REVIEW ARTICLE

Protons to Patients: targeting endosomal Na$^+$/H$^+$ exchangers against COVID-19 and other viral diseases

Hari Prasad (iD)

Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bengaluru, India

While there is undeniable evidence to link endosomal acid-base homeostasis to viral pathogenesis, the lack of druggable molecular targets has hindered translation from bench to bedside. The recent identification of variants in the interferon-inducible endosomal Na$^+$/H$^+$ exchanger 9 associated with severe coronavirus disease-19 (COVID-19) has brought a shift in the way we envision aberrant endosomal acidification. Is it linked to an increased susceptibility to viral infection or a propensity to develop critical illness? This review summarizes the genetic and cellular evidence linking endosomal Na$^+$/H$^+$ exchangers and viral diseases to suggest how they can act as a broad-spectrum modulator of viral infection and downstream pathophysiology. The review also presents novel insights supporting the complex role of endosomal acid-base homeostasis in viral pathogenesis and discusses the potential causes for negative outcomes of clinical trials utilizing alkalinizing drugs as therapies for COVID-19. These findings lead to a pathogenic model of viral disease that predicts that nonspecific targeting of endosomal pH might fail, even if administered early on, and suggests that endosomal Na$^+$/H$^+$ exchangers may regulate key host antiviral defence mechanisms and mediators that act to drive inflammatory organ injury.

Endosomal acid-base homeostasis in viral diseases

Christian de Duve *et al*. back in 1974 suggested that targeting endosomal acid-base homeostasis could confer protection against viral infections [1]. In the intervening years, a great deal of work has shown that acid pH is necessary and often sufficient to trigger the fusion reactions of many enveloped viruses that enter cells via endosomes [2–5]. As a testament to the central role of the endosomal pH in viral pathogenesis,

surface proteins of many viruses undergo acid pH-triggered large-scale conformational changes that mediate virus-cell fusion [4,5]. There has recently been a huge resurgence of interest in endosomal pH as an antiviral target due to the ongoing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, and weakly basic alkalinizing drugs, such as hydroxychloroquine and azithromycin, were rapidly

Abbreviations

APOE4, apolipoprotein ε4 allele; COVID-19, coronavirus disease 19; CTD, C-terminal cytoplasmic domain; EBOV, Ebolavirus; eNHE, endosomal Na$^+$/H$^+$ exchanger; EV, extracellular vesicles; GP, glycoproteins; GWAS, genome-wide association studies; HA, haemagglutinin; HCV, hepatitis C virus; IFNAR2, interferon alpha and beta receptor subunit 2; IFN, interferon β; NHE6, Na$^+$/H$^+$ exchanger 6; NHE7, Na$^+$/H$^+$ exchanger 7; NHE9, Na$^+$/H$^+$ exchanger 9; NHP, nonhuman primate; N-Ras, neuroblastoma RAS viral oncogene homolog; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TM, transmembrane segment; V-ATPase, vacuolar-type ATPase; VPS10, vacuolar protein sorting 10; WNV, West Nile virus.

repurposed for widespread use as therapies for coronavirus disease 19 (COVID-19), despite lack of high-quality evidence [6,7].

SARS-COV-2 infection is blocked by inhibitors of endosomal acidification, indicating that acid pH is required at an early, postreceptor engagement, stage of the viral life cycle [6,7]. Consistent with this infection model, a high-throughput loss-of-function screen to map host factors required for SARS-CoV-2 infection identified several components of the proton pump vacuolar-type ATPase (V-ATPase) responsible for endosomal acidification (Fig. 1) [8]. Indeed, studies have demonstrated that acidic pH induces conformational changes in the spike glycoprotein as well as promotes its proteolytic activation to trigger the fusion reaction [9,10]. Accordingly, excessive endosomal acidification in pro-inflammatory M1 alveolar macrophages amplified SARS-CoV-2 infection, whereas neutralization of endosomal acidity by virus-binding alkaline peptides inhibited infection [11,12]. Thus, while the targeting of endosomal pH in COVID-19 is mechanistically well grounded and supported by *in vitro* and structural data, large clinical trials of alkalinizing drugs have failed to show a clear benefit [13]. Two possible causes of therapeutic failure can be considered. First, despite the pleiotropic effects of alkalinizing drugs, they modulate endosomal pH acting indirectly and more generically, that is by sequestering into acidic compartments, and are therefore certainly not the most potent molecules for endosomal pH regulation, particularly in *in vivo* scenarios. This could explain why alkalinizing drugs with significant antiviral effects *in vitro* may fail to protect against infection and disease *in vivo* [14]. Second, like V-ATPase inhibitors and ionophores, alkalinizing drugs have multiple compartmental effects and cause unwanted, potentially harmful changes in vesicle transport, Golgi and lysosomal function, and are detrimental to the autophagy pathway that may delay viral clearance [15]. Indeed, the latter could provide a mechanistic rationale for the observed increased toxicity and mortality in COVID-19 patients receiving higher alkalinizing drug doses [16] or a combination therapy of two alkalinizing drugs, hydroxychloroquine and azithromycin [17]. To date, no drugs specifically designed to increase endosomal pH are available.

Experience with COVID-19 has underscored the need for targeted antiviral drugs to specifically and effectively modulate endosomal pH. In this regard, the discovery of endosomal Na$^+$/H$^+$ exchangers (eNHEs), namely Na$^+$/H$^+$ exchanger 6 (NHE6) and Na$^+$/H$^+$ exchanger 9 (NHE9), and recognition of their evolutionarily conserved roles as the dominant leak pathway or 'tunable valves' for luminal protons offers a unique opportunity for compartment-specific targeting of endosomal pH (Fig. 1) [18–21]. From a mechanistic
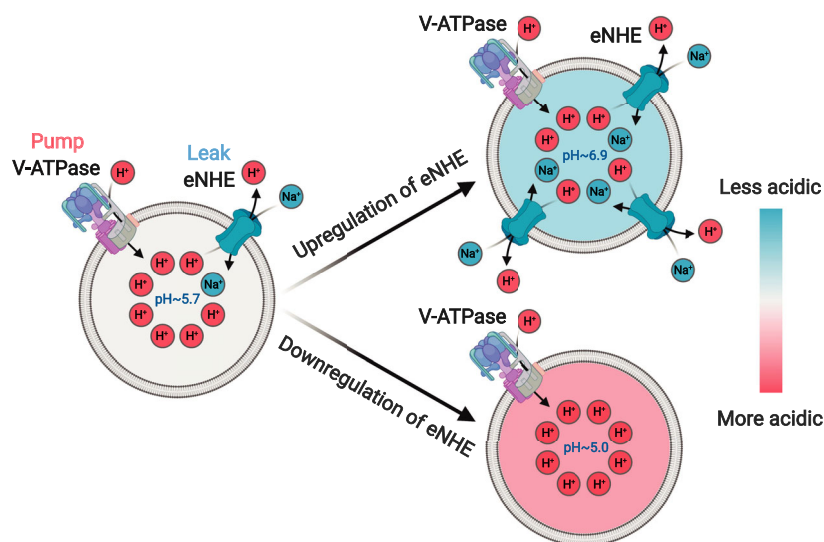

Fig. 1. Endosomal pH regulation by Na$^+$/H$^+$ exchangers. According to the 'pump-leak' model, conserved from yeast to plants and mammals, the eNHEs function as the dominant leak pathway or 'tunable valves' for luminal protons in exchange for Na$^+$ or K$^+$ ions to counteract the activity of the proton pumping V-ATPase and precisely tune the endosomal pH. The upregulation of eNHE expression or activity increases the leakage of the proton and makes the endosomal lumen more alkaline, and, conversely, the downregulation or loss of the eNHE function hyperacidifies the endosomal lumen due to the imbalance in the pump and leak pathways. The pH values indicated in the figure were obtained from experimental observations [32].

point of view, eNHEs are estimated to have exceptionally high transport rates of ∼ 1500 ions per second, so that targeting these exchangers results in robust changes in the ionic milieu within the specific confines of the endosomal space [22–24]. Studies in yeast, plants, fruit fly and mammals have established that eNHEs cause endosomal alkalization by leaking protons out of the lumen in exchange for Na$^+$/K$^+$ ions from the cytoplasm (Fig. 1) [18–21]. The significance of this mechanism has been highlighted by a number of disease states linked to mutations or altered expression levels of eNHEs, including autism, cancer and neurodegeneration [21,25,26]. This review will dissect accruing evidence linking endosomal pH and viral pathogenesis, develop eNHE as a potential antiviral target through a mechanistic understanding of their emerging role in viral diseases and discuss how this knowledge could lead to actionable therapeutic approaches.

COVID-19 and beyond: Linking NHE9 and viral diseases

Interferons are the canonical mediators of the early host response to viral infection, and they play critical roles in antiviral defence [27]. The following section summarizes the literature on NHE9, an interferon β (IFNβ)-induced protein [28,29], by examining its mechanisms of action, its potential importance in the protection against three pathogenic viruses, namely avian influenza (H5N1), West Nile virus (WNV), and SARS-CoV-2 and its broad role in the defence against diverse viral pathogens.

NHE9 and H5N1 avian influenza

Evidence for the role of endosomal pH in the pathogenic course of H5N1 avian influenza viral infection and disease was presented in the study of nonhuman primates (NHPs) infected with different human isolates [30]. Transcriptomic analysis of bronchial brush samples showed a significant induction of *SLC9A9*, the gene encoding NHE9, following the H5N1 challenge. Importantly, NHPs infected with the most virulent strain showing severe disease and death exhibited dramatic upregulation of NHE9 on days 3–7, while NHPs infected with less virulent strains showing milder disease exhibited weaker upregulation of NHE9 [30]. Notably, a similar transcriptomic analysis of peripheral blood mononuclear cells of NHPs following an Ebolavirus (EBOV) challenge identified related Na$^+$/H$^+$ exchanger 7 (NHE7; *SLC9A7*) as one of the top-10 genes that were significantly up-regulated in those who

developed severe disease and died compared to those who survived infection [31]. Hierarchical clustering performed to identify modules of co-expressed genes in response to H5N1 infection discovered a close association of NHE9 with genes involved in homeostatic process and immune response, including both anti-inflammatory (e.g. IL-10) and pro-inflammatory cytokines (e.g. IFNγ) [30]. Previous studies demonstrating the ability of NHE9 to inhibit the expression of IFNγ and to attenuate the pro-inflammatory state may support the beneficial role of NHE9 in this scenario [28].

Altogether, it is conceivable that the remarkable upregulation of NHE9 during H5N1 infection is triggered by a strong and sustained expression of antiviral innate immune genes such as IFNβ, a known inducer of NHE9 [28,30]. Given that viral infection is often seen as a competitive antagonism or evolutionary arms race between virus replication and host antiviral responses, the induction of NHE9 and resulting endosomal alkalinization may represent an antagonistic process that has evolved to limit cellular injury and disease. The well-documented role of endosomal acidification in triggering the low pH-dependent activation of the H5N1 haemagglutinin (HA) protein, which is essential for mediating membrane fusion, provides major support for this proposed adaptive mechanism [4]. However, it is not clear why the H5N1 virus, particularly the highly virulent strain, was able to replicate despite the strong and sustained upregulation of NHE9, which, as previously shown, could lead to robust endosomal alkalinization [30,32]. One possibility, based on experimental evidence, including crystallization studies, is that H5N1 viruses with higher pathogenicity were fine-tuned by mutations and evolved over time to have a higher pH optimum of HA activation than those with lower pathogenicity or, in other words, increased membrane fusion activity would be observed even at higher endosomal pH levels for highly virulent H5N1 isolates [4].

NHE9 and West Nile virus

To identify mRNAs associated with antiviral response and interferon-inducible transfer of antiviral activity to neighbouring uninfected cells (i.e. bystanders), Slonchak *et al.* [27]performed next-generation sequencing-based transcriptome-wide profiling of extracellular vesicles (EV) produced by cells infected with WNV compared to mock EV. This approach identified NHE9 as one of the most highly enriched transcripts (∼ 184-fold increase) in EV produced by WNV-infected cells, along with enrichment of mRNA coding

for well-known antiviral response components, including IFNβ, which, as previously shown, could further amplify NHE9 expression in recipient cells [27,28].

Multiple studies have established the protective role of endosomal alkalinization in the prevention of infection by WNV and other flaviviruses, such as Zika virus and yellow fever virus, by inhibiting virion-endosomal membrane fusion [3]. It is thus intriguing to speculate that the paracrine delivery of NHE9 mRNA via EV from WNV-infected cells mounts an anticipatory response in bystander cells by alkalinizing endosomal pH, thereby inhibiting virus entry into these cells. Indeed, as illustrated in the model (Fig. 2), the delivery of eNHE mRNA by EV may represent a more general and conservative antiviral mechanism against multiple viruses, and eNHEs could be considered as potential broad antiviral candidates. Importantly, eNHEs itself may play a role in the biogenesis, content and release of EV and thus modulate antiviral defence [21]. Supporting this notion, studies have demonstrated that the Na$^+$/H$^+$ ionophore monensin, which mimics constitutively activated eNHE, alkalizes endosomal pH, increases exosome secretion, and inhibits productive virus entry and cell-to-cell transmission [23,33,34].

NHE9 and SARS-CoV-2

To investigate the pathogenic process that exacerbate COVID-19 disease process and to hasten treatment development, Taylor et al. [35] employed a combinatorial high-order epistasis analysis to identify genetic risk factors that impact differential host responses to SARS-CoV-2 infection. This approach led to the discovery of *SLC9A9*, encoding NHE9, as one of 68 protein-coding genes that were strongly associated with severe COVID-19. Of note, an estimated 15% of patients with severe COVID-19 infection had variants in NHE9, independent of their pre-existing medical conditions, indicating that these variants are not associated with any particular co-morbidity, but are frequent amongst patients who develop severe life-threatening responses to the virus [35]. The Regeneron Genetics Center database of genetic determinants of COVID-19 risk and severity in 662 403 participants, including 11 356 with COVID-19, was analysed to corroborate and extend these findings [36]. Over 4000 variants in NHE9 derived from array-based imputation and exome sequences were found to be associated with COVID-19 phenotypes (including risks of infection, hospitalization and severe disease) with a nominal significance of $P \leq 0.05$. Of note, the intronic variant rs9810857 previously associated with attention-deficit/hyperactivity disorder was linked with the phenotype COVID-19 positive vs COVID-19 negative (OR = 1.4, $P < 0.05$) [37]. The emerging link between NHE9 and COVID-19 would be in line with the findings of endosomal pH-dependent conformational changes in the SARS-CoV-2 spike, which, in addition to host cell entry, is involved in evasion of the humoral immune response [9].

Importantly, multiple missense variants in NHE9 yielded a directionally consistent and statistically significant association with COVID-19 phenotypes. These substitutions are located throughout the coding frame, including the membrane-embedded transport domain and the C-terminal cytoplasmic domain (CTD; Fig. 3A–C and Table 1). It is noteworthy that an autism with epilepsy linked D496N variant in the CTD, previously reported not to alter transport activity but may be involved in regulatory functions [38], showed an association with the phenotype COVID-19-positive and severe vs COVID-19-negative or COVID-19 status unknown (OR = 123.8, 95% CI = 0.902–16991.4, $P = 5.50 \times 10^{-2}$). The G478E variant, which was mapped to a highly conserved residue in the last
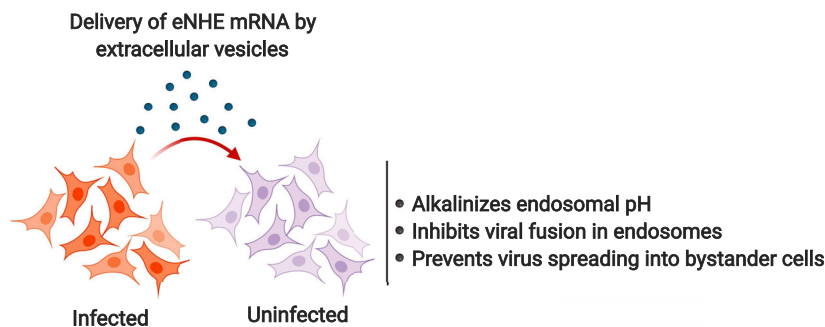

Delivery of eNHE mRNA by extracellular vesicles

- Alkalinizes endosomal pH
- Inhibits viral fusion in endosomes
- Prevents virus spreading into bystander cells

Infected   Uninfected

Fig. 2. Model for endosomal pH regulation of viral infection via EV. The delivery of eNHE mRNA by EV from infected cells may be a general and conservative antiviral mechanism against multiple viruses. Transcellular spread of eNHE mRNA would protect uninfected bystander cells by alkalinizing endosomal pH and inhibiting virus entry and cell-to-cell transmission.

transmembrane segment (TM13) and predicted to result in a loss of transport function using a structure–function approach, is of particular interest (Fig. 3A,B). Analysis of imputed data identified an association between this variant and risks of hospitalization (OR = 142.23, 95% CI = 8.11–2493.22, $P = 6.92 \times 10^{-4}$) and severe disease (OR = 27.79, 95% CI = 1.51–512.02, $P = 2.53 \times 10^{-2}$) amongst COVID-19-positive cases. The exome sequencing data also showed a reproducible association between this variant and increased risks of hospitalization (OR = 104.9, 95% CI = 6.45–1707.06, $P = 1.08 \times 10^{-3}$) and severe disease (OR = 76.55, 95% CI = 5.02–1166.78, $P = 1.80 \times 10^{-3}$) [36] (Fig. 3A–C and Table 1). Functional abnormalities such as endosomal hyperacidification, vesicular trafficking defects and reduced surface expression of membrane proteins have been reported in NHE9 patient mutations involving conserved residues in the membrane-embedded transporter domain, consistent with loss-of-function causal to disease phenotype [25,39]. Evolutionary conservation analysis in conjunction with structure-driven assessment predicted that several NHE9 variants associated with COVID-19 phenotypes would result in dysregulated transporter function (Fig. 3A–C and Table 1). Further studies are awaited to functionally evaluate the COVID-19-associated variants and to understand how NHE9 plays a role in early inflammatory pathways that are key to the progression to severe COVID-19.

Based on genetic and cellular findings, it is attractive to speculate that NHE9 plays a protective role in the prevention of adverse outcomes in COVID-19 through at least two distinct mechanisms. First, by regulating the endosomal pH of target cells, NHE9 may control the entry of SARS-CoV-2 and the initial viral load and, secondly, by its role in the regulation of type I interferon responses and polarization and differentiation of immune cells [28,29], and prevent the occurrence of a subsequent disproportional inflammatory reaction, the so-called cytokine storm [35]. In this regard, it is worth noting that a genetic variant in NHE9 that reduces its expression has been linked to an exaggerated inflammatory response in multiple sclerosis patients, as well as increased disease activity and nonresponse to IFNβ therapy [28]. Furthermore, variants in NHE9 have been also linked to N-glycosylation alterations, an important pH-linked molecular mechanism that affects viral pathogenesis, including that of SARS-CoV-2 [40–42]. Two independent genome-wide association studies of human plasma protein N-glycosylation have reported significant associations of the *SLC9A9* locus with the sialylation related traits of N-glycans [41,42]. Previous work uncovered that drugs that cause endosomal alkalization modulate the terminal glycosylation of the angiotensin-converting enzyme 2 receptor, which is the binding site for the envelope spike glycoprotein, and may contribute to its *in vitro* inhibitory activity against SARS-CoV-2 [43]. Further research is needed to determine how NHE9 regulates mechanisms that control protein glycosylation. These studies would improve our understanding of the role of NHE9 in virus-host interactions and translate into a new generation of antiviral therapeutics.

As opportunities for therapeutic intervention, it is important that eNHEs are known downstream effectors of the ϵ4 allele of apolipoprotein E (ApoE4), which is associated with severe SARS-CoV-2 infection independent of dementia and other comorbidities [21,23,44]. Studies have shown that ApoE4 potentiates presymptomatic endosomal anomalies and promotes endocytic entry of viruses, but it is unclear how these two pathways are linked at the cellular and mechanistic level [45–47]. ApoE4-associated endosomal dysfunction is mediated by pathological activation of Rab5, a master regulatory GTPase involved in early endosomal biogenesis and also important for virus-cell entry [47,48]. Notably, increased expression of Rab5 has been documented in response to SARS-CoV-2 infection, underscoring the pathogenic role of early endosomes in COVID-19 [49]. ApoE4-selective early endosomal dysfunction manifests morphologically as enlarged and amplified compartments, biochemically as hyperacidic luminal pH, and functionally as trafficking deficits, all reflect endosome impairment in their many known roles in the cell [23,47]. Building on the model developed thus far, it is intriguing to speculate that ApoE4-specific eNHE downregulation and pathological endosomal acidification, which have been documented not only in brain cells but also in peripheral cells (~ 0.9 pH unit lower) [23], may promote endosome-mediated virus entry into the cytosol. Accordingly, interventions targeting eNHE may have the potential to leverage the disease-modifying benefit of endosomal pH to reduce the risk of severe COVID-19 associated with the ApoE4 genotype.

SARS-CoV-2 variants with spike protein mutations that alter pathogenicity are becoming increasingly known [50]. It remains to be determined whether some of these mutations, similar to those found in H5N1 influenza viruses [4], could enable the spike protein to be activated even at higher endosomal pH, resulting in a novel virulence factor and increased virus fitness. While there are still outstanding questions, the association between NHE9 and COVID-19 strengthens the role of endosomal pH in the regulation of viral entry and disease course, as discussed earlier, provides a
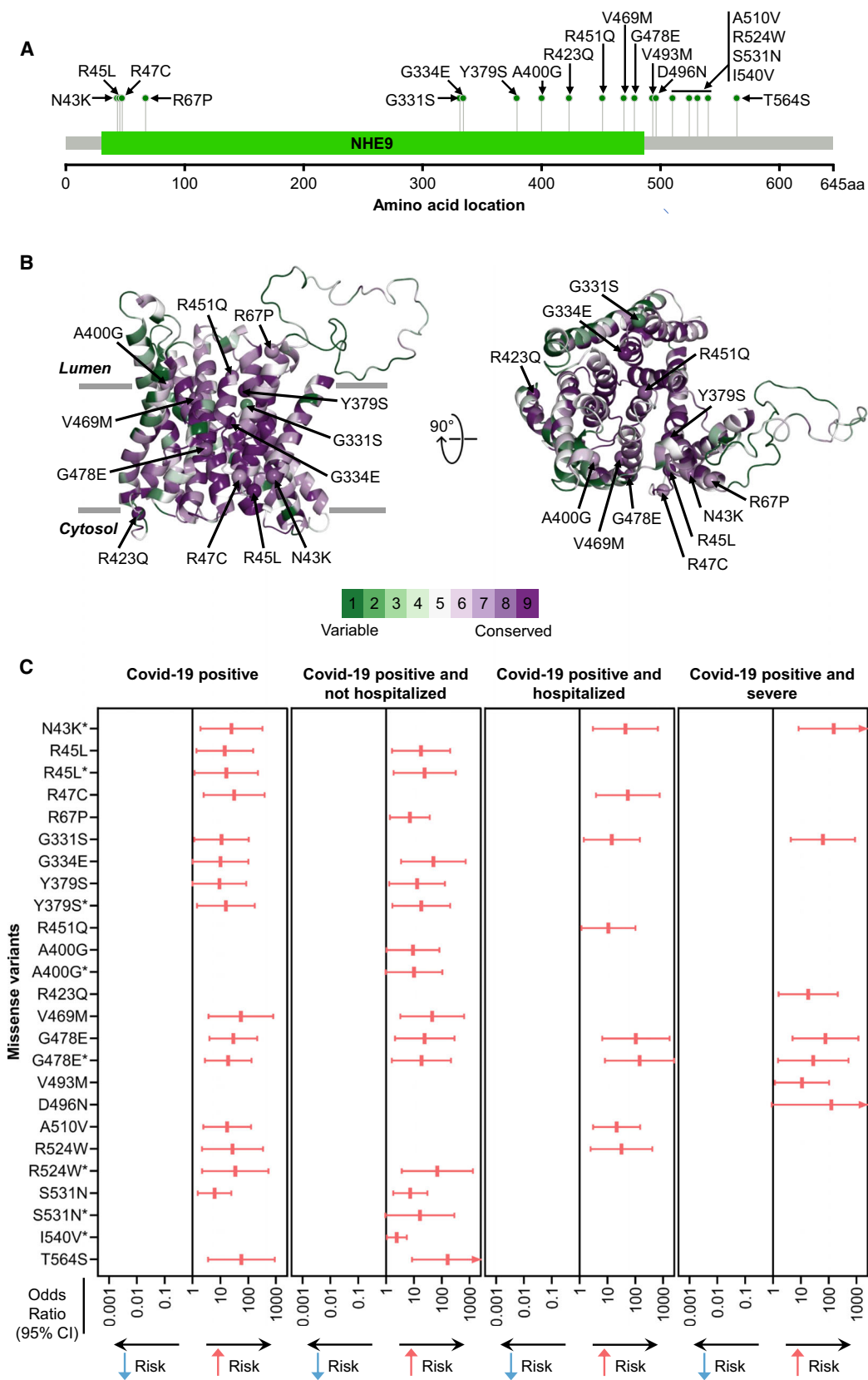
rational genetic basis for efforts aimed at the development or repurposing of drugs for therapeutic modulation of endosomal pH and sets out a strategy for stratifying individuals at the highest risk of life-threatening SARS-CoV-2 infection.

Hepatitis C and beyond: linking NHE6 and viral diseases

NHE6 is the most widely distributed eNHE and the importance of its function is reflected in humans with mutations in the *SLC9A6* gene leading to loss of function (Christianson syndrome) [18–21]. Despite overlapping endosomal location and function, NHE6 and NHE9 have nonredundant roles as evidenced by distinct and diverse clinical and cellular phenotypes [18–21]. Downregulation of NHE6 and, by extrapolation, pathological endosomal acidification has been documented *in vitro* and *in vivo* in response to a variety of human viral pathogens, namely chikungunya virus, enterovirus 71, Epstein–Barr virus, influenza A virus, Kaposi's sarcoma-associated herpesvirus, and rotavirus and a fish pathogen infectious pancreatic necrosis virus [51–57]. Of note, influenza B virus, which often causes mild illness, has been found to be associated with serious disease with myositis and encephalitis in a male with a loss-of-function mutation in NHE6 [58]. Importantly, a high-throughput RNAi silencing screen performed to map host cell factors involved in virus entry identified that NHE6 depletion enhanced human papillomavirus infection, providing a compelling indication of the protective role of eNHEs in host-virus conflicts [59]. Here is a summary of the evidence linking NHE6 to hepatitis C and Ebola viral infections that would enable us to recognize the broader role of endosomal acid-base homeostasis in viral diseases.

NHE6 and hepatitis C virus

Several lines of evidence indicate that endosomal acidification is a critical host factor for regulation of hepatitis C virus (HCV) infection [5]. Previous studies have shown that HCV viral components, specifically the core protein and nonstructural NS2 protein, directly interact with NHE6 [60], although the functional importance of this interaction for proton leak activity and to produce infectious viruses remains to be determined. Furthermore, monensin, a Na$^+$/H$^+$ ionophore that mimics NHE6, is known to inhibit HCV infection [23,34]. Importantly, analysis of publicly available microarray data showed a statistically significant reduction in NHE6 gene expression that correlated with the duration of HCV cell infection (Fig. 4) [61]. It is conceivable that sustained NHE6 depletion causes pathological endosomal acidification, which, in addition to promoting HCV infection, would result in the loss of hepatocyte polarity as previously reported and, in turn, facilitate neoplastic transformation [62]. This hypothesis will need to be tested, but the arguments put forward here will be important for future studies to better define this new relationship between endosomal pH, HCV infection and hepatocellular carcinoma.

NHE6 and Ebolavirus

Ebolavirus exploits the endosomal pathway to gain host cell entry, although the mechanism of how it fuses its envelope with endosomal membranes remains poorly understood [2]. There is consensus that endosomal acidity plays a crucial role in regulating acid-dependent proteases that cleave glycoproteins (GP) and allow fusion events [2]. Consistent with this observation, alkalinizing drug chloroquine significantly inhibited EBOV infection *in vitro*, yet failed to protect against infection and disease in an *in vivo* guinea pig model, underscoring the need for targeted antiviral strategies to modulate endosomal pH [14]. Previous research has shown that microRNA 196b, which targets NHE6 and regulates its expression, is one of the most significantly overexpressed microRNAs in cells expressing EBOV GP [63]. The downregulation of NHE6 expression observed in response to EBOV GP would cause endosomal hyperacidification and influence viral replication and pathogenesis [63]. These findings, together with the emerging role of the related

Fig. 3. Genetic association of NHE9 and COVID-19 phenotypes. (A) Lollipop representation of missense substitutions in NHE9 associated with COVID-19 phenotypes as listed in Table 1. The *x*-axis indicates amino acid locations of NHE9. (B) Side (left) and top (right) views of the NHE9 transport domain [79] and coloured according to the degree of ConSurf conservation, with green through purple, indicating variable through conserved amino acid positions. COVID-19-associated variants are represented as alpha-carbon spheres. The conservation colour bar is shown at the bottom. (C) Forest plots depicting odds ratios and 95% confidence intervals demonstrating significant association between missense variants in NHE9 derived from array-based imputation (asterisk) and exome sequences and four COVID-19 phenotypes (positive, positive and not hospitalized, positive and hospitalised, and positive and severe) vs COVID-19 negative or COVID-19 status unknown [36].

Table 1. Survey of missense variants in NHE9 associated with COVID-19 phenotypes. Missense variants in NHE9 derived from array-based imputation and exome sequences significantly associated with COVID-19 phenotypes (positive, positive and not hospitalized, positive and hospitalised, and positive and severe) vs COVID-19 negative or COVID-19 status unknown [36]. Amino acids are represented by their single letter codes in protein sequence. Evolutionary conservation (ConSurf) scores for the mutated residues were calculated using a scale ranging from 1 (highly variable) to 9 (invariant). The MutationTaster algorithm was used for functional prediction. Association analyses were carried out separately for variants derived from array-based imputation and exome sequencing using the Firth logistic regression test. AAF, alternative allele frequency; CCS, ConSurf conservation score.

Nucleotide change	Protein change	Location	CCS	dbSNP ID	Functional prediction	Phenotypes	Genetic dataset	Odds ratio (95% CI)	P-value	Cases (RR : RA : AA)	Controls (RR : RA : AA)	AAF
c.129T>A	N43K	TM1	6	rs140007028	Disease causing	COVID-19 positive	Imputed	24.99 (1.91, 327.61)	1.42×10^{-2}	1796 : 1 : 0	434 023 : 15 : 0	3.89×10^{-5}
						COVID-19 positive hospitalized	Imputed	44.29 (3.01, 652.8)	5.75×10^{-3}	1144 : 1 : 0	434 023 : 15 : 0	3.89×10^{-5}
						COVID-19 positive severe	Imputed	152.46 (8.34, 2786.62)	6.97×10^{-4}	471 : 1 : 0	434 023 : 15 : 0	3.89×10^{-5}
c.134G>T	R45L	TM1-TM2 loop	9	rs762162474	Disease causing	COVID-19 positive	Exome	14.4 (1.38, 149.91)	2.57×10^{-2}	820 : 1 : 0	109 293 : 10 : 0	4.99×10^{-5}
						COVID-19 positive not hospitalized	Imputed	16.3 (1.19, 222.54)	3.63×10^{-2}	853 : 1 : 0	112 869 : 8 : 0	7.15×10^{-5}
							Exome	18.07 (1.64, 199.59)	1.82×10^{-2}	663 : 1 : 0	109 293 : 10 : 0	5.00×10^{-5}
c.139C>T	R47C	TM1-TM2 loop	7	rs889526736	Disease causing	COVID-19 positive	Imputed	24.49 (1.87, 320.12)	1.47×10^{-2}	688 : 1 : 0	112 869 : 8 : 0	7.16×10^{-5}
							Exome	31.23 (2.53, 386.18)	7.32×10^{-3}	1672 : 1 : 0	404 287 : 13 : 0	1.72×10^{-5}
						COVID-19 positive hospitalized	Exome	54.15 (3.87, 758.39)	3.04×10^{-3}	1067 : 1 : 0	404 287 : 13 : 0	1.73×10^{-5}
c.200G>C	R67P	TM2	7	rs372258008	Disease causing	COVID-19 positive not hospitalized	Exome	7.16 (1.38, 36.99)	1.89×10^{-2}	603 : 2 : 0	403 962 : 256 : 0	3.19×10^{-4}
c.991G>A	G331S	TM8-TM9 loop	3	rs544613454	Disease causing	COVID-19 positive	Exome	11.03 (1.16, 104.99)	3.68×10^{-2}	1672 : 1 : 0	404 273 : 25 : 0	3.20×10^{-5}
						COVID-19 positive hospitalized	Exome	14.3 (1.41, 145.09)	2.45×10^{-2}	1067 : 1 : 0	404 273 : 25 : 0	3.21×10^{-5}
						COVID-19 positive severe	Exome	62.05 (4.34, 887.62)	2.36×10^{-3}	438 : 1 : 0	404 273 : 25 : 0	3.21×10^{-5}
c.1001G>A	G334E	TM8-TM9 loop	9	rs147143314	Disease causing	COVID-19 positive	Exome	10.13 (1.01, 101.57)	4.90×10^{-2}	109 : 1 : 0	8592 : 7 : 0	4.59×10^{-4}
						COVID-19 positive not hospitalized	Exome	50.04 (3.47, 721.16)	4.05×10^{-3}	38 : 1 : 0	8592 : 7 : 0	4.63×10^{-5}
c.1136A>C	Y379S	TM10	9	rs752146831		COVID-19 positive	Exome	9.25 (1, 85.13)	4.96×10^{-2}	820 : 1 : 0	109 286 : 19 : 0	9.08×10^{-5}

Table 1. (Continued).

Nucleotide change	Protein change	Location	CCS	dbSNP ID	Functional prediction	Phenotypes	Genetic dataset	Odds ratio (95% CI)	P-value	Cases (RR : RA : AA)	Controls (RR : RA : AA)	AAF
					Disease causing	COVID-19 positive	Imputed	15.71 (1.44, 170.85)	2.37×10^{-2}	853 : 1 : 0	112 858 : 19 : 0	1.04×10^{-4}
						COVID-19 positive not hospitalized	Exome	13.01 (1.31, 128.81)	2.83×10^{-2}	663 : 1 : 0	109 286 : 19 : 0	9.09×10^{-5}
c.1199C>G	A400G	TM11	6	rs187395337	Disease causing	COVID-19 positive	Imputed	18.35 (1.68, 201.04)	1.72×10^{-2}	688 : 1 : 0	112 858 : 19 : 0	1.05×10^{-4}
						COVID-19 positive not hospitalized	Exome	9.28 (1.04, 82.73)	4.60×10^{-2}	604 : 1 : 0	404 211 : 88 : 0	1.10×10^{-4}
c.1268G>A	R423Q	TM11-TM12 loop	7	rs368254745	Disease causing	COVID-19 positive hospitalized	Imputed	10.67 (1.09, 104.46)	4.20×10^{-2}	66 : 1 : 0	8720 : 18 : 0	1.11×10^{-3}
c.1352G>A	R451Q	TM12	8	rs767698612	Disease causing	COVID-19 positive severe	Exome	18.35 (1.59, 211.3)	1.96×10^{-2}	438 : 1 : 0	404 249 : 51 : 0	6.42×10^{-5}
						COVID-19 positive hospitalized	Exome	10.85 (1.15, 102.3)	3.73×10^{-2}	1067 : 1 : 0	404 251 : 49 : 0	6.17×10^{-5}
c.1405G>A	V469M	TM13	8	rs768229420	Disease causing	COVID-19 positive	Exome	54.78 (3.78, 793.3)	3.33×10^{-3}	820 : 1 : 0	109 301 : 4 : 0	2.27×10^{-5}
						COVID-19 positive not hospitalized	Exome	45.32 (3.25, 632.54)	4.57×10^{-3}	663 : 1 : 0	109 301 : 4 : 0	2.27×10^{-5}
c.1433G>A	G478E	TM13	8	rs766148122	Disease causing	COVID-19 positive	Exome	29.34 (4.09, 210.69)	7.80×10^{-4}	819 : 2 : 0	109 294 : 11 : 0	5.90×10^{-5}
						COVID-19 positive	Imputed	19.06 (2.78, 130.48)	2.68×10^{-3}	852 : 2 : 0	112 864 : 13 : 0	7.46×10^{-5}
						COVID-19 positive not hospitalized	Exome	24.48 (2.1, 285.76)	1.07×10^{-2}	663 : 1 : 0	109 294 : 11 : 0	5.46×10^{-5}
						COVID-19 positive not hospitalized	Imputed	18.6 (1.62, 213.67)	1.89×10^{-2}	688 : 1 : 0	112 864 : 13 : 0	7.03×10^{-5}
						COVID-19 positive hospitalized	Exome	104.9 (6.45, 1707.06)	1.08×10^{-3}	156 : 1 : 0	109 294 : 11 : 0	5.48×10^{-5}
						COVID-19 positive hospitalized	Imputed	142.23 (8.11, 2493.22)	6.92×10^{-4}	164 : 1 : 0	112 864 : 13 : 0	7.05×10^{-5}
						COVID-19 positive severe	Exome	76.55 (5.02, 1166.78)	1.80×10^{-3}	33 : 1 : 0	109 294 : 11 : 0	5.49×10^{-5}
						COVID-19 positive severe	Imputed	27.79 (1.51, 512.02)	2.53×10^{-3}	35 : 1 : 0	112 864 : 13 : 0	7.06×10^{-5}
c.1477G>A	V493M	CTD	6	rs775249012	Disease causing	COVID-19 positive severe	Exome	11.01 (1.18, 102.67)	3.53×10^{-3}	438 : 1 : 0	404 165 : 135 : 0	1.68×10^{-4}
c.1486G>A	D496N	CTD	4	rs111291437	Disease causing	COVID-19 positive severe	Exome	123.8 (0.902, 16991.4)	5.50×10^{-2}	32 : 0 : 0	8594 : 5 : 0	2.90×10^{-4}
c.1529C>T	A510V	CTD	1	rs140964854	Disease causing	COVID-19 positive	Exome	17.55 (2.44, 126.3)	4.44×10^{-3}	108 : 2 : 0	8589 : 10 : 0	6.89×10^{-4}
						COVID-19 positive hospitalized	Exome	21.41 (3.06, 149.97)	2.03×10^{-3}	69 : 2 : 0	8589 : 10 : 0	6.92×10^{-4}

Table 1. (Continued).

| Nucleotide change	Protein change	Location	CCS	dbSNP ID	Functional prediction	Phenotypes	Genetic dataset	Odds ratio (95% CI)	P-value	Cases (RR : RA : AA)	Controls (RR : RA : AA)	AAF
c.1570C>T	R524W	CTD	1	rs574582502	Polymorphism	COVID-19 positive	Exome	27.3 (2.19, 341.1)	1.03×10^{-2}	81 : 1 : 0	9413 : 6 : 0	3.68×10^{-4}
							Imputed	34.21 (2.2, 532.33)	1.17×10^{-2}	90 : 1 : 0	10 485 : 7 : 0	4.64×10^{-4}
						COVID-19 positive not hospitalized	Imputed	69.07 (3.64, 1310.22)	4.79×10^{-3}	41 : 1 : 0	10 485 : 7 : 0	4.65×10^{-4}
						COVID-19 positive hospitalized	Exome	31.64 (2.45, 407.8)	8.09×10^{-3}	44 : 1 : 0	9413 : 6 : 0	3.70×10^{-4}
c.1592G>A	S531N	CTD	2	rs757976578	Polymorphism	COVID-19 positive	Exome	6.2 (1.55, 24.76)	9.73×10^{-3}	818 : 3 : 0	109 242 : 63 : 0	3.00×10^{-4}
						COVID-19 positive not hospitalized	Exome	7.43 (1.81, 30.48)	5.35×10^{-3}	661 : 3 : 0	109 242 : 63 : 0	3.00×10^{-4}
							Imputed	16.37 (0.953, 281.1)	5.40×10^{-2}	687 : 2 : 0	112 826 : 51 : 0	2.38×10^{-4}
c.1618A>G	I540V	CTD	2	rs16853300	Polymorphism	COVID-19 positive not hospitalized	Imputed	2.43 (1.07, 5.53)	3.42×10^{-2}	89 : 10 : 0	8376 : 360 : 2	2.25×10^{-2}
c.1691C>G	T564S	CTD	9		Disease causing	COVID-19 positive	Exome	57.06 (3.63, 897.85)	4.03×10^{-3}	1672 : 1 : 0	404 293 : 6 : 0	8.62×10^{-6}
						COVID-19 positive not hospitalized	Exome	162.42 (8.55, 3084.09)	7.02×10^{-4}	604 : 1 : 0	404 293 : 6 : 0	8.64×10^{-6}
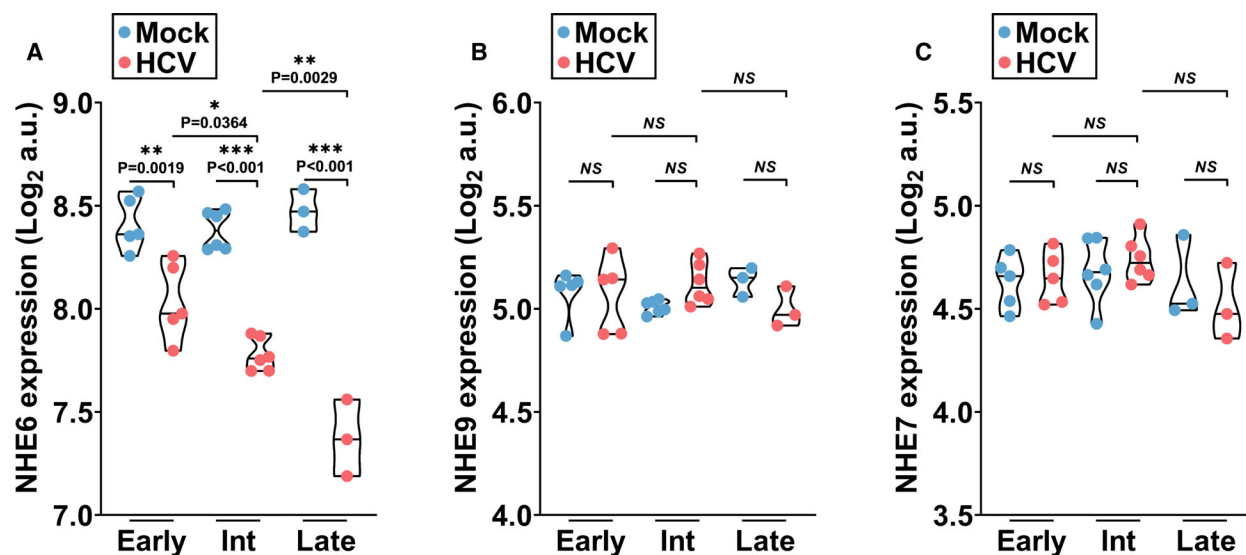
Fig. 4. Misregulated eNHE expression in hepatocytes in response to HCV infection. Analysis of publicly available microarray data showed a statistically significant reduction of NHE6 in response to HCV infection in the Huh7 hepatocyte model, correlating to the duration of infection (early, 6–12 h; intermediate/int, 18–24 h; late, 48 h) compared to mock infection [61]. The expressions of the related NHE9 and NHE7 are shown for comparison. Statistical analysis was performed by two-way ANOVA. *NS*, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

NHE7 in EBOV infection [31], highlight the importance of organellar pH in Ebola and other viral diseases.

Perspectives

Lessons learned from COVID-19

While the antiviral properties of endosomal pH have been recognized for nearly five decades, it is unclear whether and how host cells modulate endosomal acid-base balance to gain selective advantage over incoming viral pathogens [1]. The identification of variants in NHE9 associated with severe COVID-19 supports the formulation that alterations in endosomal acidification may, in principle, occur as a primary event in the viral life cycle, upstream of replication and release of new virions [35]. It is pathogenically informative that of the 68 genes associated with the severe COVID-19, only NHE9 and its conserved downstream effector vacuolar protein sorting 10 (VPS10) domain containing receptor sortilin-related Vps10p domain containing receptor 2 converge on the endosomal-lysosomal pathway, specifically the early and recycling endosomes [21,25,35]. Indeed, this provides a rational genetic basis for ongoing efforts to repurpose marketed amphipathic and weakly basic drugs, which partition into acidic compartments and alkalinize endosomes, as possible therapeutics for viral diseases (Fig. 5).

It is however critical to highlight that alkalinizing drugs are likely to have the greatest impact on the most acidic organelle, that is lysosome, and would not significantly affect endosomal pH or, in the extreme scenario, could even lead to an increase in cytotoxicity. In either case, this may explain why the clinical trials of alkalizing drugs in COVID-19 have so far been conflicting [6,7,13]. A shift in focus from pH-neutralizing agents to targeted therapy by modulating host proteins is warranted. Indeed, interventions aimed directly at increasing eNHE-mediated proton leak from early endosomes could be a better, or at least an alternate, therapeutic approach (Fig. 5). Certainly, they would be indicated against SARS-CoV-2 and other viruses entering cells via the endosomal pathway, where increasing endosomal pH would be therapeutically beneficial, and potentially also for preparedness and control of future viral pandemics of unknown origin.

Endosomal pH and interferon signalling

Regardless of whether the *SLC9A9* variants can be validated as a clinically useful marker for assessing the risk of a life-threatening hyperinflammatory disease, the emerging link between NHE9 and COVID-19 provides an intriguing insight into its role in type I interferon responses against the SARS-CoV-2 virus and the immunopathological mechanisms underlying severe disease. Further mechanistic studies are awaited to
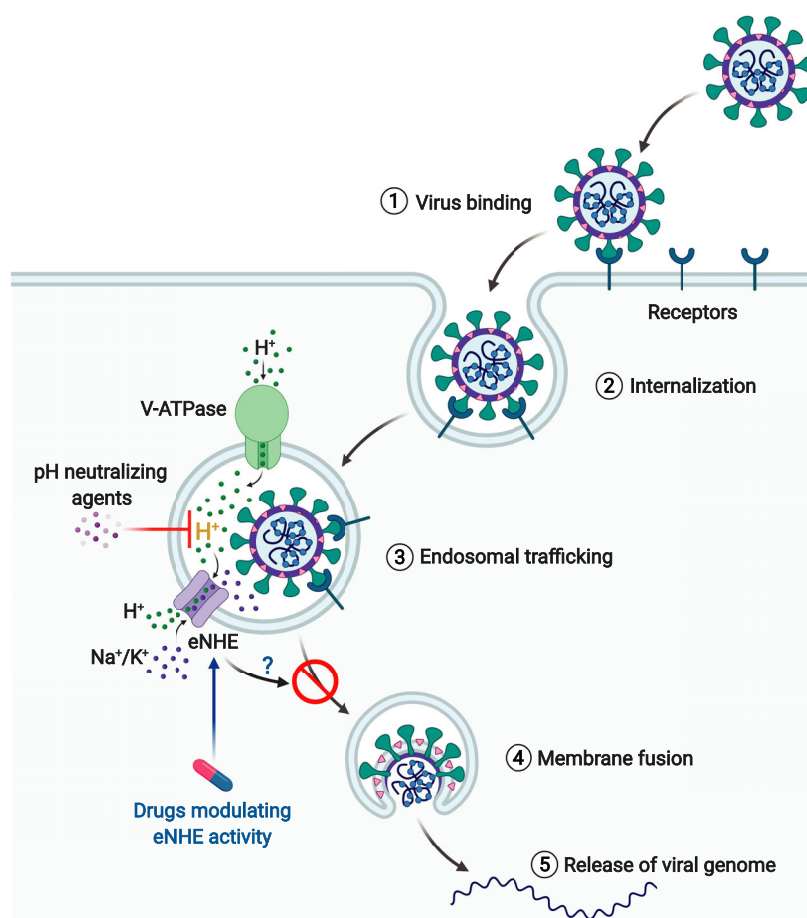
Fig. 5. Proposed role for eNHE in virion-endosomal membrane fusion. Most enveloped viruses bind to the plasma membrane receptors, enter the cell by endocytosis, traffic through the endocytic pathway and exit by acid pH-triggered membrane fusion to release the viral genome into the cytosol. pH-neutralizing agents including amphipathic and weakly basic drugs that partition into acidic compartments and alkalinize endosomes have been proposed as potential therapeutics for COVID-19 and other viral diseases. eNHEs leak protons by counteracting proton pumping V-ATPase and alkalizing endosomal lumen, which in turn could block virus-cell fusion. Interventions directly designed to increase the eNHE-mediated leakage of early endosomal protons could be attractive antiviral strategies for the specific and effective modulation of endosomal pH. The identification of variants in the eNHE NHE9 linked to severe SARS-CoV-2 infection raises the possibility that the prognostic significance of endosomal pH in COVID-19 reflects its positioning at the very intersection between resistance to virus infection and the regulation of inflammation. Mechanistically, eNHEs are the dominant leak pathway for luminal protons and strategies to target them and specifically alkalinize endosomal pH have great therapeutic potential.

understand the link between NHE9 and interferon signalling [28,29]. Based on genetic and cellular evidence, one possibility is that NHE9 activity regulates endocytic recycling and membrane persistence of interferon receptors and modulates type I interferon responses during acute COVID-19 [21,25,26].

Consistent with a beneficial role for type I interferons, loss-of-function mutations in the interferon receptor subunit gene *IFNAR2* are linked to critical illness in COVID-19 patients [64]. This suggests that administering exogenous interferons could reduce the likelihood of severe disease in COVID-19 patients, but

large clinical trials found that IFNβ treatment did not reduce mortality in hospitalized patients [65]. The reason for this inadequate treatment response has been unclear. It is possible that some patients might simply have a weak biological response to IFNβ, or they may be in the late inflammatory phase, with uncontrolled fibroproliferation and persistent lung remodelling that cannot be controlled by IFNβ treatment alone. In either case, interventions designed to amplify interferon signalling might be of therapeutic value. Given that NHE9 is a known genetic determinant of biological response to IFNβ [28,29], strategies that activate it

could potentially augment response to interferon treatment and reduce the risk of critical illness in COVID-19.

Translational insights with learning opportunities

The development of broad-spectrum antivirals is critical for combating emerging and re-emerging viral pathogens. An important therapeutic advantage of targeting eNHEs is that, in addition to potentially inhibiting a wide array of viruses, their anti-inflammatory and immunoregulatory effects may modulate both the innate and adaptive immune response. For example, NHE6 is a known downstream neuroblastoma RAS viral oncogene homolog effector in T cells that mediates protection against viral infections [66,67]. Similarly, NHE9 regulates T-cell function by modulating its response to IFNβ and can thus induce its own expression [28]. This creates a feedback loop that could be an important component of the host response to viral infection. However, given that eNHEs are predicted to play a role early in the viral cycle, interventions targeting them are likely to have an optimal time window, that is before or during the early inflammatory phase. At the late stage of hyperinflammation, their antiviral effect may be limited. Further studies are needed to substantiate this hypothesis.

Multiple intracellular organelle involvement is central to COVID-19 and critical to the mechanisms of viral entry, virus-host interactions, metabolic reprogramming and virus-induced cytopathic effects [68–71]. In addition to endosomes, other organelles such as endoplasmic reticulum, lysosomes, mitochondria and lipid droplets have been implicated in the pathogenesis [68–71]. Importantly, SARS-CoV-2-induced cytopathic effects have been shown to cause remodelling of membrane and cytoskeleton elements, as well as extensive morphological alterations of organelles to meet their biosynthetic needs, such as fragmentation of the Golgi apparatus, perturbation of the mitochondrial network, increased biogenesis of lipid droplets and peroxisome recruitment to viral replication organelles [71,72]. Additional studies will further define role of pH and ionic balance in different organelles, as well as structural reorganizations and inter-organelle communication, in the pathogenesis of COVID-19, with functional and potentially translational implications.

The role of lysosomes is of particular interest and has received a great deal of attention in the context of SARS-CoV-2 infection [68]. Indeed, the therapeutic use of lysosomotropic agents early in the COVID-19 pandemic was based on the premise that lysosomal function and autophagy promote viral infection and cytopathic effect [73,74]. However, mounting evidence suggests that inhibiting autophagy would impair cellular surveillance mechanisms and viral clearance, resulting in accelerated pathogenesis and a heightened inflammatory state involving a cytokine storm [15,75,76]. Recent studies have shown that SARS-CoV-2 reprograms cell metabolism, blocks autophagic flux and inhibits autophagosome-lysosome fusion, and that pharmacological induction of autophagy limits infection [75,76]. Continued research, as well as the development of compounds that alter the activities of eNHE and thus exhibit endosomal selectivity without impairing lysosomal function, could lead to broad-spectrum antiviral therapeutics.

Proof-of-principle for targeting endosomal pH by agents that enhance eNHE expression, such as histone deacetylase inhibitors and cAMP response element binding protein activators, exists in cell culture [23,24]. Positive reports of these drugs in human immunodeficiency virus infection support further experimental work to validate their antiviral properties and interventional trials to evaluate their efficacy [77,78]. Another strategy is to directly target eNHEs because membrane transporters are generally eminently druggable therapeutic targets. The crystal structure of mammalian eNHE has recently been determined, providing the scientific community with a long-awaited tool for drug design and setting out new opportunities for targeted therapies [79].

Importantly, because eNHEs are highly expressed in the brain and could be misregulated in viral brain infections like chikungunya [56], developing drugs that cross the blood-brain barrier and concentrate in the central nervous system would aid in the treatment of pathologies caused by direct viral neurotropism. Finally, as with viral infections, endosomal alkalization has been demonstrated to inhibit other microbial infections that hijack the endosomal pathway for pathogenesis, such as *Mycobacterium tuberculosis* and *Brucella abortus* [80,81]. It is worth noting that active infection with both of these pathogens has been shown to result in a ≥ 10-fold downregulation of NHE9 in phagocytes, indicating that interventions targeting eNHEs could have far-reaching therapeutic implications [82,83].

Conclusions

In summary, the identification of variants in NHE9 associated with severe COVID-19 underscores the prognostic significance of endosomal pH in SARS-CoV-2 infection and may reflect its positioning at the intersection of resistance to viral pathogens and the regulation of inflammation. The recent failure of

clinical trials using hydroxychloroquine for COVID-19 supports the formulation that weakly basic drugs partitioning into acidic compartments to increase pH may miss the target organelle, that is endosomes. It is worth emphasizing that conflicting reports of therapies targeting endosomal pH does not reject the importance of acid-base homeostasis in viral diseases. Rather, the above experience has spurred the need to develop interventions that specifically and effectively target endosomal pH and exhibit broad-spectrum antiviral activity. They could readily be deployed as a first line to curtail the rapid outbreak of future pandemics of known or unknown viruses.

This review highlights how eNHE may be amenable to targeted treatment for specific endosomal pH modulation that potentially delivers therapeutic benefits against a wide range of viral infections by regulating endosomal fusion reactions or by having an immunomodulatory effect that mitigates undue inflammation and benefits tissue repair. While additional work is certainly needed to dissect the role of endosomal proton leak pathways in host-viral interactions, they are unlikely to be addressed by studies in cell culture models alone. Rather, the mechanisms governing the differential host response to viral infection are best studied in animal models, including NHPs. These insights will eventually help to develop safe and effective therapies for a wide range of viral diseases of public health significance.

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Conflict of interest

The author declares no conflict of interest.

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