

## Research Article

# Dominance of SARS-CoV-2 D614G Variant Explained by the Requirement of COVID-19 for Calcium; Proximate Therapeutic Implication(s) for COVID-19

Daniel P Cashman M.D.\*

PO Box 841, Los Altos, CA, United States

### Abstract

The current dominance of D614G mutation in the SARS-CoV-2 pandemic implies increased infectivity of the virus S protein that drives cellular entry which is triggered by binding to the angiotensin converting enzyme-2 (ACE2) receptor and calcium-dependent protease-mediated activation. Understanding how the D614G spike protein mutation could produce a fitness advantage is key to therapeutic development given its epidemiological dominance. A 14-amino acid (aa) consensus sequence was found in 84 SARS-CoV-2 D614G entries from the NCBI Protein database. No other significant similarity to the D614G mutant sequence was found. A homology to the analogous wild type 14-aa consensus peptide was found in bat coronavirus (APO40579.1) and a smaller 13-aa consensus homology was found with SARS-CoV (AAP41037.1). A successive substring search constrained by the boundary of the D614G consensus peptide compared to all of the proteins in the unbiased Prosite EF-hand calcium-binding domain profile (PS50222) was undertaken because calcium triggers the protease-mediated activation of membrane fusion. A homology to single protein was found; a probable voltage-dependent N-type calcium channel subunit alpha-1B that is involved in the pore-forming regulation of transmembrane calcium transport (Uni Prot KB-P56698). A subsequent brute force Pub Med searching revealed the existence of a laboratory created aspartic acid to glycine mutation in the F protein human parainfluenza virus (hPIV-3 D104G mutation) that facilitated the spread of hPIV-3 in SPCA1 deficient cells. In humans, (SPCA1) regulates the Golgi luminal Ca<sup>2+</sup> homeostasis and is ubiquitously expressed in all tissues.

\*Corresponding author: Daniel P Cashman, PO Box 841, Los Altos, CA, United States, E-mail: dan94022@gmail.com

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Decreased SPCA1 expression causes Hailey-Hailey disease, a rare skin disorder that impairs a cells' ability to transport Ca<sup>2+</sup> (i.e., human ATP2C1 gene). Clinically, the hypocalcemia associated with hospitalized COVID-19 patients can effectively impair Ca<sup>2+</sup> transport in an analogous manner to the SPCA1 deficiency. Here, the D614G SARS-CoV-2 mutant strain can make use of clinical hypocalcemia like the D104G mutated hPIV-3 virus takes advantage of SPCA1 deficient cells to increase infectivity. These data demonstrate the critical importance of calcium for effective SARS-CoV-2/COVID-19 infection and how understanding this mechanism can be exploited for therapeutic gain to stop, or otherwise attenuate virus infection at the earliest steps. Calcium chelation by pharmaceutical EDTA, has been, and can be safely delivered via nebulizer or oral ingestion. EDTA treatment is available in outpatient and inpatient settings and can be self-administered during "quarantine" periods. These proven and safe EDTA treatments should effectively disrupt SARS-CoV-2 spread at the earliest step in the virus lifecycle and warrant investigation and optimization to save lives post haste.

**Keywords:** Calcium; Covid-19; Pandemic; SARS-CoV-2; D614G; Spike Protein Variant; EDTA; Nebulizer; Treatment; Zinc

### Introduction

The International Committee on Taxonomy of Viruses (ICTV) officially designated the COVID-virus as SARS-CoV-2 and on March 11, 2020, the WHO, has declared the COVID-19 outbreak as a global pandemic [1]. As of November 2020, the pandemic of SARS-CoV-2/ (COVID-19) coronavirus (CoV) infection has infected more than 47 million people worldwide with more than a million reported deaths [2]. Although preliminary experiments by researchers have reported that some agents may be effective against 2019-nCoV, there are currently no effective drugs targeting 2019-nCoV/SARS-CoV-2 [3,4]. Meanwhile, a SARS-CoV-2 variant in the spike protein D614G (aspartic acid to glycine mutation) has inexplicably become dominant around the world [5-7]. The CoVs are some of the largest known RNA viruses [8-10] and are enveloped pleomorphic RNA viruses with special crown-shape peplomers and a genome of 27-32 KB. Coronaviruses infect many different hosts from bats to humans and are associated with several respiratory and intestinal tract infections [11]. While there are four coronavirus genera ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ), the human coronaviruses (HCoVs) detected (to date) are either the  $\alpha$  (HCoV-229E and NL63) or  $\beta$  (MERS-CoV, SARS-CoV, HCoV-OC43, and HCoV-HKU1) genera [12]. The undisputed origin of SARS-CoV-2 and its arrival in the human population is not definitely known but sequencing analyses showed that the genome of SARS-CoV-2 shares 79.5%, 89.1%, 93.3%, and 96.2% nucleotide sequence identity with that of human SARS-CoV, bat CoV ZC45, bat CoV RmYN02, and bat CoV RaTG13, respectively, which suggests that SARS-CoV-2 probably has bat origins [13-15]. These findings are very plausible since bats serve as the natural reservoirs for two other deadly human coronaviruses (hCoVs), SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), which previously caused global outbreaks [16,17]. A recent review of the molecular basis of coronavirus entry and its replication cycle is available [18].

Animal coronaviruses can act as human pathogens due to a high mutation rate that results in disease characteristics that can range from

an asymptomatic course to death. However, the genetic mechanisms underlying cross-species adaptation remain poorly understood. Typically, successful mutations are beneficial for viral adaptation and correlate with virulence [19]. Naturally occurring small mutational changes in amino acid residues can change the cleavage site of a viral fusion protein (e.g. influenza hemagglutinin (HA)) to allow cleavage by ubiquitously expressed calcium dependent furin-like proteases. Such a mutation has been shown to have a far-reaching impact on the pathogenesis of avian influenza (HPAI) virus strains [20].

The current epidemiologic dominance of D614G implies an increased infectivity of the virus and understanding the mechanism of the D614G spike protein mutation is a potential key to therapeutic development. To date, there is no satisfying hypothesis that can explain the dominance of the SARS-CoV-2 D614G mutation [21], but there seems to be a related fitness advantage in the aspartic acid to glycine mutation (D104G) of the F (fusion) protein of human parainfluenza virus 3 (hPIV-3) in cells with impaired calcium transport. Moreover, many viruses require the calcium transporter SPCA1 for maturation and spread [22]. The aspartic acid to glycine mutation (D104G) in the hPIV-3 fusion (F) glycoprotein was discovered by passaging human parainfluenza virus 3 (hPIV-3) in the laboratory [22]. Like the SARS-CoV-2 spike protein, the hPIV-3 fusion (F) glycoprotein is surface exposed and required for membrane fusion. This parainfluenza virus D104G mutation was thought to facilitate the spread of hPIV-3 in SPCA1-deficient cells either by increasing the F protein's susceptibility for calcium-dependent furin cleavage or by creating a better substrate for a different protease [22]. SPCA1 is a secretory pathway calcium ( $\text{Ca}^{2+}$ ) transport ATPase encoded by ATP2C1 that facilitates  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$  uptake into the trans-Golgi network (TGN). SPCA1 deficiency also affects viral entry and viral spread of RSV, Flaviviridae, and Togaviridae viruses in SPCA1-deficient cells. Rescue studies using trans-complementation of SPCA1 suggested that while SPCA1 may play a role during entry, it is primarily involved in the later stages of the virus life cycle. When SPCA1 was absent or unable to transport  $\text{Ca}^{2+}$  into the TGN, furin protease levels were reduced, viral glycoprotein processing was impaired, and newly generated virus particles were less infectious. Others have also found that the SPCA1 in the Golgi network is critical for human respiratory syncytial virus (RSV) infection [23,24]. Their studies of the underlying mechanism revealed that  $\text{Ca}^{2+}$  pumped into Golgi by SPCA1 is the trigger to produce normal functional viral glycoproteins that are essential for virus spread. It is noteworthy that human furin is a calcium-dependent serine endoprotease and chelators such as EDTA and EGTA effectively suppress furin activity by removing the free calcium required for activation [25].

Similarly, the SARS-CoV-2 genome has mutated as the virus has spread [26]. Recent studies [5-7] show that a single amino acid change in the virus's spike protein from aspartic acid to glycine at residue 614, D614G has emerged to be the dominant strain in the pandemic around the world. Successful coronavirus infection requires that a virus be able to enter a host cell, replicate and then exit a cell. Coronavirus entry is a multi-step process involving several distinct domains in the spike protein that mediate virus attachment to the cell surface, receptor engagement, protease processing, membrane fusion, and internalization by a multitude of mechanisms [27]. The coronavirus spike glycoprotein contains three (S1, S2 and S2') cleavage sites that are processed by human host proteases [28]. The exact nature of these cleavage sites, and how their respective host processing

proteases can determine whether the virus can cross species is not known. There still remains some confusion on the relative roles of these calcium-dependent serine proteases involved for priming of the spike protein.

Each virus particle of SARS-CoV-2 coronavirus has approximately 24 to 26 spike (S) protein trimers that protrude from the virus surface envelope [29,30]. The spike protein of the coronavirus was shown to be a trimeric structure of the S protein of SARS-CoV-2 where each S monomer consists of an N-terminal S1 domain and a membrane-proximal S2 domain that mediate receptor binding through the ACE receptor and membrane fusion, respectively [31-34]. The receptor binding domain (RBM) is a region in the carboxy-terminal half of the S protein from amino acid (aa) residues 321 to 541 that contains all of the aa residues that interface with the host ACE2 receptor [35,36]. While the S1 subunit is responsible for receptor binding and includes the N-terminal domain and C-terminal receptor binding region (RBD), the S2 subunit facilitates membrane fusion and anchors the S protein into the viral membrane [37]. Prior to initiating the infection process, the SARS-CoV-2 spike protein exists on the surface of the virus predominantly in one of two structurally distinct conformations; prefusion and the postfusion conformation that enables membrane fusion [38]. The transition from prefusion to the postfusion conformation of the spike protein must be triggered or "primed" by a protease to be able to bind to the host receptor (ACE2) before the SARS-CoV-2 can fuse their lipid envelopes with the host cell.

Host cell fusion can occur at either the cell plasma membrane or the endosomal membrane. Cleavage of the S protein at the S1/S2 site (aa residues 676-688) and/or S2' site (aa residues 811-818) is known to activate the S protein for viral entry. The surface glycoprotein spike (S) must be cleaved at two different sites by host cell proteases, which are calcium dependent. The S protein is cleaved by the proprotein convertase furin at the S1/S2 site and the transmembrane serine protease 2 (TMPRSS2) at the S2' site [4,39]. After priming, the binding to human ACE2 receptor and internalization of the virus into the endosomes of the host cell induces conformational changes in the trimeric Spike glycoprotein. Thus, while TMPRSS2 appears essential for viral entry into primary target cells and for viral spread in the infected host, recent studies have presented perplexing and sometimes conflicting findings on how SARS-CoV-2 enters cells, raising pressing scientific questions [40]. Moreover, TMPRSS2, like furin, has calcium-binding sites that are consistent with a serine protease [41].

In addition to potential  $\text{Ca}^{2+}$  levels related to SPCA1 functionality, there are other key targets to interrupt SARS-CoV-2 infection that involve calcium. As described above, successful infection with SARS-CoV-2 requires the activity of at least two calcium-dependent peptidases, and at least two calcium-dependent fusion peptides in the S2 domain that mediate receptor binding and membrane fusion [41]. Accordingly, an experimental plan was developed to; 1) determine if there was any unrecognized homology or peptide motif(s) among the very pathologic betacoronaviruses (bat, SARS-CoV and SARS-CoV-2) in the regions of the D614G mutation, and 2), assuming there was a consensus peptide region, determine if any consensus peptide with aspartic acid to glycine mutation demonstrates sequence homology to another calcium binding protein that may improve the fitness of the D614G mutant virus.

## Bioinformatic Methods

The NCBI basic local alignment search tool (i.e., Standard Protein BLAST) was used to identify homologous regions between the

D614G mutated SARS-CoV-2 fusion protein sequence and other coronaviruses [42]. Then, the 84 sequences having the D614G mutation were searched for identical amino acid sequences in bidirectional directions that were contiguous to D614G site to identify a consensus region. This search identified a maximum of 14-amino acids VAVLYQgVNCTEVP as a consensus peptide that were found in the sequences of 84 SARS-CoV-2 Genbank entries. Either the mutant 14-aa peptide or the wild type analog (VAVLYQdVNCTEVP) was then used as a probe to search other coronaviruses for homologies.

A functional fitness of the D614G mutation to calcium binding was implied by agreement between amino acids within the consensus peptide against a library of calcium-binding proteins sequences; i.e., a homology between an internal sequences of the consensus peptide to proteins known to have an EF-hand calcium-binding domain profile [43]. A homology was found by examining a library of 1685 different EF-hand calcium-binding domains that was generated from a structural motif analysis utilizing the “Prosite” search tool [44].

## Results

No significant similarity was found with the D614G mutant consensus peptide (VAVLYQgVNCTEVP) except within the databank sequences for the D614G SARS-CoV-2 mutants. When the wild type D614 (d) consensus peptide (VAVLYQdVNCTEVP) was tested against corona virus strains HCoV-229E (CAA71056.1), HCoV-NL63 (YP\_003767.1), HCoV-OC43 (YP\_009555241.1) and MERS (AXP07355.1), no significant similarity was found [45]. However, with SARS-CoV (AAP41037.1) there were 13 matches at residues 594 VAVLYQdVNC TDV 606 and with bat (APO40579.1) there were 14 matches at residues 597 VAVLYQdVNC TDVP 610.

A Prosite search performed with the EF-hand calcium-binding domain profile (PS50222) returned 1685 sequences from the UniProtKB/Swiss-Prot release 2020\_04, which contains 563,082 sequence entries, (< <https://prosite.expasy.org/PS50222#TP>>). A sub-search of the 1685 sequences using the above peptide LYQG returned a single entry for a calcium-binding membrane protein; (P56698)CAC1B\_DIPOM Probable voltage-dependent N-type calcium channel subunit alpha-1B OS=Diplobatis ommata OX=1870830 PE=2 SV=1 [46]. This protein is thought to be involved in channel activity and is involved in the pore-forming regulation of transmembrane calcium transport and has at least 50% homology to its human homolog per UniProtKB-KW (see report < <https://www.uniprot.org/uniprot/P56698>>).

## Discussion

The lack of homology between the 14-amino acid wild type D614 consensus peptide (VAVLYQdVNC TEV) and each of the human alpha-CoV strains HCoV-229E, HCoV-NL63, and HCoV-OC43 was not totally unexpected since human alpha coronaviruses have uncleaved S proteins [47]. Similarly, the S protein naturally occurring MES-CoV is atypical with its robust furin cleavage site at S2. However, it was somewhat unexpected that the bat had a higher homology to the consensus peptide (14 amino acids) than the homology of the SARS-CoV (11 amino acids). Nonetheless, substrating analysis of the Prosite search of EF-hand calcium-binding domains revealed a homology to a protein that is thought to be involved in channel activity and is involved in the pore-forming regulation of transmembrane calcium transport [46]. This is significant in light of the analogous D104G mutation in the F (fusion) protein of human parainfluenza virus 3 (hPIV-3)

which was discovered after passage in host cells that were deficient in the calcium transporter SPCA1. In humans, SPCA1 regulates the Golgi luminal Ca<sup>2+</sup> homeostasis and is ubiquitously expressed in all tissues. Decreased SPCA1 expression causes Hailey-Hailey disease, a rare skin disorder that impairs a cells’ ability to transport Ca<sup>2+</sup> (i.e., human ATP2C1 gene) [48].

The multiple roles of calcium in facilitating the entry of a coronavirus into a host cell are both critical and complex. Calcium triggers the protease-mediated activation of membrane fusion by initiating a series of conformational changes that enable the S protein to modulate the viral entry process at multiple steps without disulfide bonds to stabilize the multiple S peptides after cleavage. That is, unlike influenza HA, the absence of disulfide bonds to covalently connect the fragments after cleavage of the coronavirus S protein and its S1 peptide and S2 peptide domains. Without disulfide bonds, the stalk domain attached to the viral membrane can be separated from the RBD region attached to the host receptor. Also, the coronavirus S protein is unusual because it can contain more than one proteolytic cleavage site and there can be cleavage of different sites at various stages of the virus life cycle. Additionally, the transient nature of the S protein cleavage means that it is difficult to observe this cleavage event based on conventional biochemical techniques (e.g., Western blots). Thus a mutation that affects the interactions between cleaved peptides of the S protein and/or membranes may improve the efficiency of the virus.

In light of the D104G mutation having arisen after virus passage in SPAC1-deficient cells, earlier studies on the calcium-dependent conformational changes of membrane-bound Ebola fusion peptide can shed light on the “fitness” provided by the D614G mutation [49]. These investigations found that the absence of Ca<sup>2+</sup> stabilizes an alpha-helical conformation and gives rise to vesicle efflux but not vesicle fusion. However, in the presence of millimolar Ca<sup>2+</sup>, the membrane-bound peptide adopts an extended beta-structure that drives vesicle fusion via a fusogenic complex. In addition, the fusogenic subunit participates actively in the formation of a functional fusion pore and the initial partitioning of the viral fusion protein into the target cell membrane [50]. Moreover, fusion peptides are different from classical trans-membrane anchors in being conformationally polymorphic due to their high content of glycine and alanine residues which facilitates permeabilization and fusion [51-53].

Physiologically, calcium is predominantly bound to albumin in the plasma, and a decrease in serum albumin will cause hypocalcemia. Moreover, low serum albumin at admission is common in community-acquired pneumonia (CAP) and independently associated with a higher risk of 30-day mortality [54,55]. Similarly, very ill COVID-19 patients are hypocalcemic and have hypoalbuminemia [56-58] Additionally, the reduced calcium levels in COVID-19 patients can be caused by the pro-inflammatory cytokines that can inhibit parathyroid hormone (PTH) secretion [57,59] and impair the response to PTH. Thus, from a clinical standpoint, hypocalcemia can effectively cause impaired transport of Ca<sup>2+</sup>-like the inability of SPCA1-deficient cells to attain millimolar concentrations of Ca<sup>2+</sup>.

In summary, this mechanistic review can be described as the COVID-19 calcium hypothesis and provides a rationale for the use of calcium-interfering drugs already approved by the FDA as therapeutics. The primary focus of this work is how to repurpose FDA-approved drugs to mitigate infection by preventing calcium from triggering the protease-mediated activation of SARS-Cov-2 entry and not

close the stable door after the horse has bolted; e.g., hydroxychloroquine (chloroquine) blocked the transport of SARS-CoV-2 from early endosomes (EEs) to late endolysosomes (ELs) resulting in failure of further transport of virions to the ultimate releasing site [60]. That is, exploit the coronavirus' dependence on calcium for therapeutic gain [41]. For other viruses, there has been an interest in repurposing  $\text{Ca}^{2+}$  channel ligands toward cell surface targets such as voltage-operated  $\text{Ca}^{2+}$  channels (VOCCs) as a way to impair viral infectivity [61,62]. Although not explicitly directed at disrupting surface channel, it is noteworthy that the cell permeable  $\text{Ca}^{2+}$  chelator BAPTA-AM also inhibited MERS-CoV pseudovirus translocation [63].

While recent experiments demonstrated Rubella infection was also inhibited by EDTA [64], in-vitro surface membrane experiments to prove that EDTA can attenuate SARSCoV-2/COVID-19 infection in Vero E6 cells in high-throughput screening is difficult, if not impossible to accomplish. First, the Vero E6 cell line is not a physiological model because Vero cells are incapable of producing type I interferon in response to viral infections [65]. This may explain why the anti-malarial drug chloroquine, which had demonstrated viral replication inhibition in Vero E6 cells [66], did not show efficacy in reducing SARS-CoV virus titers in a nonlethal mouse model [67]. Second, standard Vero E6 cells have little or no TMPRSS2 expression [68].

Another key challenge to testing the COVID-19 calcium hypothesis in tissue culture arises because one cannot lower the calcium concentration low enough to be able to juxtapose a low enough COVID-19 virus titer that would facilitate observing a quantifiable difference in cytopathic effects. Unlike NMR studies where the solvent is changed to compensate for the 110,000-fold higher signal that suppresses the signal from the peptide protons of interest, calcium is a critical component of tissue culture media. While the chemist can avoid the NMR signal arising from 110 Molar water protons that swamps-out the sample signal by dissolving a peptide at milli-molar concentration in a deuterated solvent, calcium is an indispensable component of the tissue culture media employed to propagate the SARS-CoV-2 virus.

In the absence of any effective drugs targeting SARS-CoV-2 therapy to treat COVID-19 disease, one can excuse the tissue culture limitations of Vero cells by relying on the observed natural history of SARS-CoV-2 infection (e.g. hypocalcemic and hypoalbuminemia), the lower reported prevalence of asthma in patients diagnosed with COVID-19 [41], and the ability to safely repurposing pharmaceutical EDTA. This is because there has already been much clinical experience with FDA-approved medicines and "Off-Label" IV use of  $\text{Na}_2\text{EDTA}$  for the past 50 plus years. The safety of EDTA in nebulizers has been analyzed utilizing human volunteers over 30 years ago [69]. Additionally, while not a specific recommendation herein, infusions of an edetate disodium preparation of up to 3 grams per treatment reduced recurrent cardiovascular events in the study cohort composed of nondiabetic patients (63%) and patients with type 1 and 2 diabetes (37%) with prior myocardial infarction (MI) in the 10-year National Institutes of Health-funded Trial to Assess Chelation Therapy (TACT) [70]. No acute toxic effects were observed after suitable oral intake and no adverse outcomes from intravenous disodium EDTA have been found if administered appropriately and not exceeding  $2500 \mu\text{mol L}^{-1}$  in the blood circulation [71]. In fact, for about 50 years, oral iron EDTA has been used in France in medicinal syrup for pregnant women and infants 1-6 months of age to prevent/treat iron deficiency anemia [72]. Accordingly, oral EDTA may also provide relief from the reported extra-pulmonary symptoms of COVID-19 in the GI tract [73].

Likewise, oral inhalation therapy with solutions containing EDTA delivered via nebulization is a tried and safe medical approach that can now be used to disrupt COVID-19/SARS-CoV-2 infection under medical supervision in much the same way that Albuterol nebulizer treatments are now prescribed for home use [41]. In other words, in the absence of any proven alternative therapy, the COVID-19 calcium hypothesis justifies repurposing pharmaceutical grade EDTA that the FDA previously approved for use in nebulizers as an excipient (preservative) to an active ingredient with or without Albuterol.

As described above, there was improved fitness with a D104G mutation in the F (fusion) protein of human parainfluenza virus 3 (hPIV-3) that was discovered in the laboratory [22]. By analogy to the parainfluenza D104G mutation, the aspartic acid to glycine mutation at SARS-CoV-2 Spike protein amino acid residue (614) would seem to provide the fitness to become the dominant global variant in the COVID-19 pandemic [22]. To put it another way, in the absence of any other biological "fitness" explanation, the COVID-19 calcium hypothesis does help elucidate a selective advantage that aids in the infection/transmission.

An aside of the COVID-19 calcium hypothesis would suggest that ingestion of zinc supplements is contraindicated to fight SARS-CoV-2. This is because data indicate that  $\text{Zn}^{2+}$  ion is capable of partially substituting for the ability of  $\text{Ca}^{2+}$  ion to modulate the activity of Calmodulin (CaM), which is involved in calcium-signal transduction. CaM is a canonical member of the EF-hand family of proteins, which are characterized by a helix-loop-helix calcium-binding motif [74]. Moreover, while the principle focus was on the COVID-19/SARS-CoV-2 calcium hypothesis in the previously published paper, it is noteworthy that the cleavage of influenza virus hemagglutinin (HA) by host cell proteases including TMPRSS2 is essential for virus infectivity and spread [75]. Thus EDTA treatment, may also be useful for mitigating some isolates of influenza A virus, which are cleaved by TMPRSS2 and can cause severe disease both in domestic poultry and, rarely, in humans.

The COVID-19 calcium hypothesis was based, in part, on trying to explain the prior observations that there was a markedly lower reported prevalence of asthma and COPD in patients diagnosed with COVID-19 [76,77] which were not satisfactorily being explained by any available scientific theories. The hypothesis explains that there are multiple unrecognized calcium requirements for SARS-CoV-2 infection and how the presence of EDTA excipients in nebulized  $\beta_2$ -agonist medicines can disrupt SARS-CoV-2/COVID-19 infections to explain the asthma and COPD paradox.

## Conclusion

The COVID-19 calcium hypothesis describes the key role of calcium in SARS-CoV-2/COVID-19 infection and provides viral mechanisms to elucidate why asthma patients appear resistant to COVID-19 infection. The hypothesis rests upon having discovered unrecognized calcium-dependent fusion loop domains in SARS-CoV-2 and recognizing that the substrate recognition sites for the requisite cell surface protease TMPRSS2 have a conserved SRCR (scavenger receptor cysteine-rich) domain and a LDLRA (LDL receptor class A) that utilize calcium. The COVID-19 calcium hypothesis has substantial predictive power and is consistent with clinical findings. As shown here, the COVID-19 calcium hypothesis can also provide a mechanism to explain how the D614G variant of SARSCoV-2 now dominates the pandemic based on a peptide similarity to an EF-hand calcium-modulated

protein. Based on this mechanism and the critical role of calcium in SARS-CoV-2 infection, oral and nebulized EDTA should be available to patients to disrupt virus infection at the earliest possible time; i.e. by preventing calcium from triggering the protease-mediated activation of SARS-Cov-2 entry into host cells.

The data converges on the previously unrecognized critical importance of calcium for effective SARS-CoV-2/COVID-19 infection, and how calcium chelation by EDTA can disrupt viral infection. It is noteworthy that while EDTA is only a single drug, it can still disrupt multiple key steps in the SARS-CoV-2/COVID-19 infectious process. Accordingly, repurposing EDTA from excipient to therapeutic nebulized drug with or without Beta-2 agonist supplementation logically becomes a new treatment for COVID-19/SARS-CoV-2 patients. EDTA therapy based on the COVID-19 calcium hypothesis addresses both the immediate problematic situation of finding treatment that can disrupt the calcium-dependent COVID-19/SARS-CoV-2 infectious processes and explain the worldwide dominance of the D614G variant. Historically, oral EDTA has been well tolerated “Administration of Calcium Disodium EDTA to patients in an aerosol form produced no ill-effects during and after treatment, as the therapy was well tolerated, with no changes in the respiratory organs observed. Absorption was determined to be 10% to 30% of the dose. Stippled cell counts were somewhat irregular; however, no other changes occurred in the blood analysis as a result of Calcium Disodium EDTA inhalation” [78]. Alternatively, oral EDTA may also provide relief from the reported extra-pulmonary symptoms of COVID-19 in the GI tract [79]. The potential to utilize EDTA to both reduce COVID-19 transmission and treat infection through relatively safe modalities that include nebulizer or mechanical ventilator misting of EDTA solutions (in conjunction with Albuterol/ Metaproterenol to minimize bronchoc constriction if needed) and adding EDTA to hygienic products warrant immediate further investigation(s).

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## Declaration of Competing Interest

The author declares that he has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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