosamide to infected cells abolishes accumulation of SARS-CoV proteins[171]. Niclosamide is not well absorbed orally, but research at the University of Texas is exploring administration directly to the lungs by nebulizer, and this September this group reported nebulizer-delivered niclosamide inhibited both SARS and Covid-19 viral viability and lethality in mice[172].

c. and famotidine for our heartburn

Borrell[173] is a better story teller than I am. So here it is.

> A globe-trotting infectious disease doctor named Michael Callahan was the first to call attention to the drug (famotidine = Pepsid) in the United States. Callahan is based at Massachusetts General Hospital, has extensive connections in the biodefense world, and has spent time in disease hot zones around the world, including the 2003 outbreak of SARS in Hong Kong. In mid-January, he was in Nanjing working on an avian flu project. As the Covid-19 epidemic began to explode in Wuhan, he followed his Chinese colleagues to the increasingly desperate city.
>
> They got curious about why many of the survivors tended to be poor. In reviewing 6,212 Covid-19 patient records, they noticed that many survivors had been suffering from chronic heartburn and were on famotidine rather than more-expensive omeprazole chosen by wealthier Chinese. Hospitalized Covid-19 patients on famotidine appeared to be dying at a rate of about 14% compared with 27% for those not on the drug, although the analysis was crude and the result was not statistically significant.
>
> But that was enough for Callahan to pursue the issue... with Robert Malone, (who) is part of a classified project called DOMANE, that uses computer simulations, artificial intelligence, and other methods to rapidly identify FDA-approved drugs that can be repurposed against threats such as new viruses. Malone had his eyes on the viral papainlike protease (in Nsp3). To see whether famotidine

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168 Lei J *et al*. Nsp3 of coronaviruses: Structures and functions of a large multi-domain protein. Antiviral Res 2018; 140:58-74.

169 Zhang X and Yap Y. Old drugs as lead compounds for a new disease? Binding analysis of SARS coronavirus main proteinase with HIV, psychotic and parasite drugs. Bioorganic & Medicinal Chemistry 2004; 12: 2517–2521.

170 Bailey-Elkin BA, *et al*. Crystal structure of the Middle East respiratory syndrome coronavirus (MERS-CoV) papain-like protease bound to ubiquitin facilitates targeted disruption of deubiquitinating activity to demonstrate its role in innate immune suppression. J Biol Chem 2014; 289: 34667–34682. doi:10.1074/jbc.M114.609644.

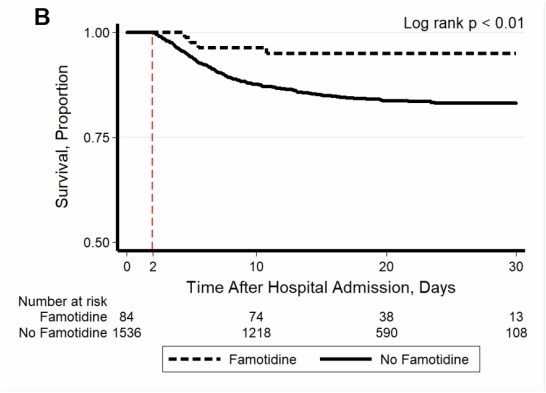
171 Wu C-J *et al*. Inhibition of severe acute respiratory syndrome coronavirus replication by niclosamide. Antimicrobial Agents & Chemotherapy 2004; 48: 2693–2696.

172 Brunaugh A *et al*. Broad-spectrum, patient-adaptable inhaled niclosamide-lysozyme particles are efficacious against coronaviruses in lethal murine infection models. BioRxiv 2020; doi: https://doi.org/10.1101/2020.09.24.310490.

173 Borrell B. New York clinical trial quietly tests heartburn remedy against coronavirus. April 26, 2020. https://www.sciencemag.org/news/2020/04/new-york-clinical-trial-quietly-tests-heartburn-remedy-against-coronavirus.

> binds to the protein, he would ordinarily need the enzyme's 3D structure, not available for months. So Malone recruited computational chemist Joshua Pottel to predict it from crystal structures of the 2003 SARS protease and the new coronavirus' RNA sequence.
>
> Pottel then tested how 2,600 different compounds interact with the new protease. The modeling yielded several dozen promising hits that pharmaceutical chemists and other experts narrowed to three. Famotidine was one.
>
> Callahan began work toward a double-blind randomized study. After getting FDA approval, Northwell (hospital) used its own funds to launch the effort. Just getting half of the needed famotidine in sterile vials took weeks, because the injectable version is not widely used. On 14 April, the U.S. Biomedical Advanced Research and Development Authority (BARDA) awarded a $20.7 million contract to pay Northwell's trial costs.
>
> Timothy Wang, head of gastroenterology at Columbia University Medical Center, saw more hints of famotidine's promise in his own retrospective review of records from 1,620 hospitalized COVID-19 patients. He shared the results with Callahan, and they are co-authors on a paper.

This is not a critical journal club read. Rather, the crucial elements are observation, curiosity, and gathering collaborators to look more deeply even if a first analysis isn't "statistically significant." Remember with a smile Mark Twain saying, "There are liars, damn liars, and statisticians." Statistics are useful tools, especially for studying patients who start out widely distributed. But a very good molecular biologist once reminded me that, "if you understand orbital mechanics and see the sun rise in the east today, you don't need statistics to predict where it will rise tomorrow!" In that approach the trick is to correctly comprehend the underlying causes of what we observe.



An initial clinical study of famotidine in Covid-19 was reported (above)[174]. It included 1,620 hospitalized Covid-19 patients of whom 84 started famotidine with 24 hours of admission. Famotidine significantly reduced mortality (from 22% to 10%, $p<0.01$) and peak ferritin levels (from median 846 ng/ml to 708 ng/ml, $p<0.03$). Proton-pump inhibitors were ineffective, as also indicated by the prior data from China. Mather *et al.*[175] have also reported a retrospective cohort study in hospitalized patients with Covid-19 that observed famotidine was associated with mortality reduction from 26% to 14.5% and also reduction of some inflammatory markers.

A RCT of famotidine for Covid-19 is now underway, although Northwell Medical Center was slowed in recruiting patients by the low level of Covid-19 summer cases in New York. Moreover, new work from Malone *et al.* shows that the Covid-19 Nsp3-PL^{PRO} protease activity is

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174 Freedberg D *et al*. Famotidine use is associated with improved clinical outcomes in hospitalized Covid-19 patients: A propensity score matched retrospective cohort study. Gastroenterology 2020; doi: https://doi.org/10.1053/j.gastro.2020.05.053.

175 Mather J *et al*. Impact of famotidine use on clinical outcomes of hospitalized patients with Covid-19. Am J Gastroenterology 2020; doi: 10.14309/ajg.0000000000000832.

not inhibited by famotidine[176]. Famotidine also did not exhibit any direct antiviral activity against Covid-19 in infected Vero cells. These authors reviewed unique characteristics of famotidine and argued that famotidine's very high affinity for binding host histamine H2 receptors can account for its improvement of Covid-19 clinical outcome. They suggested that Covid-19 lung pathology may be enhanced by Mast-cell degranualation. We have seen evidence above implicating abnormal glycosylation of Fc on anti-**S**pike IgG in induction of MAS and showing that such pro-inflamatory IgG immune complexes can also bind Mast-cell FcγRs and induce histamine release. This connection to the pathophysiology supports the interpretation of famotidine effect advanced by Malone *et al*. Plasma histamine tests are available; thus it seems strange that I cannot find any measurements of histamine levels in coronavirus patients! The antihistamine story is continuing to develop with evidence that azelastine (FDA-approved H1 receptor blocker widely used as a nasal spray for allergic rhinitis) is effective against SARS-CoV-2 infection[177]. Daily treatment with azelastine spray diluted to 1/5th Rx concentration caused a >99.9% inhibition of viral replication in human nasal epithelium at both 48 and 72 hours post-infection.

5. Colchicine

Tatdiff *et al*.[178] have recently reported a double-blind RCT of colchicine 0.5 mg bid for 3 days and then qd for 30 days versus placebo given to 4,159 outpatients with PCR-confirmed Covid-19. The studied endpoints were hospitalization, mechanical ventilation, and death. The odds ratios for colchicine treated patients were 0.75 for hospitalization (93 vs 123), 0.50 for mechanical ventilation (10 vs 20), and 0.56 for death (5 vs 9). Pneumonia developed in 2.9% of those treated with colchicine and 4.3% of controls. Subgroup analysis showed somewhat larger protective effects for diabetic patients.

The stated rationale of this study was to use colchicine to target the NLRP3 inflammasome. NLRP3 protein is predominantly expressed in macrophages and is kept in an inactive state by association with the chaperone protein HSP90, which is a member of the protein-folding machinery that was discovered in the response to heat stress (Heat-Shock Proteins). NLRP3 activation is induced by Fcγ2 receptor activation of the Syk kinase[179], which Hoepel *et al*.[36] showed to be the signaling pathway by which macrophage Fcγ2 receptors activated the hyper-inflamatory response to IgG-**S**pike immune complexes. Active NLRP3 assembly of the "inflamasome" is spatially coordinated by microtubules[180], and the primary mechanism of action of colchicine is inhibition of tubulin polymerization for microtubules in cells[181]. Moreover, the Syk kinase is closely associated with tubulin captured by colchicine linked to agarose, and α-tubulin undergoes site-specific phosphorylation by Syk[182]. So both the Syk kinase and assembly of the NLRP3 inflamasome are closely associated with tubulin, and colchicine appears to inhibit the Covid-19 MAS activation sequence (IgG-**S**pike

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176 Malone R *et al*. Covid-19: Famotidine, histamine, mast cells, and mechanisms. 2020; DOI: https://doi.org/10.21203/rs.3.rs-30934/v1.

177 Konrat R *et al*. The anti-histamine azelastine, identified by computational drug repurposing, inhibits SARS-CoV-2 infection in reconstituted human nasal tissue *in vitro*. BioRxiv 2020; doi https://doi.org/10.1101/2020.09.15.296228.

178 Tardiff JC *et al*. Efficacy of colchicine in non-hospitalized patients with Covid-19. MedRxiv 2021; https://doi.org/10.1101/2021.01.26.21250494.

179 Duffy E *et al*. FcγR mediates TLR2- and Syk-dependent NLRP3 inflammasome activation by inactivated Francisella tularensis LVS immune complexes. J Leukocyte Biol 2016; 100:1335–1347.

180 Misawa T *et al*. Resveratrol inhibits the acetylated α-tubulin-mediated assembly of the NLRP3-inflammasome. International Immunology 2015; 27:425-434.

181 Ying Y *et al*. Colchicine - update on mechanisms of action and therapeutic uses. Semin Arthritis Rheum. 2015; 45:341–350. doi:10.1016/j.semarthrit.2015.06.013.

182 Fernandez J *et al*. Phosphorylation- and activation-independent association of the tyrosine kinase Syk and the tyrosine kinase substrates Cbl and Vav with tubulin in B-Cells. J Biol Chem 1999; 274: 1401-1406.

immune complex→ macrophage Fcγ2 receptor→ Syk kinase→ NLRP3→MAS) by preventing assembly of the NLRP3 inflamasome on tubulin. Although colchicine has no known direct antiviral activity against SARS-CoV-2, it has now been clinically shown to be a useful anti-inflamatory treatment for outpatients to reduce significantly patient deterioration during the infection.

6. Anticoagulation

a. Aspirin

Platelet activation is inhibited by aspirin through its induction of irreversible acetylation of serine-530 on cycloxygenase-1, thus inhibiting platelet production of thromboxane A_2 (TXA_2) that would activate more platelets[183]. We've reviewed compelling evidence of inappropriate platelet activation and autopsy findings of numerous platelet-fibrin microthrombi in the lungs following patients' deaths from Covid-19. This led Manne *et al.*[42] to suggest the use of aspirin as a platelet stabilizing agent, and Chow *et al.*[184] have examined the clinical implications of low-dose (81 mg) aspirin taken by Covid-19 patients before any intubation. They utilized a 4-hospital consortium and 4 months of patient data on 412 Covid-19 inpatients, of whom 98 received aspirin and 314 did not. Of the 98 aspirin patients, 74 were taking it before admission, and this group had enhanced risk factors including higher rates of hypertension, diabetes, coronary artery disease, and renal disease ($p<0.001$). The qSOFA scores at admission were not different between the groups, and the proportions of patients in the aspirin and no-aspirin groups receiving various other treatments were not different. Patients on aspirin had lower progression to mechanical ventilation (35.7% vs 48.4%, $p=0.03$) and ICU admission (38.8% vs 51%, $p=0.04$). When examined by Cox proportional hazards, aspirin patients had a hazard ratio (HR) of 0.56 for mechanical ventilation ($p=0.007$), 0.57 for ICU admission ($p=0.005$), and 0.53 for in-hospital mortality ($p=0.02$). There was no difference in bleeding risk between the 2 groups. Similar data showing the substantial protective effect (>50% reduced mortality) of aspirin has recently emerged from a review of 28,350 Covid-19 patients cared for in the U.S. VA system[185]. Routine aspirin use in Covid-19 appears well justified by the pathophysiology, histopathology, and growing clinical experience.

b. Dipyridamole

Platelet activation can also be inhibited by increased concentrations of adenosine and cyclic AMP, both of which are enhanced by the multi-target activity of dipyridamole (Persantin), which is also a potent radical scavenger[186]. Dipyridamole also stabilizes PMNs, inhibits formation of NETs[187], and has important antiviral properties; at a 500 nM concentration it inhibits the SARS-CoV-2 main-protease (M^{Pro}), and at a 100 nM concentration it blocks *in vitro* replication of the virus[188]. Both those concentrations are well within plasma levels achieved by routine doses of dipyridamole. However, clinical reports are sparse. Liu *et al.*[188] reported on 22 Covid-19 patients with lymphopenia, reduced platelet counts, and bilateral pneumonia by CT. All were treated with

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183 Warner T *et al*. Anti-platelet therapy: cyclo-oxygenase inhibition and the use of aspirin with particular regard to dual anti-platelet therapy. British J Clin Pharmacol 2011; 72:619-633.

184 Chow J *et al*. Aspirin use is associated with decreased mechanical ventilation, ICU admission, and in-hospital mortality in hospitalized patients with COVID-19. Anesthesia Analgesia 2020; ePub Ahead of Print doi: 10.1213/ANE.0000000000005292.

185 Osborne T *et al*. Association of mortality and aspirin prescription for Covid-19 patients at the Veterans Health Administration. MedRxiv 2020; https://doi.org/10.1101/2020.12.13.20248147.

186 Gresele P *et al*. Anti-platelet therapy: Phosphodiesterase inhibitors. British J Clin Pharmacol 2011; 72:634–646.

187 Zuo Y *et al*. Neutrophil extracellular traps (NETs) as markers of disease severity in Covid-19. MedRxiv 2020; https://doi.org/10.1101/2020.04.09.20059626.

188 Liu X *et al*. Therapeutic effects of dipyridamole on Covid-19 patients with coagulation dysfunction. MedRxiv 2020; https://doi.org/10.1101/2020.02.27.20027557.

ribivirin, methylprednisolone, and supplemental O_2, and 12 patients were also treated with dipyridamole 50 mg TID. Dipyridamole was associated with significantly improved D-dimer and lymphocyte and platelet counts; the study was too small to infer significant outcome differences between standard care and dipyridamole-treated patients. Besisik *et al.*[189] also report dipyridamole reduced lab-evidence of coagulopathy (Hazard Ratio = 0.62) and progressive severity (HR=0.12) in a retrospective cohort study of 462 Covid-19 patients. The University of Michigan on May 18 undertook a 100 patient RCT (NCT04391179) of dipyridamole, but has reported no results by the end of January 2021.

c. Heparin and enoxaparin

The evidence is unclear for thrombin inhibitors like heparin or Factor Xa inhibitors like enoxaparin. The pathophysiology does not fit such a coagulation etiology, and I can't find moderately convincing cohort studies or RCTs in which the raw data shows obvious mortality reduction by heparin or enoxaparin. In large series I tend to resist extensive statistical manipulation when basic outcome differences by treatment are grossly modest. Early in the pandemic (March & April) an Italian cohort study of 1,376 Covid-19 patients (596 without enoxaparin) reported in-hospital mortality of 25.5% without and 25% with 40 mg daily enoxaparin[190]. More recently, Rentsch *et al.*[191] reported 30-day mortality in a nationwide VA cohort of 4,297 Covid-19 patients, with 3,627 having received prophylactic heparin (30.2%) or enoxaparin (69.1%) at admission. After 30 days, raw mortality was 14.1% with anticoagulation and 16.3% without; this study found no difference between heparin or enoxaparin. Nobody wants patients to have DVT or PE, but reported mortality differences with systemic anticoagulation are so far very modest, and these mortality differences do not approach the mortality reduction achieved by early use of aspirin.

7. Convalescent plasma

One can't make this stuff up. On August 12 Mayo Clinic published a preprint[192] reporting on non-randomized multicenter convalescent plasma transfusions to 35,322 Covid-19 patients, of whom 52.3% were in ICU and 27.5% were on ventilators. There was no control group not receiving the treatment, and plasma antibody content was unknown at time of transfusion. Overall 30-day mortality was 24.5%, and for patients on ventilators it was 41%. Transfused plasma IgG concentrations were subsequently obtained for only 8.7% (3,082) of the patients (Supplement 2 in[192]), and for that subgroup 30-day mortality was examined as a function of IgG concentrations (Supplement 3[192]) - low (29.6%), medium (27.4%), and high (22.3%). In this subgroup these IgG-stratified 30-day mortality data appear to oscillate near the overall 30-day mortality (24.5%), and I cannot find any IgG-stratified data for patients on ventilators. On August 26 FDA issued an EUA based on this report with the commissioner at a Trump press conference stating that convalescent plasma reduced mortality 35% (my BS detector screams). On September 1, the NIH Covid-19 Treatment Guidelines Panel announced that after review of all available evidence on convalescent plasma treatment, it is not standard care because, "There are currently no data from well-controlled, adequately powered randomized clinical trials that demonstrate the efficacy and safety." Then on

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189 Besisik S *et al*. Dipyridamole added to anticoagulant prophylaxis: Decline in poor outcome of clinically severe ill Covid-19 Patients. Internat Soc Thrombosis Hemostasis Abstract 2021; PBCO01.

190 Albani F *et al*. Thromboprophylaxis with enoxaparin is associated with a lower death rate in patients hospitalized with SARS-CoV-2 infection. A cohort study. EClinicalMedicine 2020; 27:100562.

191 Rentsch C *et al*. Early initiation of prophylactic anticoagulation for prevention of Covid-19 mortality: A nationwide cohort study of hospitalized patients in the United States. MedRxiv 2020; doi.org/10.1101/2020.12.09.20246579.

192 Joyner M *et al*. Effect of convalescent plasma on mortality among hospitalized patients with Covid-19: Initial three-month experience. MedRxiv 2020; https://doi.org/10.1101/2020.08.12.20169359.

September 8, Agarwal *et al.*[193] reported a randomized trial of convalescent plasma in 464 Covid-19 patients requiring oxygen supplement (235 treated and 229 control). The mortality rates were not even close to significantly different (13.6% treated and 14.6% control). Now the New England Journal of Medicine has published (November 24) a RCT of convalescent plasma in 333 patients[194] (228 treaated and 105 placebo); this study concluded, "No significant differences were observed in clinical status or overall mortality between patients treated with convalescent plasma and those who received placebo." Dr. Fauci was kind when he noted that there is little convincing evidence of safety and effectiveness here. See AAAS Science commentary at https://blogs.sciencemag.org/pipeline/archives/2020/08/24/convalescent-plasma-the-science-and-the-politics. This looks like another FDA political debacle.

Furthermore, evaluation of convalescent plasma is considerably more complicated than 3-strata of IgG concentration – like which antibody and what's going on at the Fc end. A collaboration by Dartmouth and Johns Hopkins[195] studied samples from 136 convalescent and 15 naïve control donors. Antibody isotype against **S**, **N**, and the **S**-receptor-binding-domain (**RBD**) and the Fc binding to cellular Fc-receptors were examined. Convalescent samples had variably increased IgG, IgA, and IgM targeting **S** and **N** and to a lesser extent the **RBD**, where neutralization of virus binding would be expected. Donors who had been hospitalized showed higher anti-Covid-19 IgG than less sick subjects. Elevated antibodies against endemic (non-Covid-19) coronavirus epitopes were also found in convalescent donors, suggesting amplification of pre-existing coronavirus immunity. IgG Fc with FcγR binding (remember macrophage activation) was higher in older male donors, consistent with **"the possibility that IgG responses may drive disease enhancement**[195]**."** In fact, virus neutralization is most associated with IgM[196,197], and Covid-19 specific IgM has been associated with reduced mortality[196]. So if we're successfully and safely to use convalescent plasma treatment, we need to know what is in the bag, and so far no one has.

8. Monoclonal antibodies

A monoclonal antibody originates from a single plasma cell that is then expanded into many cells to produce that single antibody; a pathological example of this is multiple myeloma. Scaling up to produce clinically useful amounts of monoclonal antibodies is a major undertaking. President Trump's serious Covid-19 infection was treated in early October with (1) supplemental oxygen, (2) remdesivir, (3) famotidine, (4) continuing low-dose aspirin, (5) dexamethasone, and (6) 8 grams of Regeneron's "antibody cocktail" (REGN-COV2). His doctors were working to kill the virus and quiet the storm.

REGN-COV2 contains 2 monoclonal IgG antibodies, one from a recovered human and the other from mice genetically altered to produce human antibody. The antibodies target 2 non-overlapping epitopes on the Covid-19 **S**pike protein. The idea is that the 2 antibodies will forestall mutational escape and cover all the **S**pikes to prevent the virus binding to cells. In an August 3 report, preclinical study of REGN-COV2 in animals produced evidence of effectiveness in both

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193 Agarwal A *et al*. Convalescent plasma in the management of moderate COVID-19 in India: An open-label parallel-arm phase II multicentre randomized controlled trial (PLACID Trial). MedRxiv 2020; doi: https://doi.org/10.1101/2020.09.03.20187252.

194 Simonovich V *et al*. A randomized trial of convalescent plasma in Covid-19 severe pneumonia. NEJM 2020; DOI: 10.1056/NEJMoa2031304.

195 Natarajan H *et al*. SARS-CoV-2 antibody signatures robustly predict diverse antiviral functions relevant for convalescent plasma therapy. MedRxiv 2020; https://doi.org/10.1101/2020.09.16.20196154.

196 Atyeo, C. *et al.* Distinct early serological signatures track with SARS-CoV-2 survival. Immunity 2020; doi:10.1016/j.immuni.2020.07.020.

197 Gasser R *et al*. Major role of IgM in the neutralizing 1 activity of convalescent plasma against SARS-CoV-2. BioRxiv https://doi.org/10.1101/2020.10.09.333278.

prophylaxis and treatment of infections without evidence of antibody-mediated disease exacerbation[198]. On September 29 a Regeneron press release[199] had announced "the first data from a descriptive analysis of a seamless Phase 1/2/3 trial of its investigational antibody cocktail REGN-COV2 showing it reduced viral load and the time to alleviate symptoms in non-hospitalized patients with COVID-19." The Regeneron study had examined doses of zero, 2.4 and 8 grams among 275 patients and observed with only the larger dose significantly reduced viral load 7 days after treatment.

Although this treatment approach looked promising, the Eli Lilly trial in hospitalized Covid-19 patients of treatment with both remdesivir and the monoclonal antibody bamlanivimab was halted by NIH on October 26 because there was no evidence of efficacy[200]. Furthermore, on October 30, the Safety Monitoring Board for the study of REGN-COV2 stopped use in participants needing high-flow oxygen or ventilation because of a negative safety signal and an unfavorable risk/benefit profile[201]. So, problems continue to appear with antibodies against **S**pike in SICK Covid-19 patients, and there is no clinical report showing reduction of Covid-19 morbidity or mortality by monoclonal antibody therapy. If such evidence develops, production limitations mean it would be some time before monoclonal antibody therapy became available for wider clinical use.

Closing thoughts in early March 2021

Kill the virus and quiet the storm. We certainly need multi-drug protocols to be consistently successful in treating Covid-19. That reality greatly magnifies the difficulty for RCTs attempting to establish efficacy. But there are some inexpensive, very safe, and widely available repurposed drugs with which we have extensive experience and that show promise at the levels of both basic science and clinical studies. These include early outpatient ivermectin, colchicine, antihistamines (famotidine and perhaps azelastine spray), and aspirin and for admitted patients cyclosporine, dipyridamole, and steroids. Additional study of all these is needed, and additional work on both the antiviral mechanism of niclosamide and its clinical effect is essential. The clinical trials of remdesivir by Beigel *et al.*[111], Spinner *et al.*[112], and WHO[96] really "dampen my enthusiasm" (a lethal comment on a NIH grant review). It appears ineffective, and it is expensive.

In the meantime, eventually as physicians we have to make a commitment as to how we will provide care to patients. That commitment is based on our best knowledge, and it can also change as new knowledge and experience emerge. In fact, as physicians gained experience and better approaches emerged to treating both outpatients and those hospitalized, the United States (and also other nations) has had a considerable reduction in Case Fatality Rate (% CFR = Deaths/Cases X 100), as seen in the following graph. However, current U.S. CFR remains 1.8%.

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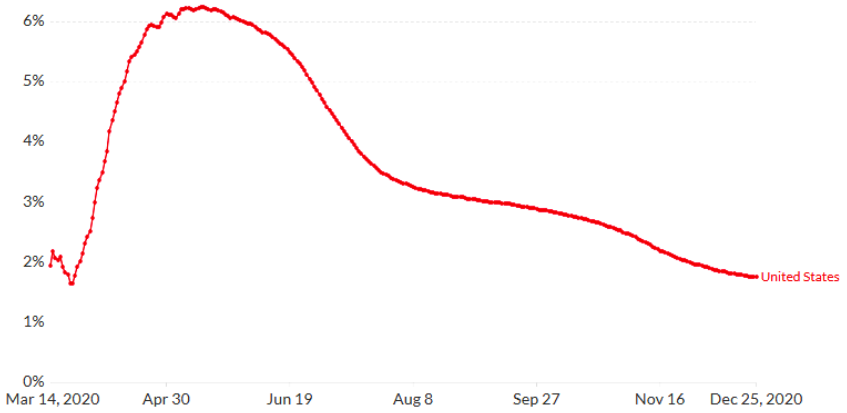
198 Baum A *et al*. REGN-COV2 antibody cocktail prevents and treats SARS-CoV-2 infection in rhesus macaques and hamsters. BioRxiv 2020; https://doi.org/10.1101/2020.08.02.233320.

199 https://investor.regeneron.com/news-releases/news-release-details/regenerons-regn-cov2-antibody-cocktail-reduced-viral-levels.

200 https://blogs.sciencemag.org/pipeline/archives/2020/10/27/more-antibody-data.

201 https://investor.regeneron.com/news-releases/news-release-details/regn-cov2-independent-data-monitoring-committee-recommends.



Your treatment path is going to be guided by your institutional protocols. But at the end of January 2021, this is the approach I would choose when the History & Exam suggest possible Covid-19. Please be forgiving of my including a bit of very basic stuff for completeness.

1. EXAMINE PATIENTS. Of course look right at the heart-rate and room-air O_2 saturation on vital signs. A Covid-19 patient with heart rate > ~90, resp > 20, and O_2 sat less than 92% should make us start thinking inpatient admission and maybe ICU. These patients need EKG, chest CAT scan (X-ray is poorer for finding early Covid-19 pneumonia), cardiac ultrasound to look at RV function regarding possible pulmonary microthrombi, and blood tests including CBC, HbA1c, ferritin, d-dimer, vWF, troponin, and liver enzymes. Of course, IV, O_2, and monitor.

2. DIAGNOSTIC COVID-19 TEST. Abbot provides a FDA-approved 15-minute bedside test that appears to have good sensitivity and specificity. A positive test can guide initiation of treatment even before a rapid confirming RT-PCR

3. WITH POSITIVE COVID-19 TEST, STAGE THE DISEASE. Normal O_2 saturation, HbA1c, d-dimer, and ferritin are not at a stage of MAS, and can be best **treated** as outpatients. Daily O_2 saturation checks should be done. This instrument (pulse oximeter) is relatively inexpensive (~$40.00) at most pharmacies. Elevated HbA1c increases risk, and these patients and all diabetics should have close glucose monitoring (newer continuous personal monitors) and daily follow-up aimed at **TIGHT** glucose control. NO MAS – NO MAS!

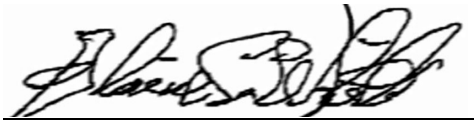
4. TREAT THE DISEASE. I would use what we have rather than delay treatment of infected outpatients who come to an ED. I strongly agree with the non-partisan parts of the very sharp criticism of avoiding outpatient treatment while waiting to see if SARS-CoV-2 patients get worse, that was published by Dr. Joseph Ladapo (UCLA) in a Wall Street Journal opinion on November 25, 2020. In the above review and an Epocrates interaction check, I found no reason not to use ivermectin, famotidine, and aspirin for outpatients. Cyclosporine and dexamethasone both appear helpful for sicker patients, and I would add those and consider dipyridamole on hospital admission. This approach is summarized below and is similar to the Broward, East Virginia Medical College, Front-line Covid Critical Care Alliance, and Baylor protocols aimed first of all at treating outpatients so they never require hospitalization.

Proposed Rx protocol:

Drug	Dosage	Rationale	Monitor
All: Ivermectin	0.4 mg/kg p.o. every 72 hours up to 3 doses.	Blocks importin and viral RdRp & helicase.	O_2 saturation.
Outpatient Consider Colchicine	0.5 mg p.o. qd	Inhibits formation of inflamasone in macrophages	O_2 saturation
All: Famotidine	80 mg p.o. tid[202]	H2 receptor blocker	O_2 saturation
All: Aspirin	1 adult tab, then 81 mg qd	Stabilizes platelets.	Perfusion distal extremities.
Inpatient: Consider dipyridamole	50 mg p.o. bid	Stabilizes Platelets & PMNs ANTIVIRAL	Platelet count, D-dimer, watch for bleeding.
Inpatient: Cyclosporine-MODIFIED	100 mg p.o. bid	Blocks Nsp1 & viral replication. Modulates MAS.	Cyclosporine & Interferon levels. BUN, creatinine, & for ^ K.
Inpatient: Dexamethasone	6 mg p.o. qd if O_2 sat falls.	Modulates MAS.	Watch for GI bleed.

This core treatment approach uses antiviral, anti-inflamatory, and platelet-stabilizing treatment continuously with escalation to cyclosporine, dipyridamole, and steroids if disease progression requires hospitalization.

Even though it's now up to 200 footnote references, I again hope this review is not too "research-opaque" and helps to expand our underlying understanding of this very dangerous pandemic that you are providing care for. And I will always be very proud of you for being there.


M.D.

An Old Emeritus ES Thomas Professor of Emergency Medicine, Wayne State University, and Emeritus Member, (Medicine) U.S. National Academies of Science, Engineering, and Medicine

[202] Janowitz T *et al*. Famotidine use and quantitative symptom tracking for COVID-19 in non-hospitalised patients: A case series. Gut 2020; 69:1592–1597.