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THE ROLE OF CERAMIDE IN VIRAL INFECTIONS

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بسم الله الرحمن الرحيم

صدق الله العظيم

DEDICATION

TO THE KINDEST HEARTS IN MY LIFE MY MOTHER AND MY FATHER... WHO GIVE ME ALL THE SUPPORT AND CARE IN MY LIFE.

LAST BUT NOT LEAST TO ALL HEALTHCARE WORKERS WHO FIGHTING AGAINST COVID -19 WITH GREAT PERSON RISK.

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Abstract

Sphingolipids are structural molecules of cell membranes with important roles in maintaining barrier function. Ceramides are the basic structural units of all sphingolipids. This study is aimed to assess the role of ceramide in a variety of diseases, in particular, viral infection. Ceramide is a lipid messenger at the heart of sphingolipid metabolism. In concert with its metabolizing enzymes, particularly acid sphingomyelinases(ASM), it has key roles in regulating the physical properties of biological membranes, including the formation of membrane microdomains which are implicated in signal transduction and membrane trafficking . Thus, ceramide has been attributed significant roles in the viral life cycle: they may serve directly as receptors or co-receptors for viral entry, it forms microdomains that cluster entry receptors and/or enable them to adopt the required conformation or regulate their cell surface expression. ceramide can regulate viral uptake, often through sphingomyelinase activation, and mediate endosomal escape and intracellular trafficking. Ceramide can be key for the formation of viral replication sites. Interaction with host cell membranous compartments is of key importance for replication of viruses and this therefore is highly dependent on SL metabolism. However, significant changes in ceramide levels and composition have been noted in a number of severe diseases including cancer, inflammatory conditions and infectious diseases. The ASM/ceramide system provides a framework for a better understanding of the infection of cells by SARS-CoV-2 and the clinical, antiviral, and anti-inflammatory effects of functional inhibitors of ASM. This framework also supports the development of new drugs or the repurposing of “old” drugs against COVID-19. Ceramide contributes to all stages of the viral life cycle : viral entry, replication and release. It controls inflammatory cytokine signaling and is implicated in hepatic pathology, cardiovascular pathophysiology, diabetes, cancer and infectious diseses.

1. Introduction

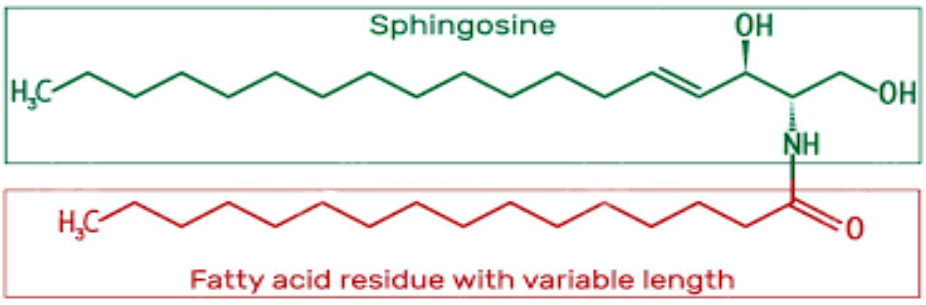
Sphingolipids are structural molecules of cell membranes with important roles in maintaining barrier function and fluidity (1). Sl are essential for the growth of not only mammalian cells but also invertebrate and fungal cells (65). Sphingolipid metabolites modulate various cellular events including proliferation, differentiation, and apoptosis (1).

Sphingolipids [e.g., ceramides, sphingomyelins, sphingosine, sphingosine-1-phosphate (S1P) and gangliosides] Despite being relatively minor components of membranes, they have potent biological activities, altering the physiochemical properties of lipid bilayers and regulating the activity of receptors and intracellular proteins (2). Sphingolipids, including the two central bioactive lipids ceramide and sphingosine-1-phosphate (S1P), act as highly potent signaling molecules themselves, regulating, for instance, cellular apoptosis and autophagy, or activation and survival, respectively (3).

alterations in SL biosynthesis or accumulation are of crucial importance in the pathophysiology in severe diseases including lysosomal storage diseases and cancer, and pharmacological interference with SL metabolism has already been proven as an effective target for treatment of major depression, cancer and inflammation (4).

2. Ceramide

Ceramide is an important lipid messenger at the heart of sphingolipid metabolism, that consists of a sphingosine backbone, which is acylated with one of several possible acyl coenzyme A molecules by a ceramide synthase (5) (Fig1) . Thus, the term “ceramide” technically comprises a whole class of molecules that differ in their acyl chain and can have different biological functions as a result (5).



. Figure 1: ceramide structure

Ceramide is the central hub of sphingolipid metabolism. De novo biosynthesis of sphingolipids in mammalian cells is initiated in endoplasmic reticulum (ER) (6).The first step is the condensation of L-serine and palmitoyl CoA to generate 3-keto dihydrosphingosine, which is reduced to dihydrosphingosine (6) (Fig 2). Dihydrosphingosine undergoes N- acylation followed by desaturation to generate ceramide (6). These reactions to produce ceramide occur at the cytosolic surface of the endoplasmic reticulum (ER) (6).

After de novo synthesis in the ER, ceramide is transported to the Golgi apparatus via Cert (7). Ceramide is provided for the synthesis of complex sphingolipids such as sphingomyelin (SM) by sphingomyelin synthase (SMS) and glycosphingolipids (GSL) by glycosyl transferase (GST) (8). Complex sphingolipids are transported from the Golgi apparatus to the plasma membrane or lysosomes (7). There the breakdown of SM to Cer takes place by sphingomyelinase (SMase), degradation of GSL occurs by the stepwise action of specific hydrolases. Subsequently, Cer can be degraded by ceramidase (CDase) to sphingosine (Sph) (9). S1P can be formed by phosphorylation via sphingosine kinase (SphK1/2) (52). Degradation of S1P occurs through S1P phosphatase (SPP) or S1P lyase (SPL) forming Sph or hexadecenal (2EHD) and phospho-ethanolamine (P-EA) (10).

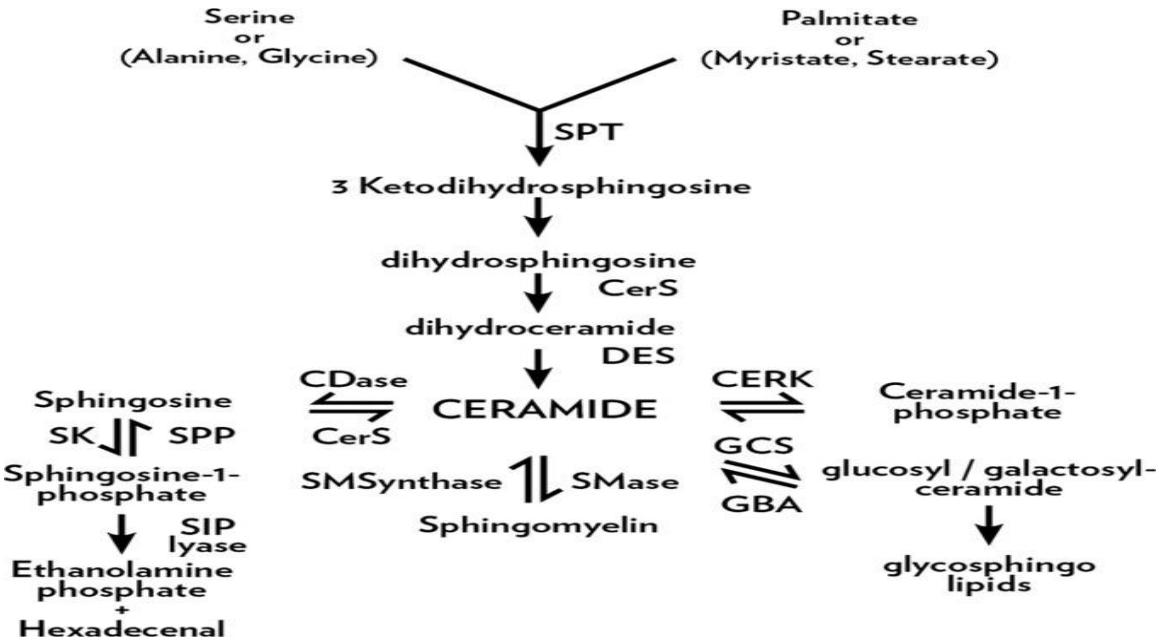


Figure 2: Metabolic pathway of ceramide

Surface ceramide is generated by the acid sphingomyelinase (ASM), which is a lysosomal protein that catalyzes the conversion of sphingomyelin into ceramide (11). Since lysosomes are constantly recycling to the plasma membrane, the ASM can be also found on the cell surface and binds to the outer leaflet of the plasma membrane (12). Surface ASM acts as a signaling molecule and generates ceramide in the outer leaflet of the cell membrane (13).

Ceramide molecules are very hydrophobic and spontaneously associate with each other to form small ceramide-enriched membrane domains that fuse and form large highly hydrophobic, tightly packed, and gel-like ceramide-enriched membrane domains termed “platforms” (14). Thus, the generation of ceramide by the ASM dramatically alters the biophysical properties of the plasma membrane (14). These large, distinct, ceramide-enriched membrane domains serve to cluster, aggregate and reorganize activated receptor molecules such as CD95, CD40 (13) (Fig 3).

Ceramide-rich platforms were also shown to mediate a variety of stress stimuli such as γ-irradiation (15), ultraviolet light (16), or Cu2+ intoxication (17), as well as infection of cells with at least some pathogenic bacteria and viruses (18). The high density of activated receptors upon trapping and clustering in ceramide-enriched membrane domains and the proximity to signaling molecules facilitates and amplifies signaling via the specific receptor, as shown for CD95 (19).

Over the past decades numerous studies have demonstrated that depending on their molecular structure, ceramides confer unique biophysical properties to the biological membranes (20). While the mechanisms of ceramide function in biological membranes and the biological consequences of ceramide alterations for cellular processes are still poorly understood (20). ceramides were established as second messengers regulating key cellular processes including cytoskeleton dynamics, endocytosis, protein transport and subcellular localization, cell cycle, autophagy, and apoptosis (21). Therefore, ceramides are indispensable for cellular homeostasis and control of fundamental functions, such as proliferation, migration, differentiation, adaptation to stress, survival, and senescence (22).

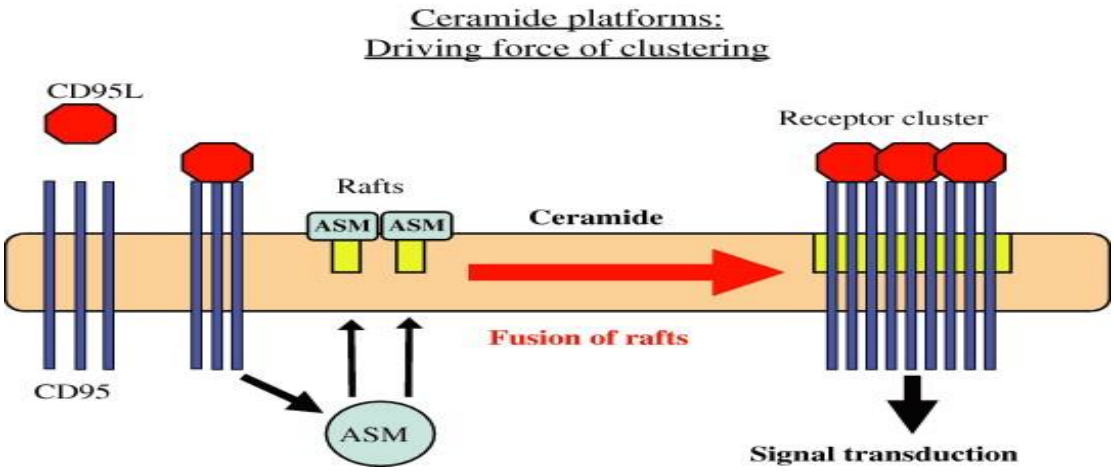


Figure 3: ASM-mediated platform formation

3. The role of ceramide in diseases

Ceramides are believed to have much less benign effects in many other disease states, and as discussed above ceramide-enriched membranes can promote the entry of viruses and bacterial pathogens into cells (23). Whether ceramide modulation in disease occurs as a side effect in response to a pathological mechanism, such as cell stress or inflammation, or whether they are involved actively in the development of disease does not always appears to be clear (24). However, significant changes in ceramide levels and composition have been noted in a number of inflammatory conditions, including metabolic diseases, irritable bowel syndrome, asthma, arthritis, multiple sclerosis, retinal degeneration and cystic fibrosis, and the mechanistic implications are under investigation (24).

Ceramides in the circulation, especially those linked to saturated fatty acids (C16 to C22), may be biomarkers of these diseases (25). In obesity and dyslipidemia, sphingolipids such as ceramide and its metabolites induce the cellular dysfunctions that underlie diabetes and cardiovascular disease in that they disrupt insulin sensitivity, pancreatic β‑cell function, vascular reactivity and mitochondrial metabolism (23). Thus, ceramides synthesised de novo promote apoptosis of pancreatic β-cells in both types 1 and 2 diabetes (26). 16:0-Ceramide especially has profound effects upon adipose tissue metabolism, and it has been identified as the main mediator of obesity-derived insulin resistance, together with impaired fatty acid oxidation and hepatic steatosis (26).

Ceramides have cardiotoxic properties and are reported to be drivers of cardiovascular disease (27). In mice and rats, inhibition or reduction of enzymes involved in ceramide synthesis by pharmacological means prevents the development of diabetes, atherosclerosis, hypertension and heart failure (23). In cultured cells and isolated tissues, ceramides perturb mitochondrial functions that may contribute to heart disease by inducing cell death and inflammation; by accumulating in mitochondria of cardiomyocytes, they increase their permeability and lead to apoptosis and cell death (23). In humans, ceramide levels in serum, and in particular an elevated ratio of very-long-chain to long-chain ceramides, are considered to be good biomarkers for adverse outcomes in cardiovascular disease (25). There are also reported effects upon neurological diseases, such as Alzheimer's disease, Parkinson's disease and depression, and 18:0-ceramide is reported to be a potential contributor to human aging (29).

4. The role of ceramide in cancers

In 1993, the induction of apoptosis by ceramide was first demonstrated in leukaemic cells by treatment with exogenous ceramide (30). Since then, there have been myriad studies showing that endogenously generated ceramide is a bona fide inducer of apoptosis that is regulated by various mechanisms in a cell-type-dependent and/or context-dependent manner (31). However, there are also studies that have demonstrated that induction of ceramide might protect some cancer cells from cell death (32). For example, ceramide synthase 6 (CERS6)-generated C16 ceramide was shown to be important in protecting head and neck squamous cell carcinoma (HNSCC) cells from endoplasmic reticulum (ER) stress-mediated apoptosis (33).

Ceramide-producing enzymes CerS2 (ceramide synthase 2) and CerS6 (ceramide synthase 6) were shown to be elevated in cancerous breast tissues as compared with normal breast tissues (34). Endogenous C16-, C24-, and C24:1-ceramide levels were increased in human head and neck squamous cell carcinomas, compared to normal tissues levels (35). Importantly, C16- and C18-ceramides were demonstrated to play two opposing roles in human head and neck squamous cell carcinomas, prosurvival and proapoptotic, respectively (36). While initially ceramides were regarded as death-promoting signaling molecules, it is now believed that effect of the ceramide elevation depends on the specific ceramide structure and the cellular context (37).

Another important aspect of ceramide in cancer, specific elevation of ceramide-metabolizing enzymes, is connected to cancer resistance, a major obstacle in cancer therapy (38). Thus, elevation of SPHK1 and GCS in prostate cancer cells was found to be responsible for resistance to paclitaxel (38). Elevated GCS expression has been demonstrated to correlate with progression of breast, urinary, ovarian cancers, and leukemia (39), whereas upregulation of acid ceramidase on irradiation conferred prostate cancer resistance (40). This opens an opportunity to target these enzymes for treatment of resistant tumors (41).

5. The role of ceramide in inflammations

regards to sphingolipids, some of them have been described as important mediators of inflammatory responses, which in principle is beneficial for protecting the organism against infection or injury (42). Inflammatory mediators include chemokynes, cytokines, vasoactive amines, products of proteolitic cascades, phospholipases, different forms of eicosanoids, and some sphingolipids (43). Generation of proinflammatory metabolites, however, should be blocked or at least reduced when inflammation becomes out of control, so as to protect the organism from major damage (43). Concerning phospholipases, a key mediator of inflammatory responses is cytosolic PLA2 (cPLA2), an enzyme that has been involved in receptor-dependent and independent release of arachidonic acid and eicosanoid production (44).

ceramide was initially described as pro-inflammatory for different cell types (45), and more recently it has been implicated in the development of allergic asthmatic responses and airway inflammation (46). In addition, exogenous addition of the short-chain cell permeable C2-ceramide, to cultured astrocytes upregulated the expression of 12-lipoxygenase, thereby leading to generation of reactive oxygen species (ROS) and the initiation of inflammatory responses (47). Acid sphingomyelinase-derived ceramide has also been involved in PAF-mediated pulmonary edema (48). Subsequently, it was proposed that at least some of the pro-inflammatory effects of ceramides might in fact be mediated by its conversion to C1p which potently and specifically stimulated AA release and prostanoid synthesis in A549 lung adenocarcinoma cells (48).

the mechanism whereby C1P stimulates AA release occurs through direct activation of cPLA2 (49) (Fig 4). The interaction between C1P and $cPLA_2\alpha$ is a crucial link in

eicosanoid synthesis (49). Following an inflammatory stimulus, Ca^{2+} activated $cPLA_2\alpha$ translocates to the golgi membrane where it binds phosphatidylcholine (PC)(50). CERK-derived C1P directly interacts with $cPLA_2\alpha$, thereby enhancing the association of cPLAα to the PC-rich membrane (49). $cPLA_2\alpha$ hydrolyzes PC to produce arachidonic acid (AA), which is further metabolized to several different eicosanoids, one of which is prostaglandin (PGH_2) (49). Prostaglandins are involved in various biological processes associated with the inflammatory response (51).

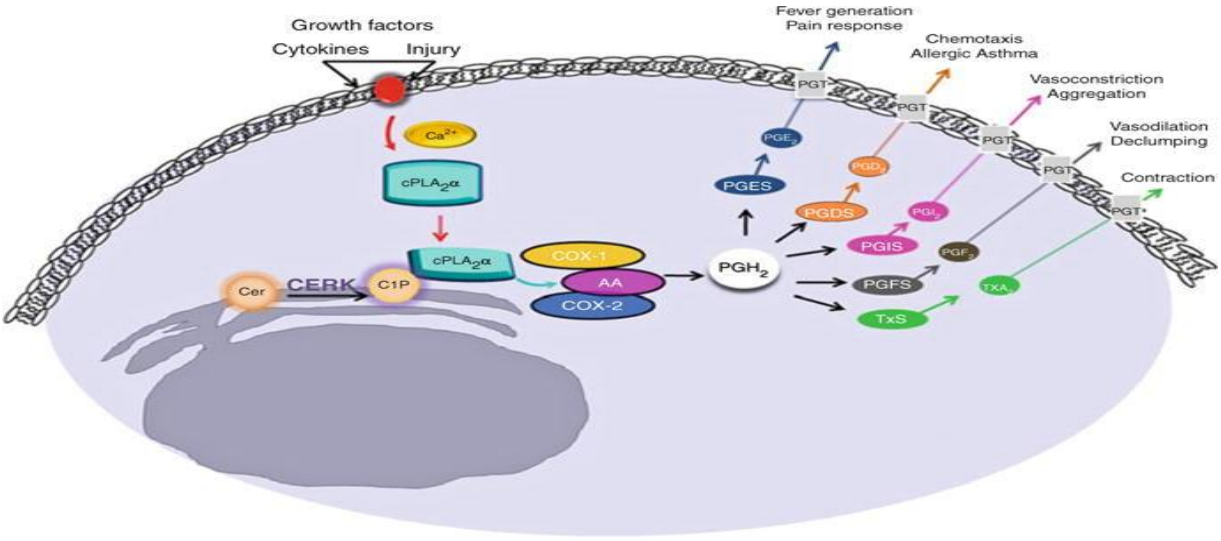


Figure 4: The interaction between C1P and $cPLA_2\alpha$

6. The role of ceramide during viral infections

Several viruses use ceramide core as host cell receptors or co-receptors for infection. ceramide- enriched membrane microdomains can behave as moving platforms, allowing the recruitment of co-receptors after the initial virus–receptor interaction (52). Microdomains could thus either stabilize the attachment of the virus to the cell surface through multiple low affinity interactions between the viral glycoprotein and lipid headgroups and/or convey the virus to an appropriate co-receptor by clustering and activating receptor molecules (52). Based on their biophysical properties, ceramide-enriched membrane domains which condense into larger platforms in response to sphingomyelinase activation or ceramidase inhibition, respectively, are sites of endocytic uptake of pathogens (53). In addition to biophysical alterations promoting membrane vesiculation and fusogenicity (54), concentration of pathogen receptors and membrane proximal signaling complexes is believed to aid in pathogen uptake (54). Therefore,

conditions favoring the generation of these domains (mainly by activation of sphingomyelinases or inhibition of ceramidase especially by inflammatory signals or viruses themselves on receptor interaction) would create a favorable environment enhancing viral infection (54).

This has in fact been verified for several viruses. For instance, the ability of CD300lf to support murine Norovirus entry was found to depend on SL biosynthesis, and more specifically, on ceramide generation (55). Thus, exogenous addition of ceramide restored susceptibility of serine palmitoyl-transferase deficient cells, and this relied on both formation of ceramide-enriched membrane domains and ceramide induced conformational changes of surface resident CD300lf proteins (55).

infection of human epithelial cells with several rhinovirus strains triggers a rapid activation of the acid sphingomyelinase correlating with microtubules- and microfilament-mediated translocation of the enzyme from an intracellular compartment onto the extracellular leaflet of the cell membrane (56). The activity of the acid sphingomyelinase results in the formation of ceramide in the cell membrane and, finally, large ceramide-enriched membrane platforms (57). Rhinoviruses colocalize with ceramide-enriched membrane platforms during the infection (56). The significance of ceramide-enriched membrane platforms for rhinoviral uptake is demonstrated by the finding that genetic deficiency or pharmacological inhibition of the acid sphingomyelinase prevented infection of human epithelial cells by rhinoviruses (56).

Promotion of viral entry by ASM activation through host cell surface interaction was mechanistically investigated for the measles virus (MV, an enveloped virus) in dendritic cells (DCs)(58) and for adenovirus (a non-enveloped virus) in epithelial cells (59). Interaction of MV glycoproteins with DC-SIGN on the DC surface activates translocation of ASM along with CD150 from intracellular endosomes to the cell surface (60) (Fig 5). This makes the CD150, the MV entry receptor on hematopoetic cells, available to promote viral infection of DCs (60). Thus, activation of ASM subsequently promotes ceramide release in the membrane, making ASM activation a promising target in measles infection (60). MV-induced immunosuppression, which occurs despite efficient virus-specific immune activation (61). MV causes immunosuppression mainly through suppression of T cells: ceramide generation by ASM upon contact with MV contributes to

actin cytoskeletal paralysis, resulting in the loss of T cell polarization, adhesion and motility (62).

Ebola virus (EBOV), which enters cells through micropinocytosis, has also been reported to result in ASM recruitment to the site of viral attachment, and ASM activity is required for EBOV infection (63). EBOV was shown to bind to SM-rich regions in the plasma membrane and the depletion of SM strongly reduced infection (63). As DC-SIGN also binds EBOV glycoproteins (64), it is possible that both MV and EBOV recruit ASM from the lysosome through a DC-SIGN-mediated signaling pathway (64).

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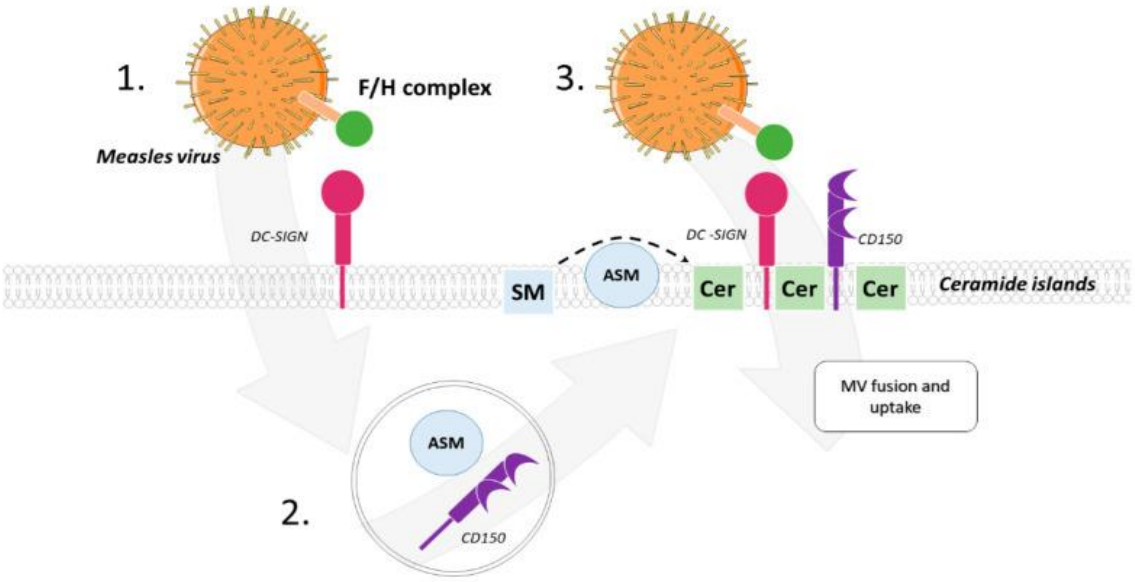

Figure 5: Ceramide-enriched membrane domains in MV infection

Membrane rupture is a key entry mechanism for many non-enveloped viruses (66) (Fig 6). Adenovirus lytic protein-VI pierces the membrane, stimulating a calcium-influx and lysosomal exocytosis (66). Subsequently, endocytosis occurs to maintain the cell surface area, which the adenovirus hijacks for cell entry (66). Interaction adenovirus particle with its surface receptors CAR and α3 integrin causes limited uncoating at the cell surface which leads to exposure of the adenoviral membrane lytic protein-IV (66), this protein causes membrane lesions followed by Ca2+-influx promoting a wound repair process by subsequent lysosomal exocytosis along with ASM surface display and formation of ceramide-enriched membrane domains (59). These act to enhance viral

endocytosis and to recruit and concentrate lytic protein-VI in endosomes, thereby catalyzing endosomal leakiness and finally rupturing as required for release of the viral capsid into the cytosol (67). In this study, ceramide release and concentration within endosomes has clearly been revealed as crucial for protein-IV recruitment and subsequent viral release from these compartments (60). Human norovirus similarly commandeer calcium- and ASM-dependent cellular wound removal processes (68) .

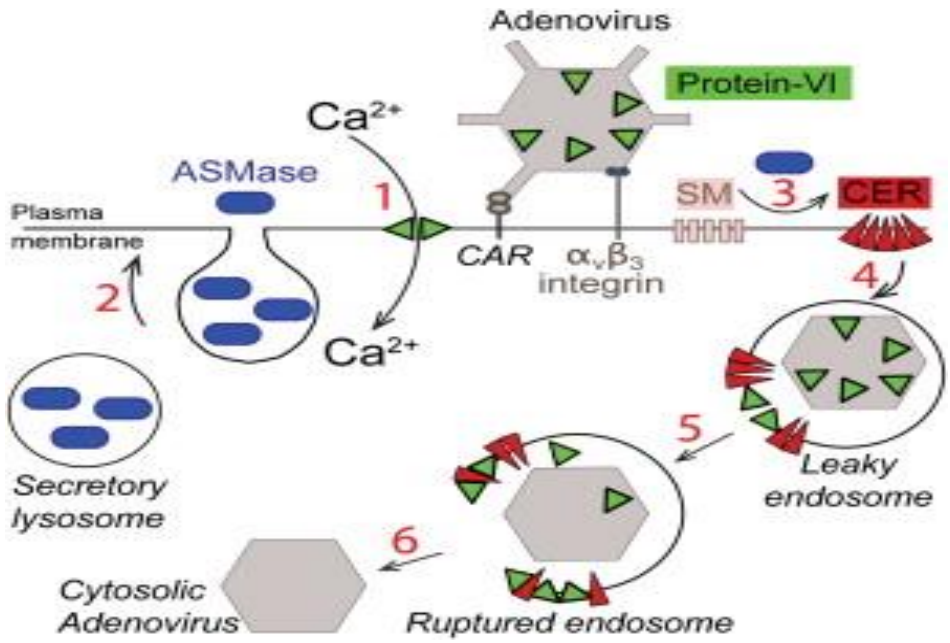

Figure 6: Lipid Signaling-Enhanced Adenovirus Penetration of Endosomal Membranes

Viruses can remodel intracellular membranes to form replication sites. Zika virus (ZIKV), for example, dysregulates the lipid landscape of infected host cells, particularly with regard to sphingolipids (71). Ceramide redistributes to the ZIKV replication site and the disruption of sphingolipid biosynthesis blocks ZIKV infection (71).Ceramide also redistributes to West Nile virus (WNV) replication sites and ceramide production via the de novo and salvage pathways necessary for WNV replication (72). SM and ceramide transfer protein (CERT) are required for the biosynthesis of double-membrane vesicles that serve as HCV replication sites (73). In contrast, ceramide does not redistribute into the replication sites of another flavivirus, dengue virus, and the inhibition of ceramide synthase actually enhanced dengue virus production (72), demonstrating that even viruses from the same genus can have different sphingolipid-requirements for replication (74).

Bile acids facilitate the endosomal escape of calciviruses by triggering ceramide formation by ASM (75). Inhibition of ASM results in the retention of porcine enteric calcivirus, feline calcivirus and murine norovirus in the endosomes and reduces viral replication (75). The downstream effects of ceramide generation were not studied further, but as ceramide is a known activator of cathepsin proteases (76), the authors hint that ceramide generation may lead to the activation of cathepsin L, which cleaves the calcivirus capsid protein, enabling replication (77). For a human norovirus strain (GII.3), however, blocking cathepsin activity had no effect on viral replication, whereas ASM inhibition did significantly reduce replication (68).

Ceramide can also regulate intracellular transport, both of incoming virions to their replication site, as well as of new viral products for the assembly of new virions (78) . One example is the ceramide-mediated trafficking of the M glycoprotein of infectious bronchitis virus (IBV) (78).

7. The role of ceramide in covid-19

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is closely related to the deadly coronaviruses SARS-CoV-1 and Middle East respiratory syndrome coronavirus (MERS-CoV) (79). The 2019 outbreak of coronavirus disease (COVID-19), caused by SARSCoV-2, has become a public health emergency of international concern (79). Infection with SARS-CoV-2 often results in mild respiratory tract disease, but a substantial number of patients also experience severe symptoms and pneumonia (79). A high proportion of critically ill patients require intensive care and ventilator treatment, with a high mortality rate (80). The total infection death rate is approximately 0.66%, rising sharply to 7.8% in people aged over 80 (81). This has led to excess mortality in many countries (82). Risk factors for severe/fatal COVID-19 are advanced age, obesity, chronic respiratory disease, hypertension, cardiovascular disease, kidney disease, cerebrovascular disease, malignancy, and diabetes (83). Severe COVID-19 courses are characterized by hyperinflammation and cytokine storms, with significantly higher serum levels of interleukin (IL)-6, IL-8, IL-10, IL-2R and tumor necrosis factor (TNF)- alpha (84).

Infection of epithelial cells with SARS-CoV-2 is initiated by binding of the S protein of the virus to ACE2 (85) (Fig 7). Binding is followed by fusion of the viral and cellular membrane, which requires priming of spike by cellular proteases that cleave spike into

the S1 and S2 subunits (85). Spike-protein cleavage is mediated by TMPRSS2, but also by cathepsin B and L (86). Ceramide may have several functions in the infection with SARS-CoV-2: We have shown that these ceramideenriched membrane domains trap and cluster ACE2 upon cellular infection with SARS-CoV-2, which is very likely a pre-requisite for signaling via this receptor and therefore a pre-requisite for the infection (87). It is possible that ceramide-mediated clustering of ACE2 in large membrane domains amplifies signaling via ACE2 and is thereby required for internalization of ACE2 and SARS-CoV-2 into endosomes (87).

However, it might be also possible that ceramide generated within endosomes or on the outer leaflet of the cell membrane upon infection with SARS-CoV-2 binds to cathepsins in endosomes and thereby triggers spike-protein priming and membrane fusion (86). Previous studies using TNF already demonstrated an activation of cathepsins by ceramide (88). In line with a direct ceramide−protein interaction, it might be also possible that ceramide binds to and directly activates TMPRSS2 and thereby facilitates membrane fusion (86). Alternatively, ceramide-enriched membrane domains might trap ACE2 and TMPRSS2 within a small, distinct area of the plasma membrane resulting in a high concentration of TMPRSS2 and thereby S-protein priming, membrane fusion and infection (86) .

Many clinically approved medications functionally inhibit ASM and are called FIASMAs (functional inhibitors of acid sphingomyelinase) (89). The FIASMA fluvoxamine showed beneficial effects on COVID-19 in a randomized prospective study and a prospective open-label real-world study. Retrospective and observational studies showed favorable effects of FIASMA antidepressants including fluoxetine, and the FIASMA hydroxyzine on the course of COVID-19 (89).The destruction of ceramide-enriched membrane domains by means of anti-ceramide antibodies or neutral ceramidase treatment also prevents infection with SARSCoV-2 (90). Likewise, genetic downregulation of ASM abrogates infection with SARS-CoV-2 (90). The reconstitution of ceramide in cells treated with a FIASMA, anti-ceramide or ceramidase by the addition of exogenous ceramide restores infection with SARS-CoV2 (90). In humans, oral application of amitriptyline very efficiently blocks the infection of freshly isolated nasal epithelial cells with SARS-CoV-2 (91).

Interaction of the viral spike protein with its receptor-binding domain of ACE2 was found to be prevented upon exogenous supply of sphingosine, which directly associated with this receptor (92). Thus, sphingosine, known for its bacteriocidal activities in the respiratory tract, might also exert antiviral activities at the level of entry in this particular compartment (93).

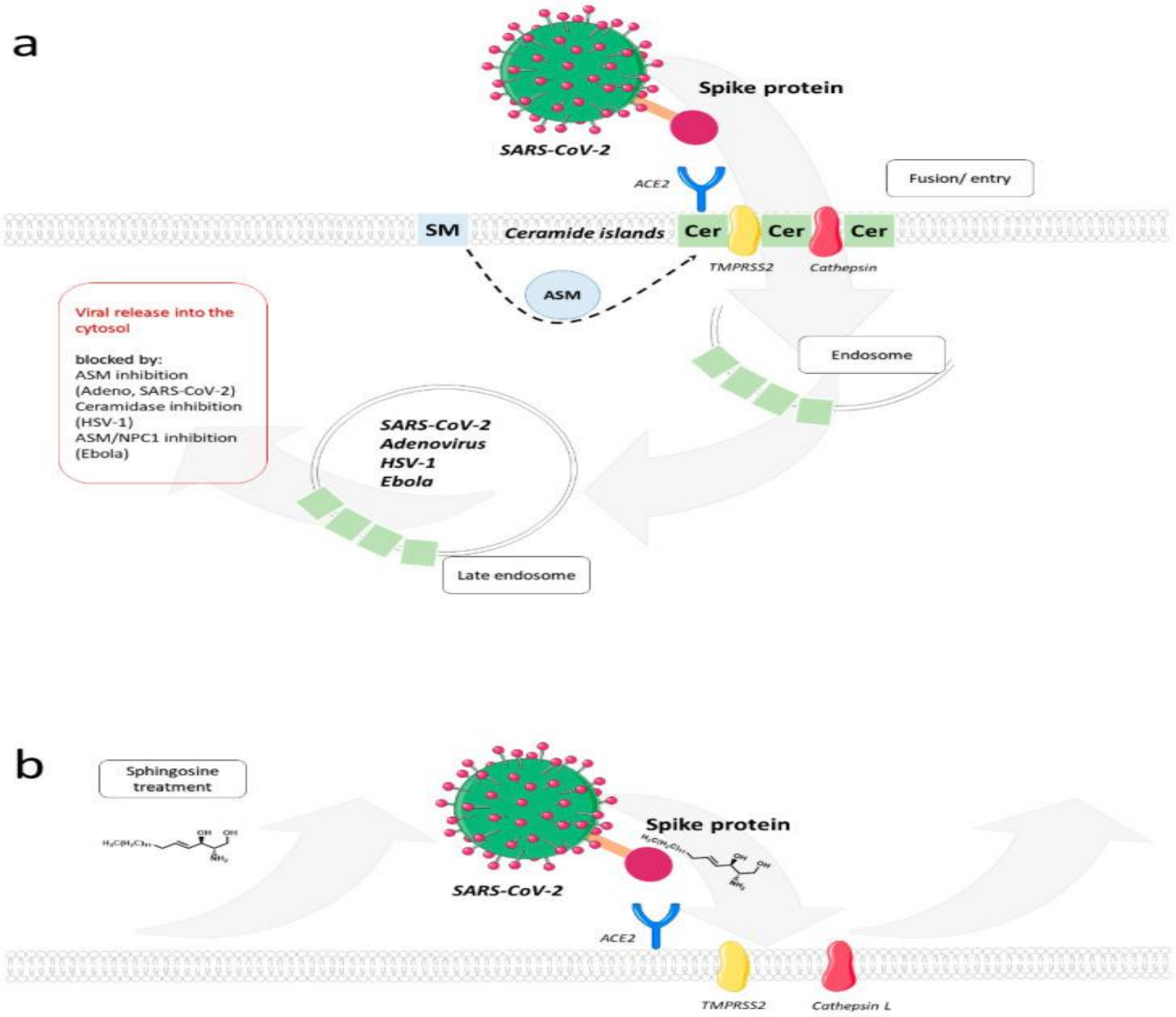

Figure7: Ceramide-enriched membrane domains in SARS-CoV-2 infection

8. Conclusion

Ceramide contributes to all stages of the viral life cycle : viral entry, replication and release. It controls inflammatory cytokine signaling and is implicated in hepatic pathology, cardiovascular pathophysiology, diabetes, cancer and infectious diseses.

Inhibitors of ceramide metabolism like functional inhibitor of acid sphingomyelinase (FIASMAs) have been reported to have antiviral properties against a multitude of different viruses including measles virus, Norovirus, Rhinovirus and SARS-CoV-2. Future work should focus on further defining the involvement of sphingolipids in viral entry, replication and release with the hope of identifying new antiviral therapeutic targets.

Of particular current interest in light of the SARS-CoV-2/COVID-19 pandemic is the repurposing of FIASMAs for the inhibition of SARS-CoV-2 entry**.** The long-standing clinical experience with these drugs and their favorable pharmacological properties, including good absorption, distribution, metabolism and excretion, lack of habituation, reversible inhibition and lack of rebound effects make them ideal candidates for a swift indication expansion to manage SARS-COV-2 infection/COVID-19. They may also provide an economic treatment option, particularly in countries that struggle with financing the vaccination program and where SARS-COV-2 will likely become endemic**.**

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