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Impaired ACE2 glycosylation and protease activity lowers COVID-19 susceptibility in Gitelman's and Bartter's syndromes

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Abstract

Introduction: Gitelman's and Bartter's syndromes (GS/BS) are two rare genetic tubulopathies which present with metabolic alkalosis and increased ACE2 levels. ACE2 serves as the entry point of the SARS-CoV-2 virus. The virus attaches to its target cell via its surface spike (S) protein binding to ACE2 and spreads into the cells through the action of specific proteases, such as cathepsin (Cat-L), whose activity relies on an acidic enviroment. During the first two years of the COVID 19 pandemic it has been assessed via telephone surveys the impact of COVID-19 on a cohort of 128 GS/BS patients living in the main northern Italy: none of them suffered major syntoms suggesting a natural protection from the disease. Given that blocking ACE2/viral S protein interaction is effective against SARS-COV-2 infection and that increased pH, a feature of GS/BS, has been shown to interfere with ACE2 glycosylation and protease activity, we recruited 20 GS/BS patients (13 females, 7 males, 32–68 years), with either GS (n = 19) or BS (n = 1) and 15 healthy controls (7 females, 8 males, 29–52 years) and assessed the levels of mononuclear ACE2 and its glycosylation alongside plasma Cat-L activity.

Material and methods: ACE2 profile of protein expression was assessed using Western Blot analysis**.** Cat-L activity was measured using a commercially available fluorescence-based assay. Metabolic alkalosis, in terms of bicarbonate blood levels, was assessed through hemogasanalysis.

Results GS/BS patients had higher nonglycosylated ACE2 levels (0.82 ± 0.19 d.u. vs. 0.67 ± 0.13 $p = 0.01$) and lower Cat-L activity (3.91 ± 1.13 r.f.u. vs. 5.31 ± 0.8 $p < 0.001$) compared to healthy subjects. In addition, GS/BS's Cat-L activity inversely correlated ($p < 0.001$, $r = 0.78$) with blood bicarbonate (HCO_3^-), while a negative correlation between ACE2 glycosylated isoform and HCO_3^- approaches statistical significance ($p = 0.08$)**.**

Discussion/Conslusions: Endo-lysosomal pH plays a critical role for the endocytic uptake of SARS-CoV-2. Increased intracellular organelle pH interferes with both ACE2 glycosylation and the binding via S protein as observed in experiments with chloroquine (CQ). The inverse correlation observed in GS/BS between blood HCO_3^- and Cat-L activity, alongside the trend toward a negative correlation between blood HCO_3^- and the glycosylated isoform of ACE2 suggest that GS/BS patients' metabolic alkalosis underlies these effects; this explains the protection against COVID 19 that has been observed in telephone surveys. These data have been replicated in patients with Fabry disease (FD) confirming that an alteration in the endosomal system can determine protection against the virus. The altered endosomal processing provides a robust mechanistic rationale for the effects of the combination of nirmatrelvir-ritonavir (Paxlovid), which exerts its effect via inhibition of proteins involved in lysosomal processes key for SARS-CoV-2 cell

entry and replication; furthermore it provides an “in vivo” human model where the effects of endosomal pH, ACE2 glycosylation status and Cat-L activity alter SARS-CoV-2 infection rate and severity and point to these as new potential targets to fight COVID-19.

1 INTRODUCTION

1.1 Bartter's Syndrome

Bartter's syndrome (BS) includes five different types of inherited salt-losing tubulopathies all characterized by hypokalemia, hypochloremic metabolic alkalosis, activated RAS, high Ang II levels yet normotension or hypotension, and a blunted cardiovascular effect of Ang II. The prevalence of the disease is 1/100000 (1, 2).

The electrolyte abnormalities of BS are similar to those induced by treatment with furosemide or other drugs that inhibit the Na-K-2Cl cotransporter of the thick ascending limb of Henle's loop (2).

1.1.1 Clinical manifestations

Clinical BS manifestations are muscle weakness, anorexia, polydipsia, polyuria, failure to thrive and growth retardation with salt-wasting and high plasma levels of prostaglandins. Biochemical explanation can be related to the impaired tubulo-glomerular feedback when chloride is not reabsorbed in the macula densa and the ensuing activation of cyclooxygenases promotes prostaglandins-induced renin release and aldosterone production in order to recover normal intravascular fluid balance. Other consequences of impaired sodium reabsorption are hypercalciuria and progressive medullary nephrocalcinosis and the reduced capacity to concentrate urine.

1.1.2 Genetic defect

Mutations in different genes results in 5 different BS types with considerable phenotypic overlap.

BS type 1: Mutations in the NKCC2 cotransporter have classified, with typical Na+ and K+ waste and systemic alterations from fetal gestation onwards. The clinical manifestations are severe salt-wasting hypokalemic metabolic alkalosis, hypercalciuria, high levels of prostaglandins in urines associated with progressive medullary nephrocalcinosis. Further, the excess of prostaglandins E2 leads to fever, hypocalcemia, hyponatremia and severe dehydration (3).

Other types are caused by mutations in genes that regulate the activity of NKCC2.

BS type 2: It is caused by mutations in genes that regulate the activity of NKCC2. The KCNJ1 (Kir1.1) encodes for the apical ATP-sensitive K+ channels ROMK that recycles K+ from the cell back to the lumen. A nonfunctional ROMK identifies BS type 2. The falling luminal K+ shuts down

NKCC2 activity and induces salt-wasting with a transient hyperkalemia associated to a pseudo hypoaldosteronism type 1 phenotype in the antenatal form (4).

BS type 3: It is caused by mutations in the chloride channel CLCNKb and is characterized by salt wasting and hypovolemia. A necessary -subunit for CLCNKb activity is barttin encoded by the BSND gene. Mutations affecting the barttin interacting motifs result in severe phenotype contrarily to the milder or even undetected form caused by mutations in the alpha-helices (5).

BS type 4°: It is caused by mutations in the barttin protein which is required for the basolateral location of the CLCNKb and the CLCNKa isoform. Digenic mutations might inactivate all the 4 alleles of the both CLCNKb and CLCNKa resulting in different BS subtype named BS type 4b with nerve deafness and defect in the sensory-neural transduction of sound, especially for the high presence of CLCNKa in the inner ear (6).

BS type 5°: It is caused by the mutations in the MAGED2 gene. It is a severe antenatal form of BS which resolves spontaneously during the first weeks to months of life. Originally was referred to patients with alterations of the Ca2+ -sensing receptor (CaSR) with autosomal dominant hypocalcemia (7).

1.1.3 Therapy

The current therapy for BS is the correction of electrolyte abnormalities, with supplementation of sodium chloride, potassium chloride and fluids based on the severity of the disease. For most BS patients in case of severe hypokalemia unsolvable with dietary supplementation, the use of potassium sparing diuretics, renin-angiotensin system blockers or nonsteroidal anti-inflammatory drugs have been proposed (1,2).

1.2 Gitelman's syndrome

Gitelman's syndrome (GS) is a genetic characterized by the simultaneous presence of hypokalemia with hypomagnesemia and hypocalciuria in addition to the occurrence in early adulthood and a generally milder clinical presentation distinguishes GS from BS. The prevalence of the disease is 1/40000, making it one of the most common tubulopathy (2).

1.2.1 Genetic defect

GS is caused by loss-of-function mutations in the SLC12A3 gene, which encodes the Na^+-Cl^- cotransporter and is characterized by hypokalemic metabolic alkalosis, hypocalciuria, hypomagnesemia, activated Renin-Angiotensin System (RAS) and high Angiotensin II (Ang II)

levels (8). The cloning and characterization of the gene SLC12A3 encoding for the sodium chloride cotransporter NCC, showed that several mutations can be detected in GS patients, most of them impairing NCC function with ensuing sodium wasting and the activation of adaptive mechanisms such as the aldosterone-driven increased excretion of K+ in exchange for Na+ and the flux-dependent K+ secretion along the collecting duct. GS patients are either normo or hypotensive and represent a model of endogenous Ang II antagonism as Ang II cardiovascular effects are blunted (9).

1.2.2 Clinical Manifestations

GS typical clinical features are salt-craving, muscle weakness, fatigue and cramps with electrolyte imbalance associated to the side effects of treatment with thiazide diuretics that target the distal convoluted tubule (DCT). Specifical signs and simpthoms of GS are hypokalemia, metabolic alkalosis, hypomagnesemia, hypocalciuria, normal or low blood pressure absence of morphological and functional renal abnormalities. Onset time of the symptoms can vary but mostly appear in the early adulthood as less severe, while in young patients causes growth retardation, pubertal delay and short stature. Specifically, in the early portion of the DCT the transport of Na+ is electrogenic for the concomitant release of Cl- through the NCC while in the late portion of the tubule, the activation of the epithelial sodium channel (ENaC) in order to restore the water balance leaves a negative charge in the lumen. As a counterpart, to restore the neutral charge, the renal outer medullary potassium channel (ROMK) drives out K+ inducing hypokalemia. Hypocalciuria is another feature of GS, in fact impaired function of the NCC leads to major structural remodeling of the distal convoluted tubule. Calcium imbalance is associated to arthropathy with calcium pyrophosphate crystal deposition or chondrocalcinosis. Other clinical symptoms of GS are dizziness and prolongation of the QT interval on electrocardiogram that can causes arrhythmia (10, 11).

1.2.3 Therapy

GS is usually managed by free sodium chloride intake together with oral magnesium and potassium supplements The use of potassium-sparing diuretics and renin angiotensin system inhibitors has been can be recommended in GS, however evidence supporting the efficacy, safety and tolerability of these options either in GS is limited and for their nature, they might aggravate renal sodium wasting and increase the risk of hypotension secondary to hypovolemia (10).

1.3 Bartter and Gitelman Syndromes a model to study hypertension

The RAS regulates blood pressure, fluid and electrolyte balance and systemic vascular resistance. The signalling cascade triggered by the binding of Ang II with its cell-surface Ang II subtype-1 or

subtype-2 receptors (AT1R, AT2R) mediate several physiological effects. Depending on the receptor involved, Ang II can promote either vasoconstriction, inflammation, fibrosis and cellular growth or vasodilation, insulin sensitivity, anti-remodeling, and anti-atherogenesis effects. Persistent activation of the RAAS in healthy individuals leads in fact, to hypertension and target organ damage. Patients affected by these two rare genetic syndromes exhibit endogenously activated RAS and high Ang II levels, yet blunted Ang II mediated cardiovascular-renal effects and normotension or hypotension. Calò et al demonstrated that these patients have higher levels of both angiotensin converting enzyme 2 (ACE2) and Ang 1-7, activation of anti-inflammatory, antiapoptotic, antiproliferative and antiatherosclerotic defenses, reduced oxidative stress and blunted ROCK signaling compared to controls. They have also upregulated regulator of G-protein signaling (RGS)-2. RGS-2 acts as a negative regulator for Ang II signaling via AT1R which significantly extends to Ang II mediated activation of RhoA/ROCK system. RGS-2 acts as a negative regulator for Ang II signaling via AT1R which significantly extends to Ang II mediated activation of RhoA/ROCK system (9, 12).

1.4 COVID 19

Coronavirus disease 19 (COVID -19) is an infectious respiratory disease that emerged at the end of the year 2019 caused by Coronavirus 2, a highly transmissible virus that has had a pandemic spread causing a global health emergency. It causes a severe acute resporatory syndrome known as SARS CoV 2 (13).

1.4.1 Epidemiology

In December 2019, the first cases of atypical pneumonia were described in Wuhan (China), to an exponential growth of new cases, initially limited in the Chinese country and later extended to other states. 24 On 11 March 2020, the WHO defined officially the COVID-19 outbreak a pandemic. As of early May 2023, more than 750 millions of COVID-19 cases worldwide and a total of 6.9 million deaths (WHO Coronavirus (COVID-19) Dashboard Accessed 4 May 2023,https://covid19.who.int).

1.4.2 Pathogenesis

CoVs, including SARS-CoV-2, are enveloped viruses with positive-sense single-stranded RNA that possess the largest genomes (~30 kb) among the known RNA viruses, which belong to the Betacoronavirus genus of the family Coroaviridae. Four structural proteins of spike (S), envelope (E), membrane (M), nucleocapsid (N)and 16 nonstructural proteins (nsp1–16) are encoded by the viral genome. SARS-CoV-2 is able to recognize and bind the host receptor ACE2 at its extracellular domain via its spike (S) glycoproteins. S is a transmembrane protein formed by a homotrimer, which protrudes from the surface of the virus in numerous copies and provides it with the classic crown appearance (14). It is composed of two non-functional subunits covalently bound: S1 and S2. S1 has the function of binding the receptor and is composed of the N-terminal domain (NTD) and the receptor binding domain ACE2 (RBD). S2, on the other hand, is used for the fusion of the viral membrane with the host cell's membrane. RBD is the first molecular target of neutralising antibodies formed by a natural or vaccine infection. The conformational changes in the S2 unit can be triggered by either the transmembrane serine protease TMPRSS2 or lysosomal cysteine protease cathepsin L in the endosomal compartment following ACE2-mediated endocytosis. After protease-preactivation of the S1/S2 cleavage, SARS- CoV-2 can use mutually exclusive routes to penetrate cells: TMPRSS2-mediated plasma membrane entry, regardless of the pH conditions or clathrin-mediated endocytosis where S2 cleavage is performed by cathepsin L which requires low-pH as in the endosomes (15, 16).

1.4.3 Transmission

The contagion occurs mainly through the expulsion of droplets, direct contact or respiratory secretions during unprotected close contact with a subject infected. However, viral particles were also isolated from faecal swabs and blood implying alternative routes of contagion. The main source of infection are the infected subjects both asymptomatic and symptomatic. In addition, the data indicated that the transmission of SARS-CoV-2 can also occur following contact with fomits (14).

1.4.4 Clinical manifestations

SARS-CoV-2 infections often lead to “flu-like” symptoms such as headache, fever, sore throat, backache, cough, and loss of taste or smell. Although plenty of infection cases are asymptomatic or mild, there are certain cases that show severe outcomes and are associated with systemic inflammation, acute respiratory distress syndrome and cardiac injury. The severe COVID-19 disease with multiorgan damage can be fatal, and its risk largely depends on comorbidities including diabetes, obesity, hypertension, and chronic kideny disease among others (14).

1.4.5 Treatments

All the treatments can divided into two big groups on the basis of their targets: antiviral agents and therapies targeting host.

Antiviral agents include polymerase inhibitors, protease inhibitors, inhibitors of nucleoside and nucleotide reverse transcriptase, entry and uncoating inhibitors, and other antivirals (17). Here some of the most important drugs used during the pandemic.

Remdesivir, is a broad-spectrum antiviral drug with activity against viruses from several families, including coronaviruses. It is a nucleotide prodrug, and its active metabolite can inhibit the activity of RNA polymerases, which is a key enzyme for the replication of many viruses, including coronaviridae. Acting as an adenosine triphosphate (ATP) analogue, remdesivir triphosphate has favourable selectivity over the natural ATP substrate for incorporation into nascent viral RNA chains by the SARS-CoV-2 RNA-dependent RNA polymerase. The efficacy of remdesivir for the treatment of COVID-19 was demonstrated in a large number of key clinical trials, including in hospitalized patients, as combination therapy, in out patients at high risk for disease progression, in paediatric patients and in renally impaired patients (18).

Paxlovid is a new oral antiviral drug, produced by Pfizer for use against COVID-19, given for 5 consecutive days to patients with mild to moderate disease. Paxlovid consists of nirmatrelvir, a novel SARS-CoV-2 main protease inhibitor targeting 3CLpro of SARS-CoV-2, plus ritonavir for its

action as an inhibitor of cytochrome P450 3A4 to decrease nirmatrelvir metabolism and increase its serum levels (19). Paxlovid treatment is effective for patients with COVID-19, reducing the mortality or hospitalization rate by 78% (20).

Molnupiravir, an orally active RNAdependentRNApolymerase (RdRp) inhibitor active against SARS CoV 2 infection. Molnupiravir is the isopropyl ester prodrug of the ribonucleoside analogue β-D-N4-hydroxycytidine (NHC). An in vitro evidence shows that molnupiravir is a potent inhibitor of SARS-CoV-2 replication with an EC50 in the submicromolar range; the effect of this antiviral injection was also observed in animal models (19).

Fluvoxamine is a selective serotonin reuptake inhibitor and σ-1 receptor agonist which has shown potential of early outpatient treatment of COVID-19 with good safety and effectiveness in patients in intensive care unit (ICU) (19).

Other drugs that are not primarily antivirals but that have been studied for their characteristics that make them potentially useful against SARS CoV 2 are:

Azithromycin, an antibiotic medication used for the treatment of a number of bacterial infections, has been administred to patients with COVID 19 infection because it seemed to slow down virus replication. A larege trial showed that it did not reduce the recovery time or risk of hospitalization for people who were suspected with COVID-19 (17).

Hydroxychloroquine (HCQ) and chloroquine (CQ), used to treat malaria and rheumatologic conditions, have been suggested as potential treatments for COVID-19. In 2005 Vincent et al. had studied its properties in vitro. This drug showd anti SARS CoV activity since it altered the endosomal pH altering the trans Golgi network (TGN). In this way it reduced the glycosilation capacity of proteins, in particular ACE2 which appare to be critical to the viral entry (21). In one study of 1,561 patients with COVID-19 treated with hydroxychloroquine and 3,155 in usual care, HCQ did not lower patient mortality compared with usual care (22). Moreover, HCQ did not provide significant improvement in symptom severity for early, mild COVID-19 outpatients, and could not prevent symptomatic infection after SARS-CoV-2 exposure. HCQ has also proved dangerous as it can trigger arrhythmias, even potentially fatal (23).

Covalescent plasma did not show clear results from various clinical trials. Although convalescent plasma showed partial effectiveness in selected patients, its potential is still controversial. In addition, only part of plasma antibodies will be neutralizing, and those non-neutralizing antibodies will bind to non-spike protein viral antigens, which will sabotage antibody reactions to further cause tissue damage (17).

Bamlanivimab, a neutralizing antibody showed strong binding to ACE2 and neutralizing activity. Monoclonal antibodies are restricted only to the same or single epitope due to their monovalent

affinity, which may be ineffective against the virus variance. To solve this problem, researchers combined two monoclonal antibodies. Omicron variant of SARS CoV2 showed resistance to 17 neutralizing antibodies (17).

JAK inhibitors have proven effective on improving clinical outcomes of hospitalized patients with COVID19. These kinase inhibitors are used as treatments for COVID-19 because they inhibit virus-induced immune activation and signaling of inflammation (17).

Corticosteroids have been proved to be able to improve survival in severe COVID-19 (17).

1.5 ACE 2

ACE2 is well known to play a critical role in the renin-angiotensin system, modulating the detrimental effects of angiotensin (Ang) II via conversion of Ang II into Ang 1–7, which has opposite effects to Ang II. ACE2 also converts angiotensin I to angiotensin (1–9) and ultimately stimulate Mas-related G protein-coupled receptors (9).

The ACE2 receptors are ubiquitous within the human body, particularly overexpressed on intestinal epithelial cells of the gut, endothelial and smooth cells of the blood vessels, heart (epicardia, adipocytes, fibroblasts, myocytes, coronary arteries), lung (macrophages, bronchial and tracheal epithelial cells, type 2 pneumocytes), brain, testis, and on tubular epithelial cells of kidney (9).

ACE2 is involved in counterbalancing the function of ACE: ACE2 removes the carboxyl-terminal amino acid phenylalanine from vasoconstricting angiotensin II and hydrolyses it back into angiotensin. ACE2 can also participates in cleaving bradykinin, apelin, neurotensin, dynorphin A and ghrelin (24).

ACE2 is a type I transmembrane protein with a molecular weight (mw) of 120 kDa in its most common glycosylated form. It has six potential N-glycosylation sites in its primary structure. The de-glycosylated form has a mw of ~90 kDa. Mutation at ACE2 glycosylation sites has been reported to impact SARS-CoV-2 binding Viral S-protein physically interacts and utilizes membrane bound TMPRSS2 and ACE2 to get primed and enter into the human cell (25).

These high-affinity interactions between the S protein and ACE2 receptor occur due to numerous interacting residues in SARS CoV 2 - S-ACE2 protein–protein interaction, which form the strong (multi-epitope) adhesion synapse between the viral surface and epithelial layer of the host (15).

ACE2 receptor has N-terminal peptidase domain (PD); PD of ACE2 facilitates binding of highly conserved receptor binding domain (RBD) present at the distal part of S1 domain of virals S-protein. hACE2 glycans located at the interface with the CoV2-S RBD make distinct site-specific contributions

to CoV2-S interactions and their net effect determines the efficacy of virus entry into the host cell (26).

1.5.1 ACE 2 and RAS inhibitors in COVID 19

Increased ACE2 resulting from ACEi or ARB treatment of hypertensive patients might increase their SARSCoV-2 infection rate. This higher SARS-CoV-2 infection rate related to increased ACE2 has been criticized, while there are suggestions that administering ACEi and ARBs might be benefcial in treating COVID-19 (27).

The potential, protective role of ACE2 in SARS-CoV-2 infection-induced COVID-19 morbidity and mortality has recently been reviewed. Upregulation of ACE2 can be potentially benefcial by maintaining Ang II conversion to the vasodilatory, anti-infammatory and antiatherosclerotic Ang 1–7, while the use of ARBs could be benefcial by blocking excessive Ang II type-1 receptor (AT1R)-mediated Ang II activation, thereby increasing Ang 1–7 levels by upregulating ACE2 activity (28).

The downregulation of ACE2 by virus causes an upsurge in Ang II, which by acting on AT1 receptor may enhance systemic injury, pulmonary fibrosis, pulmonary inflammation, and ARDS (27). In vitro studies demonstrated that downregulation of ACE2 could worsen alveolar damage in different pulmonary injury models (29). Simultaneous injection of recombinant ACE2 and AT1 receptor blockers showed effectiveness in weakening the magnitude of lung injury in another study (30).

1.5.2 ACE 2 And Bartter's and Gitelman's Syndromes

The centrality of ACE2 and therefore of the RAS to COVID-19 provides a clear rationale for the study of systems, particularly human ones, in which ACE2 and other RAS components are altered.

Gitelman's and Bartter's syndromes (GS/BS) patients have endogenously increased levels of ACE2 and Ang 1-7. GS/BS represent a human model of endogenous Ang II antagonism with activated RAS and high Ang II levels, yet blunted cardiovascular effects. GS/BS patients have increased and correlated levels of ACE2 and Ang 1-7, compared to hypertensive patients and healthy subjects, which may be useful in assessing the potential role of RAS and the ACE2/Ang 1-7 axis in COVID 19 (28).

1.5.3 ACE2 and Chloroquine

Of particular interest are the reports detailing the effects of chloroquine (CQ), which is a weak base that, when unprotonated, can diffuse across membranes and accumulate in acidic cellular compartments (21).

The potential contribution of these drugs in the elevation of endosomal pH and its impact on subsequent virus entry or exit could not be ruled out. Endosomes have an acidic pH due to the activity of a proton pump, and endosomal acidification is closely interlinked with endosomal, intracellular messenger. Inhibition of the proton pump prevents calcium release from endosomes and reduction of extracellular calcium blocks endosomal acidification (31). These processes have been shown to be critical for SARS-CoV-2 entry in the cell, which has been shown to be blocked when the proton pump is inhibited (21).

Kellokumpu suggested that Golgi-localized glycosylation is a pH-sensitive process (32) and used CQ to change intracellular pH. He found a 0.2 unit increase in pH which interfered with both mucin type O-glycosylation and terminal a-2,3-sialylation of N-linked glycans without changing overall Golgi morphology (33). Vincent et al. reported that CQ induced elevations of endosomal pH and interfered with terminal glycosylation of ACE2 (34). Isobe et al highlighted the importance of ACE2 glycosilation on ACE2/SARS CoV 2 spike interaction (26).

In summary the alteration of the acidifcation process of Trans Golgi Network (TGN)/post-Golgi leave ACE2 membrane expression unaltered; the impairment of terminal glycosylation of ACE2 may result in reduced binding affinities between ACE2 and SARS Co V 2 spike protein and adversly affects the spread of SARS-CoV 2 infection.

1.6 Cathepsin L

Cathepsins are non-specific proteases with endo- and exopeptidase activity that participate in the degradation of proteins in late endosomes and lysosomes as well as being present in the Golgi apparatus. To best perform their catalytic activity, cathepsins require the presence of an acidic pH following which autoactivation from the pro enzyme to the functioning enzyme occurs (35). Cathepsin L (Cat L) a cysteine protease is expressed in all tissues and cell types and the main function concerns the proteolysis of protein antigens generated by endocytosis of pathogens. Cat-L is a critical protease involved in S protein processing and thereby enhances SARS-CoV2 viral entry, although the precise cutting sites of Cat L have not yet been fully understood, cutting at the CS 2 level seems to represent an essential step for the entry of the SARS CoV 2 virus into the cell. In particular in the clathrin-mediated endocytosis where S20 cleavage is performed by Cat L (L (15, 35).

As it was already demonstrated for SARS-CoV-1, Cat-L was found to be highly correlated also with SARS-CoV2 infection and associated with the severity of COVID-19 and its inhibition has been suggested as a possible therapeutic approach (15).

1.7 SURVEYS

We made a telephonic survey regarding COVID-19 among our 128 GS/BS patient cohort who live in Lombardia, Emilia-Romagna, and Veneto, the hot spot regions of COVID-19 pandemic in Northern Italy. During the first wave (april 2020) we found a complete absence of COVID-19 symptoms, making the number of positives in the GS/BS patients cohort 0.00% (95% CI 0.00–3.62%). Using the survey population size (n = 128) and assuming that the surveyed population represented a random sample of the "hot spot regions," the probability of obtaining zero COVID-19-infected subjects upon surveying 128 subjects using the realistic prevalence based on the analysis of Signorelli et al. (36), the probability resulted statistically significant ($p < 0.01$) (37).

A second survey on the same cohort one year later during the third wave of COVID-19 in April 2021, found that only 8 patients tested positive for COVID-19, of which 4 were asymptomatic and 4 had very mild symptoms (38). A third survey was assessed on the same cohort in December 2021-January 2022 during the peak of the fourth wave of COVID-19 induced by the Omicron variant, prevailing at 95% in Italy. The results of this latter survey confirmed the data of the previous surveys, showing that 14 patients tested positive, 9 asymptomatic and 5 with mild symptoms (39).

Accepting that we may have missed some SARS-CoV-2 infected patients, this occurrence would thus suggest that the effects of GS/BS renders SARS-CoV-2 infections asymptomatic and that COVID-19 symptoms are associated to ACE2 levels. These surveys indicate that GS/BS patients appear to be protected from either infection by SARS-CoV-2 itself or from the resulting clinical disease.

2 EXPERIMENTAL PROTOCOL

Based on the data obtained from telephone surveys, it appeared that patients suffering from BS/GS are protected from the most severe forms of SARS CoV 2 infection. These patients tend to have, as an effect of the pathology, metabolic alkalosis.

Either ion transport issues or chronic state of metabolic alkalosis in GS/BS patients, jointed with the blunted/reduced intracellular calcium signaling due to reduced second messenger induced intracellular calcium release, can drive changes in the pH of the TGN/endosome system and result in altered ACE2 protein glycosylation which have been shown to be critical for the binding with the RBD of the S viral protein (35).

Brufsky suggested that a different glycosylation pattern of ACE2 could be involved in SARSCoV-2 infection (40).

Moreover the amplification of the RAS provides an explanation as to how GS and BS phenotypes can exhibit overlapping clinical findings despite their different mutations.

Metabolic alkalosis in GS/BS patients may reproduce the same pH-dependent effects on ACE2 glycosylation induced by CQ in vitro, thereby impacting not only the GS/BS phenotypes, but in the case of COVID-19, blocking/inhibiting SARS-CoV-2 binding and the resulting COVID-19 disease (21).

As well metabolic alkalosis can alter pH in the endosomes resulting in compromised activity of pH-dependent proteases, like cathepsin-L which relies on an acidic enviroment to be activated (15).

We recruited 20 GS/BS patients from the previous survey (13 females, 7 males, 32–68 years), with either GS (n = 19) or BS type III (n = 1) and 15 healthy controls (seven females, eight males, 29–52 years) (Table 1) and assessed the levels of mononuclear ACE2 and its glycosylation alongside plasma Cat-L activity.

	Male	**Female**	**Age (yo)**
Healthy Control	8	7	29-52
Sdr Bartter	1	-	36
Sdr Gitelman	6	13	32-68

Table 1

3 MATERIALS AND METHODS

3.1 Mononuclear cells preparation

Peripheral blood was collected in tubes containing EDTA as an anticoagulant (6 ml Vacutainer tubes ®) filled completely. The blood samples were processed immediately after the collection. Plasma fractions were isolated by centrifugation from 35 mL of EDTA anticoagulated blood and immediately stored at -80˚C for the following procedures. Peripheral blood mononuclear cells were isolated by Lympholyte-H gradient (Cedarlane, Burlington, Canada). Total protein extracts were obtained by cell lysis using an ice-cold buffer (Tris-HCl 20mM, NaCl 150 mM, EDTA 5.0 mM, Niaproof 1.5%, Na_3VO_4 1.0 mM, SDS 0.1%) added with protease inhibitors (Complete Protease Inhibitor Cocktail, Roche Diagnostics, Manheim, Germany). Protein concentration was then evaluated by bicinchoninic acid assay (BCA Protein Assay, Pierce, Rockford, USA).

3.2 Assessment of ACE2 protein expression

ACE2 profile of protein expression was assessed using western blot analysis. Equal amount of proteins (60 mg) were separated by SDS-PAGE (8% acrylamide gel), transferred onto nitrocellulose membranes (Hybond ECL, Amersham, Uppsala, Sweden) and blocked with BSA (5% in Tween-PBS). Membranes were probed overnight with a primary monoclonal antibody against ACE2 that recognizes a nondisclosed epitope located in the region of ACE2 (aa 631–805) that includes part of the ectodomain (aa 18–740), the transmembrane domain (aa 741–761), and the cytoplasmic domain (aa 762–805) (sc-390851, Santa Cruz Biotechnologies, Santa Cruz, CA, USA).

De-glycosylation experiments have shown that ACE2 can be recognized at 100-90kDa as partial or non-glycosylated ACE2 isoform [1,2]. The antibody used in this study is able to recognize both 90 kDa ACE2 and 120KDa ACE2 isoforms, this latter widely recognized as the glycosylated form. The Amersham ECL™ Rainbow Marker-Full Range (12000-225000 Da, RPN800E, GE Healthcare, Amersham, Uppsala, Sweden) has been loaded as marker of molecular weight.

Specific anti-mouse HRP-conjugated secondary antibody (Amersham Biosciences, Uppsala, Sweden) was added. Membranes were then incubated overnight with a primary monoclonal antibody against b-actin as housekeeping gene (A5441, Sigma Aldrich, St.Louis, MO, USA) and the corresponding anti-mouse HRP-conjugated secondary antibody (Amersham Biosciences, Uppsala, Sweden) was added afterwards. Finally, immunoreactive proteins were visualized with chemiluminescence using SuperSignal WestPico Chemiluminescent Substrate (Pierce, Rockford, USA) at the Amersham Imager 600 (GE Healthcare UK Limited, Buckinghamshire, UK). Protein

immunocomplex were evaluated by a PC based densitometric semiquantitative analysis using NIH ImageJ software (NIH, Bethesda, MD, USA) and quantification of targeted proteins were normalized using housekeeping b-actin detected in the same membrane.

3.3 Cathepsin L activity

Cat-L activity was measured using a commercially available fluorescence-based assay (ab65306, Abcam, Discovery Drive, Cambridge, UK). Briefly, plasma aliquots (50 mL) from patients and controls were incubated on a 96-well plate with a synthetic FR-AFC substrate at 37˚C for 2 hours. A background control and a negative control were also included in the plate. Finally, the free fluorescent AFC released was detected (excitation 400 nm and emission 505 nm) using the EnSight Multimode Plate Reader instrument (PerkinElmer, Waltham, MA, USA)

Cat-L activity has been determined by comparing the relative fluorescence units (r.f.u.) with the r.f.u. level of the negative control sample.

3.4 Bicarbonate blood levels

Metabolic alkalosis, in terms of bicarbonate blood levels, was assessed through hemogasanalysis using RAPIDPoint® 500 Blood Gas System (Siemens Healthineers, Erlangen, Germany).

3.5 Statistical analysis

Data are presented as scatter dot plot and expressed as mean ± SD.

The normal distribution of the variables was formally verified beforehand by Shapiro-Wilk test and statistical analysis using parametric unpaired Student t test was performed using GraphPad Prism version 9.0.1 for macOS (GraphPad Software, San Diego California USA, www.graphpad.com). P - Values at 5% levels or less ($p<0.05$) were considered significant.

4 RESULTS

4.1 Assessment of glycosylated ACE2 levels in healthy controls and GS/BS patients

GS/BS patients had higher non-glycosylated ACE2 levels (0.82 ± 0.19 d.u. vs. 0.67 ± 0.13 p = 0.01) compared to healthy subjects (Fig. A). A negative correlation between ACE2 glycosylated isoform and HCO3 − approaches statistical significance (p = 0.08).

4.2 Assesment of Cathepsin-L activity levels in healthy patients and GS/BS

GS/BS patients had lower Cat-L activity (3.91 ± 1.13 r.f.u. vs. 5.31 ± 0.8 p < 0.001) (Fig. B) compared to healthy subjects. In addition, GS/BS's Cat-L activity inversely correlated (p < 0.001, r = 0.78) (Fig. C) with blood bicarbonate (HCO3 −).

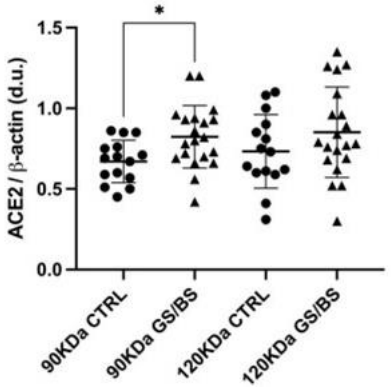

Figure A

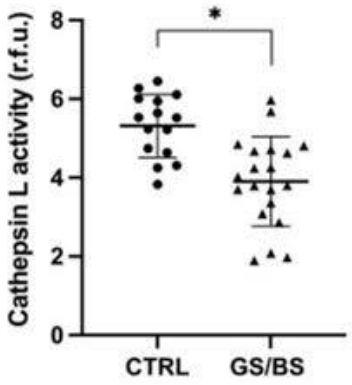

Figure B

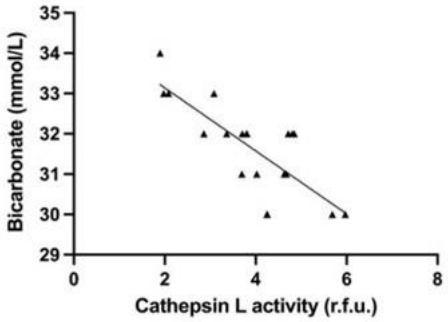
Bicarbonate (mmol/L)
35
34
33
32
31
30
29
0
2
4
6
8
Cathepsin L activity (r.f.u.)

Figure C.

5 DISCUSSION

COVID-19 is an infectious respiratory disease that emerged at the end of the year 2019 and caused by the SARS-CoV-2 virus, which has had a pandemic spread and has been the subject of intense scientific interest in recent years. The cellular mechanism of entry and spread of the virus occurs thanks to the binding of the SARS-CoV-2 Spike protein to the glycosylated ACE2 enzyme and to the intervention of several proteases: TMPRSS 2 and Cat L. Cat L relies on an acidic enviroment to be activated (15, 21).

ACE2 is a type I transmembrane protein with a molecular weight (mw) of 120 kDa in its most common glycosylated form, the de-glycosylated form has a mw of ~90 kDa (25). ACE2 participates in the counter-regulation of the RAAS system by converting Ang I and Ang II, produced by renin and ACE, into Ang (1–9) and Ang (1–7) respectively (9). It has several potential sites of N-glycosylation, a process that occurs at the level of the endoplasmic reticulum and in the Golgi apparatus and needs and acidic enviroment. Mutation at ACE2 glycosylation sites has been reported to impact SARS-CoV-2 binding Viral S-protein physically interacts and utilizes membrane bound TMPRSS2 and ACE2 to get primed and enter into the human cell (25, 26).

Recently, protection against infection has been observed and studied in patients with GS/BS, two rare genetic tubulopathies, which have among the clinical features a chronic metabolic alkalosis and elevated levels of ACE2 (9).

The group of professor Calò run three telephonic surveys over 128 Bartter's and Gitelman patients finding surprising results. Only few patients have been infected over 2 years, none of them died nor suffered major syntoms or needed to be hospitalized during the pandemic (37-39).

The studies in GS/BS to explore and better define the human RAS and RhoA/ROCK systems provide further background as to the protective effects of increased levels of ACE2 along with ROCK inhibition and how those might be of use against SARS-CoV-2 infection-induced respiratory complications. Specifically, GS/BS patients have an activated RAS and high Ang II levels, yet blunted Ang II-mediated cardiovascular effects and normotension or hypotension. Moreover, they have increased and correlated levels of both ACE2 and Ang 1-7 accompanied by activation of anti-inflammatory, antiapoptotic, antiproliferative, and anti-atherosclerotic defenses, reduced oxidative stress, and blunted Rho kinase signaling (2, 9).

Both glycosylated ACE2 and Cat-L activity are critical for SARS-CoV-2 binding and infection (14-16). The genetic defects of GS/BS inducing metabolic alkalosis alter chloride transport (2).

Chloride anion (Cl^-) is a key factor in cellular homeostasis as changes in intracellular Cl^- concentration drive gene and protein expression, post-translational modification, and intracellular/extracellular pH. Endo-lysosomal pH plays a critical role for the endocytic uptake of SARS-CoV-2 (15). Increased intracellular organelle pH, in fact, interferes with both ACE2 glycosylation and the binding via S protein as observed in experiments with CQ)/HCQ. Vincent et al. (15) reported that CQ induced elevations of endosomal pH and this interfered with terminal glycosylation of ACE2. The importance of ACE2 glycosilation on ACE2/SARS CoV 2 spike interaction has been recently highlighted (26).

The inverse correlation in GS/BS between blood HCO_3^- and Cat-L activity alongside the trend toward a negative correlation between blood HCO_3^- and the glycosylated isoform of ACE2 that have been found in our study suggest that GS/BS patients' metabolic alkalosis underlies these effects and that the endosomal processing system in GS/BS patients is impaired.

Fabry disease (FD), one of the most prevalent lysosomal storage disorder (LSD), is a monogenic inherited X-linked disease caused by mutations in the alpha galactosidase gene (41) which lead to accumulation of glycosphingolipids, mainly globotriaosylceramide (Gb3) and lyso-Gb3, in the lysosomes, producing a multisystemic storage disorder. FD patients have an impaired intracellular biochemistry characterized by oxidative stress, mitochondrial dysfunction, impaired autophagy, and endolysosomal maturation that contribute to FD adverse outcomes (42).

From a recent publication of the group of professor Calò emerged that FD patients seem to be protected from the SARS CoV 2 infection (43). These data are supported by another survey performed on 243 FD patients that during the first wave of the COVID 19 pandemic in 2020 appared to be protected from the disease (44). It has been shown that the impariment in the endosomal maturation alters the ACE2 glycosilation process and Cat L activity in FD patients in comparison to healthy subjects (43).

The results of this study parallel those reported in GS/BS patients and provide a mechanistic explanation for the protection from SARS-CoV2 infection and severe manifestations of COVID-19 observed in FD patients. The endosomal disruption in FD patients likely negatively affects viral entry and infection(43).

Other scientific publication explained how targeting Cat L may lead to reduced viral loads through reducing viral replications in mice (45). In another study CQ has shown potential in blocking the SARS-CoV-2 infection cycle by releasing basic side chains that raise the endosomal pH and inactivate Cat-L (46) supporting the findings in the GS/BS patients.

These observations reinforce the pathophysiological hypothesis underlying the fact that pH would determine an alteration in the protease-mediated entry mechanism.

The altered endosomal processing highlited in FD and GS/BS patients provides a robust mechanistic rationale for the effects of the combination of nirmatrelvir-ritonavir (Paxlovid), a new antiviral drug, which exerts its effect via inhibition of proteins involved in lysosomal processes key for SARS-CoV-2 cell entry and replication (19). These findings provide a rationale to fuel the pharmaceutical reserch towards new drugs that specifically target ACE2 glycosylation and proteases involved in SARS-CoV-2 infection.

6 CONCLUSIONS

GS/BS patients' physiology provides an "in vivo" human model where the effects of endosomal pH, ACE2 glycosylation status, and Cat-L activity alter SARS-CoV-2 infection rate and severity. These results have been confirmed and reproduced in patients affected by FD confirming that the disruption of the endo-lysosoms activity protects from the most severe form of the infection. These findings could fuel the search of new drugs targeting specific proteins involved in lysosomal processes and point to these as targets to fight COVID-19.

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