The Evidence which Suggests that This Is No Naturally Evolved Virus

A Reconstructed Historical Aetiology of the SARS-CoV-2 Spike

Birger Sørensen, Angus Dalgleish & Andres Susrud
Immunor & St Georges University of London

ABSTRACT

To discover exactly how to attack SARS-CoV-2 safely and efficiently, our vaccine candidate Biovacc-19 was designed by first carefully analysing the biochemistry of the Spike. We ascertained that it is highly unusual in several respects, unlike any other CoV in its clade. The SARS-CoV-2 general mode of action is as a co-receptor dependent phagocyte. But data shows that simultaneously it is capable of binding to ACE2 receptors in its receptor binding domain. In short, SARS-CoV-2 is possessed of dual action capability. In this paper we argue that the likelihood of this being the result of natural processes is very small. The spike has six inserts which are unique fingerprints with five salient features indicative of purposive manipulation. We then add to the bio-chemistry a diachronic dimension by analysing a sequence of four linked published research projects which, we suggest, show by deduction how, where, when and by whom the SARS-CoV-2 Spike acquired its special characteristics. This reconstructed historical aetiology meets the criteria of means, timing, agent and place to produce sufficient confidence to reverse the burden of proof. Henceforth, those who would maintain that the Covid-19 pandemic arose from zoonotic transfer need to explain precisely why this more parsimonious account is wrong before asserting that their evidence is persuasive, most especially when, as we also show, there are puzzling errors in their use of evidence.

Introduction: Why does this matter?

No-one has ever produced a safe and effective vaccine against a coronavirus. In the context of a forthcoming paper addressing contingency actions cognizant of this fact, the potentialities for 'trained immunity' from 'new old friends' in the form of *Bacillus Calmette–Guérin* (BCG), *Microbacillus vaccae* (IMM-102) and most especially *Microbacillus obuense* (IMM-101) by stimulating the innate immune system and especially Delta Gamma T cells are explored; and a salutary review of failed vaccine programmes is included (Kleen *et al*., 2020). On 28th April 2020, *Nature* published a graphical guide to eight conceptual approaches featuring in current explorations of around 90 vaccine development programmes intended to counter SARS-CoV-2 (Callaway, 2020).

We have just (2nd June 2020) published Biovacc-19 in *QRB-Discovery*: a candidate vaccine for this daunting task (Sørensen *et al.*, 2020). Its mode of action is unique and therefore is not included in the *Nature* review. In our paper we gave reasons why the virus vector or RNA vector based approaches that are the basis of the eight methodologies reviewed in *Nature* are unlikely to prove immunogenic and why either, but especially RNA vectored models, may carry significant risk of Antibody Dependent Enhancement (ADE). As we have detailed in *QRB-D,* we have seen such a story before over thirty years in the failure of all three mainstream vaccine approaches to HIV, which we predicted but were disbelieved.

As with our HIV vaccine, the methodology underpinning Biovacc-19 first analysed fully the virus target. In this case we published the general mode of action for infectivity of SARS-CoV-2. Doing this took us into a fundamental exploration of the biochemistry and structure of the SARS-CoV-2 Spike which is highly singular, possessed of features that we have not seen before and which are not present in other SARS viruses of that clade. We posited that the SARS-CoV-2 general mode of action is as a co-receptor dependent phagocyte. But unusually, simultaneously, data shows that it is capable of binding to ACE2 receptors in its receptor binding domain. In short, SARS- CoV-2 is possessed of dual action capability. How do we think this was made possible? That is the subject of this paper. We shall argue from evidence below that the likelihood of this being the result of natural processes is very small.

The co-receptor dependent phagocytic general method of action for infectivity and pathogenicity of SARS-CoV-2 appears to be specifically related to cumulative charge resulting from inserts placed on the surface of the Spike receptor binding domain, right next to the receptor binding motif. That SARS-CoV-2 has charged inserts is not in dispute (Zhou *et al.*, 2020) What we have shown that is new is that the SARS-CoV-2 Spike carries significant additional charge (isoelectric point (pI) pI=8.2) compared to human SARS-CoV Spike,(pI = 5.67) and the implications thereof. Basic domains - partly inserted, partly substituted amino acids and partly redistributed from outside the receptor binding domain - explain the salt bridges formed between the SARS-CoV-2 Spike and its co-receptors on the cell membrane. We comment further on the significance of this in the next section.

Puzzling features

An influential paper was published in *Nature Medicine* on 17 March 2020. Andersen *et al* observed that several mutations have occurred in the receptor binding domain of SARS-CoV-2. These, they suggested, therefore sustain an hypothesis of natural evolution (Andersen *et al.*, 2020). We do not agree. We do agree that it is indeed correct that several such mutations are to be seen and in a forthcoming companion article to this one, about three other viruses of interest, we will discuss further Andersen *et al's* evidence and argumentation in that context. But here we observe only that the contention that it is improbable that Covid-19 emerged through laboratory manipulation of a related SARS-CoV-like coronavirus because the ACE2 binding is not ideal is weakened because Andersen *et al* cite two authorities which actually say the reverse of what they say that they say.

Wan *et al* are cited by Andersen *et al* but offer them no support (Wan *et al.*, 2020). Wan *et al* say, correctly in our view, that computational structural modelling of complex virus-receptor interactions can be used for structural predictions and that such models can potentially be used for Gain-Of-Function modelling. It is well known that models have been developed from data generated in animal model systems such as the palm civet. Wan *et al* say that the SARS-CoV-2 binding to the ACE2 receptor confirms the accuracy of the structural predictions. Therefore the data and conclusion in Wan *et al* contradicts Andersen *et al's* opinion that it is improbable that the virus could have emerged through laboratory manipulation.

There is a similar problem with (Sheahan *et al*., 2008). This deals with research on a civet strain SZ16 and the infective strain SARS-CoV Urbani. These strains were used to create a chimeric virus icSZ16-S. Sheahan *et al* go on to explain that by *in vitro* evolution of the chimeric virus icSZ16-S on human airway epithelial (HAE) cells in the lab, they have been able to produce two new viruses binding to such HAE cells. Therefore this reference supports the very opposite of the Andersen *et al* hypothesis. We are immediately wary of any paper containing such egregious errors.

Our discovery of the high pI number, the high accumulated charge and how it comes about, in the course of our bio-chemical analysis, suggested several features which individually seem unlikely to be the result of natural evolution and which, taken together, and applying Occam's Razor to hone the most parsimonious hypothesis, make natural evolution a less likely explanation than purposive manipulation, specifically for Gain of Function.

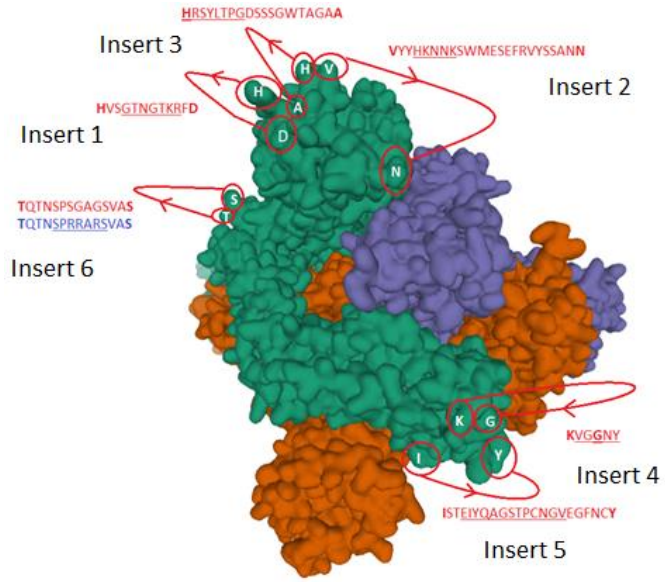

Figure 1: The identified inserts examined in the PDB 6VXX electron microscopy structure (Walls *et al*., 2020) The sequences highlighted in red could not be found in the cryo-electron microscopy structure data. The 6 aligned sequences in Fig. 1 in (Sørensen *et al.*,2020) are underlined in the missing sequences. Bold amino acids indicate first and last amino acids used to build the structure where the missing part is in between. Insert 6 did not have the same sequence in 6VXX as in the reference Sars-CoV-2 sequence. The authors stated that a designed mutated strain lacking the furin cleavage site residues was used.

To recapitulate Fig 2 from our vaccine paper, there are 6 inserts which make the SARS-CoV-2 Spike structurally special. They are unique fingerprints of the SARS-CoV-2 Spike which deserve to be highlighted in support of this view; and there are five salient features that strengthen the case for purposive manipulation in the laboratory.

1. *A major part of the spike protein has human-like domains with matured transmission adaption*. Blasting the Spike protein with a rolling window of 6 amino acids showed that 78.4% of 6 amino acid windows are human like. This means that with nearly 80% of the spike protein has a built-in stealth property by having high human similarity. Therefore, it is remarkably well-adapted virus for human co-existence. Such high human similarity also implies a high risk for the development of severe adverse events/toxicity and even Antibody Dependent Enhancement (ADE) unless specific precautions are taken when using the Spike protein in any vaccine candidate: precautions that might not suggest themselves to designers employing conventional methodologies and innocent assumptions about the target virus, lacking our detailed anatomisation of it. Furthermore and significantly, Zhan *et al* also note that, surprisingly, this characteristic is present from the very first isolate (Zhan *et al*, 2020). This is something that does not sit well with an hypothesis of natural evolution.

2. *The Spike displays new amino acid inserts with condensed cumulative charge, all of which are surface exposed (please refer to the reproduced figure from the vaccine paper, above).* This is a most significant finding as we mentioned in opening. Being physically located on the surface of the Spike protein greatly increases the infectivity and pathogenicity of the virus, enabling these inserts to participate in binding to co-receptors/negatively charged attachment receptors or even, as we have discovered, to the negatively charged phospholipid heads on the cell membrane. Such a result is typically the objective of gain of function experiments to create chimeric viruses of high potency. Therefore this is a strong indicator of manipulation

3. *The concentration of positive charge is on the receptor binding domain near the receptor binding motif at the top of the Spike protein.* As with (2) this is more elegantly explained by an hypothesis of purposive manipulation than one of natural evolution. As can be seen in Figure 2 (side view) of the Spike trimer, the majority of the positive charged amino acids are located near or on the top of the spike protein giving the receptor binding domain a pI=8.906, while the Cov-2 specific Cys538-Cys590 bridge brings in additional charge from 526-560 (with even higher pI=10.03) via the Cys391-Cys525 to positions right next to the receptor binding motif (where the ACE2 receptor is located). It is this which facilitates the dual mode capability, allowing binding to ACE2 and/or to co-receptors/attachments receptors. We posit that such ACE2 independent attachment and infectivity is happening and is evidenced clinically by the Covid-19 disease pattern. It is also reported by Zhou *et al* (2018). The receptors that are the most likely to be involved are CLEC4M/DC-SIGN (CD209) – see discussion point (5) below.

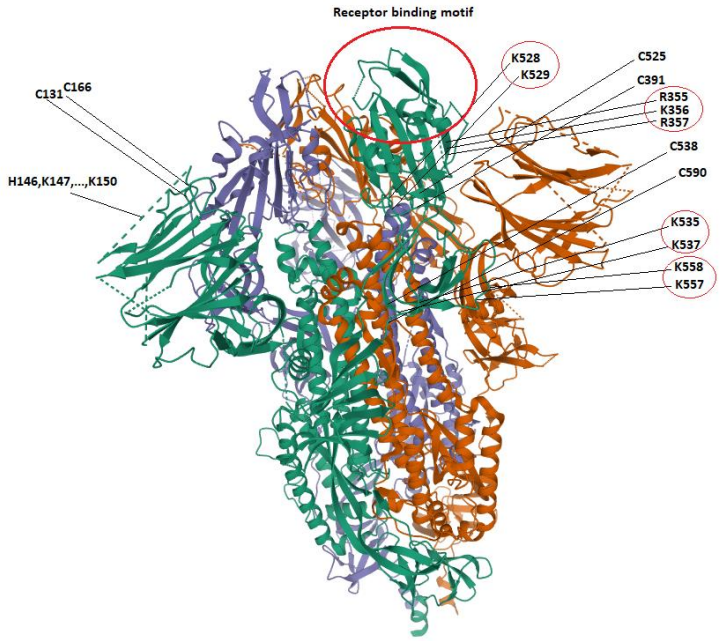

Figure 2: The positive charged domains associated with cysteine loops Cys131-Cys166, Cys336-Cys361, Cys391-Cys525, Cys538-Cys590. As can be seen, there is a high concentration of positive charged surface exposed amino acids within the receptor domain next to the receptor binding motif at the top of the spike. The location of the positive charged amino acids in red circles on the right-hand side of the figure points out their surface exposure making them available for cell attachment as discussed in (5) below. Insert 2 (HKNNK) in Figure 1 above is located within the Cys131-Cys166 loop but was omitted in the Cryo-EM structure shown in dashed lines (Walls *et al*, 2020). However, charged amino acids belong to the hydrophilic group of amino acids and are most likely surface exposed.

4. *The Spike is so configured that it can bind to cell tissue without use of the ACE2 receptor*. Clinically it is widely observed that the Covid-19 virus compromises the functions of olfaction and bitter/sweet receptors, erythrocytes, t-cells, neurons and various tissues such as intestine epithelia. These different targets do not engage and use ACE2 receptor binding. The concentration of high positive charge in and around the top of the Spike protein and the potential to use opposite charged attachment-/co-receptors can facilitate binding and infection in the general mode of action for infectivity that we published in detail in QRBD. In 2018 Zhou P et.al. 2018 found that a new Corona virus which they named SADS (Swine Acute Diarrhoea Syndrome) could infect the intestine and kill piglets without use of ACE2, aminopeptidase N (APN) or dipeptidyl peptidase 4 (DPP4) receptors.[9] We have done a blast analysis of the SADS Spike S1 protein and could find no trace of ACE2 RBM. The significance of this will become clear in the next point and the next section.

5. *Location and concentration of charge on the attachment receptor CLEC4M/DC-SIGN (**C**-type **Lec**tin domain family **4** member **M** (CLEC4M)/ **D**endritic **C**ell-**S**pecific **I**ntercellular adhesion molecule-3-**G**rabbing **N**on-integrin(DC-SIGNR) also known as CD209) (Marzi et al., 2004).* Analysis of the CLEC4M attachment receptor shows an overall pI=5.23 where the C-type lectin tail 274-390 has a pI=4.4. However, due to the two disulfide bonds Cys296-Cys389 and Cys368-Cys381 the C-terminal part of the tail is pulled back to a domain around position 296. This condensed negatively charged domain is ready for formation of salt-bridges with similar condensed opposite charged amino acids structures on the S1 RBD of SARS-CoV-2. This finding is fascinating and significant for a different reason to the others. It is not about Spike manipulation itself: in the next section we will explain that and how we believe that these capabilities were developed between 2008 - 2015. This finding points to something else: a trial to demonstrate a newly discovered attachment/co-receptor by field testing and verification. The context was the 2018 Swine Acute Diarrhoea Syndrome (SADS) outbreak in Guangzhou province.[10] Assuming that the Wuhan Institute of Virology team had discovered the functionalities of CLEC4M/DC-SIGN/CD209 receptors in the new SADS-CoV isolate and the fact that it could bind to positive charge (Ref: https://www.uniprot.org/uniprot/Q9NNX6 (CD209) and https://www.uniprot.org/uniprot/Q9H2X3) and that they wanted to do a field test of the described functionalities, the best conditions for doing so would be in connection with an ongoing viral infection. If this SADS originally did not have a ACE2 receptor binding motif

(RBM), then a binding capacity verification of these attachment receptors could be done straightforwardly. But if SADS did have an ACE2 RBM, then it would be necessary to remove or disable the RBM of the Spike protein on this CoV isolate and execute the experiment in piglets including the formal Cox postulate verification of infection as described in the 2018 paper.

We postulate that there are 2 charged domains on SADS that are likely to contribute to attachment receptor binding located in domains 330-360 and 540-560 respectively. Recollect that we have identified a similar highly charged structure on SARS-CoV-2 within the edge of the RBD domain (526-560) with pI=10.03 which is brought right into the core of the RBD (to approximately position 400) by Cys-Cys bridging of the domain (538-590). This domain can contribute binding similar to that which can be observed for SADS. This new Cys-Cys property inserted into the SARS-CoV-2 Spike does not exist in SARS-CoV and hence could not provide such charge enhancement onto the RBD and co-receptor binding by natural evolution.

Taken all together, we suggest that our research findings on the general mode of action for infectivity of SARS-CoV-2 and the further puzzling features just mentioned, justify the question of the historical aetiology of these manipulations.

We did not need to address this issue diachronically for the purposes of vaccine design. However, it is important for a soundly based understanding of the present and potential future epidemiology of the Covid-19 pandemic and for strategies for its management. Therefore to our earlier amino-acid level of biochemical analysis we now add here a forensic analysis of published research literature concerning SARS-CoV-2. We will extend this type of analysis to three other viruses in the companion article.

Since, regrettably, international access has not been allowed to the relevant laboratories or materials, since Chinese scientists who wished to share their knowledge have not been able to do so and indeed since it appears that preserved virus material and related information have been destroyed, we are compelled to apply deduction to the published scientific literature, informed by our own biochemical analyses. We refute pre-emptively objection that this methodology does not result in absolute proof by observing that to make such a statement is to misunderstand scientific logic. The longer the chain of causation of individual findings that is shown, especially converging from different disciplines, the greater the confidence in the whole. We posit that the evidence below attains a high level of confidence.

A sequence of four linked research papers is explained

A comprehensive review of the relevant literature shows that a substantial amount of directly relevant gain-of function research has been undertaken. Four studies are especially noteworthy. They are linked in two ways: scientifically, in that the third and fourth build upon the results of the first and second, and in continuity of the institution and personnel across all four. The Wuhan Institute of Virology is a key collaborator in all these projects and Dr Zheng-Li Shi is one of the Institute's most experienced virologists and bat specialists. She is a common thread through all the key research projects.

1. In 2008, Dr Shi was in the team whose research was an enabling pre-cursor to the two linked gain-of-function projects which lead to SARS-CoV-2's exact functionalities, including functionalities discovered via SADS and potentially field-tested in the 2018 study as suggested above. The 2008 Ren W *et al* project successfully demonstrated technical capabilities to interchange RBD's between bat SARS-like and human SARS viruses: "... a minimal insert region (amino acids 310 to 518) was found to be sufficient to convert the SL-CoV S from non-ACE2 binding to human ACE2 binding, indicating that the SL-CoV S is largely compatible with SARS-CoV S protein both in structure and in function. The significance of these findings in relation to virus origin, virus recombination, and host switching is discussed" (Ren *et al*, 2008). Dr Shi is next a lead author of the second paper in this sequence, (Hou *et al*, 2010) and a co-author and the senior Chinese author of the third, (Menachery *et al*, 2015). She is also a co-author of the fourth (Zhou P. *et al*, 2018)

2. In 2010 scientists from the 'Special Viruses' section of the Wuhan Institute of Virology were engaged in 'gain of function' experiments, jointly with international collaborators, to increase SARS-CoV infectiousness for humans. They used an HIV pseudo virus to express seven bat ACE2 receptors and compared their binding properties to human ACE2 receptors in order to pick the best for further optimizing a SARS-like coronavirus's ability to bind to human cells. They also found that some bat ACE2 receptors are very close to human ACE2 receptors. This study provided a model system for testing the most infectious of SARS-CoV-like viruses which already had been selected in a vast survey of Chinese bat populations between 2005 – 2013.(Xu L *et al, 2016*). These viruses were potentially

infectious to humans via the ACE2 receptor. Further new viruses were identified between 2012-2015 (Lin *et al,2017*).

3. In 2015 scientists from the 'Special Viruses' section of the Wuhan Institute of Virology were engaged in 'gain of function' experiments jointly with a majority team from the University of North Carolina Chapel Hill. Together, they manipulated bat viruses to create a mouse adapted chimeric virus SHC014-MA15 which binds to and can proliferate on human upper airway cells (2B4 Calu-3 - a cell line contributed by Chapel Hill): ("group 2b viruses encoding the SHC014 spike in a wild type backbone can efficiently utilize multiple ACE2 receptor orthologs, replicate efficiently in primary human airway cells, and achieve *in vitro* titers equivalent to epidemic strains of SARS-CoV"). We suggest that it is a high priority in further investigations to ascertain precisely from Chapel Hill lab records the exact donor provenance of 2B4 Calu-3. The lead Wuhan scientist, who provided the CoV material, was Dr Zheng-Li Shi ("provided SHC014 spike sequences and plasmids"). We note that what is described here are, in fact, precisely SARS-CoV-2 properties. *In vivo* experiments at Chapel Hill replicated the chimeric virus in mouse lung which showed significant pathogenesis which was the opposite of what the team had expected ("the creation of chimeric viruses like SHC014-MA15 was not expected to increase pathogenicity"). Menachery *et al* reported that it may be hard to develop a vaccine against SHC014-MA15. We can see, therefore, that the 2015 experiment advanced the 2010 work by perfecting in animal trials a virus optimised to infect the human upper respiratory tract. The 2015 authors were well aware that the chimeric virus which they had created was very dangerous because they discussed this fact. Of the opportunity/costs of their research, they suggested that "while offering preparation against future emerging viruses, this approach must be considered in the context of the US government-mandated pause on Gain Of Function (GOF) studies" (which has since been lifted). They also speculated that " review panels may deem similar studies too risky to pursue as increased pathogenicity in mammalian models cannot be excluded." It is certainly the case that this experiment created a chimeric virus with very high infectivity potential targeted to the human upper respiratory tract. Yet a surprising observation is that the paper states that this research consortium has permission to continue this research. It appears that optimisation gain of function work on this chimeric virus did continue. We deduce from paper authorships that this was done in the Wuhan Institute of Virology.

4. In 2018, as discussed earlier, Dr Shi's close colleague Peng Zhou, with others, investigated a coronavirus outbreak associated with a fatal Swine Acute Diarrhoea Syndrome (SADS) in Guangdong Province. This paper relates that piglets had a tissue specific infection site located in the intestine and that verification of the Bat Covid nature of this new SADS as the disease-causing agent was confirmed. 25,000 piglets died. However, the really interesting part of this study reports that in order to identify the receptor(s) used by the SADS CoV, known coronavirus host cell receptors were investigated: Angiotensin Converting Enzyme 2 (ACE2), Amino Peptidase N (APN), and Di-Peptidyl Peptidase 4 (DPP4). None of these receptors worked. But indirectly in their paper, the authors revealed their ability to express and to test new receptors in the ways posited earlier. Recollect that the model to do this was proven and reported in the 2010 work. Thus it is plain that SADS is a CoV infection utilising new tissue-specific binding domains; but the authors provide no hint about which receptor the virus is using in piglets except that it is not any of the best known three. We have offered our deduction above. Pigs, of course, have immune systems very similar to humans.

Now recollect that Menachery V.D *et al* in 2015 had shown that their chimeric virus SHC014-MA15 could, against their prediction, very successfully infect primary human upper airway epithelial cells (HAE) from the cell-line 2B4 Calu-3. With this in mind, we next observed that in the Covid-19 pandemic, a well-reported symptom in the early phase of the infection is loss of taste, headache and a sore throat. We have discussed this issue in the QRBD article in detail. But to summarise: in 2015 in a research review (Workman *et al,* 2015*)* discussed bitter/sweet taste receptors and the role these receptors play in mediating airway immune functions. They concluded thus: "Over the past several years, taste receptors have emerged as key players in the regulation of innate immune defenses in the mammalian respiratory tract. Several cell types in the airway, including ciliated epithelial cells, solitary chemosensory cells, and bronchial smooth muscle cells, all display chemoresponsive properties that utilize taste receptors."

Therefore we hypothesise the reconstructed historical aetiology of the Spike as follows:

In 2008, Dr Zheng-Li Si and WIV colleagues successfully demonstrated technical capabilities to interchange RBD's between bat SARS-like and human SARS viruses. Building upon this, the 2010 work (Hou *et al*, 2010) perfected the ability to express receptors on human cells. On these foundations, the central Gain of Function work that underpins the functionalities of SARS-CoV-2 took place, carrying the WIV spike and plasmid materials to bond successfully to a UNC Chapel Hill human epithelial cell-line. This work (Menachery *et al*) produced a highly infectious chimeric virus optimised to the human upper respiratory tract. In convergent support of this hypothesis, both Lu (Lu *et al*, 2020*)* and Jia (Jia *et al*, 2020) have now, in January and April 2020, shown that SARS-CoV-2 has a bat SARS-like backbone but is carrying an RBD from a human SARS and Zhan *et al* have, like us, noted unusual adaption to humans from the first isolate. In the 2015 Chapel Hill work it was only ACE2 receptors that were discussed. However, in 2018 Zhou P. *et al* demonstrated capabilities to clone other

receptors like APN and DPP4 and to test and compare these against the (intestine) tissue specific SADS-CoV identified. Then, in the 2019-20 Covid-19 pandemic, profuse symptoms indicating compromise of the bitter/sweet receptors are reported. Taken all together, this implies that by employing insights gained after 2015, as just deduced, a further optimization of the 2015 chimeric virus for additional binding to receptors/co-receptors such as bitter/sweet specific upper airway epithelia receptors occurred. That would help to explain the otherwise puzzling high infectivity and pathology associated with SARS-CoV-2 and hence also help to explain the social epidemiology of its spread.

Conclusion

We have deduced the internal logic of published research which resulted in the exact functionalities of SARS-CoV-2, including the convergence of agreement from difference classes of source, the timings of the stages of the research and the development of documented capabilities by named institutions and individuals. These meet the criteria of means, timing, agent and place in this reconstructed historical aetiology to produce sufficient confidence in the account to reverse the burden of proof. Henceforth, those who would maintain that the Covid-19 pandemic arose from zoonotic transfer need to explain precisely why this more parsimonious account is wrong before asserting that their evidence is persuasive, most especially when, as we have indicated, we note puzzling errors in their use of evidence. In our companion article, in a similar forensic manner we will explore the primary evidence used to sustain the hypothesis of zoonotic transfer. In neither this article nor the next do we speculate about motive.

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