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Yash Gupta
Dawid Maciorowski
Brian Medernach
Daniel P. Becker Ph.D.
Loyola University Chicago, dbecke3@luc.edu
Ravi Durvasula

See next page for additional authors

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Iron Dysregulation in COVID-19 and Reciprocal Evolution of SARS-CoV-2: natura
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Yash Gupta, PhD1, Dawid Maciorowski, BS2, Brian Medernach, MD3, Daniel P. Becker, PhD4, Ravi Durvasula, MD1, Claudia R. Libertin, MD1, Prakasha Kempaiah, PhD1*

1 Infectious Diseases, Mayo Clinic, Jacksonville, FL, USA; 2 University of Wisconsin-Madison, School of Medicine and Public Health, Madison WI, USA; 3 Department of Medicine, Loyola University Medical Center, Chicago, IL, USA; 4 Department of Chemistry and Biochemistry, Loyola University Chicago, Chicago, IL, USA

*Address correspondence: Dr. Prakasha Kempaiah, Ph.D., e-mail:
kempaiah.prakasha@mayo.edu

Running title: Role of Covidin in iron dysregulation and pathogenesis

KEYWORDS: Iron homeostasis, Ferritin-transferrin paradox, Hypoxia, Evolved Variants, Host proteases, and MD simulations
ABSTRACT: After more than a year of the COVID-19 pandemic, SARS-CoV-2 infection rates with newer variants continue to devastate much of the world. Global healthcare systems are overwhelmed with high positive patient numbers. Silent hypoxia accompanied by rapid deterioration and some cases with septic shock is responsible for COVID-19 mortality in many hospitalized patients. There is an urgent need to further understand the relationships and interplay with human host components during pathogenesis and immune evasion strategies. Currently, acquired immunity through vaccination or prior infection usually provides sufficient protection against the emerging variants of SARS-CoV-2 except Omicron variant requiring recent booster. New strains have shown higher viral loads and greater transmissibility with more severe disease presentations. Notably, COVID-19 has a peculiar prognosis in severe patients with iron dysregulation and hypoxia which is still poorly understood. Studies have shown abnormally low serum iron levels in severe infection but a high iron overload in lung fibrotic tissue. Data from our in-silico structural analysis of the spike protein sequence along with host proteolysis processing suggests that the viral spike protein fragment mimics Hepcidin and is resistant to the major human proteases. This functional spike-derived peptide dubbed ‘Covidin’ thus may be intricately involved with host ferroportin binding and internalization leading to dysregulated host iron metabolism. Here, we propose the possible role of this potentially allogenic mimetic hormone corresponding to severe COVID-19 immunopathology and illustrate that this molecular mimicry is responsible for a major pathway associated with severe disease status. Furthermore, through 3D molecular modeling and docking followed by MD simulation validation, we have unraveled the likely role of Covidin in iron dysregulation in COVID-19 patients. Our meta-analysis suggests the Hepcidin mimetic mechanism is highly conserved among its host range as well as among all new variants to date including Omicron. Extensive analysis of current mutations revealed that new variants are becoming alarmingly more resistant to selective human proteases associated with host defense.
Key points:

- The dysregulation of iron homeostasis appears to be a hallmark feature of severe SARS-CoV-2 infection

- The decreased availability of free iron outside of the cell is protective against many bacterial infections, but the importance of intracellular iron for SARS-CoV-2 is well established

- In addition to increasing intracellular iron for viral replication, the presence of both host Hepcidin and its molecular mimic Covidin at high levels will likely result in toxic levels of intracellular iron (Ferroptosis)

- Ferroptosis leads to the release of free radicals which also have a toxic effect on surrounding cells

- SARS-CoV-2 variants are becoming resistant to host proteolysis innate defense mechanisms.

- The Y→N 501 mutation confers site loss of the cathepsin G enzyme susceptibility

- An alarming aspect is that loss of lysosomal protease sites on spikes may enable SARS-CoV-2 variants to infect macrophages in the future opening the possibility of antibody-mediated enhancement of COVID-19
Introduction:
The COVID-19 pandemic is caused by the SARS-CoV-2 virus, which belongs to the Coronaviridae family. Other members of the family have phylogenetic similarities, including SARS-CoV, which causes Severe Acute Respiratory Syndrome (SARS), and MERS-CoV, which causes Middle Eastern Respiratory Syndrome (MERS) [Gupta et al., 2021a]. The clinical spectrum of COVID-19 infection ranges from asymptomatic to critical illness with typical fever, malaise, cough, gastrointestinal symptoms, shortness of breath, and myalgias. The mean incubation period for COVID-19 is currently understood as between 5-12 days [Lauer et al., 2020] while new variants, such as Delta and Omicron, have even shorter incubation times [COVID and Team, 2021; Homma et al., 2021; Snell et al., 2021]. Patients who progress to severe COVID-19 disease develop dyspnea and hypoxia with rapid progression to respiratory failure and commonly meet the criteria for acute respiratory distress syndrome (ARDS) [Control et al., 2021]. Severe COVID-19 also leads to multi-organ failure hallmarked by the cytokine release syndrome characterized by fever, thrombocytopenia, and markedly elevated inflammatory markers [Chen et al., 2020; Maciorowski et al., 2020; Onder et al., 2020].

A commonality among members in the Coronaviridae family is the viral spike (S) protein, the principal viral surface glycoprotein responsible for host membrane attachment. The S protein is a transmembrane trimer in a metastable prefusion conformation that undergoes structural rearrangement and peptidase processing to fuse the viral membrane with the host cell membrane [Gupta et al., 2021b]. This protein mediates viral attachment to the host cell surface receptors and is responsible for the consequent fusion between the viral and host membranes to enable viral entry [Plante et al., 2021a]. The S protein has two subunits, S1 and S2 (Fig. 5.). When the S1 subunit binds to a host cell receptor, the interaction causes shedding of the destabilized S1 subunit and transitions to the S2 subunit that maintains a stable post-fusion conformation [Wrapp et al., 2020]. In terms of viral attachment mechanisms, it is clear that both SARS-CoV and SARS-CoV-2 recognize the angiotensin-converting enzyme II (ACE2) receptor as the host receptor that binds to the S protein [Hoffmann et al., 2020].

Recent studies note a significant similarity between the SARS-CoV-2 spike glycoprotein cytoplasmic tail region and the amino acid sequence of the Hepcidin protein [Ehsani, 2020].
There is a lacuna of understanding the role of molecular mimicry by an intracellular portion of spike protein and the soluble human analog Hepcidin. In this context, manipulating host iron regulation may be a key component in understanding the pathogenesis, lung fibrosis, hypoxemia, inflammation, and cytokine release syndrome associated with serious COVID-19 infection. The dysregulated iron state in COVID-19 pathogenesis has not been fully explored. However, there are links to iron and its dysregulation in the paradox of hyperferritinemia[Ruan et al., 2020] and anemia status[Zhao et al., 2020], which are seen together in COVID-19, particularly in severely infected patients [Sarode et al., 2021]. This dysregulation and iron overload causing ferroptosis may explain other symptomatology of COVID-19 pathogenesis including multi-organ pathology[Jacobs et al., 2020; Yang and Lai, 2020], and explain neuroprotection by vitamin E, a known ferroptosis blocker[Tavakol and Seifalian, 2021]. Iron dysregulation has been linked to neurological disturbances including cognitive impairment, ageusia, and anosmia which are common manifestations of severe COVID-19 disease[Edeas et al., 2020].

Hepcidin is a liver-derived peptide hormone that is a crucial regulator of systemic iron homeostasis[Girelli et al., 2021]. Hepcidin was first isolated in the year 2000 as a peptide with antimicrobial activity and independently described in the literature after being isolated from both human dialysate ultrafiltrate as well as from urine[Wojciechowska et al., 2021]. Hepcidin is encoded by the Hepcidin antimicrobial peptide (HAMP) gene and is initially synthesized as an 84 amino acid pre-pro-Hepcidin. This molecule is then processed to the 60 amino acid pro-Hepcidin, and is ultimately cleaved to a mature C-terminal 25 amino acid active peptide[Leong and Lönnerdal, 2004]. In 2004, Nemeth et al. described the target site of Hepcidin as ferroportin[Nemeth and Ganz, 2021]. Ferroportin is an iron exporter on the surface of absorptive intestinal enterocytes, macrophages, hepatocytes, and placental cells, responsible for releasing iron into plasma.

Hepcidin-ferroportin homeostasis is central to iron regulation and plays a role in several disease states. By acting on ferroportin, Hepcidin controls the flow of iron into plasma from duodenal enterocytes absorbing dietary iron, from macrophages involved in recycling of iron from senescent erythrocytes, and hepatocytes involved in iron storage. When Hepcidin
concentrations are low, iron enters the blood plasma at a high rate. When Hepcidin concentrations are high, ferroportin is internalized and iron is trapped in enterocytes, macrophages, and hepatocytes [Nemeth and Ganz, 2021]. Hepcidin synthesis is regulated at the transcriptional level by multiple stimuli. HAMP gene expression is upregulated by iron overload, inflammation, and decreased iron-deficient states, and hypoxia [Agarwal and Yee, 2019]. Iron affects gene expression via BMP/SMAD pathways, while inflammation and IL-6 utilize the JAK/STAT pathway [Varga et al., 2021]. Iron is essential for high load viruses including SARS-CoV-2, which is inhibited by iron chelators \textit{in-vitro} [Salaris et al., 2021]. But excess intracellular iron accumulations lead to apoptosis (ferroptosis) as seen in COVID-19 patient biopsies [Jacobs et al., 2020; Manivannan and Sundaresan, 2021]. The host Hepcidin protein does not instigate iron accumulation localized near the infection site, in contrast to patients with severe pneumonitis. Furthermore, hypoxemic hypoxia blocks Hepcidin formation completely via multiple pathways [Choi et al., 2007].

Host proteases create a hostile environment for pathogens and thus play an important role in innate immunity. They are also critical for antigenic processing and adaptive immunity. The variations in the substrate site, as well as protease polymorphisms, alter the processing [Pereira et al., 2021; Rawat et al., 2013]. A zoonotic pathogen that lacks co-evolutionary history with a new host needs to modify and adapt through molecular evolution in order to thrive [Plante et al., 2021b]. SARS-CoV-2 has demonstrated multiple instances of species' jump [Ekstrand et al., 2021] and the rapid evolution for adapting to the new hosts, for example, the Mink variant [Bayarri-Olmos et al., 2021]. Another peculiar feature of SARS-CoV-2 is the advent of convergent mutations, i.e. the same mutations among different lineages [Cherian et al., 2021; Martin et al., 2021; Zhou et al., 2021]. However, antibody response specificity varies significantly among individuals and cannot exert a selective pressure specific enough for site-specific convergent mutations.

This manuscript investigates the mechanism through which SARS-CoV-2 utilizes host hormone mimicry as demonstrated through protein modeling, docking, and MD simulations. We found that spike protein degradation by host proteases leads to the release of a Hepcidin-like peptide. We hypothesize that infected cells with surplus viral proteins when ultimately
degraded through various pathways involving proteases can release a Hepcidin mimetic peptide dubbed Covidin. We hypothesize that a Hepcidin-like overload profile is caused by a virus-derived Covidin protein which is supported by the clinical data reported from numerous studies. Furthermore, analysis of the proteolytic fate of the spike protein and release of Covidin among all the variants reported so far, reveal an important association of mutations with proteolytic sites.
Methods

Multiple sequence alignment (MSA) of SARS-CoV-2 spike mimetic peptide (Covidin) with Hepcidin sequence from different mammals. The NCBI databank was used to retrieve protein sequences from mammals recorded for Hepcidin hormone orthologues structurally related to SARS-CoV-2. The analysis aimed at investigating the conservation of Hepcidin in reference sequences vs clinical and environmental strains of SARS-CoV-2 from different countries (GISAID)[Kalia et al., 2021] using bioinformatics tools. T-COFFEE and PRALINE software [Bawono and Heringa, 2014; Dereeper et al., 2008] were used for alignment.

Worldwide mutation rates for Covidin peptide. GSAID database global SARS-CoV-2 sequence analysis available from the Nextrain server was used to map mutation rates in the Hepcidin mimetic region (Covidin) of the spike protein[Hadfield et al., 2018].

Analysis of host protease activity on the SARS-CoV-2 Spike protein. The host protease specificity and spike protein cleavage site locations were predicted using the iProt-Sub Server[Song et al., 2019]. The iProt-Sub server employs an algorithm based on specificity information from the MEROPS database[Rawlings et al., 2016] that has been validated for 38 different proteases from the four major protease families (aspartic, cysteine, metallo-, and serine) and was used to identify substrate protein cleavage sites for each of the enzymes examined. The amino acid residue N-terminal to the cleavage site is color-coded by protease family; the color code assigned with the iProt-Sub server was red for aspartic, yellow for cysteine, blue for metallo-, and green for serine, with multiples assigned to the highest scoring family at a given site (Fig.4). iProt-Sub is considered the most advanced server with greater accuracy and coverage due to its more comprehensive server capabilities and adoption of machine-learning techniques.

The Procleave server[Li et al., 2020], a more advanced version of iProt-sub, has implemented a probabilistic model trained with both sequence and structure feature information. The Procleave database consists of AI trained with 66,441 protein-substrate complexes. The scoring matrix was used to shortlist affected protease sites. We have analyzed spike protein
sequences of representative isolates from highly prevalent lineages of SARS-CoV-2. Data were obtained from the NIAID Virus Pathogen Database and Analysis Resource (ViPR)[Pickett et al., 2012]. The different spike proteins were mapped for polymorphisms and analyzed by the Procleave server selecting from most of the significant human proteases. The differences in the scores for all the protease substrate sites analyzed were compared among all variants. A more than 50% drop in score indicated a difference in the target peptides’ cleaved or partially cleaved status by specific proteases. Even though the mutants have only a few substitutions, they have significantly altered protease specificity landscapes (Table 1).

**Protein & peptide modeling.** For ferroportin protein structures, we first determined if there are any homologous proteins with known structures. This was attempted using sequence-based searches on the Protein Data Bank (PDB)[PDBe-KB: a community-driven resource for structural and functional annotations, 2020]. The search did not yield any feasible homologs with available 3D structures. To circumvent this, a de-novo/fragment modeling approach was performed using the I-Tasser server [Yang et al., 2015] and the human ferroportin amino acid residue sequence was uploaded to the server. COACH predictions[Wu et al., 2018] for ligand and metal ion binding characteristics and OPM servers’ prediction[Lomize et al., 2012] for extracellular domain orientation were used to predict the binding site for the peptides. Small peptide modeling for Hepcidin and Covidin was performed using the Phyre2 server[Kelley et al., 2015]. All models were validated and corrected by the FGMD server[Zhang et al., 2011].

**Protein-peptide docking.** The ClusPro server was selected due to its prior success in predicting at least one near-native complex within its top 10 predicted interfaces[Kozakov et al., 2017]. We uploaded our structure files from the I-Tasser and Phyre2 modeling to the ClusPro server. Once the server had completed its predictions, we then selected the top 30 predicted interfaces to be investigated further. Following the data from the COACH and OPM servers, the top docking cluster for each peptide showed interactions with the extracellular domain and blocked the central channel. The top 30 interfaces between the Ferroportin structure and the peptides showed high similarity with each other in terms of binding interactions. The top cluster was selected for further investigation. To map out the interactions in 2D, we used LigPlot+ v.2.2 software[Laskowski and Swindells, 2011], which superimposes
the interactions of the two peptide ligands with ferroportin demonstrating the same binding core space and interacting residue pairs.

**MD simulations of docked complexes.** All calculations, simulations, and visualizations for this work were conducted on a Dell Precision T3430 running Ubuntu Linux version `bionic beaver` on an Intel Xeon E-2174G processor. All simulation preparations and simulations were conducted using the Desmond Molecular Dynamics System, with code available from D. E. Shaw Research, integrated with the Maestro molecular modeling environment provided by Schrödinger, LLC[Schrodinger, 2020b]. Visualization and trajectory analyses were conducted using the Maestro GUI interface to Schrodinger using the Simulations interaction diagram wizard[Schrodinger, 2020a]. As no ferroportin structure is available from *Homo sapiens* or any other mammal, the I-TASSER server modeled the structure [Yang et al., 2015]. The topology of ferroportin in a lipid bilayer membrane was obtained from the Orientations of Proteins in Membranes database (OPM)[Lomize et al., 2012]. The top model from I-TASSER was pre-processed using Maestro’s ‘Protein Preparation Wizard.’ During pre-processing, all non-protein crystal artifacts including waters (>3 non-water hydrogen bonds), ions, and any ligands were removed, and all hydrogens in the model were deleted to minimize mistakes in bond orders and hydrogen atoms added to all protein residues as warranted. After pre-processing, the Protein Preparation Wizard noted any overlapping atoms or missing residue side chains. The errors if any were resolved using Optimization/minimization steps using the OPLS3e force field. The Maestro System Builder tool was employed to construct a multi-molecular system for the Molecular Dynamics simulation. This tool primarily performed six tasks to prepare the modeled system as follows: (1) a water box encompassing the protein docked with either of the peptides (Hepcidin/Covidin) was created to provide at least a 10 Å buffer for the protein in all directions, in reference to whole complex spacial dimentions; (2) a lipid bilayer membrane was placed around the protein according to OPM coordinates utilizing POPC phospholipid models and extending to the simulation box in the x and y directions; (3) the system was solvated in a solution of TIP3P water models; (4) 0.1 M NaCl was placed in the solution to mimic physiological conditions; (5) ions and water molecules placement were excluded within 3 Å of the protein; and (6) OPLS3e force field parameters were selected for utilization for both this preparation and all later simulations.
Results and Discussion

**Phylogenetic analysis.** The Hepcidin mimetic peptide at the C-terminal region of the SARS-CoV-2 Spike protein is usually omitted from protein structure determinations because the second heptad repeat domain destabilizes the spike structure to form a mature fusion protein with the first heptad domain. Not much is known about the function of this highly conserved portion of the spike protein in the literature. This homologous peptide is more similar to its accepted primary sequence in bat and pangolin hosts (Figs 1 & 2). The mimicry appears to be highly conserved among the spectrum of hosts suggesting a functional role in pathogenesis typical of Coronaviruses (Fig 2). There is a grouping observed among mammals susceptible to severe SARS-CoV-2 infection (Fig 1). While the peptide present in primates and bats seems to be more evolved, the similarity between the two groups is noteworthy due to the apparent evolutionary distance between the two groups of species.

The global mutant distribution shows multiple prevalent mutations in the spike protein. The mutant/variants of the spike protein are known to have an advantage against detection by several monoclonal antibodies[Greaney et al., 2021], but variants are primarily neutralized by original Wuhan strain-based vaccines or previous infection[Bertoglio et al., 2021; Stamatatos et al., 2021]. Additionally, there is an underlying founder effect at play due to several seeding infections in each geographical area at the beginning of the pandemic and the reintroduction of more virulent variants. The appearance of convergent mutations among various distinct lineages suggests a common selective pressure that is very site-specific. While antibody specificity dramatically varies from individual to individual, it is not expected to elicit single amino acid level changes. Compared to mutation rates for the whole spike protein, the Covidin region is highly conserved (Fig 3). It is to be noted that most of the mutations were for amino acids positioned on the surface of the structure (Fig 5). The viral S protein displays the highest degree of genetic variability in the virus genome, however, the region encoding the Covidin peptide has proven highly conserved among the identified SARS-CoV-2 variants (Fig 3). This relationship of hepcidin and molecular mimicry of the SARS-CoV-2 Covidin peptide with host ferroportin may have significant ramifications on iron dysregulation and may be a key to understanding severe COVID-19 human disease.
Protease site mapping. Upon whole sequence protease site mapping by the most advanced servers including iProt-Sub and Procleave, the fate of spike in the human body was revealed to be excessive fragmentation due to proteolysis by various resident human proteases (Fig. 4). Interestingly, the Covidin peptide was resistant to human proteolytic machinery suggesting persistence of this peptide post spike degradation in infected individuals and upon autophagy/apoptosis or necrotic degradation of dead infected cells with surplus unassembled viral proteins. The Covidin peptide region was 100% conserved in all the major variants including the recent highly mutated Omicron strain[Karim and Karim, 2021] analyzed (Table 1) and therefore, proteolytic resistance was likewise conserved. There was, however, specific and repetitive protease site loss by the mutations in the variants (Table 1.) (The Omicron protease map is presented separately in Supplementary table 1 because of too many unique mutations). While the spike protein is heavily glycosylated protecting it from proteases, a few sites on the protein surface are still vulnerable to proteolysis and were protected by polymorphisms in the variants. A spacial clustering i.e., the structural proximity of mutations conferring site loss for proteases from the same cellular/extracellular compartment/location was observed. (Fig 5).

As there is no evolutionary advantage to 'lethal' strains, the appearance of variants with a more severe prognosis instead of asymptomaticity, which is more advantageous for unchecked spread, is perplexing. The mutations have a few unique characteristics such as they seem to be scattered and there is no hotspot to evade antibody binding though, all are on the surface i.e. exposed outside protein structure. There is little correlation between the known neutralizing antibody binding sites and emerging mutations[Cameroni et al., 2021; Chauhan et al., 2021]. The loss of the Meprin A and matrix metallopeptidase protease (MMPs) sites were either at the RBD or towards the N-terminus of the S1 cleavage site. Meprin A and MMPs are involved in fibrosis, tissue injury, and inflammation, all of which are hallmarks of severe COVID-19 disease. Meprin A enzymes are expressed to remodel epithelial cells and collagen and to help in macrophage infiltration to the alveoli sac[Bonnans et al., 2014]. Cross cleavage of such enzymes might have a detrimental effect on viral potency and RBD integrity. The earliest and most prevalent mutation of D→G at 614 has been an enigma concerning the mechanism of
benefit to the variant as it causes a site loss to Meprin A subunit-beta. Loss of Thrombin and Furin cleavage sites are also toward the N-terminal spike region and might help respective variants with a more stable spike protein.

The site loss for cathepsins is nearby or on the viral fusion domains. These enzymes are aspartic proteases optimally working at acidic pH and many are present in the lysosome. Such site loss makes sense to keep viral fusion machinery active in the endosome from where the nucleocapsid can be delivered to the cytoplasm through viral fusion. The most omnipresent Y→N 501 mutation confers the site loss of the 'cathepsin G' enzyme which is an inflammation-associated enzyme involved in eliminating intracellular pathogens. The Y→N 501 mutation has been attributed to enhanced virulence in many lineages with unknown mechanisms [Callaway, 2021]. Lysosomal enzyme site loss may also be advantageous to the virus as these proteases are responsible for antigen processing. These polymorphisms should also have poorer antigen presentation in the variants and could be an immune evasion strategy. Antigen processing has already been reported to correlate with COVID-19 severity. Genome-wide association studies (GWAS) have shown the gene ERAP2 associated with one of the high-risk variants [Lu et al., 2020], which has been shown to affect adaptive immune response by altered antigen processing[Chen et al., 2016]. This suggests that the variants are becoming hypo-immunogenic and may have more severe disease and not confer much protection against re-infection. Antibody-dependent enhancement (ADE) caused by enhanced viral replication has been reported for viruses that infect macrophages, including SARS-CoV[Weingartl et al., 2004] and MERS-CoV[Agrawal et al., 2016] both in vitro and in vivo[Wan et al., 2020]. It has been reported that there is no macrophage persistence from infection by SARS-CoV-2 and hence no ADE [García-Nicolás et al., 2021; Hui et al., 2020]. But if the variants are becoming more resistant to lysosomal proteases including Cathepsin G, then more severe infection observed in the variants could be attributed to ADE. As these mutations are present on different lineages, and we have seen many convergent mutations in past with SARS-CoV-2, these strains might be evolving in the same direction. Such possibilities are alarming since a considerable fraction of the population now has antibodies against SARS-CoV-2 through infection or vaccination. ADE was recently observed with the Delta variant with a cross-reactive antibody failing to neutralize the mutant and resulting in macrophage infection through the IgG
receptors[Maemura et al., 2021; Yahi et al., 2021]. The recent emergence of the Omicron strain[Karim and Karim, 2021] which is the most infectious of the lineage is heavily mutated in the spike protein region and is responsible for a breakthrough as well as reinfections of SARS-CoV-2 [Pulliam et al., 2021; Wilhelm et al., 2021]. While Omicron variant causes mild disease it reduces protection from vaccines and prior infections[Cele et al., 2021], and due to the Omicron-spike protein epitope gap with other variants, the Omicron-specific antibodies could aggravate ADE in subsequent Delta variant infection which is still in circulation.

**Protein modeling, protein-protein docking, and MD simulations.** The peptide models of Covidin were highly similar to Hepcidin (Fig. 6). The docking with modeled ferroportin showed biochemical conservancies, and the interactions observed also have significant physiological mimicry of host Hepcidin. The interaction MAP revealed 85% spacial overlap in the binding site, and Covidin has more interactions with the target ferroportin (Fig. 7). It is noteworthy that this is a conserved interaction among different hosts due to exponentially faster turnover of the viral peptide and is expected to become more evolved.

Long MD simulations (100ns) could not reveal how Hepcidin or Covidin promote ferroportin ubiquitination and degradation, as the system seem to be highly stable for both the complexes (Figs 8&9). But as seen with the interaction map, the Covidin-ferroportin complex was more stable than the Hepcidin-ferroportin complex. As both peptide and receptor were modeled, the confidence in the structure is limited, however, the critically important receptor ferroportin should be crystallized and structurally characterized with urgency.

**Relationship of Iron transport, Hepcidin, and Covidin.** Lung fibrosis observed in COVID-19 patients and resulting hypoxia is the main reason for mortality in severe cases which can also be complicated by secondary bacterial and fungal infections[Wang et al., 2020]. COVID-19 infection in humans is primarily asymptomatic or exhibiting a mild disease unless it manifests with the onset of hypoxemia from pneumonitis which may progress to acute respiratory distress syndrome (ARDS) with similarities to toxic shock syndrome(TSS)[Chambers, 2020]. There is still a lack of understanding as to why some individuals are asymptomatic, some require low-oxygen supplementation to then recover,
while others rapidly decompensate into ARDS [López-Escobar et al., 2021]. It is possible that once a threshold is crossed by either uncontrolled hyperinflammation and/or stimulation of the signaling pathways comodulated by hypoxia and the result is ARDS [Helms et al., 2020]. The vast quantity of Covidin released from dying lung fibroblasts caused by the SARS-CoV-2 virus may be a contributing factor (Fig. 10.).

Patients infected with the Delta variant have documented higher viral loads in bronchial alveolar lavages [Malik et al., 2021]. Similarly, the presentations of those infected with the Delta variant are described to have fewer co-morbidities and more rapid decompensation than those with the original Wuhan strain or alpha strain [Twohig et al., 2021]. The virulence-associated with the Delta strain is due to the higher viral burden, higher particle infectivity, and hypoantigenicity [Mclochova et al., 2021]. COVID-19 associated lung fibrosis is not governed by classic pathways and a deviation has been reported by multiple studies [de Paula et al., 2020]. While Ferritin is shown to be highly expressed in COVID-19 patients due to cytokine storm, IL-6 induction, or increased release from damaged cells, contrastingly the serum iron and transferrin levels were both very low [Bellmann-Weiler et al., 2020]. Reduction in transferrin levels indicates cellular iron accumulation through this carrier and can even activate platelets (Fig. 10.). Under hypoxic conditions, the normal response of the organism is to increase the number of red blood cells (RBCs), thereby increasing the delivery of oxygen to starved tissues and organs. During hypoxic conditions, the signaling pathway involving hypoxia-inducible factors (HIFs) is also activated. HIFs are known to reduce Hepcidin levels thereby increasing the extracellular iron levels, stimulating erythropoiesis, and boosting active hemoglobin levels through increased erythropoietin (EPO) release [Liu et al., 2012]. An elaborate histopathological analysis of a 44-year old victim of SAR-CoV-2-ARDS showed multi-organ ferroptosis primarily in the lungs [Jacobs et al., 2020]. Sphingosine-1 is one of the markers of severe COVID-19 [Brondani et al., 2020; de Paula et al., 2020] and is induced by high serum iron or Ferroptosis [Thayyullathil et al., 2021]. ABO gene loci have been associated with the severity of COVID-19 [Buti Ferret et al., 2020; Thibord et al., 2021; Zhao et al., 2021], and these same gene loci have been implicated in iron dysregulation diseases e.g. hemochromatosis [Benyamin et al., 2014]. Severe COVID-19 is accompanied by hypoxia which strongly reduces Hepcidin levels through erythropoietin (EPO) hormone [Liu et al., 2012], and such iron dysregulation can
be attributed to Covidin peptide which we have found to be proteolysis resistant against common and major human proteases and thus can present in very high concentrations once excess spike protein is degraded in dying/dead infected cells through phagocytosis. Hypoxia is essential in escalating viral infection, as it induces surface localization of Furin protease[Arsenault et al., 2012], bypassing the endosomal route of nucleocapsid to plasma membrane fusion through cell surface spike processing by furin enzyme. Also, SARS-CoV-2 and hypoxia-induced ACE2 overexpression should depress TGF-β and thereby reduce fibrosis, the opposite of what is observed.

Further, Vitamin D and Mn^{2+} have shown to be effective in reducing the severity, and in the case of Vitamin D, reduced mortality has been seen in large trials[Griffin et al., 2021]. However, the mechanism of action is still not well established. As these agents induce overexpression of ferroportin, the reduction of ferroptosis and thereby Sphingosine-1 mediated fibrosis could be a plausible mechanism of action for the observed protection. This can be further coupled with Hepcidin hormone antagonists like Fursultiamine[Hawula et al., 2019], an FDA-approved vitamin supplement, or LY2928057[Witcher et al., 2013], a monoclonal antibody in Phase 2 clinical trials to reverse the severe fibrosis and ferroptosis in lung alveoli epithelia. As Covidin mimics Hepcidin, it should therefore cause ferroportin degradation via the ubiquitin proteasomal pathway, whereby antagonists alone cannot reverse the intracellular iron overload. The hypoxia might also be accelerated by reduced erythropoiesis due to low serum iron, rapidly deteriorating a patient’s condition, and aggravating COVID-19 morbidity. This hypoxia may be further aggravated based on recent reports of SARS-CoV-2 invading erythropoietic cells[Shahbaz et al., 2021].

The dysregulation of iron homeostasis appears to be a hallmark of severe SARS-CoV-2 infection. Several studies noted elevated serum ferritin levels correlate to severe disease, anemia, and elevated Hepcidin levels, and may be helpful as clinical predictors for severe disease[Taneri et al., 2020; Zhou et al., 2020]. Furthermore, there is a concern that Hepcidin overexpression could play a role in SARS-CoV-2 infection specifically in those with high-risk comorbidities[Banchini et al., 2021]. Under infectious pro-inflammatory conditions, the innate immune system responds with increased host Hepcidin production, ultimately decreasing the
bioavailability of iron. The decreased availability of free iron outside of the cell is protective against many bacterial infections, but the importance of intracellular iron for SARS-CoV-2 is well established[Perricone et al., 2020]. In addition to increasing intracellular iron for viral replication, if both host Hepcidin and its molecular mimic Covidin are at high levels, this will likely result in toxic levels of intracellular iron(Fig.11). In effect, there is a “Hepcidin overdose” which leads to an intracellular iron burden that overwhelms host cytoplasmic ferritin with high levels of iron-mediated free radicals leading to cell death via ferroptosis. Ferroptosis leads to the release of free radicals which also have a toxic effect on surrounding cells. Iron chelators have been shown to play a promising protective role in reducing/reversing fibrosis[Birlutiu et al., 2021; Serrano et al., 2020]. In addition to Covidin’s effect on iron regulation, a downstream effect of the iron released post ferroptosis in the form of hemin can activate platelets as has been seen in COVID-19 patients[Pretorius and Kell, 2014; Schmaier, 2020].

**Future scope.** The effects of COVID-19 infection and the role of Covidin homology need further investigation to determine its precise role in the inflammatory processes of severe COVID-19 infection. Ferroportin-Hepcidin/Covidin complex structures need to be experimentally characterized. Iron chelation and Vitamin D induced ferroportin overexpression during the early stages of infection need to be trialed as prophylactic from the ferroportin-hypoxia-COVID trifecta. Additional mechanistic studies are warranted to understand this complex disease and improve patient management. Also, global strain monitoring is more paramount than ever. Epidemiology of severity, especially in resource-poor settings is required to quarantine any ‘superbug’ with more virulence or capable of ADE 'in time'. Personalized and more specific quarantine and containment measure guidelines for close contacts of severe COVID-19 patients might aid in restricting the spread of superbugs, as we have experienced failures in that regard with the currently dominant Delta variant and soon to be dominant Omicron. Uninterrupted development of vaccines tailored to the latest strains to combat variants capable of immune evasion or ADE should be of paramount importance. Small molecule SARS-CoV-2 essential enzymes inhibitors could also help control novel strains as functional sites have lower mutation rates than the spike proteins.
CONCLUSION. Our proteolysis, protein modeling, peptide docking, and MD simulation experiments strongly support a functional biological mimicry by Covidin of the natural host Hepcidin, an iron homeostatic hormone. Further, this association seems to be highly conserved within the established primary hosts of SARS-CoV-2, consistent with supporting evidence of the high iron requirement by relatively large and resource-intensive elevated viral turnover of SARS-CoV-2. Other hypoxic conditions created by ferroptotic de-epithelization and fibrosis of lungs fuel COVID-19 severity by modulating proteases such as furin, TMPRSS2, and increasing infectivity. As in many viral respiratory diseases, the role of the possibly hypoxic state that ensues during or after viral pathology must be considered. This is where the relationship between hepcidin, the master regulator of iron hemostasis in the body, and the pathogenesis of SARS-CoV-2 and its iron-dependent replication limitation must come under scrutiny.

Thus, early control of ferroptosis or direct countering of Covidin-ferroportin interactions might provide a key intervention to reduce the mortality and suffering of COVID-19 ARDS patients. The longer phasing of host iron efflux utilized in SARS-CoV-2 replication can prolong the rapid replication and escape of the virion particles, thus allowing the possibility of therapeutic interventions to reduce SARS-CoV-2 induced pathologies.

SARS-CoV-2 variant evolution has led to critical protease resistance in the virus and has conferred higher infection rates and a more severe disease prognosis. This may have facilitated the selection of new variants that are hypoinmunogenic in terms of adaptive immunity and thereby leading to reduced protection against reinfection by variant disease. An alarming aspect is that loss of lysosomal protease sites on spikes may enable SARS-CoV-2 variants to infect macrophages in the future opening the possibility of antibody-mediated enhancement in COVID-19. Therefore, as long as the virus is allowed to spread globally relatively unrestrained, the danger will persist. With the emergence of new and more infective strains, ADE reported in the Delta variant, and the immunogen gap increasing between variants calls for universal treatments. The 100% conserved Covidin peptide sequenced could provide a key to developing a cure by alleviating the root cause of severe disease that results in mortality.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.
ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

YG and PK conceived and designed the study. YG and DM performed the sequence and *in-silico* target analysis. DPB helped to interpret the interactions and all in-silico analyses. BM, RD, and CRL covered the clinical aspect of COVID-19 and the role of iron/Hepcidin homeostasis. All authors contributed to writing and reviewing the manuscript.

REFERENCES


Laskowski RA, Swindells MB. 2011.


**Figure legends**

**Fig.1.** Phylogenetic analysis of Hepcidin hormone reveals a grouping among mammals with severe SARS-CoV-2 infection, while the peptide in primates and bats seems to be more evolved. Still, the similarity between the two groups is surprising due to the actual species evolutionary distance between the two.

**Fig.2.** Multiple sequence alignment of spike fragment homologous to Hepcidin we now call Covidin with different mammals. The hotter regions (towards the red spectrum) are highly conserved while colder (towards the blue spectrum) are the least conserved. Hepcidin seems to be highly conserved among all mammals and has high homology with the Covidin. Interestingly the Covidin homology is higher for pangolin and bat which are postulated to be viral reservoirs suggesting an evolutionary advantage conferred by this peptide for viral pathogenesis.

**Fig. 3.** Current mutation rates of different regions of the SARS-CoV-2 genome (GSAID-Nextstrain). Data from 4298 genomes sampled between Dec 2019 and Aug 2021 shows the Covidin peptide has 0.0028 average mutational diversity making it highly conserved compared to 0.2 and 0.05 average mutational diversity for whole-genome and spike protein, respectively. Mutations are represented by vertical bars with sizes proportional to percent frequencies. (A) Genome-wide mutation rates, (B) Mutation rates in Spike protein, and (C) Mutation rates in the Covidin region.

**Fig.4.** The protease map of Spike protein (N terminal on the left and C terminal on the right). This analysis suggests that if a spike protein is degraded by human protease, there is a high probability of releasing Hepcidin like a peptide fragment i.e. Covidin. This spike degradation could result following normal endosomal degradation of the spike, post nucleocapsid delivery in the cytoplasm, or necrosis of the dead virus-infected cell with the unassembled surplus spike. The sequence of the peptide at position 1214-1255 w.r.t. Ref:UniProt ‘P0DTC2’ (Wuhan isolate) is “N-term-‘WYIWLGFIAGLIAIVMVTIMLCCMTSCCSCLKGCCSCGSCCK’-C-term.”
Fig. 5. Procleave prediction of variation in protease site scores due to mutations in various variant spike sequences (Table.1) relative to the Wuhan sequence mapped on 3D structure (PDB ID: 7KJ2). Proteases act as innate immune components by degrading foreign peptides thus rendering them ineffective. The variant mutations, though mainly on the protein surface, do not seem to confer much immunological advantage, as only a few seem to improve receptor binding. Mutations have long been attributed to greater stability and transmissibility of viruses, in part by enabling the survival of proteases present in the host system.

Fig.6. Schematic representation of Comparative 3D structure of interacting (A) Natural Hepcidin and (B) Covidin docked at the central pore of ferroportin.

Fig.7 (A): Comparative amino acid interaction map of Hepcidin vs. Covidin with Ferroportin central pore extracellular domains. The colored interactions are host Hepcidin with Ferroportin and grayscale are Covidin with ferroportin in the same space. (B) Comparative amino acid interaction map of Covidin vs. Hepcidin with Ferroportin central pore extracellular domains. The colored interactions are Covidin with Ferroportin and grayscale are Hepcidin with ferroportin in the same space. Both Hepcidin and Covidin bind strongly to the central pore from the extracellular space of a host iron transporter `ferroportin` and the resulting complex has similar interaction features and binding space as the natural hormone Hepcidin.

Fig.8. MD simulation (100 ns) of natural Hepcidin hormone bound to ferroportin. The docking was highly stable with negligible deviation from original throughout the simulation and the OPM predicted membrane topology chosen for simulation was also highly stable.

Fig.9. MD simulation (100 ns) of Covidin viral origin peptide bound to ferroportin. The docking was highly stable with negligible deviation from the original dock throughout the simulation, and the OPM predicted membrane topology chosen for simulation was also highly stable.
Fig. 10. Cartoon representation of overlap of hypothetical iron dysregulation by Hepcidin-like peptide Covidin and the actual clinical picture of COVID-19 patients. The lung fibrosis observed in COVID-19 patients and resulting hypoxia is the main reason for mortality in severe cases now seconded by secondary bacterial and fungal infections [Yang and Lai, 2020]. Classic pathways do not govern COVID-19 associated lung fibrosis and a deviation has been reported by multiple workers [Rodriguez-Duran et al., 2019]. Ferritin is shown to be highly expressed in COVID-19 patients due to inflammation mediators such as IL-6 and cytokine storm or increased release from damaged/ferroptosis cells, contrastingly the serum iron and transferrin levels are very low. Sphingosine-1 is one of the markers of severe COVID-19 is induced by high serum iron or ferroptosis. But as severe COVID-19 is accompanied by hypoxia which strongly reduces Hepcidin levels through erythropoietin (EPO) hormone, such iron dysregulation can be attributed to Covidin peptide which we have found to be proteolysis resistant against common and major human proteases and thus can be present in very high concentrations once excess spike protein is phagocytosed in dying infected cells. Hypoxia is essential in escalating viral infection, and it induces surface localization of Furin protease bypassing the endosomal route of nucleocapsid but rather plasma membrane fusion by cell surface spike processing by furin enzyme. Also, SARS-CoV-2 and hypoxia-induced ACE2 overexpression should repress TGF-β and reduce fibrosis, the opposite of what is observed. Our proteolysis, protein modeling, peptide docking, and MD simulation experiments strongly support functional biological mimicry of Covidin with the natural host Hepcidin hormone. Further, this association seems to be more conserved with the proposed primary hosts of SARS-CoV-2, supporting evidence of high iron requirement by relatively large and resource-intensive high viral turnover of SARS-CoV-2. Further, Vitamin D and Mn²⁺ have been effective in reducing the severity, and in the case of Vit. D, reduced mortality as seen in large trials. However, the mechanism for which is still not well established. As these agents induce overexpression of ferroportin, the reduction of ferroptosis and thereby Sphingosine-1 mediated fibrosis could be a plausible mechanism of action for observed protection. This can be further coupled with Hepcidin hormone antagonists like Fursultiamine (FDA approved vitamin supplement) or LY3127804 (monoclonal antibody, Phase2) to reverse the severe fibrosis and possibly ferroptosis in lung alveoli epithelia. As Hepcidin/Covidin causes ferroportin degradation by ubiquitin proteasomal pathway, antagonists alone cannot reverse the
intracellular iron overload. Reduced erythropoiesis due to low serum iron might also accelerate hypoxia, rapidly deteriorating patient condition, and aggravating COVID-19.

**Fig.11.** Overview of the deciphered relationship of different subsets of COVID-19 pathology and evolution.
Key points:

- The dysregulation of iron homeostasis appears to be a hallmark feature of severe SARS-CoV-2 infection.

- The decreased availability of free iron outside of the cell is protective against many bacterial infections, but the importance of intracellular iron for SARS-CoV-2 is well established.

- In addition to increasing intracellular iron for viral replication, due to presence of both host Hepcidin and its molecular mimic Covidin at high levels, this will likely result in toxic levels of intracellular iron (Ferroptosis).

- Ferroptosis leads to the release of free radicals which also have a toxic effect on surrounding cells.

- SARS-CoV-2 variants are becoming resistant to host proteolysis innate defense mechanism.

- Y→N 501 mutation confers the site loss of the `cathepsin G` enzyme.

- Alarming aspect is that loss of lysosomal protease sites on spikes may enable SARS-CoV-2 variants to infect macrophages in the future opening the possibility of antibody-mediated enhancement in COVID-19.
### Fig. 2

The image presents a sequence alignment of different species, highlighting conserved and unconserved regions. The alignment includes sequences from various species such as Covidin, HUMAN_Hepcidin, Macaca_fascicularis, Macaca_mulatta, Ailuroprocta_melae, Felis_catus_hyp, Manis_javanica, MOUSE_Hepcidin, Myotis_brandtii, Canis_familiaris, and Callithrix_jaccus. The alignment is color-coded to indicate conserved and unconserved positions. The consistency across the sequences is also noted at the bottom of the alignment.
Fig. 4
Fig. 5

- N-terminal domain
- Connector domain
- ACE-II
- Central helix
- Heptad repeat 1
- Heptad repeat 2
- S1-cleavage site
- S2-cleavage site
- Fusion peptide
- Subdomain 1
- Subdomain 2
- Connector domain
- Meprin A
- Cathepsin
- Matrix metallopeptidase
- Variant proteolysis site loss

- Receptor-binding motif
- Receptor-binding domain
- Covidin
- TM
Fig. 6
Fig. 7
Fig. 8

Protein RMSD (Å) vs. Time (nsec)

(A)

RMSF (Å) vs. Residue Index

(B)
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**Fig. 10**

- **Healthy Lungs** to **Fibrotic Lungs**
  - Hypoxemia
  - Alveoli de-epithelialization
  - Overexpression of RIG-I
  - Hypoxia induces relocalization of furin to the cell surface and TMPRSS2 overexpression

- **SARS-CoV-2**
  - Late, severe COVID-19 phase in adults: cytokine storm, hyperinflammation, and multiorgan damage
  - Multisystem inflammatory syndrome in children (MIS-C), similar to Kawasaki disease

- **Infected Cell**
  - ACE2
  - Chloroquine
  - Vitamin D
  - Ferritin
  - Dead cell post viral infection (Surplus viral protein)

- **Ferroptotic cell**
  - Ferritin leakage from damaged cells
  - Iron overload
  - Localized thrombocytosis typical to Covid-19

- **Liver**
  - Ferritin
  - Hepcidin
  - Covidin (Protect against proteolytic attack)
  - Ferroportin
  - Pro-inflammatory cytokines (IL-4 & IL-13)
  - Pro-fibrotic cytokines IL-4 & IL-13

- **Hepatocyte**
  - Hepcidin
  - COVID-19 (Proteolytic resistant peptide similar to hepcidin)

- **Spike Mutant**
  - Spike trimer
  - Low serum iron in severe Covid-19 patients
  - Ferroptosis

- **Sphingosine-1**
  - Direct antagonist of hepcidin

- **LY3127804**
  - Direct antagonist of hepcidin

- **ANG 1-7**
  - TGF-β downregulation blocks fibrosis

- **ACE2**
  - Overexpression of ACE2

- **Angiogenesis**
  - Neovascularization

- **Spike**
  - Spike trimer
  - COVID-19 (Proteolytic resistant peptide similar to hepcidin)
Hypoxia

Severe COVID-19 triad

Ferroptosis

Highly conserved, hepcidin mimicking proteolysis resistant spike protein fragment

Hepcidin mimicking proteolysis resistant spike protein fragment

Epithelial erosion and increased Intracellular iron for viral replication

Variants

De-epithelialization and lung fibrosis
Due to rapid Higher loads and more severe infections

Selective pressure due to protease
Overexpression and increased phagocytosis

Host protease
Site loss

COVID-19

Shorter incubation time, breakthrough infections, Hypoimmunogenic, and environmental stability

Highly global burden, high loads, founders’ effect

Fig. 11
Iron Dysregulation in COVID-19 and Reciprocal Evolution of SARS-CoV-2: *natura nihil frustra facit*

Yash Gupta, PhD¹, Dawid Maciorowski, BS², Brian Medernach, MD³, Daniel P. Becker, PhD⁴, Ravi Durvasula, MD¹, Claudia R. Libertin, MD¹, Prakasha Kempaiah, PhD¹*

¹ Infectious Diseases, Mayo Clinic, Jacksonville, FL, USA; 2 University of Wisconsin-Madison, School of Medicine and Public Health, Madison WI, USA; 3 Department of Medicine, Loyola University Medical Center, Chicago, IL, USA; 4 Department of Chemistry and Biochemistry, Loyola University Chicago, Chicago, IL, USA

**Supplementary Table 1.** Prediction of protease site loss/gain due to amino acid polymorphisms among strains from the new omicron (B.1.1.529) lineages of SARS-CoV-2. There are around 30 mutations in the first reported omicron variant which is mutating further at an unprecedented rate. Also, there is a gain of predicted protease cleavage sites unlike other lineages, possibly explaining milder infections from omicron. The adjacent mutations thus could be in response to immunological selective pressure given more clustered mutations compared to other lineages. Also, there are several structural deviations in spike protein exposing new sites that need to be modified in order for the virus to resist host proteases.

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