Manuscript ID: 102308

Manuscript title: Emerging roles of the acid sphingomyelinase/ceramide pathway in metabolic and cardiovascular diseases: mechanistic insights and therapeutic implications

1 Peer-review report

Reviewer #1:

Scientific Quality: Grade D (Fair)

Language Quality: Grade B (Minor language polishing)

Conclusion: Major revision

Specific Comments to Authors:

I have reviewed your manuscript " Emerging Roles of the Acid Sphingomyelinase/ Ceramide Pathway in Metabolic and Cardiovascular Diseases: Mechanistic Insights and Therapeutic Implications". Here are some of the key shortcomings I would point out to the authors regarding the submitted manuscript:

General Resonse: Thanks very much for taking your time to review this manuscript. I really appreciate all your comments and suggestions! Please find my itemized responses in below and my revisions/corrections in the re-submitted files.

Abstract: (1) You mention that the role of the ASMase/Cer pathway in metabolic diseases remains underexplored. Can you elaborate on the specific gaps in the existing literature that your review aims to address, and how your findings contribute to filling these gaps? (2) The abstract suggests that inhibiting the ASMase/Cer pathway has potential therapeutic implications. What specific evidence do you present in the review that supports the efficacy of ASMase/Cer inhibition in the treatment of metabolic diseases? (3) You discuss the impacts of the ASMase/Cer pathway on various metabolic processes. Can you detail any particular mechanisms by which this pathway

influences insulin resistance and mitochondrial homeostasis, and how these insights could lead to new therapeutic approaches? (4) How do you envision the translation of your findings regarding the ASMase/Cer pathway into clinical practice? Are there any specific clinical trials or studies currently underway that you believe will validate your proposed therapeutic strategies?

Response to (1):

The direct link between ASMase activity and metabolic disorders remains unclear. Changes in ASMase activity under different metabolic conditions (such as fasting, postprandial states, or exercise) and their effects on ceramide (Cer) production are not well understood. Further studies are needed to investigate the dynamic changes in ASMase activity and reveal its role under various metabolic states.

The potential of ASMase/Cer as early diagnostic biomarkers has not been fully explored. Further research is needed to examine the temporal changes in ASMase activation and Cer accumulation across different metabolic diseases, to better understand the viability of the ASMase/Cer pathway as an early diagnostic marker for metabolic disorders.

There is insufficient research on the association between ceramide subtypes and disease subtypes. Systematically evaluating the specific expression of ceramide subtypes (e.g., Cer16:0, Cer18:0, Cer24:1) in different metabolic disease subtypes (such as obesity-related diabetes vs. non-obese diabetes), and their correlation with clinical phenotypes, could provide more precise biomarkers for the ASMase/Cer pathway, offering new directions for personalized treatment and disease monitoring.

There is also a lack of clinical translation research on the ASMase/Cer pathway. A comprehensive review of the current progress on ASMase inhibitors, including their efficacy in metabolic and cardiovascular diseases, is needed. This should also address the major challenges in translation, such as drug safety, effectiveness, and the definition of clinical indications.

Response to (2):

The review mentions several tricyclic antidepressants, including imipramine, amitriptyline, and doxepin, with distinct effects on ASMase and ceramide (Cer) pathways. Imipramine triggers ASMase-mediated lysosomal degradation and improves diabetic cardiomyopathy. Amitriptyline, which inhibits ASMase activity and lowers Cer levels, exhibits dual effects. Doxepin improves hyperglycemia and fatty degeneration in obese diabetic mice by modulating ASMase and Cer levels.

Another compound discussed in the review is the natural product α-mangostin (α-MG), which inhibits the ASMase/Cer pathway in the aorta of mice, reduces oxidative stress, and promotes vasodilation, suggesting its potential as a therapeutic agent for cardiovascular diseases. The review also mentions the drug enalapril, which lowers sphingosine-1-phosphate (S1P) levels in the blood and kidneys of hypertensive rats, and decreases ASMase levels in cardiac tissue, indicating its potential for treating cardiovascular diseases.

Response to (3):

Ceramide causes insulin resistance by PP2A and PKCζ mediated inhibition of Akt and MLK-3 mediated inhibition of IRS. Ceramide may also decrease Glut-4 gene transcription (*Lipids in Health and Disease, 2013, 12: 98*) .

ASMase can impact IR by causing endoplasmic reticulum stress and the release of inflammatory factors, as evidenced by the fact that it causes prolonged endoplasmic reticulum stress in ASH/NASH and also accelerates the progression of NAFLD through periodontitis (*Int J Mol Sci, 2023, 24(9):8322; J Hepatol, 2015, 62(1):219-33*). Ceramides produced by ASMase on lysosomes or cell surfaces reduce insulin signaling by blocking IRS-1 phosphorylation, which in turn causes insulin resistance and lipotoxicity (*J Hepatol, 2015, 62(1):219- 33*). Consequently, insulin signaling is decreased (*J Hepatol, 2015, 62(1):175-*

81).Activation of ASMase and the subsequent accumulation of ceramide (Cer) lead to mitochondrial dysfunction, while downregulation of ASMase expression and reduction in Cer production improve cellular energy metabolism. Ceramide promotes mitochondrial fission by upregulating the expression of dynamin-related protein 1 (DRP1), while inhibiting mitochondrial fusion by modulating key fusion proteins, such as mitofusin 1 (MFN1) and optic atrophy 1 (OPA1).

Given the role of the ASMase/Cer pathway in insulin resistance and mitochondrial homeostasis, the development of ASMase inhibitors, ceramide synthesis inhibitors, mitochondrial fusion activators, and mitochondrial fission inhibitors presents a promising therapeutic strategy. These approaches hold significant potential in regulating insulin resistance, mitochondrial function, and related diseases, including metabolic and neurodegenerative disorders.

Response to (4):

Translating the discovery of the ASMase/Ceramide (Cer) pathway into clinical practice is a complex yet promising process. A deep understanding of how this pathway functions in different diseases is essential for successful translation. The regulation of ASMase activity and ceramide levels may vary across disease types, determining whether activators or inhibitors of the ASMase/Cer pathway should be developed. For example, in cancer, ceramide typically induces apoptosis (*Mol Metab,2024,83:101936*), which requires the development of drugs that activate ASMase to increase ceramide production. In contrast, in neurodegenerative diseases, the accumulation of ceramide can lead to neuronal damage and dysfunction (*Int J Mol Sci,2022,23(15):8082*), suggesting the need for ASMase inhibitors to reduce ceramide generation and alleviate its toxic effects on the nervous system. Currently, no clinical trials targeting ASMase are underway.

Introduction: (1) The introduction discusses metabolic diseases but does not provide clear definitions or examples of specific metabolic disorders. Including a brief definition could help readers unfamiliar with the topic better understand the context. (2) While the introduction mentions the rising prevalence of metabolic diseases, it lacks a comprehensive review of recent studies that have explored the ASMase/Cer pathway. A more thorough literature review would strengthen the rationale for focusing on this pathway. The introduction introduces the ASMase/Cer pathway but does not adequately explain its biological significance or how it fits into the broader context of metabolic disease mechanisms. A clearer connection between this pathway and metabolic disorders would enhance the argument for its relevance. The rationale for focusing on the ASMase/Cer pathway over other potential pathways implicated in metabolic diseases is not clearly articulated. The authors should justify why this pathway is particularly important to study. The introduction lacks a clear statement of the hypotheses or specific aims of the review. Outlining these objectives would provide a clearer direction for the readers and establish the purpose of the manuscript. (3) The introduction primarily focuses on biological aspects without acknowledging interdisciplinary approaches (e.g., lifestyle factors, socioeconomic influences) that contribute to the prevalence of metabolic diseases. Including these perspectives could enrich the discussion. Although the introduction notes the global challenge posed by metabolic diseases, it does not discuss the implications for different populations or healthcare systems. A more global perspective would highlight the urgency of addressing these issues. (4) The introduction uses terms like "insulin resistance" and "oxidative stress" without sufficient explanation. Providing brief definitions or context for these terms would enhance clarity, especially for readers from diverse backgrounds.

Response to (1):

Metabolic diseases typically result from disruptions in the body's

metabolic processes, leading to issues in energy metabolism, substance conversion, or waste excretion. Specific metabolic disturbances include: Carbohydrate metabolism disorders (insulin resistance（IR）, insufficient insulin secretion, and hyperglycemia), Lipid metabolism disorders (hypercholesterolemia and hypertriglyceridemia), Protein metabolism disorders (amino acid metabolism abnormalities and urea cycle defects), Energy metabolism abnormalities (mitochondrial dysfunction), Endocrine imbalances (thyroid and adrenal dysfunction), Vitamin and mineral metabolism disorders (vitamin D and iron metabolism issues).

Response to (2):

The section titled "1. The ASMase/Cer pathway" in the main body of the review covers the composition and research progress of the ASMase/Cer pathway. In the revised manuscript, we have integrated this content into the Introduction.

The ASMase/Cer pathway plays a critical role in numerous physiological and pathological processes. The importance of studying this pathway is highlighted by several key points: (1) Ceramide, as a vital cellular signaling molecule, profoundly influences cell survival, death, and other physiological functions by regulating various signaling pathways (such as PI3K/Akt, MAPK, JNK, and NF-κB); (2) Ceramide accumulation is closely linked to metabolic diseases, neurodegenerative diseases, cancer, and immune regulation. Investigating this pathway helps elucidate the molecular mechanisms of these diseases and may provide new therapeutic targets. This review aims to summarize recent findings on the role of the ASMase/Cer pathway in cell signaling and the mechanisms of metabolic diseases, enhancing our understanding of the potential mechanisms behind these conditions and offering a theoretical foundation for potential therapeutic strategies.

Response to (3):

The prevalence of metabolic diseases is influenced not only by biological factors but also by interdisciplinary factors such as lifestyle, socioeconomic status, regional differences, population susceptibility, and healthcare systems. Unhealthy lifestyles significantly increase disease risk, with populations in poor conditions and lacking health education being at higher risk. Urbanization and industrialization also face higher risks of metabolic diseases, while incidence and disease progression vary significantly across different age groups, genders, and other populations. Regions with poor healthcare access and inadequate health education are more prone to the spread of metabolic diseases (*Cell Metab,2023,35(3):414-428.e3;Front Public Health,2023, 11:1253506*). Understanding the multidimensional factors behind the prevalence of metabolic diseases helps provide a more comprehensive view of their complexity and the challenges in prevention and treatment.

Response to (4):

Insulin resistance refers to the reduced responsiveness of body cells to insulin, preventing effective glucose uptake and leading to elevated blood glucose levels (*Metabolism,2021,119:154766*). Oxidative stress occurs when the accumulation of free radicals, such as reactive oxygen species (ROS), exceeds the capacity of the antioxidant defense system, resulting in cellular damage(*Metabolism,2021,119:154766*).

1. The ASMase/Cer pathway

(1) While the section mentions different types of sphingomyelinase (SMase), it lacks a detailed explanation of how these enzymes interact within the ASMase/Cer pathway. A more comprehensive overview of the pathway's dynamics would enhance understanding. (2) The section does not sufficiently address the regulatory mechanisms that control ASMase activity and ceramide production. Discussing factors that influence these processes would provide

deeper insights into their biological significance. (3) The theoretical discussion around the ASMase/Cer pathway would benefit from specific examples or case studies illustrating its role in metabolic diseases. This would help to contextualize the pathway within real-world scenarios. (4) The section references various functions of ASMase and ceramide but does not include specific studies or experimental evidence that corroborate these claims. Citing relevant research would strengthen the arguments made.

Response to (1):

Different types of sphingomyelinases (SMases) function in various cellular environments, collectively regulating ceramide production. ASMase, located in the cell membrane and lysosomes, converts sphingomyelin into ceramide (*Biomolecules,2020 ,10(11):1497;Cancer Res,2020,80(12):2651-2662*). nSMase, found in the cell membrane and cytoplasm, is involved in sphingomyelin metabolism on the cell surface (*Biomolecules,2020,10(11):1497; Blood,2023,142(20):1708-1723*). The hydrolytic enzyme Alk-SMase aids in the body's digestion of sphingolipids in the intestines and catalyzes the body's production of phosphorylcholine and ceramides from sphingolipids, both of which are vital biological processes in phospholipid metabolism(*Am J Physiol Gastrointest Liver Physiol,2019,316(4):G425-G445;Cancer Cell Int,2022,22(1):344*).

While the location, activation conditions, and functions of different SMases (ASMase, nSMase, Alk-SMase) vary, they often collaborate in cellular responses, immune reactions, apoptosis, and metabolic regulation, collectively influencing cellular health and pathological states.

Response to (2):

The regulation of the ASMase/Ceramide (Cer) pathway is complex, involving multiple intracellular and extracellular signaling pathways, enzyme activity modulation, and external stimuli. The main regulatory factors and mechanisms are as follows:

Cellular Stress and Environmental Stimuli: Oxidative stress can activate ASMase by generating free radicals (*J Cell Biol,2020,219(4):e201903176*). Ultraviolet (UV) radiation induces ASMase activation in skin cells (Front Immunol,2022,13:880850;Photochem Photobiol,2024 ,100(6):1894-1901). Cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) can activate ASMase (*Cardiovasc Drugs Ther,2024,38(1):43-56;Arch Toxicol, 2023,97(8):2069-2087;Am J Physiol Endocrinol Metab,2013,305(7):E853-67*). Extreme temperature changes, such as heat stress or cold exposure, can also activate ASMase, altering ceramide levels (*Neuroscience,2017,358:79-92;Mol Cell Proteomics,2023,22(4):100525*).

Signaling Pathway Regulation: MAPK and NF-κB pathways can activate ASMase or induce its expression (*Adv Sci (Weinh),2024,11(40):e2405759*). The PI3K/Akt pathway inhibits ASMase activity, reducing ceramide production (*Int J Biochem Cell Biol,2021,134:105952;Thyroid,2019,29(5):700-713*).

Lipid Metabolism Regulation: Sphingomyelin is the substrate for ASMase, and the intracellular levels of sphingomyelin directly influence ASMase activity (*Chinese Pharmacological Bulletin, 2023, 39(1): 57-65;Blood,2019,134(7): 645-655;Biomolecules,2020,10(11):1497*).

Post-translational Modifications: ASMase activity is regulated by phosphorylation, with certain kinases (such as PKC and AMPK) enhancing its activity through phosphorylation (*J Biol Chem,2007,282(15):11549-61*). ASMase stability and activity can also be regulated via the ubiquitin-proteasome pathway. Acetylation of ASMase affects its function, with deacetylation enhancing its activity (*J Clin Invest,2019,129(2):786-801;Mol Biol Cell,2015,26(1):1-14.*).

Gene Expression Regulation: Transcription factors such as Sp1 and SREBP-1 increase the transcription of the ASMase gene (*Biochem Biophys Res Commun,2016,470(4):851-6;Oxid Med Cell Longev,2022,2022:8688643*).

Response to (3)（4）:

The following major text section describes the roles of ASMase and ceramide and particular instances of the ASMase/Cer pathway in metabolic disorders.

2. The ASMase/Cer pathway in metabolic and cardiovascular diseases

2.1 Disruption of glucose homeostasis and insulin resistance

(1) Can you provide more detailed mechanistic insights into how the ASMase/Cer pathway specifically contributes to insulin resistance in different tissues (e.g., muscle, liver, adipose)? Are there tissue-specific differences in the pathway's activation and effects? (2) Your section describes associations between elevated ASMase activity, ceramide levels, and various metabolic conditions. What experimental evidence do you provide to support these causal relationships? Are there any longitudinal studies that corroborate these findings? (3) Ceramides exist in various species with differing biological effects. How do different ceramide species specifically influence metabolic and cardiovascular diseases? Have you considered the potential for specific ceramide species to act as biomarkers for these conditions? (4) You mention that inhibiting the ASMase/Cer pathway has therapeutic potential. What specific interventions have been tested in clinical settings? Can you summarize any notable outcomes or challenges faced in translating this research into clinical practice? (5) The section discusses the pathway's role in inflammation. How do you propose to differentiate between the pathological effects of ceramide accumulation versus its potential protective roles in immune responses? What evidence supports your claims? (6) You highlight mitochondrial dysfunction as a consequence of ceramide accumulation. Can you elaborate on the specific mitochondrial pathways affected by ceramide, and how these alterations contribute to the progression of metabolic diseases?

Response to (1):

Although ceramide is produced by the ASMase/Cer pathway and can lead to insulin resistance in all of these tissues, ceramide's mode of action differs depending on the tissue. Ceramide interferes with insulin receptor signaling (such as IRS-1 and the PI3K/Akt pathway) in muscle to prevent glucose uptake (*Physiol Rev, 2018, 98(4):2133-2223*). Ceramides raise blood glucose levels in the liver by influencing insulin receptor function and encouraging gluconeogenesis (*Trends Pharmacol Sci, 2017, 38(7):649-665*). Ceramides worsen insulin resistance in adipose tissue by activating pro-inflammatory signaling pathways such as NF-κB and blocking insulin signaling (*Nat Rev Mol Cell Biol, 2021, 22(11):751-771*).

Response to (2):

ASMase activity and ceramide buildup in liver, muscle, and adipose tissue is markedly elevated by a high-fat diet (HFD), according to animal studies (*J Hepatol, 2014, 61(5):1126-34; Inflammation, 2023, 46(5):2037-2054; Physiol Rev, 2018. 98(4):2133-2223*). According to clinical research, people with type 2 diabetes mellitus and obesity had higher levels of ceramide in their plasma and adipose tissue (*Obesity (Silver Spring), 2015, 23(7):1414-21; J Lipid Res, 2020, 61(7):1065-1074*).In insulin tolerance tests, ASMase-/- mice fed a high-fat diet had superior glucose tolerance than control mice and maintained lower levels of ceramide in the liver and adipose tissue (*J Hepatol, 2014, 61(5):1126-34; J Biol Chem, 2009, 284(13):8359-8368*).

When combined, these experimental findings imply that ceramide buildup and increased ASMase activity are significant contributors to metabolic disorders (such as diabetes and insulin resistance).

Response to (3):

Patients with insulin resistance and type 2 diabetes mellitus had higher levels of C16-Cer and C18-Cer (*Diabetes Res Clin Pract, 2021, 179:108991*). By inhibiting the PI3K/Akt signaling pathway and lowering the expression of GLUT4

transporter proteins, which impact glucose absorption and metabolism, C16-Cer accumulation results in insulin resistance (*Mol Metab, 2019, 21:36-50*). By controlling fatty acid release and changing adipocyte differentiation in adipose tissue, C18-Cer encourages fat formation and raises insulin resistance (*Cell Rep, 2019, 26(1): 1–10.e7*). C16-Cer and C18-Cer levels can be used to gauge the degree of metabolic abnormalities in the condition.

By activating inflammatory pathways (e.g., NF-κB) and NADPH oxidative enzymes, the accumulation of C16-Cer and C18-Cer in arterial endothelial cells raises oxidative stress, resulting in endothelial dysfunction and vascular endothelial cell injury (*Cardiovasc Diabetol, 2023, 22(1):25*). Increased SAMase and C6-Cer levels impact vasoconstriction and endothelial cell function (*Int J Mol Sci, 2019, 20(2):411*). Atherosclerosis, coronary heart disease, and heart failure are among the cardiovascular disorders that can be predicted by looking for changes in C16, C18, SAMase, and C6 ceramide.

Response to (4):

For the solution, see Response in Abstract (4).

Response to (5):

The term "pro-inflammatory effects" describes how elevated ceramide levels in pathological states (such as tissue damage or pathogen infection) encourage the production of inflammatory mediators and the activation of inflammatory cells (such as neutrophils and leukocytes), which in turn increases the inflammatory response. For instance, increased ceramide levels encourage neutrophil infiltration and the release of inflammatory factors in a mouse model of myocardial infarction (*Circulation, 2020*). Cer and inflammatory markers (leukocytes and neutrophil to lymphocyte ratio) have been found to positively correlate in clinical investigations of acute chest pain (PEACP) (*Cardiovasc Diabetol, 2023, 22:92*). Contrarily, immunoprotection refers to the protective action of ceramides in physiological settings, potentially through the

modulation of immune cell activity (e.g., T-cells, NK-cells) to aid in the removal of pathogens or injured cells.

In summary, ceramides' effects vary depending on their concentration and the physiological or pathological setting in which they are present; they may be immunoprotective in healthy settings and more pro-inflammatory in pathological ones.

Response to (6):

In addition to impairing ATP synthesis and the mitochondrial respiratory chain, ceramide buildup also compromises the integrity and functionality of the mitochondrial membrane, which encourages the production of ROS, causing oxidative stress, insulin resistance, and problems with lipid metabolism. In addition to directly harming cells and mitochondria, excessive ROS generation triggers inflammatory reactions by activating signaling pathways like NF-κB and MAPK, which further disrupts the insulin signaling pathway and fat metabolism (*Front Endocrinol (Lausanne), 2021, 12: 635175; Circ Res, 2021, 128: 335-357; J Appl Toxicol, 2022, 42: 1057-1066*). Ceramides also prevent mitochondrial autophagy by changing the activity of autophagy-related proteins like mTOR and ULK1 (Unc-51-like kinase 1). This results in the intracellular buildup of damaged mitochondria, which worsens insulin resistance and dysfunctional lipid metabolism and raises the risk of type 2 diabetes and obesity (*Autophagy, 2022, 18(3):703-704;Int J Mol Sci, 2024, 25(15):8061;Autophagy, 2021, 17(9):2528-2548*).

2.2 Triacylglycerol (TAG) accumulation and steatosis

(1) Can you clarify the specific mechanisms by which ceramide influences triacylglycerol (TAG) synthesis and breakdown? What roles do key enzymes involved in lipid metabolism play in this process? You mention that ceramide accumulation leads to reduced TAG breakdown. How do you differentiate between the effects of ceramide on TAG synthesis versus TAG catabolism? Are

there specific studies you can reference that illustrate this relationship? (2) How does the dysregulation of the ASMase/Cer pathway contribute specifically to the progression of NAFLD? Are there critical thresholds of ceramide levels that correlate with disease severity in NAFLD? (3) How do ceramides interact with other lipid species (e.g., free fatty acids, cholesterol) in the context of TAG accumulation and steatosis? What implications does this have for overall lipid metabolism? What clinical evidence supports the role of the ASMase/Cer pathway in TAG accumulation and steatosis in human populations? Are there cohort studies or clinical trials that have explored this relationship?

Response to (1):

By blocking insulin signaling, Cer lowers the absorption of glucose and amino acids, hence influencing the production of triglycerides (TAGs). In particular, Cer accelerates the transfer of free fatty acids (FFA) across membranes by promoting the production and activity of the fatty acid transporter protein CD36. Furthermore, Cer can stimulate the expression of the cholesterol regulatory element binding protein (SREBP), which allows enzymes like Fatty Acid Synthase, Glycerol-3-Phosphate Acyltransferase, and Diacylglycerol Acyltransferase to convert free fatty acids (FFA) to triglycerides. Glycerol 3-phosphate acyltransferase and diacylglycerol acyltransferase convert TAG to TAG and encourage its storage in lipid droplets. Cer also inhibits the activation of hormone-sensitive lipase (HSL), slowing TAG metabolism (*Nature Metabolism Volume, 2019, 1: 1051-1058; Int J Mol Sci, 2018, 19:3995*). Collectively, these processes impact total lipid metabolic balance and lead to intracellular fat storage.

Response to (2):

By controlling several facets of glucolipid metabolism, insulin resistance, inflammatory response, oxidative stress, and mitochondrial homeostasis, the ASMase/Cer pathway collectively influences the course of non-alcoholic fatty

liver disease (NAFLD) (*Int J Mol Sci, 2023, 4:8322; J Hepatol, 2014, 61:1126-34*). Although specific essential limits have not been established, studies have demonstrated that ASMase expression and activity are raised in NAFLD in both liver and blood samples, indicating that changes in Cer levels correspond with the severity of the illness (*Arch Toxicol, 2023, 7:2069-2087*).

Response to (3):

Inhibiting ceramide synthesis or encouraging ceramide breakdown in adipocytes reduces inflammation and hepatic steatosis, which improves systemic metabolism. According to this, Cer and FFA may interact to modify systemic metabolism by influencing steatosis and TAG accumulation (*Nature Metabolism volume, 2019: 1051-1058; TDiabetes Metab J, 2020, 44: 222-233*). Cer may enter the artery walls and introduce LDL cholesterol particles, causing atherosclerosis and the development of plaque (*Antioxidants (Basel), 2023, 12:143*). The function of the ASMase/Cer pathway in TAG buildup and steatosis in humans has not been investigated in any clinical trials.

2.3Inflammation and immune dysregulation

(1) Can you elaborate on the specific mechanisms by which ceramide accumulation triggers inflammation and immune dysregulation? What pathways are activated in macrophages, and how do these contribute to the development of insulin resistance and metabolic diseases? (2) How do you differentiate between the pro-inflammatory effects of ceramide and any potential protective roles it may have in immune responses? What evidence supports your assertions regarding ceramide's role in promoting inflammation? (3) You mention the involvement of pro-inflammatory cytokines like IL-6 and TNF-α. How do these cytokines interact with the ASMase/Cer pathway, and what feedback mechanisms might exist that could further exacerbate inflammation? (4) The section touches on the role of gastric microbiota in immune dysregulation. Can you provide more detailed insights into how

alterations in gut microbiota affect the ASMase/Cer pathway and subsequent inflammatory responses? What clinical studies or epidemiological evidence support the connection between ceramide levels, inflammation, and metabolic disease outcomes? Are there specific patient populations that exhibit these associations more prominently?

Response to (1):

Pathogens attach to the extracellular segment of the Toll-like receptor (TLR) on macrophages due to altered cell membrane composition and permeability caused by excess Cer. This attracts bridging proteins like MyD88, activates the nuclear factor-κ B (NF-kB) and mitogen-activated protein kinase (MAPK) pathways, and activates the NOD-like receptor protein 3 (NLRP3) inflammasome, which in turn activates caspase-1 and releases inflammatory cytokines (TNF-α and ILs) . Caspases 1 are activated and inflammatory cytokines (TNF-α and ILs) are released when the NOD-like receptor protein 3 (NLRP3) inflammasome is activated (*Inflamm Res,2019, 68: 915-932;Nat Rev Gastroenterol Hepatol, 2023,20: 633-646;Int J Mol Sci, 2023, 24(21):15824*).

Through the upregulation of inflammatory factors, the NF-κB and MAPK pathways indirectly cause the dephosphorylation of IRS-1's Ser307 site. This decreases IRS-1's binding to the insulin receptor and prevents the activation of the downstream PI3K/Akt pathway, which in turn decreases glucose transport and metabolism (*J Appl Toxicol,2022,42: 1057-1066;Int J Mol Sci,2023, 25(10):5104*).

Response to (2):

Response Refer to Response in 2.1-(5).

Response to (3):

Response Refer to Response in 2.1-(5). The activation of transcription factors like NF-κB may be one way that IL-6 and TNF-α contribute to elevated ASMase

gene expression during inflammation (*Inflamm Res,2019, 68: 915-932;Nat Rev Gastroenterol Hepatol, 2023,20: 633-646;Int J Mol Sci, 2023, 24(23):16633*). When ASMase is activated, sphingolipids in cell membranes are converted to ceramides (Cer), which has a direct impact on the fluidity and functionality of the membranes. This in turn influences receptor signaling, such as TLRs, on the membranes, which in turn influences the production of downstream inflammatory factors, creating a positive feedback loop (*Nat Rev Gastroenterol Hepatol, 2023,20: 633-646;Int J Mol Sci, 2023,24(23):16633*).

Response to (4):

Through several pathways (such as the release of LPS, disruption of bile acid metabolism, creation of inflammatory factors, etc.), changes in the gut microbiota may impact the ASMase/Cer pathway, thereby exacerbating both local and systemic inflammatory responses. Through positive feedback processes, this process may further accelerate the course of metabolic disorders, such as diabetes mellitus and non-alcoholic fatty liver disease (NAFLD).

According to a clinical study, astragalus and nicotinamide riboside decreased harmful lipid ceramide levels and indicators of hepatic inflammation in NAFLD (*Hepatology, 2023, 78:863-877*);An additional clinical study demonstrated that fenofibrate and omega-3 fatty acids can lower plasma ceramide levels and lessen the inflammatory response linked to cardiovascular disease in NAFLD patients, improving heart and liver health (*FASEB J, 2021, 35:e21976; Lipid Re, 2020, 61:1065-1074.*).

2.4Mitochondrial dysfunction and oxidative stress

(1) Can you provide a more detailed explanation of the specific mechanisms through which ceramide accumulation leads to mitochondrial dysfunction? Which mitochondrial processes (e.g., oxidative phosphorylation, ATP production) are most affected, and how? How do you quantify the contribution

of ceramide-induced ROS production to mitochondrial dysfunction? What experimental models or methods did you use to measure ROS levels and their effects on mitochondrial function? (2) What specific areas of future research do you believe are necessary to further elucidate the relationship between the ASMase/Cer pathway, mitochondrial dysfunction, and oxidative stress? How can these areas be prioritized in the context of developing therapeutic interventions?

Response to (1):

Ceramide buildup's impacts on mitochondrial function primarily show up in the following ways: (1) Electron transport chain (ETC) damage includes reduced ATP generation, elevated reactive oxygen species (ROS), reduced respiratory capacity, and inhibition of oxidative phosphorylation. (2) ADP/ATP transport impairment: Cer decreases microtubule protein binding to VDAC1, which disrupts ADP/ATP transport in the mitochondria and further impacts ATP synthesis. (3) VDAC2 channel opening: Cer causes the voltage-dependent anion channel 2 (VDAC2) on the outer membrane of the mitochondria to open, which causes pro-apoptotic substances (such as cytochrome c) to be released from the mitochondria and initiates apoptosis.(4) AMPK pathway activation: Cer activates p38 MAPK and AMPK, which upsets the mitochondrial membrane potential and triggers apoptosis. Overall, Cer buildup affects cell survival and function by impairing mitochondrial activity, inhibiting ATP generation, and activating the apoptotic pathway through numerous mechanisms. The entire process of Cer's impact on mitochondria is interconnected, making it challenging to determine whether the effect—that of apoptosis on mitochondria or that of Ceramide on ATP—is stronger.

One popular technique for measuring the impact of ROS is to measure changes in ROS concentration and mitochondrial functional activity. Techniques for measuring ROS content include flow cytometry, spectrophotometry, chemiluminescence, and fluorescent labeling. tests for mitochondrial oxygen

consumption include spectrophotometric assessments of the activity of the mitochondrial respiratory chain complex, enzymatic tests for NADH absorbance, and dye-based assays for mitochondrial membrane potential.

Response to (2):

Further research into how the ASMase/Cer pathway mediates cellular activities like cellular immune regulation, inflammation, apoptosis, etc., as well as an examination of the precise mechanism of its role in metabolic diseases like cardiovascular diseases, can help clarify the relationship between the ASMase/Cer pathway, mitochondrial dysfunction, and oxidative stress. With an emphasis on how reactive oxygen species (ROS) disrupt DNA and RNA replication, oxidize mitochondrial proteins, cause membrane lipid peroxidation, and trigger the opening of the mitochondrial permeability transition pore (mPTP), oxidative stress damages the mitochondrial structure and causes mitochondrial dysfunction. Learn more about the relationship between oxidative stress and mitochondrial dysfunction. Prioritize therapeutic therapies that can target important ASMase/Cer pathway nodes and combined approaches that can reduce ROS, dampen inflammation, and apoptosis all at once.

3. Therapeutic strategies targeting the ASMase/Cer pathway

(1) Can you provide specific data or studies that demonstrate the efficacy of ASMase inhibitors in reducing ceramide levels and improving metabolic outcomes? What endpoints were measured in these studies, and how do they support the proposed therapeutic strategies? (2) What specific future research directions do you believe are most critical for advancing therapeutic strategies targeting the ASMase/Cer pathway? How should funding and research priorities be aligned to support these initiatives? How might ASMase inhibitors be integrated into existing treatment regimens for metabolic diseases? Are

there specific combination therapies that you believe could enhance the therapeutic effects of ASMase targeting? What are the major challenges in translating findings from preclinical models to clinical settings? How do you propose to address these challenges in future research? (3) What are the proposed mechanisms by which ASMase inhibition exerts beneficial effects on metabolic diseases? How do these mechanisms differ across various tissues affected by metabolic disorders?

Response to (1):

ASMase may be a therapeutic target for alcoholic hepatitis, according to experimental research that showed promethazine improved hepatic steatosis in ethanol-fed mice and inhibited ethanol-induced ASMase activation, ceramide production, and PP2A activation (*Am J Physiol Gastrointest Liver Physiol, 2012, 302(5): G515–23*). Improvements in hepatic steatosis, modifications in ceramide levels, and impacts on inflammatory, insulin, and apoptotic signaling pathways were among the endpoints that were assessed in these investigations. According to a different study, promethazine mitigated heart hypertrophy and cardiac dysfunction in mice fed a high-fat diet (HFD) and decreased cardiomyocyte apoptosis and fibrosis (*Cardiovasc Diabetol, 2023, 22(1):25*). Cardiomyocyte apoptosis, fibrosis, and improvement in heart hypertrophy were the study's evaluated objectives.

Response to (2):

An in-depth investigation of ASMase's mechanism of action in various metabolic diseases and the investigation of ASMase inhibitor combination therapies with other metabolic disease treatment regimens to improve the effectiveness of ASMase-targeted therapies are potential important future research directions to advance therapeutic strategies targeting the ASMase/Cer pathway. Drug specificity, adverse effects, dosage control, and the safety and effectiveness of long-term therapy are the primary obstacles in the clinical

translation research of ASMase inhibitors. According to recent research, desipramine (DMI) and N-acetylcysteine (NAC) together can inhibit ASMase, shield mouse cells from oxidative stress, inflammation, and lung fibrosis, and improve the therapeutic effects of ASMase (*Ecotoxicol Environ Saf, 2022, 242:113914*). The mechanism of NAC resistance to ASMase inhibitors, the efficacy of NAC in combination with other ASMase inhibitors, determining the ideal dose and dosing schedule for NAC combination therapy, and tailoring treatment to lessen the incidence of resistance are the primary obstacles to bringing this therapy to the clinic.

Response to (3):

The following are hypothesized methods by which ASMase inhibition improves metabolic diseases: inhibition of ASMase lowers Cer levels, which in turn lowers inflammatory reactions and apoptosis. By altering the autophagy process, ASMase inhibition enhances intracellular lipid and protein metabolism and slows the development of metabolic disorders. Alcohol-induced liver damage and steatosis are lessened by altering the endoplasmic reticulum stress response. These pathways may vary depending on the tissue affected by metabolic diseases. For example, in the liver, they may largely show up as a reduction in endoplasmic reticulum stress and steatosis, whereas in muscle and adipose tissue, they may show up as decreased inflammation and increased insulin sensitivity.

3.1 Functional inhibitors of ASMase

Have any studies explored the effects of combining ASMase inhibitors with other therapeutic agents? What were the outcomes, and how might combination therapies enhance or mitigate the effects of ASMase inhibition? Are there potential mechanisms of resistance that could limit the effectiveness of ASMase inhibitors in long-term treatment scenarios? How do you propose to address these challenges in future research?

Response to (1):

Although it has been demonstrated that desipramine (DMI) and N-acetylcysteine (NAC) together may slow the development of pulmonary fibrosis in rats by reducing oxidative stress and ASMase activity in vivo, the precise mode of action of the combined approach has not been investigated (*Ecotoxicol Environ Saf, 2022, 242:113914*). The mechanism of resistance to ASMase inhibitor treatment is thought to be lessened by NAC, as both NAC and ASMase inhibition diminish oxidative stress injury (*Pharmacol Ther, 2021, 228:107916*). The investigation of resistance mechanisms, combination tactics, ideal therapy dosages, and customized approaches all present formidable obstacles.

3.2 Natural compounds

Can you provide specific evidence from studies that demonstrate the efficacy of the natural compounds discussed in inhibiting ASMase activity? What experimental models were used, and what were the key outcomes? How do the natural compounds identified specifically inhibit ASMase? Are there known pathways or molecular targets that these compounds interact with, and how do these interactions lead to the desired therapeutic effects? What is known about the bioavailability and metabolic stability of the natural compounds mentioned? How might these factors influence their effectiveness in clinical settings? Have there been studies exploring the potential synergistic effects of combining these natural compounds with other ASMase inhibitors or conventional therapies? What were the findings, and how might they inform future treatment strategies? While natural compounds are often perceived as safe, what are the potential side effects or interactions with other medications that could arise from their use? Are there any documented adverse effects in clinical or preclinical studies?

Response to (1):

Typically, ultra-performance liquid chromatography (UPLC) equipment is used to measure how the natural substance α-mangostin inhibits ASMase activity. By attaching itself directly to the ASMase active site and blocking the binding of the substrate sphingolipids to the enzyme, α-mangostin inhibits the catalytic activity of ASMase in a diabetic mouse model. Furthermore, α-mangostin may bind to DNA or control the activity of transcription factors to prevent the transcription of the ASMase gene.

α-Mangostin's known mechanisms of action include inhibiting SIRT-1 and TLR4/NF-κB signaling pathways to reduce inflammatory responses and causing PARP cleavage and apoptosis via the RXRα-AKT signaling pathway (*Front Pharmacol, 2021, 12:739658*； *J Exp Med, 2003, 198:1043-1055; J Cell Physiol, 2016, 231: 2439-51*). It might be able to treat inflammatory reactions in metabolic disorders through these mechanisms.

Response to (2):

α-Mangostin is not soluble in water and is somewhat soluble in organic solvents like methanol. α-Mangostin has little oral absorption and a low absolute bioavailability. The efficiency of α-mangostin's clinical applications is limited by its low solubility and metabolic stability. However, formulation techniques like soft gels can improve their solubility and bioavailability, potentially increasing the efficacy of their therapeutic treatment (*Int J Mol Sci, 2020, 21:6211*). The use of these natural compounds in conjunction with other ASMase inhibitors has not been studied; however, α-Mangostin has been used in conjunction with traditional cancer treatments like chemotherapy or radiation therapy, which may increase therapeutic efficacy while lowering toxicity to healthy cells (*Biomolecule, 2024, 14:1382*). It is necessary to conduct additional research to investigate and confirm whether α-Mangostin can work in concert with other ASMase inhibitors.

Response to (3):

The negative effects of α-Mangostin were primarily linked to its effects on mitochondrial function and oxidative stress in preclinical or clinical trials (*Phytother Res, 2023, 37:3394-3407; Future Med Chem, 2021, 13:1679-1694*). α-Mangostin has been shown to cause oxidative damage, mitochondrial malfunction, and apoptosis in a triple-negative breast cancer model (*Phytother Res, 2023, 37(8):3394-3407*), which could potentially affect cells.

3.3 Emerging therapeutics

Can you elaborate on what makes the emerging therapeutics discussed in this section innovative compared to existing treatment options for metabolic diseases? What specific advancements do they offer in terms of efficacy or mechanism of action? What are the primary challenges in translating these emerging therapeutics from bench research to clinical practice? How do you propose to address these challenges in further studies? Have any studies investigated the use of these emerging therapeutics in combination with existing treatments for metabolic diseases? What were the outcomes, and how could these combinations enhance treatment efficacy?

Response to (1):

According to the article, a number of metabolic illnesses may be influenced by targeting the ASMase/Cer pathway through a number of mechanisms, including changes in mitochondrial function, inflammation and immunology, and glycolipid metabolism. Furthermore, by selectively blocking ASMase in cardiac tissues, lowering Cer levels, and reducing cellular stress and inflammatory reactions, empagliflozin offers a unique treatment strategy for CVD and metabolic diseases. In order to treat metabolic illnesses, targeting ASMase/Cer methods offers a numerous mechanism of action that could result in greater efficacy.

The requirement for extensive clinical trials to validate efficacy, safety, and side effects, as well as customized treatment plans and medication interactions, are

significant obstacles to clinical translation.

According to certain research, empagliflozin and GLP-1 receptor agonists, such as liraglutide, can further enhance blood glucose, body weight, and cardiovascular health while lowering the risk of complications from diabetes. Furthermore, empagliflozin has demonstrated encouraging synergistic effects when used in conjunction with cardiovascular drugs (such as beta-agonists, ACE inhibitors, etc.) to treat individuals with diabetes and cardiovascular disease.

4. Conclusion

In the conclusion, how do you plan to succinctly summarize the most critical findings of your review? What specific insights do you believe are essential for readers to retain regarding the ASMase/Cer pathway and its implications for metabolic diseases?

Response to (1):

According to this review, Cer is not only closely linked to metabolic diseases and their symptoms, but it also affects the mechanism of action of cellular glycolipid metabolism, oxidative stress, inflammatory response, and mitochondrial homeostasis. These factors have a significant impact on the development of metabolic diseases and their symptoms, including cardiovascular diseases. Thus, ongoing investigation and study have raised hopes for the creation of novel medications that target the ASMase/Cer pathway as well as for the development of fresh approaches and treatment plans. The ASMase/Cer pathway may therefore be a viable target for the management of metabolic disorders like diabetes and obesity.

Response to (2):

The reader should, in my opinion, remember the following particular insights: The relationship between the ASMase/Cer pathway and metabolic diseases is

discussed in this paper, with particular attention to the mechanisms through which the pathway influences oxidative stress, inflammation, mitochondrial homeostasis, and cellular glucose-lipid metabolism. It is also shown that activation of the ASMase/Cer pathway based on metabolic disorders impacts metabolically relevant glucose-lipid metabolism pathways. Additionally, it is mentioned that the dysregulation of the ASMase/Cer pathway is impacted by diseases of these glycolipid pathways, creating a positive feedback loop. Consequently, the ASMase/Cer pathway offers novel concepts and treatment approaches as well as a possible therapeutic target for metabolic disorders.

Revision reviewer:

Dear Authors, I have reviewed your manuscript titled Emerging Roles of the Acid Sphingomyelinase/Ceramide Pathway in Metabolic and Cardiovascular Diseases: Mechanistic Insights and Therapeutic Implications (Manuscript Number: 102308). I am pleased to inform you that you have adequately addressed all previous concerns and made the necessary corrections. Thank you for your efforts. Good luck

Response:

Thanks for your comments.