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The role of sphingolipids in multi-organ lipotoxicity in metabolic syndrome.

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1. Abstract

The global incidence of metabolic dysfunction–associated steatohepatitis (MASH) and heart failure with preserved ejection fraction (HFpEF) is rising due to the increasing prevalence of metabolic syndrome (MetS). Both MASH and HFpEF share comorbidities such as obesity, type 2 diabetes, and hypertension, which suggest common pathophysiological mechanisms. These diseases are characterized by lipotoxicity and mitochondrial dysfunction. Current treatments for MetS, MASH, and HFpEF are limited and often target specific organs, increasing the risk of drug interactions and side effects. This study examines the role of sphingolipids, particularly ceramides, in multi-organ lipotoxicity within MetS. Preliminary data and current literature indicate that ceramides play a crucial role in metabolic diseases. By modeling biosynthetic pathways for ceramide, we aimed to identify new intracellular targets to modulate ceramide content. Given the lower SPTLC1 levels (key enzyme in ceramide de novo synthesis) in HFpEF hearts, reducing ceramide content using SPTLC1 inhibitor like myriocin, presents a challenge. Alternatively, adiponectin receptors have emerged as potential therapeutic targets, acting after ceramide synthesis. JT003, an AdipoR1/2 dual agonist, has proven its effectiveness improving lipid metabolism, reducing fibrosis and inflammation via AMPK and PPARα signaling pathways. Whether JT003 impact on ceramide metabolism has never been previously explored. In this study, we first investigated ceramide metabolism and triglyceride accumulation in the hearts of C57BL-6J-mice on standard (STD) or high-fat diets (HFD), with or without myriocin treatment. We used Oil Red O staining and immunofluorescence to quantify triglycerides and ceramides, respectively. Our results showed that HFD significantly increased both triglycerides and ceramides levels in WT mice, which were both reduced by myriocin treatment, further emphasizing the role of ceramide metabolism in the heart. Secondly, in *in vitro* experiments, we modeled ceramide accumulation using palmitate treatment on various cell lines, including 3T3-L1, HEK293, AC16, and HepG2 cells. JT003 treatment significantly reduced ceramide content across all cell lines by enhancing ceramide degradation through ceramidase activity without altering the transcription of *de novo* synthesis enzymes. Our findings suggest that JT003 could be a promising therapeutic compound for targeting

ceramide accumulation in MetS, MASH, and HFpEF, providing a comprehensive treatment strategy for these complex and interconnected conditions.

2. Acknowledgments

I would like first to express my deepest gratitude to Elisabetta Trevellin. Her support and mentorship have been pivotal throughout my journey in the lab. From teaching me the fundamental techniques to guiding me through the complexities of research, her expertise has shaped my academic and professional growth. Moreover, her assistance in the writing of this thesis has been significant. I also extend my sincere thanks to Federico Capone, whose expertise in data analysis and interpretation has been crucial in bringing this project to fruition. His insightful feedback and collaborative spirit have greatly enhanced the quality of this work. Thanks also to Professor Vettor for opening the doors of his laboratory to me and for providing me with the opportunity to contribute to such a project.

Beyond the laboratory, I want to express my appreciation to everyone who has accompanied me during my two years of master's studies in Italy. Moving to a foreign country alone was a challenging yet enriching experience, and I am leaving with not only a wealth of new knowledge but also with cherished memories and lasting friendships. The support and kindness I received from colleagues, friends, and mentors have made this journey unforgettable, and I am truly grateful for the opportunity to grow both personally and professionally in such a vibrant and welcoming environment.

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3. Introduction

3.1. Metabolic syndrome (MetS)

The pathophysiology of the metabolic syndrome (MetS) is complex and still poorly understood, but it has been established that chronic low-grade inflammation is a key factor. This chronic inflammation is generally linked to excessive caloric intake, leading to obesity. Although obesity plays a central role in MetS, it is crucial to consider the distribution of adipose tissue. Indeed, visceral obesity, characterized by an accumulation of fat in the intra-abdominal area, is the main cause of chronic systemic inflammation, even in normal weight subjects. Thus, MetS is more common in individuals with visceral obesity.

Adipose tissue, now recognized as an endocrine organ, plays a pivotal role in this process. Excessive caloric intake causes hypertrophy and hyperplasia of adipocytes, leading to metabolic disorders and structural changes in adipose tissue. Initially, these changes induce hypoxic conditions for the adipocytes furthest from the blood vessels, resulting in the release of fatty acids and signaling molecules triggering pro-inflammatory pathways (Ambroselli, D. et al, 2023). Secondly, there is an ectopic accumulation of lipids, i.e. storage of triglycerides in tissues other than adipose tissue, which promote inflammation in insulin-sensitive organs such as muscle and liver, predisposing individuals to insulin resistance (Snel, M. et al, 2012). Insulin resistance in different tissues alters insulin modulation of lipolysis, inhibiting insulin's anti-lipolytic effect and increasing the concentration of circulating free fatty acids (FFAs). In muscle, FFAs prevent insulin-dependent glucose uptake, while in muscles and liver, insulin resistance affects glucose transport and glycogen synthesis. This deficiency leads to increased insulin secretion by beta cells to maintain normal blood glucose levels. Insulin resistance also generates oxidative stress, which activates and promotes atherogenesis and tissue fibrosis.

As a result, insulin resistance is central to the development of MetS, and promotes the progression of other diseases, comorbidities, and complications such as cardiovascular diseases, hypertension, dyslipidemia, type 2 diabetes, non-alcoholic fatty liver diseases, major depressive disorders, infertility, polycystic ovary syndrome, cognitive impairment, and sarcopenia.

MetS is recognized as a set of metabolic abnormalities resulting from acquired genetic and environmental influences, contributing to chronic inflammation. Early diagnosis underlines the importance of lifestyle modification and risk factor management, while drug therapy targets the organs specifically affected by MetS.

In 1999, the Diabetes Group of the World Health Organization (WHO) defined the MetS as the presence of insulin resistance, in combination with at least two other risk factors in obese individuals. These risk factors include waist-to-hip ratio, body mass index, hypertension, dyslipidemia, and microalbuminuria. In 2005, this definition was revised by the Joint Interim Statement, whereby MetS is diagnosed if three of the following criteria are met:

- Elevated waist circumference, with population- and country-specific thresholds (≥102 cm for European men and ≥88 cm for European women).
- Blood triglycerides ≥ 150 mg/dL.
- Blood HDL cholesterol < 40 mg/dL in men and < 50 mg/dL in women.
- Blood pressure ≥ 130/85 mmHg.
- Fasting blood glucose ≥ 100 mg/dL.

In 2015, Scuteri, A. et al, analyzed the prevalence and distribution of MetS across 34,821 individuals from different European and U.S. cohorts, finding an overall prevalence of 24.3%. Specific clusters of MetS components, linked to higher cardiovascular risk, were more common in specific regions, such as the UK, Southern Europe, and Belgium. The prevalence of MetS highlights the urgent need to develop strategies to counter this emerging health crisis (Ambroselli, D. et al, 2023).

In this work, we will focus on two complications of the MetS: metabolic dysfunction–associated steatohepatitis (MASH) and heart failure with preserved left ventricular ejection fraction (HFpEF), which affects the heart as a cardiovascular disease.

3.1.1. Metabolic dysfunction–associated steatohepatitis (MASH)

The liver is a crucial organ with a fundamental role in metabolism. However, it is highly susceptible to damage from harmful substances such as drugs, alcohol, and unhealthy foods. These detrimental exposures can lead to a number of liver diseases and the accumulation of fat within the liver, known as steatosis. This condition often marks the onset of fatty liver disease, which can significantly impair liver function and overall

health. A new nomenclature for fatty liver diseases has been established in 2023, proposing the term "metabolic dysfunction–associated steatotic liver disease" (MASLD) to replace non-alcoholic fatty liver disease (NAFLD). This new definition reflects the underlying metabolic dysfunctions and eliminates the assumption of alcohol use, which can be misleading. This shift is expected to improve the diagnosis and management of liver conditions linked to metabolic syndromes, promoting a more precise understanding of the disease and fostering better patient care (Rinella, M. E., et al, 2023).

Metabolic dysfunction–associated steatohepatitis (MASH) is part of the spectrum of metabolic dysfunction–associated steatotic liver disease (MASLD), which encompasses a range of liver conditions related to metabolic dysfunction. The initial stage typically manifests as metabolic dysfunction–associated steatosis, which can progress to MASH, MASH-related cirrhosis, or even hepatocellular carcinoma (HCC). MASH is characterized by steatosis accompanied by lobular inflammation and ballooning of hepatocytes, with or without fibrosis (Han, S. K. et al, 2023).

In the initial stages, hepatocytes accumulate triglycerides through de novo lipogenesis, driven by the presence of glucose and FFAs in the blood. The progression to MASH is marked by multiple cellular stresses, particularly at the level of the endoplasmic reticulum and mitochondria, such as oxidative stress due to reactive oxygen species (ROS) and lipotoxicity, which may or may not lead to hepatic fibrosis (Huby, T., & Gautier, E. L., 2022). Inflammation in MASH is classified as mild (grade 1), moderate (grade 2), or severe (grade 3), with further sub-classifications based on the degree and location of fibrosis (Han, S. K. et al, 2023). Intra-lobular inflammation is characterized by infiltration of a mixture of immune cells. Hepatic leukocytes, such as macrophages, secrete inflammatory cytokines such as tumor necrosis factor-alfa (TNF-α). Hepatocytes, stressed by these mediators, also release pro-inflammatory molecules such as adenosine triphosphate (ATP), extracellular vesicles, and chemokines, amplifying inflammation and leading to fibrosis. This cycle of stress and inflammation can worsen, leading to cirrhosis and hepatocellular carcinoma (HCC) in some patients.

A high-fat, high-cholesterol diet can also promote the development of MASH and induce stress in the endoplasmic reticulum and mitochondria, leading to liver dysfunction via the production of pro-inflammatory mediators. This process can result from the production of fatty acids via hepatic lipogenesis, but can also take place via the

release of FFAs circulating in the blood, originating from adipose tissue in a context of MetS and/or obesity. The lipid stress thus generated can ultimately lead to hepatocyte apoptosis, which can be observed following hepatocyte ballooning, when hepatocytes are hypertrophic due to increased triglyceride storage, with a rarefied cytoplasm, thus characterizing the steatosis stage (Huby, T., & Gautier, E. L., 2022). As a result, liver steatosis, characterized by the abnormal accumulation of lipids in liver tissue, serves as the initial stage of lipotoxicity in the progression of MASH.

3.1.1.2. MASH associated lipotoxicity

The term "lipotoxicity" was introduced in the early 1990s by Roger Unger. He observed that the main dysregulations associated with MetS could be reproduced by increasing the delivery of fatty acids to skeletal muscle. Over the years, a growing body of evidence has shown that promoting ectopic fat deposition leads to metabolic disorders, and that to improve these conditions, it is essential to promote healthy fat storage or oxidation to improve these conditions.

Lipids entering cells are metabolized by mitochondria (oxidized) or converted into complex lipids, notably triglycerides (TGs), by the endoplasmic reticulum (ER). Although triglycerides are markers of lipotoxicity, they are generally not directly harmful and may even offer protection (Chaurasia, B., & Summers, S. A., 2015). Instead, FFAs rather than TGs leads to the formation of toxic metabolites via the hepatic metabolism (Bessone, F., et al, 2019). Therefore, the true origin of MASH pathology lies in the insulin resistance associated with MetS. Although the liver is not the first organ to manifest symptoms, once affected, it intensifies the effects induced by glucose and insulin, promoting the overproduction of FFAs. This leads to an imbalance between lipid accumulation and elimination in the liver.

In a healthy context, glucose and insulin activate sterol regulatory binding protein-1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP), leading to the production and storage of FFAs in the form of triglycerides (TGs). These are then exported as very low-density lipoprotein (VLDL) or degraded by lipophagy. However, in MASH, this lipophagy process is disrupted. An excess of FFAs and TGs initially leads to an overload of β-oxidized FFAs, contributing to oxidative stress. At the same time, lipid export via VLDL is impaired, leading to a dramatic increase in toxic fatty acid

metabolites in the liver. More specifically, overactivation of the SREBP-1c protein induces excessive production of malonyl-CoA by acetyl-CoA carboxylase 2 (ACC2). This metabolite inhibits the β-oxidation-regulating enzyme, carnitine palmitoyltransferase 1 (CPT1), and causes the accumulation of toxic lipids such as oxidized cardiolipins and ceramides. Ceramides specifically play a significant role in liver lipotoxicity. Indeed, these molecules increase oxidative stress within mitochondria by altering the respiratory chain and mitochondrial DNA, further impeding β-oxidation. In the context of MASH, toxic lipids like ceramides also interfere with insulin signals, leading to degradation of insulin receptors (IRS1/2). This contributes to impaired glucose metabolism and increased lipid accumulation (**Figure 1**) (Bessone, F., et al, 2019).

As a result of these mechanisms, only a small fraction of lipids is converted into ceramides. Roger Unger's research had also demonstrated that blocking ceramide biosynthesis could inhibit the negative lipotoxic effects of fatty acids in the pancreas of rats. Subsequently, more detailed studies on ceramides, facilitated by technological advances, strongly corroborated Unger's initial observations who even described ceramides as 'the most important of the deleterious routes' underlying their lipotoxicity. These studies have shown that reducing ceramide levels in rats prevents insulin resistance, diabetes, MASLD and cardiovascular disease. Thus, ceramide lipotoxicity linked to MetS does not affect only the liver (Chaurasia, B., & Summers, S. A., 2015).

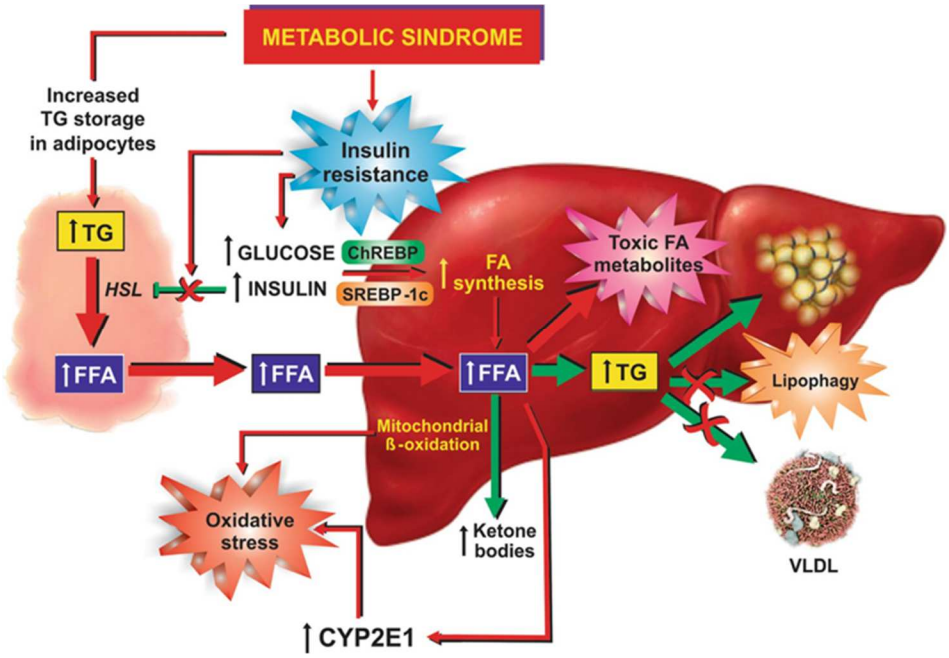

Figure 1 : *Metabolic basis of NAFLD (Bessone, F. et al, 2018)*

3.1.2. Heart failure with preserved left ventricular ejection fraction (HFpEF)

Heart failure (HF) is a cardiac disorder in which the heart is unable to pump blood adequately to meet the body's needs. When considering the left ventricular function, HF is divided into two categories: heart failure with reduced ejection fraction (HFrEF) and heart failure with preserved ejection fraction (HFpEF). In this section, we will focus on HFpEF, which accounts for over 50% of HF cases (Nair, N., 2020). The left ventricle (LV) undergoes two phases during each beat: filling (diastole) and contraction (systole). The volume ejected during LV systolic contraction is critical for cardiac output, which depends on cardiac contractility, ventricular preload, afterload (the force required to expel blood) and heart rate. Cardiac output can be simplified to LV stroke volume (the amount of blood ejected by the LV with each beat) multiplied by heart rate. Low cardiac output is a key feature of HF and can be caused by any alteration in the above-mentioned determinants (Biga, L.M., et al, 2019). HFpEF is primarily a problem of diastolic function and cardiac filling. One of the most important causes of HFpEF is hypertension, which leads to increased afterload. Increased afterload reduces systolic ejection volume and, consequently, cardiac output. To compensate, the sympathetic nervous system increases contractility, heart rate and venous tone. In the long term, however, these mechanisms lead to ventricular remodeling and dysfunction. In addition, reduced cardiac output leads to decreased renal perfusion, which activates the renin-angiotensin-aldosterone system (RAAS), a mechanism that also increases cardiac output, but leading in the long term to ventricular remodeling and dysfunction (Aryee, E. K. et al, 2023). This ventricular remodeling results in LV hypertrophy, causing a decrease in LV size and increase in LV fibrosis. As a result, the LV become rigid and less compliant, reducing filling volume during diastole. Ultimately, this rigidity increases LV filling pressures, which in turn increases atrial pressure and can lead to pulmonary hypertension. However, since diastolic function is impaired, the problem lies in filling the ventricles, while the ejection fraction (the percentage of blood volume ejected by the heart during each cardiac cycle) remains normal and preserved at over 50%, as the heart still manages to pump the small volume of blood during systole (Nair, N., 2020).

As previously mentioned, HFpEF is mainly caused by hypertension, and can therefore be linked to conditions such as obesity, MetS, obstructive sleep apnea and diabetes. Indeed, these conditions lead to the deposition of ectopic fat in areas such as the myocardium, which can profoundly affect cardiac function. These fat deposits increase myocardial rigidity, thus contributing to the hypertension responsible for HFpEF (Oduah, M. T., et al 2023). They also exacerbate systemic inflammation. Adipose tissue produces pro-inflammatory cytokines and adipokines, such as TNF-α and interleukin-6, increasing the risk of HFpEF. Chronic inflammation linked to these fatty deposits is also associated with insulin resistance and oxidative stress. Excess lipids inflow in cardiomyocytes exceed their oxidative capacity, leading to lipid accumulation and eventually mitochondrial dysfunction. This causes the accumulation of toxic metabolites, increases oxidative stress and leads to myocardial dysfunction (Aryee, E. K. et al, 2023). Therefore, inflammation and lipotoxicity play crucial roles in the progression of HFpEF, making the management of these fatty deposits and inflammatory processes essential to improving clinical outcomes in affected patients.

3.1.2.1. HFpEF associated lipotoxicity

As previously mentioned, lipid overload in the heart contributes to the development of HFpEF by causing hypertension. A diet rich in saturated fats and refined carbohydrates promotes lipid absorption by non-adipose tissues, leading to ectopic lipid accumulation. In the hearts of subjects with obesity, increased fatty acid oxidation is often insufficient to prevent lipid deposition, leading to severe diastolic dysfunction. This ectopic fat reaches the heart in the form of FFAs. Once in the heart, these fatty acids can either undergo oxidation within the mitochondria or be esterified into compounds such as triglycerides (TG) for storage. Long-chain fatty acids require the CPT1 system to enter the mitochondria. An insufficient CPT1 activity diverts FAs from oxidation to esterification, leading to an increase in TGs storage. High levels and accumulation of TGs in the myocardium are associated with their toxicity, however the way in which lipids induce myocardial toxicity is not yet fully elucidated, but TGs *per se* do not appear to be directly responsible for it. What is known is that reactive lipid species such as ceramides and diacylglycerols (DAGs) are produced when TGs are overproduced (Capone, F., et al,

2022). Lipid intermediates, such as DAGs and ceramides, can contribute to cardiac dysfunction by regulating NADPH oxidase (NOX2) through activation of protein kinase C (PKC), thereby increasing the production of reactive oxygen species (ROS). ROS can disrupt myocardial function in several ways. They reduce the availability of nitric oxide (NO) by converting it into peroxynitrite. In addition, peroxynitrite activates protein phosphatase 2a, leading to impaired calcium management in the sarcoplasmic reticulum, further aggravating HFpEF. ROS can also activate the ryanodine receptor (RyR), increasing cytosolic calcium and potentially leading to chronic activation and cardiac tension. This increased calcium release can further stimulate ROS production, creating a vicious circle. In addition, ROS activate hypertrophic and pro-fibrotic pathways, including NF-κB and MAPK, and increase myocardial stiffness by modifying titin, contributing to diastolic dysfunction. Finally, PKC signaling, potentiated by ROS, can independently modify pathways regulating diastolic function, activating fibroblasts, promoting fibrosis and leading to impaired calcium management, increased cardiomyocyte necrosis and ventricular wall thickening. This complex interaction underlines the multifaceted role of lipid intermediates and ROS in the pathogenesis of HF (**Figure 2**) (Leggat, J., et al, 2021). Overall, excess long-chain fatty acids (LCFAs) in HFpEF lead to pathological storage of toxic fatty acid esters and ceramides, resulting in lipotoxicity and diastolic dysfunction (Capone, F., et al, 2022).

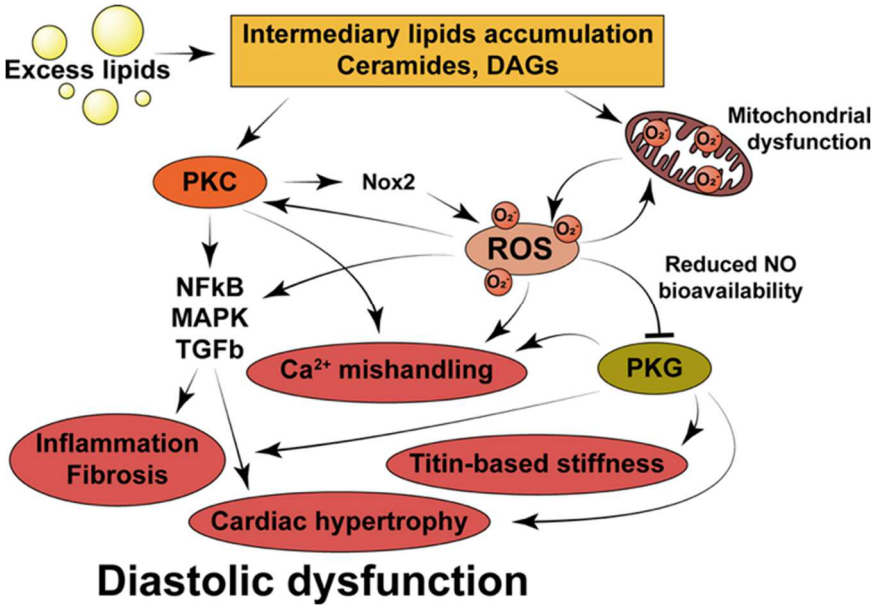

Figure 2 : *Mechanisms in which lipid intermediates can promote the generation of ROS, and by which ROS may go on to elicit diastolic dysfunction. (Leggat J. et al, 2021)*

3.1.3. Similarities between MASH and cardiometabolic HFpEF

If we analyze MetS, obesity and hypertension, they are all characterized by ectopic lipid accumulation and the activation of inappropriate inflammatory pathways. They lead to structural and functional alteration of cells by oxidative stress, progressive fibrosis and organ dysfunction. This description parallels the pathogenesis of MASH but is also very often associated with a prevalent phenotype of HFpEF (**Figure 3**).

The incidence of MASH and HFpEF is increasing worldwide, driven by the growing prevalence of MetS. The main comorbidities in MASH patients are the same observed in HFpEF patients, such as obesity (~80%), type 2 diabetes (~50%), hyperlipidemia (~80%) and hypertension (~70%) (Capone, F. et al, 2023). Data on the prevalence of NASH in HFpEF are still limited, however, both diseases are often associated. Indeed, one study of 181 HFpEF patients revealing a NAFLD in 27% of the cohort, half of whom had advanced liver fibrosis. In the overall cohort, patients with advanced fibrosis or cirrhosis were older, had a higher BMI, and were more likely to have diabetes, atrial fibrillation and chronic kidney disease. Multivariable analysis of this study also showed that advanced age, higher BMI and the presence of diabetes were independently associated with advanced fibrosis or cirrhosis (Miller, A., et al, 2020).

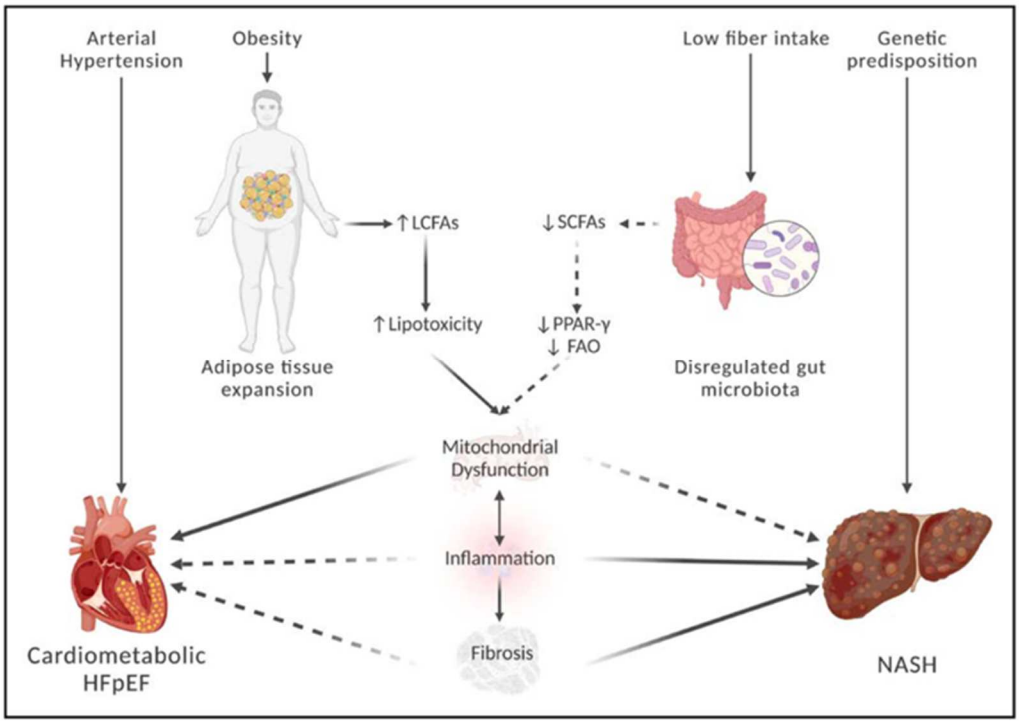

Figure 3 : *Key shared pathogenic pathways between NASH and HFpEF (Capone F. et al, 2023)*

The progression from subclinical intramyocardial lipid accumulation to HFpEF is not well understood, but lipotoxicity is a crucial factor in MASH, arising from adipose tissue dysfunction, lipid spillover, ectopic lipid deposition, and organ damage.

Metabolically active lipid species like ceramides often cause liver injury in MASH, with similar mechanisms suggested in HFpEF, though the specific myocardial lipid species remain unclear. Short-chain fatty acids (SCFAs), produced by gut microbiota, play a role in MASH by promoting lipid oxidation through their anti-inflammatory and antihypertensive properties. Dysregulation of SCFA-producing bacteria has been noted in HFpEF, but its impact is still unknown. Lipotoxicity leads to mitochondrial dysfunction and structural abnormalities in both MASH and HFpEF, contributing to tissue damage. Inflammation is also involved, with elevated proinflammatory cytokines affecting both hepatocytes and cardiomyocytes. Patients with HFpEF and obesity show increased inflammatory biomarkers and a proinflammatory myocardial transcriptomic signature. Despite these similarities, MASH and cardiometabolic HFpEF have distinct features: the liver synthesizes storage molecules while the heart oxidizes energy substrates to generate ATP, limiting the applicability of pathophysiological insights from MASH to HFpEF (Capone, F. et al, 2023).

3.1. Sphingolipids

Sphingolipids, first identified by Johann L.W. Thudichum in 1884, are a unique class of lipids characterized by their amphipathic nature, containing both hydrophilic and hydrophobic regions. They all share a common backbone called a sphingoid base, which is attached to fatty acid chains. This group includes several important molecules such as sphingosine, ceramide, sphingosine-1-phosphate (S1P), ceramide-1-phosphate (C1P), and sphingomyelin (SM). These sphingolipids can be categorized into three main groups based on their structure: sphingoid bases and their derivatives, ceramides, and complex sphingolipids (**Figure 4**). They play crucial roles in regulating cellular processes such as proliferation, apoptosis, and differentiation. They influence lipid bilayer properties and regulate cellular functions, playing critical roles by triggering stress responses (Wang, J., et al, 2021).

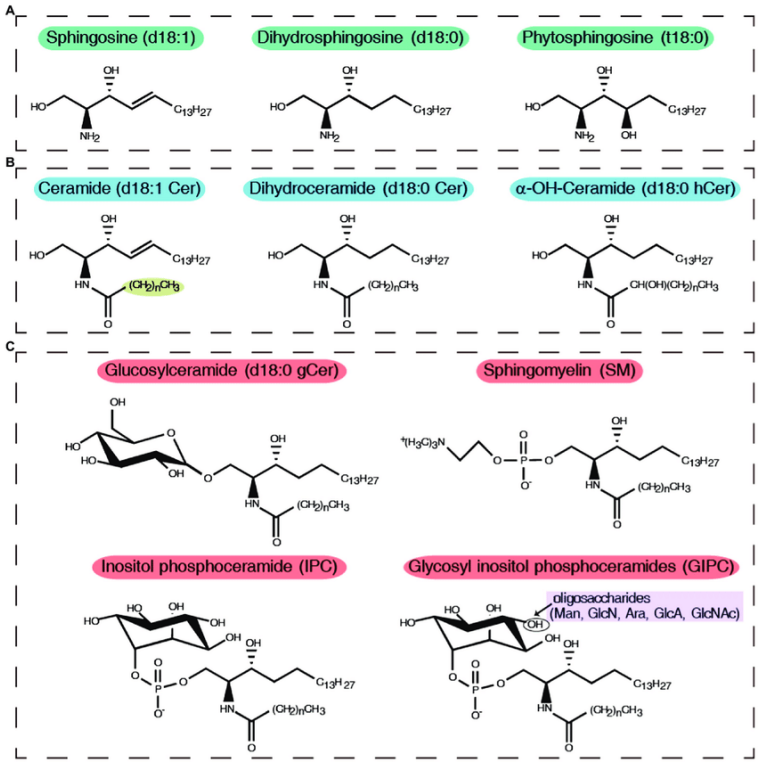

***Figure 4**: General structures, nomenclature, and abbreviated names of sphingolipids. (A) Sphingosine and its hydroxylation and saturation derivatives; (B) Ceramide and its derivatives; (C) Complex sphingolipids, including glucosyl sphingolipids, sphingomyelin, inositol phospho ceramide, and glycosyl inositol phospho ceramides. (CH2)n, chain length of the N-acyl sphingolipid moiety. Oligosaccharides: mannose (Man), glucosamine (GlcN), arabinose (Ara), glucuronic acid (GlcA), and N-acetylglucosamine (GlcNAc). (Wang, J., et al, 2021)*

Among these, ceramides are particularly significant due to their involvement in lipotoxic pathways in MetS-related diseases such as MASH and HFpEF. Structurally, ceramides are synthesized by binding a long-chain fatty acid to the amine group of a sphingoid base, making them integral components of cell membranes in eukaryotic cells (Quinville, B. M., et al, 2021). Ceramides are synthesized through three primary pathways: *de novo* synthesis, sphingomyelin hydrolysis, and the salvage pathway. The *de novo* synthesis, which occurs in the endoplasmic reticulum (ER), serine and palmitoyl-CoA are condensed by serine palmitoyltransferase (SPT) to form 3-ketodihydrosphingosine, which is then reduced to dihydrosphingosine by 3-ketodihydrosphingosine reductase (KDSR). Dihydrosphingosine is acylated into dihydroceramide by ceramide synthases (CerS), with six isoforms (CerS1 to CerS6) producing ceramides with varying acyl-chain lengths from C14 to C30, and CerS2, synthesizing long-chain ceramides. Dihydroceramide desaturases (DES1 and DES2) then convert dihydroceramides into ceramides by introducing a double bond. Ceramides

serve as precursors for complex sphingolipids, being transported to the Golgi apparatus where they can be glycosylated into glucosylceramide, modified to form sphingomyelin, or phosphorylated into ceramide-1-phosphate (C1P). In the plasma membrane, sphingomyelin can be hydrolyzed back to ceramide by sphingomyelinases, or ceramides can be converted into sphingosine by ceramidase and further phosphorylated into sphingosine-1-phosphate (S1P) by sphingosine kinase. Additionally, sphingomyelin and glycosphingolipids internalized into lysosomes are hydrolyzed to ceramide and subsequently to sphingosine, which can be recycled to the ER via the salvage pathway (Yu, X. D., & Wang, J. W., 2022). Palmitate, or palmitic acid, is a crucial precursor for ceramide *de novo* synthesis. It is commonly used in cell cultures to induce the ceramide biosynthesis pathway, enabling the study of lipid metabolism and its effects on various metabolic diseases (**Figure 5**). Despite their low abundance, ceramides have profound effects on cellular health and disease mechanisms, with disruptions in their metabolism significantly affecting cellular homeostasis and influence lipid bilayer properties (Blachnio-Zabielska, A., et al, 2022). This study aims to elucidate the pathways of ceramides metabolism in adipose tissue, liver, and the heart to better understand their lipotoxic effects in MetS-related diseases.

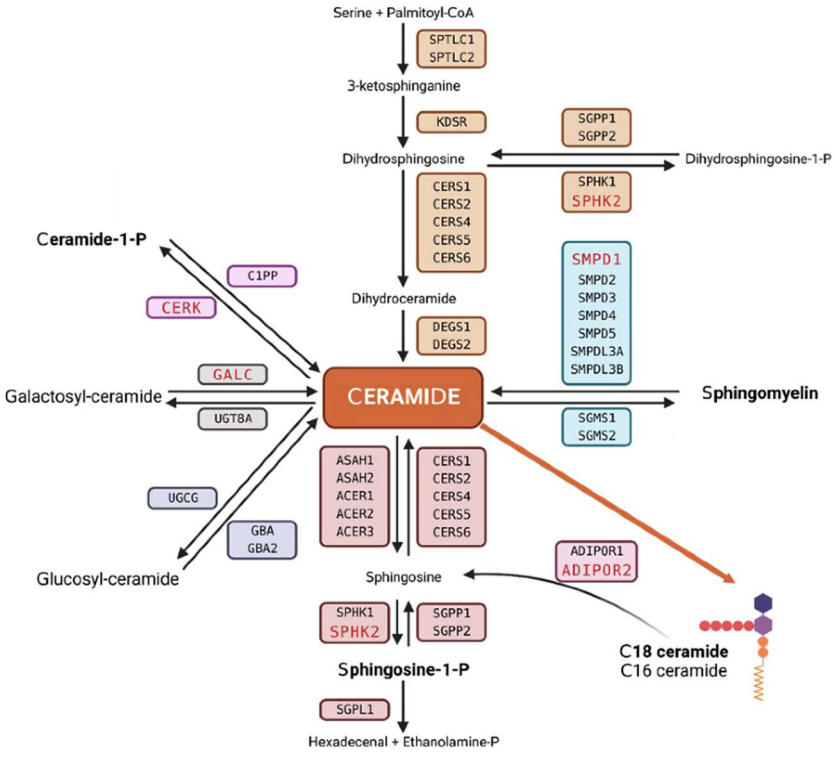

***Figure 4**: Ceramide biosynthetic pathways*

3.1.1. Sphingolipids mediated lipotoxicity in adipocytes

Ceramides play a crucial role in lipid uptake and storage within adipocytes, significantly influencing their metabolic functions. But they are also contributing to lipotoxicity in the context of obesity, overnutrition and high saturated fat intake, which elevate ceramide levels. Their metabolism involves both *de novo* synthesis pathways, where ceramides are synthesized from serine and palmitoyl-CoA, and the other pathways where ceramides are derived from other sphingolipids (**Figure 5**).

In healthy metabolic conditions, ceramide level is tightly regulated by systemic or intracellular pathways in the adipose tissue. Indeed, they act as an important secondary messenger capable of sensing the nutrient status changes and optimally regulate the whole-body metabolic homeostasis. They are directly associated with hormonal factors implicated in obesity and metabolic diseases, independent of the dietary content.

In MetS, the ability of adipose tissue to store and sense nutrients abundance is compromised, causing a spillover of the intermediate lipid metabolites into circulation, resulting in ectopic fat deposition, and causing lipotoxicity. Ceramides are, in those conditions, among the most deleterious intermediates as they modulate the signaling pathways of glucose metabolism, apoptosis, fibrosis and triglycerides synthesis. For example, several data support the hypothesis that due to the inflammation of the adipose tissue in obese subjects, inflammatory cytokine TNF-α induces ceramide accumulation by altering ceramide-generating and metabolizing enzymes. This drives the inhibition of Akt/PKB activation in macrophages and adipocytes, leading to inflammation-induced insulin resistance from the inflamed adipose tissue, thus inhibiting glucose uptake and metabolism by impairing the function of glucose transporter type 4 (GLUT4) (Yazıcı, D., & Sezer, H., 2017). Moreover, ceramides also inhibit the hormone-sensitive lipase (HSL) activation in response to β-adrenergic receptor stimulation, which attenuates lipolysis and reduces the release of FFAs from the lipid droplets within adipocytes. This leads to even more lipid accumulation contributing to a cascade of adverse metabolic effects, including impaired energy homeostasis, increased fatty acid accumulation, and systemic insulin resistance (**Figure 6**).

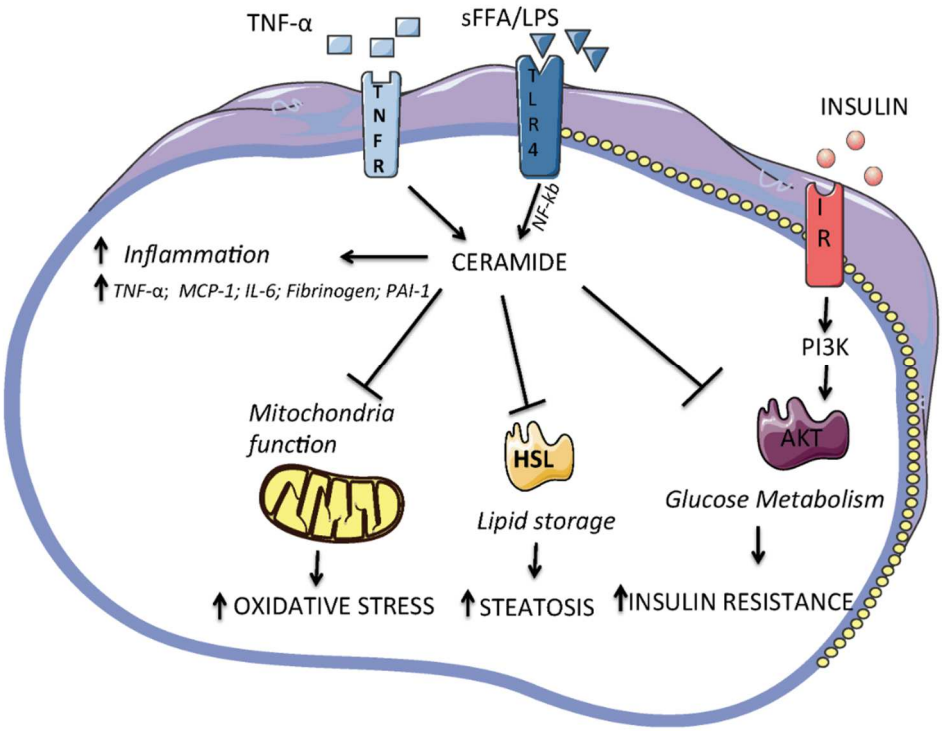

***Figure 6**: Mechanisms of ceramide induced lipotoxicity in adipocytes. Akt: protein kinase B; PI3K: phosphatidylinositol-3-kinase; LPS: lipopolysaccharide; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; PAI-1: plasminogen activator inhibitor 1; sFFA: saturated fatty acids; TLR4: toll-like receptor 4; TNF-α: tumor necrosis factor alpha; IL-6: interleukine6; TNFR: tumor necrosis factor alpha receptor; MCP-1: monocyte chemoattractant protein-1; IR: insulin receptor (Gaggini, M., et al, 2022).*

Targeting the regulation of ceramide synthesis and accumulation in adipocytes through dietary interventions or pharmacological means offers potential therapeutic avenues to restore metabolic health and mitigate the harmful effects of lipotoxicity associated with obesity and related metabolic diseases (Li, Y., et al, 2020).

3.1.2. Sphingolipids mediated lipotoxicity in cardiomyocytes

HFpEF is a clinical syndrome often associated with lipotoxicity, characterized by the toxic accumulation of lipids in the heart. The specific lipid species involved in HFpEF and their precise toxic mechanisms remain largely unknown, it is hypothesized that ceramides play a crucial role in its development. Ceramides in cardiomyocytes are also synthesized *via* the three primary metabolic pathways: 1) *de novo* synthesis in the cytosolic layer of the endoplasmic reticulum *via* SPT, 2) hydrolysis of sphingomyelin via sphingomyelinase, and 3) production from sphingosine via sphinganine N-acyltransferase (ceramide synthase), known as the salvage pathway (**Figure 5**).

Ceramides are pivotal in healthy cardiomyocytes metabolism, acting as essential components of the cell membrane and secondary messengers in various protective responses, including apoptosis, cell growth, proliferation, differentiation, maturation, senescence, necrosis, and stress response. In pathological conditions, ceramides' relationship with apoptosis is significant. Indeed, they mediate lipotoxic signaling pathways that connect lipid-induced inflammation with insulin signaling inhibition. One mechanism involves the acid sphingomyelinase-produced ceramides, crucial for toll-like receptor 4 (TLR4)-induced insulin resistance by inhibiting insulin signaling, activating protein phosphatase 2A (PP2A) and preventing Akt translocation to the plasma membrane, mirroring the effects seen in adipocytes (Stratford, S., et al, 2004). Ceramides also disrupt insulin signaling by activating p38 MAPK and JNK, which are implicated in HF and cardiomyopathy (Chen, C. L., et al, 2008). JNK activation by ceramides can directly induce mitochondrial apoptosis through cytochrome c release and caspase 9 activation (Aoki, H., et al, 2002). Moreover, ceramides also induce apoptosis via caspase 3 and 8 pathways in cardiomyocytes (**Figure 7**) (D'Souza, K., et al, 2016). These molecular signals underscore ceramides' role in cardiomyocyte dysfunction and the development of heart diseases. In conditions like MetS, disrupted ceramide balance in cardiomyocytes due to lipotoxicity accelerates apoptosis through those mechanisms and contributes to disease pathogenesis. Therefore, pharmacological interventions targeting sphingolipid metabolism, such as inhibiting *de novo* ceramide synthesis, could potentially improve myocardial function in ischemic heart disease, including HFpEF (Borodzicz, S. et al, 2015).

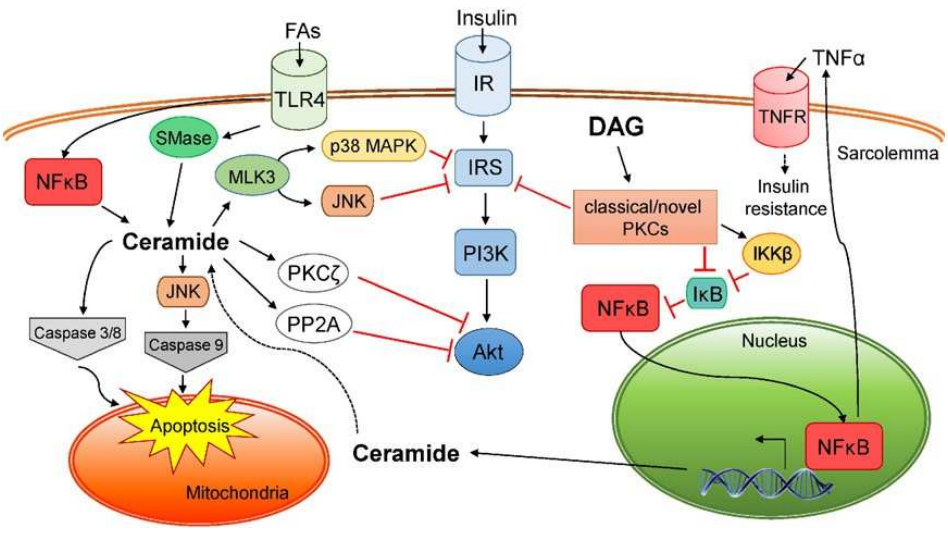

***Figure 7**: Mechanisms of cardiac lipotoxicity induced by ceramides (D'Souza, K., et al, 2016).*

3.1.3. Sphingolipids mediated lipotoxicity in hepatocytes

In healthy hepatocytes, ceramides play a crucial role in maintaining cellular integrity by participating in various cellular processes such as cell signaling, apoptosis, regulation of cell growth, differentiation, and stress responses. They are essential components of the structure of cell membranes and are synthesized through three primary pathways: *de novo* synthesis, sphingomyelin hydrolysis, and the lysosomal salvage pathway (**Figure 5**). In the context of MetS, specifically in MASLD, ceramide metabolism becomes dysregulated, leading to adverse effects on liver function.

In MetS, ceramides exacerbate hepatic insulin resistance, a condition characterized by impaired insulin signaling and glucose uptake. They can bind Akt/PKB via activation of protein kinase Cζ (PKCζ), obstructing Akt/PKB translocation to the plasma membrane, where it mediates insulin response (Stratford, S., et al, 2004). Additionally, ceramides activate protein phosphatase 2A (PP2A), which further impairs Akt/PKB translocation (Summers, S. A., et al, 1998). Ceramides also activate the RNA-dependent protein kinase (PKR) and phosphorylate insulin receptor substrate 1 (IRS1) through JNK-dependent pathway, resulting in IRS1 inactivation and subsequent insulin resistance (Yang, X., et al, 2010). Moreover, ceramides increase oxidative stress, disrupting the mitochondrial electron transport chain, accelerating ganglioside GD3 synthesis in the endoplasmic reticulum, which then promotes ROS production in mitochondria (Rippo, M. R., et al, 2000). Furthermore, ceramides influence fatty acid uptake and *de novo* lipogenesis in hepatocytes. They enhance the plasma membrane localization of CD36, a fatty acid transporter, leading to increased fatty acid uptake (Xia, J. Y., et al, 2015). Ceramides also modulate *de novo* lipogenesis by upregulating sterol regulatory element-binding protein-1c (SREBP-1c), a key regulator of triglyceride and cholesterol synthesis (Worgall, T. S., et al, 2004). In addition, ceramides promote liver inflammation and fibrosis, increase hepatic ceramide levels can lead to inflammation via TLR4 activation, similarly as in the cardiomyocytes (Wan, X., et al, 2016). Finally, ceramides activate hepatic stellate cells (HSC), promoting their transformation into profibrotic myofibroblasts and enhancing collagen production, which contributes to liver fibrosis (Moles, A., et al, 2010).

Overall, the dysregulated ceramide metabolism observed in MetS, particularly in MASLD, underscores their role in exacerbating liver dysfunction. Targeting ceramide synthesis and signaling pathways offers a promising therapeutic strategy for mitigating liver diseases associated with MetS by reducing ceramide levels and their toxic effects (Yu, X. D., & Wang, J. W., 2022) (**Figure 8**).

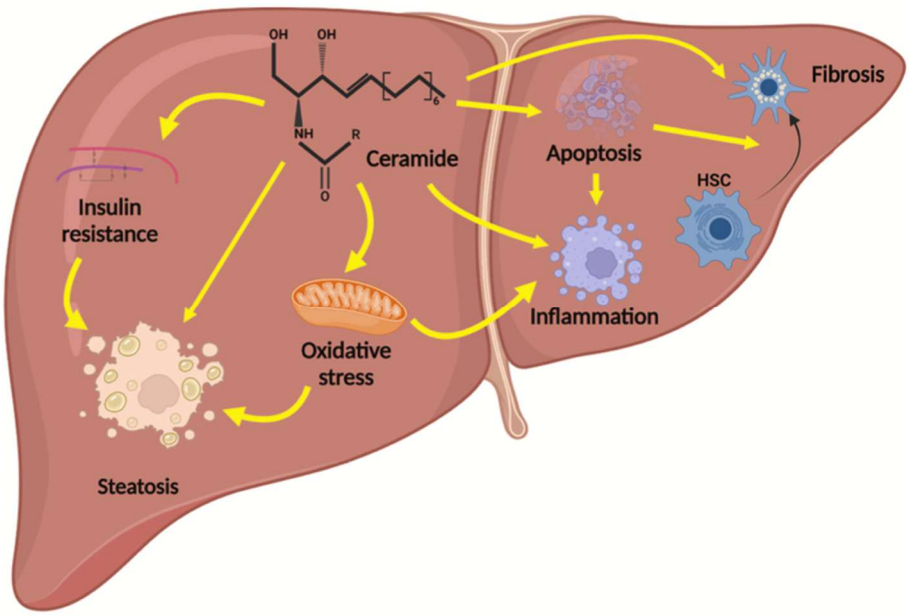

***Figure 8**: Mechanisms of ceramides mediated lipotoxicity in hepatocytes, inducing the development of NAFLD, insulin resistance, increasing oxidative stress, promoting apoptosis, and ultimately leading to steatosis, inflammation, and fibrosis. HSC: hepatic stellate cells. (Yu, X. D., & Wang, J. W. 2022).*

3.3. Exploring therapeutic strategies to target sphingolipids lipotoxicity

MetS comprises a set of conditions that increase the risk of cardiovascular disease (CVD), chronic kidney disease, and type 2 diabetes (T2D). The mechanisms underlying MetS are complex and not fully elucidated, but systemic inflammation, or chronic low-grade inflammation, is recognized as a crucial factor. Adipose tissue expands in response to excess caloric intake, leading to hypertrophy and hyperplasia. Excess visceral adipose tissue, typical of MetS, causes metabolic disorders and structural changes. Ectopic lipid accumulation in muscle and liver predisposes individuals to insulin resistance (IR), which is central to the onset and progression of MetS, CVD, and T2D. Insulin resistance in adipose tissue disrupts insulin-mediated lipolysis, increasing circulating FFAs and inhibiting insulin's antilipolytic effect. In skeletal muscle and liver, Insulin resistance impairs glucose transport and glycogen synthesis, prompting increased

insulin secretion, which over time leads to T2D. Additionally, obesity and insulin resistance-induced systemic oxidative stress activate signaling cascades that promote tissue fibrosis. The pro-inflammatory state associated with MetS also contributes to increased cardiovascular risk. Treatments for MetS aim to reduce and prevent chronic metabolic disorders, with different dietary patterns and nutrients studied for their therapeutic potential. However, aside from lifestyle modifications, a global MetS treatment does not exist, and existing drugs target specific organs, increasing the risk of drug interactions and side effects pathways (Ambroselli, D. et al, 2023).

One of the comorbidities associated with MetS that we focus on this study is MASH. It is a progressive form of MASLD, characterized by inflammation and fibrosis, which can anticipate severe liver conditions such as cirrhosis and hepatocellular carcinoma (HCC). The pathogenesis of MASH involves several pathways including insulin resistance, oxidative stress, inflammasome activation, endoplasmic reticulum (ER) stress, and mitochondrial dysfunction. These factors collectively lead to the activation of hepatic stellate cells (HSCs) and the development of liver fibrosis. Despite significant research efforts, there are no approved therapy available for MASH. Treatments targeting different pathways, such as peroxisome proliferator-activated receptors (PPARs) agonists, farnesoid X receptor (FXR) agonists, and glucagon-like peptide-1 receptor (GLP-1R) agonists, have been tested in clinical trials but often exhibit unacceptable side effects, including cardiovascular risks, pruritus, and severe gastrointestinal disorders, or fail to demonstrate significant efficacy in improving MASH (Alzahrani, B., et al, 2018). Consequently, there is an urgent need for novel therapeutic approaches to treat this devastating disease.

The second condition associated with MetS we are aiming to treat is HFpEF, which is a cardiovascular disease mainly caused by hypertension and can be linked to conditions such as obesity, obstructive sleep apnea, and diabetes. HFpEF arises from hypertension-induced increased afterload, leading to diastolic dysfunction and triggering compensatory mechanisms that cause ventricular remodeling, hypertrophy, and fibrosis. The rigidity induced increases pressure in the ventricles, which in turn increases arterial pressure and can lead to pulmonary hypertension. Although diastolic dysfunction is the main mechanism responsible for HFpEF, there are multiple cardiac, vascular, and non-cardiac abnormalities that contribute. Current treatments for HFpEF

are limited. The lack of therapeutic options is largely related to the complexity and heterogeneity within the HFpEF syndrome (Omote, K., et al, 2022).

Given the limitations of current treatments for MetS, MASH, and HFpEF, there is an urgent need to find effective therapeutic strategies. Recent studies have identified adiponectin receptors (AdipoR1 and AdipoR2) as potential therapeutic targets for MASH. Adiponectin, an adipocytokine primarily produced by mature adipocytes, plays a crucial role in cellular energy management, extracellular matrix (ECM) metabolism, and regulation of proliferation and migration. Adiponectin levels are decreased in individuals with MASLD, and this evidence suggests that activating AdipoRs may have beneficial effects on lipid metabolism and liver fibrosis. AdipoR1 and AdipoR2 mediate fatty acid oxidation and glucose metabolism mainly through the AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor α (PPARα) signaling pathways. The activation of these pathways not only improves mitochondrial function but also reduces inflammation and fibrosis in the liver (Xu, H., et al, 2020). Adiponectin is also relevant in HFpEF. Just like in individuals with MASLD, adiponectin levels decrease with an excessive body weight and obesity-related cardiovascular diseases. There are several mechanisms through which adiponectin may prevent most of the pathophysiologic mechanisms underlying diastolic dysfunction and HFpEF, including the prevention of myocardial hypertrophy, cardiac fibrosis, oxidative stress, atherosclerosis, and inflammation, while promoting angiogenesis (Negi, S. I., et al, 2012). Understanding the mechanisms underlying adiponectin-mediated improvement of diastolic function has become an exciting field of research, making adiponectin a promising therapeutic target (Francisco, C., et al, 2016). Moreover, AdipoRs and AdipoR2 possesses intrinsic basal ceramidase activity that is enhanced by adiponectin (Vasiliauskaité-Brooks I, et al. 2017). This ability depends on the structure of the cytosolic domain of the molecule, which presents as an enzyme with a binding site for C18 ceramide. When activated by adiponectin, AdipoR2 and transform C18 ceramide into FFA and sphingosine (Vasiliauskaité-Brooks I, et al. 2017).

Considering these findings, we have focused on JT003, an AdipoR1/2 dual agonist, as a potential therapeutic agent for MASH via ceramides clearance. JT003 binds to both AdipoR1 and AdipoR2, enhancing their activation and subsequent signaling pathways. JT003 exhibited potent anti-lipogenesis and anti-fibrogenesis effects,

ameliorated MASH progression and related fibrosis via AMPK and PPARα signaling pathways (Xu, H., et al, 2020). Additionally, JT003 activation of the PI3K-Akt pathway contributed to the improvement of insulin resistance. Whether JT003 also reduce ceramides content in treated cells is unknown. Given the complex pathophysiology of MASH, JT003 may offer a comprehensive treatment strategy, addressing the underlying mechanisms of the disease and providing a rational pharmacological approach to improving MASH and related fibrosis. Therefore, we hypothesize that beneficial effects of JT003 in MASH may depends also on ceramides clearance. We hypothesize that JT003 could also be a good therapeutic target for HFpEF due to the similar mechanisms underlying both conditions. JT003's ability to activate both adiponectin receptors and the subsequent beneficial effects on metabolic pathways, fibrosis, and inflammation suggest that it may offer a comprehensive treatment strategy for HFpEF as well. Therefore, JT003 may provide a novel and effective approach to managing both MASH and HFpEF, addressing the urgent need for new therapeutic options in these challenging diseases both involved in MetS.

4. Materials and Methods

4.1. Cell Cultures and Treatment

In this study, we cultured three different cell types: 3T3-L1 preadipocytes (ATCC CL-173), HEK293 human embryonic kidney cells, AC16 human cardiomyocyte cells, and HepG2 human hepatocyte carcinoma cells, each under specific conditions.

The 3T3-L1 preadipocytes were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 150 units/mL streptomycin, 200 units/mL penicillin, 2 mmol/L L-glutamine, and 1 mmol/L HEPES, all sourced from Life Technologies. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. Upon reaching confluence, adipogenic differentiation was initiated using a cocktail comprising 1 mmol/L dexamethasone (DEXA), a glucocorticoid that stimulates differentiative processes through activation of transcription factor C/EBPβ; 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich), which increases cAMP turnover by inhibiting the degradation enzyme cyclic AMP phosphodiesterase, resulting in the activation of transcription factor C/EBPδ; and 70 nmol/L insulin (Novo Nordisk), which stimulates differentiation into adipocytes through the induction of phospho-kinase cascade activity. IBMX was removed after three days, while insulin treatment continued until full differentiation. HEK293 cells were cultured in DMEM supplemented with 10% FBS, 150 units/mL streptomycin, 200 units/mL penicillin, and 2 mmol/L L-glutamine under the same environmental conditions (37°C, CO2 5%). AC16 cells were grown in DMEM supplemented with 12% FBS, 150 units/mL streptomycin, 200 units/mL penicillin, 2 mmol/L L-glutamine, and 1 µL/mL amphotericin B. HepG2 cells were cultured in MEM supplemented with FBS 10%, 150 units/mL streptomycin, 200 units/mL penicillin, 2 mmol/L L-glutamine and 1% non-essentials amino-acids.

Treatments for the cells were conducted at 100% confluence for 3T3-L1, AC16 and HepG2 cells, and at 80% confluence for HEK293 cells. 3T3-L1 cells were treated with either 300 µM of BSA Control for BSA-Fatty Acid complexes (Caymon Chemical), 5 µM of myriocin (M1177 from Merck) combined with 300 µM of BSA Control, 300 µM of BSA-Palmitate saturated fatty acid (Caymon Chemical), or 5 µM of myriocin combined with 300 µM of BSA-Palmitate. Additionally, 3T3-L1 cells received treatments of either 300 µM of BSA Control, 100 µM of JT003 trifluoroacetate (SML-3078 from Sigma-Aldrich)

combined with 300 µM of BSA Control, 300 µM of BSA-Palmitate, or 100 µM of JT003 combined with 300 µM of BSA-Palmitate.

Similarly, HEK293 cells were treated with either 300 µM of BSA Control, 5 µM of myriocin combined with 300 µM of BSA Control, 300 µM of BSA-Palmitate, or 5 µM of myriocin combined with 300 µM of BSA-Palmitate. HEK293 cells also received treatments of either 300 µM of BSA Control, 100 µM of JT003 combined with 300 µM of BSA Control, 300 µM of BSA-Palmitate, or 100 µM of JT003 combined with 300 µM of BSA-Palmitate.

AC16 cells were treated with either 300 µM of BSA Control, 50 µM of JT003 combined with 300 µM of BSA Control, 300 µM of BSA-Palmitate, or 50 µM of JT003 combined with 300 µM of BSA-Palmitate. All treatments were performed for 24 hours.

Similarly, HepG2 cells were treated with either 300 µM of BSA Control, 25 µM of JT003 combined with 300 µM of BSA Control, 300 µM of BSA-Palmitate, or 25 µM of JT003 combined with 300 µM of BSA-Palmitate. All treatments were performed for 24 hours.

Post-treatment, cells were either harvested for real-time quantitative PCR analysis or fixed in PFA 4% for immunofluorescence. The focus of these analyses was to assess endpoints such as gene expression, as well as lipid accumulation.

4.2. RNA Isolation and Real-Time Quantitative PCR

Total RNA was isolated from cultured cells using the RNeasy Mini kit (Qiagen). Briefly, cells were lysed in 300 µL of RLT Buffer, and an equal volume of 70% ethanol was added to the lysates. Each sample was then transferred to a RNeasy Spin Column and centrifuged at 10 000 rpm for 15 seconds at room temperature. The liquid was discarded, and the RNA-binding membrane was washed with two different buffers before the RNA was eluted with 30 µL of RNase-free water. The RNA concentration and quality were assessed using a DeNovix QFX fluorometer. Each sample was then diluted in RNAse-free water to have 26 µL of total final volume and a homogenous amount of RNA in each tube, based on the results of the quantification previously performed. Therefore, tests on cells with myriocin and JT003 were performed on specific quantities of RNA in the tubes. To eliminate any residual DNA, the RNA samples were treated with TURBO DNase (Ambion). Specifically, 2 µg of RNA was mixed with 3 µL of DNase reaction buffer and 1µL

of DNase, and the volume was adjusted to 30 µL with RNase-free water. The mixture was incubated at 37°C for 30 minutes, followed by the addition of 3 µL of DNase Inactivation Reagent, incubation at room temperature for 2 minutes, and centrifugation at 10 000g for 90 seconds. The treated RNA was then reverse-transcribed into cDNA using 150 ng of random primers (Promega Corporation, Madison, WI, USA) and 4,5 µL of RNase-free water were added. The samples were placed at 70°C for 5 min, after which the temperature was lowered to 4°C. At this point, in each reaction were added 10 µL of M-MLV RT Buffer, 0,5 mmol/L dNTPs, 200 U of M-MLV RT, and 20 U of RNAsin (Promega), in a final volume of 50 µL. The reaction was incubated at 37°C for 1 hour, followed by 95°C for 2 minutes, and the synthesized cDNA was stored at -20°C. The levels of mRNA were quantified by Real-time quantitative PCR (qPCR), performed using a StepOne™ Real-Time PCR System (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems) and specific intron-spanning primers (detailed in Table 7). Each 20 µL qPCR reaction included 5 µL of cDNA (5 ng/µL), 10 µL of SYBR Green PCR Master Mix, and 5 µL of forward and reverse primers solution (300 µM each). The qPCR protocol consisted of an initial denaturation at 95°C for 2 minutes, followed by 45 cycles of 95°C for 15 seconds, annealing for 30 seconds (temperature specific to each primer set), and 72°C for 30 seconds.

***Table 1**: Intron-spanning primers used in the gene expression analysis*

Gene	Forward primer 5' – 3'	Reverse primer 5' – 3'
GAPDH	CTCTCTGCTCCTCCTGTTCGAC	TGAGCGATGTGGCTCGGCT
CERS2	CCGAGATGGACGTGTCTACG	CCAGGGGCAGCGTGATATAG
CERS5	AATTCTGTGAAAGCATGTGGAGAT	TCGGATGTCCCAGAACCAAG
CERS6	GCTGACGAGGTTCTGTGAGAG	CCACAACCAGGGGGTCTTTTT
ADIPOR2	CCCGGATCCCCGAACGCTTTTT	CCCCCGCCGATCATGAAACGAA
DEGS1	AAGACTTCGAGTGGGTCTACA	GGATCAGGTTTCATCAAGGACTTT
KDSR	CTGGTTGCACGAAATGAGGATAAG	ATCAACTGATATGCAAAGCACCAC
SPTLC1	GAAGTATGGCGTGGGGACTT	CAGGCGGTCTTCCAAATCCA
ACER2	TGCTATCGCCGAGTTCTACA	GTTGAAGCATGTTGCATACTGA
ACER3	GGTACATCGCCGAGTTCTTGGT	ACAGCTGTATATCATTGGGAGTTCA
SMPD1	ATGCTCTTTCCCCATACCCC	AAGAGCCAGAAGTTCTCACGG

Melting curve analysis was performed to verify the specificity of the amplifications. Gene expression levels were quantified using the delta-delta Ct method, normalized by dividing the arbitrary units of mRNA from each gene of interest by the amount of the housekeeping gene GAPDH. Each sample was analyzed in duplicate, with negative controls included in each run.

The delta-delta Ct method, also known as the $2^{(-\Delta\Delta Ct)}$ method, is a technique for analyzing relative changes in gene expression from real-time quantitative PCR experiments. This method involves three main steps: first, the cycle threshold (Ct) values of the target gene and the housekeeping gene (here GAPDH) are determined for each sample. Next, the difference in Ct values (ΔCt) between the target and housekeeping genes is calculated for each sample. Finally, the ΔCt values of the experimental samples are compared to the ΔCt values of a control or reference sample to obtain the ΔΔCt value. The relative expression level of the target gene is then calculated as $2^{(-\Delta\Delta Ct)}$, providing a measure of fold change in gene expression normalized to the housekeeping gene and relative to the control sample.

4.3. Ceramides staining

After the 24 hours of treatment with Palmitate or BSA in combination with either myriocin or JT003, cells were fixed on coverslips using 500 µL of 4% paraformaldehyde (PFA) for 30 minutes at room temperature and permeabilized with 0,25% Triton X-100 for 10 minutes. For ceramide detection, the cells were incubated with a monoclonal primary antibody (C8104 Anti-Ceramide, Merck) at a 1:100 dilution in PBS-BSA 5% for 3 hours at room temperature. Following incubation, cells were washed three times with PBS-BSA 0,2% for 5 minutes each. The binding of primary antibodies was visualized using Alexa-488–conjugated secondary antibodies (Life Technologies) at a 1:2000 dilution in PBS-BSA 5% for 1 hour at room temperature, protected from light. After incubation with the secondary antibody, cells were washed three times with PBS-BSA 0,2% for 5 minutes each, followed by a 2-minute incubation with Hoechst-33342 dye diluted 1:6000 in PBS for nuclear staining. Subsequent washes were performed three times with PBS-BSA 0,2% for 5 minutes each, followed by a final wash in distilled water. The coverslips were then mounted using 30 µL of Fluorescence Mounting antifade medium (Life Technologies) and

examined using a Leica DMI6000 CS SP8 laser scanning microscope (Leica Microsystems). Image analysis was performed using ImageJ software (https://imagej.nih.gov/ij/).

4.4. Mice's heart slices Oil Red O and ceramides staining

C57BL-6J-mice were provided with water and standard diet (STD) or high-fat diet (HFD), were maintained in a 12h light/dark cycle, and housed in enriched cages. After 8 weeks of HFD, mice were treated with myriocin (0.3 mg/kg every other day, intraperitoneal injection) or vehicle (PBS in control group) and maintained in HFD for additional 8 weeks. All experimental animal procedures were performed in accordance with local regulations, rules, and guidelines. Mice used in this study were 15 weeks old. Left ventricles (LVs) were isolated from mice hearts, immediately fresh frozen or embedded in Optimum Cutting Temperature compound (OCT), and stored at -80°C. Slices of 9 µm from the LVs were made with a Leica CM 1950 OUV cryostat (Leica Biosystems), mounted on glass slides, and stored at -80 °C.

For the Oil Red O (ORO) staining, slides were fixed with formalin, incubated at room temperature (RT) with Oil Red O solution for 20 min, rinsed with PBS and nuclei were counterstained with hematoxylin for 1 min. The slides were then rinsed with running tap water for 5 minutes, rinsed in distilled water and covered with an aqueous mounting medium.

For immunofluorescence staining of ceramides, heart slices were fixed with 4% paraformaldehyde, permeabilized with Triton X-100, and incubated with a monoclonal anti-ceramide antibody. This was followed by washing and incubation with an Alexa-488-conjugated secondary antibody. The process included nuclear staining with Hoechst-33342 and mounting with antifade medium for microscopy. This method mirrors the one used for cells, ensuring consistency in detecting ceramide levels.

The images of Oil Red O staining were taken with an optical microscope and analyzed with ImageJ. Images of immunofluorescent staining were taken with a Leica DMI6000 CS SP8 laser scanning microscope (Leica Microsystems) and analyzed with ImageJ.

4.5. Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 9 software. Results are presented as means ± SEM. Depending on the experimental design, data were analyzed using an unpaired Student's t-test, one-way ANOVA, or two-way ANOVA with Newman-Keuls post hoc test, as appropriate.

5. Results

5.1. The effect of HFD and myriocin on mice hearts

To investigate the role of ceramide metabolism and triglyceride accumulation we examined the hearts of mice on either a standard diet (STD) or high-fat diet (HFD). This study aimed to understand the underlying mechanisms of lipid dysregulation, particularly focusing on the ceramide biosynthesis pathway.

To explore lipid accumulation, we employed Oil Red O staining to quantify triglycerides and immunofluorescence to assess ceramides content. These methods provided rapid insights into lipid content, complementing more detailed lipidomic analyses. We found that STD-fed mice had lower triglyceride levels compared to HFD-fed mice, and myriocin treatment reduced triglyceride levels in HFD-fed WT mice (**Figure 9**).

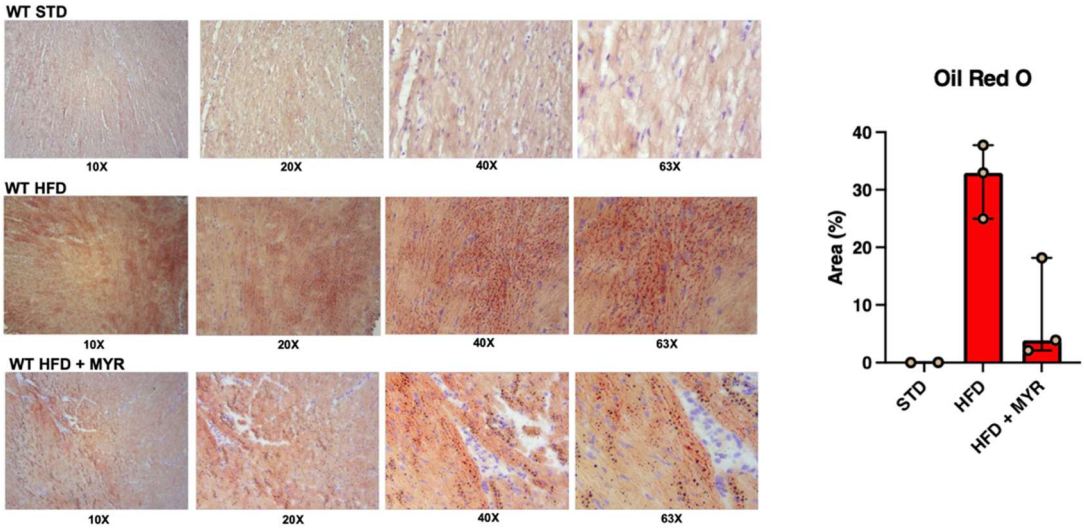

***Figure 9**: LVs from WT mice treated with STD or HFD with or without myriocin, stained with Oil Red O. Pictures were taken with a light microscope at various magnifications and quantification of the triglyceride accumulation within the heart (shown by the red droplets).*

We performed immunofluorescence staining on the hearts of these mice to evaluate ceramide levels. The results indicated an increased ceramide signal in HFD treatment, which was reduced by subsequent myriocin treatment (**Figure 10**).

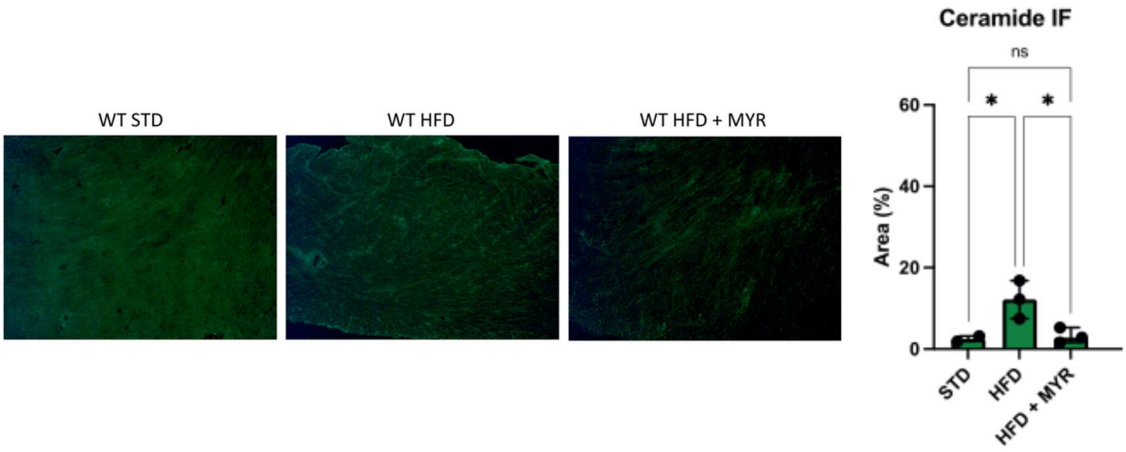

***Figure 10**: LVs from WT mice treated with STD or HFD with or without myriocin, stained with immunofluorescence, viewed under laser scanning microscope and quantification of the ceramide accumulation within the heart (shown in green).*

Our results indicate that a HFD promotes the accumulation of triglycerides and ceramides in myocardial tissue. Treatment with myriocin effectively reduced these lipid levels, highlighting the complex interplay between triglyceride accumulation and ceramide biosynthesis. Immunofluorescence analysis further demonstrated increased ceramide levels in HFD-fed mice, emphasizing the role of ceramide metabolism in lipid homeostasis. The reduction of ceramide levels by myriocin suggests that systemic inhibition of SPTLC1 regulates both triglyceride and ceramide levels in the heart. Whether this depends on cardiac specific inhibition of SPTLC1 or is a consequence of liver/adipose tissue inhibition of ceramides de novo biosynthesis is unclear.

5.2. The effect of palmitate and myriocin on different cell lines

To investigate and modulate ceramide metabolism *in vitro*, we modeled ceramide accumulation using palmitate treatment (300 μM) on 3T3-L1 and HEK293 cells for 24 hours. The use of palmitate as a substrate allowed us to induce ceramide synthesis and subsequently evaluate the modulation of ceramide levels. Immunofluorescence (IF) was employed to detect ceramide concentration using a ceramide-specific antibody (Monoclonal Anti-Ceramide Ab, Sigma #C8104). Given that myriocin is a known inhibitor of SPTLC1 enzyme in the *de novo* ceramide synthesis, acting before the production of ceramides, we treated 3T3-L1 cells and HEK293 cells with myriocin to demonstrate the effects of pre-synthesis inhibition.

On 3T3-L1 cells, palmitate treatment did not significantly affect the gene transcription of key enzymes involved in the ceramide *de novo* synthesis pathway. Similarly, myriocin treatment did not alter mRNA levels of these enzymes (**Figure 11**). Immunofluorescence analysis showed that palmitate induced an increase in cellular ceramide content, whereas myriocin did not significantly reduce ceramide levels (**Figure 12**). These findings suggest that while palmitate promotes ceramide accumulation, myriocin does not effectively counteract this increase in 3T3-L1 cells.

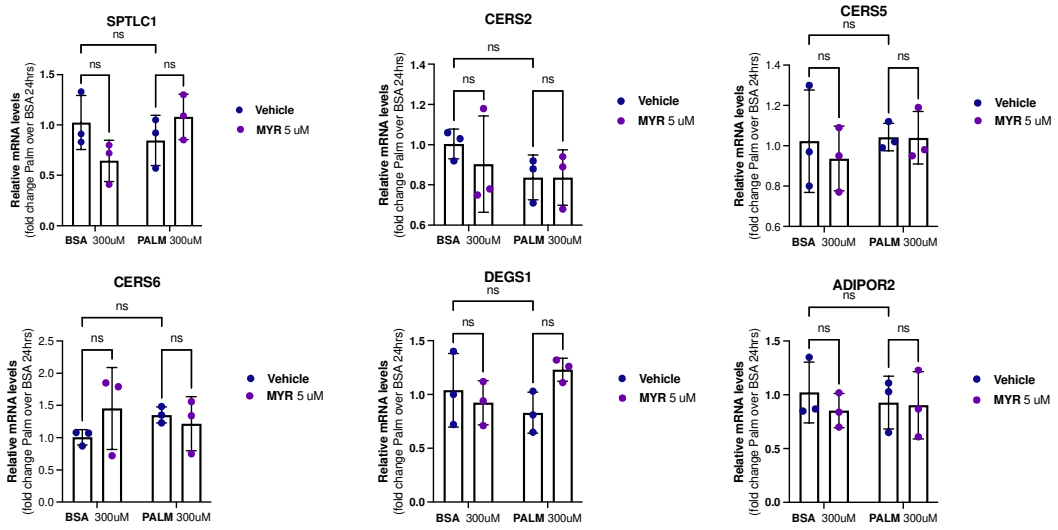

***Figure 11**: Gene expression in 3T3-L1 cells of enzymes involved in ceramide biosynthetic pathways treated with palmitate and myriocin.*

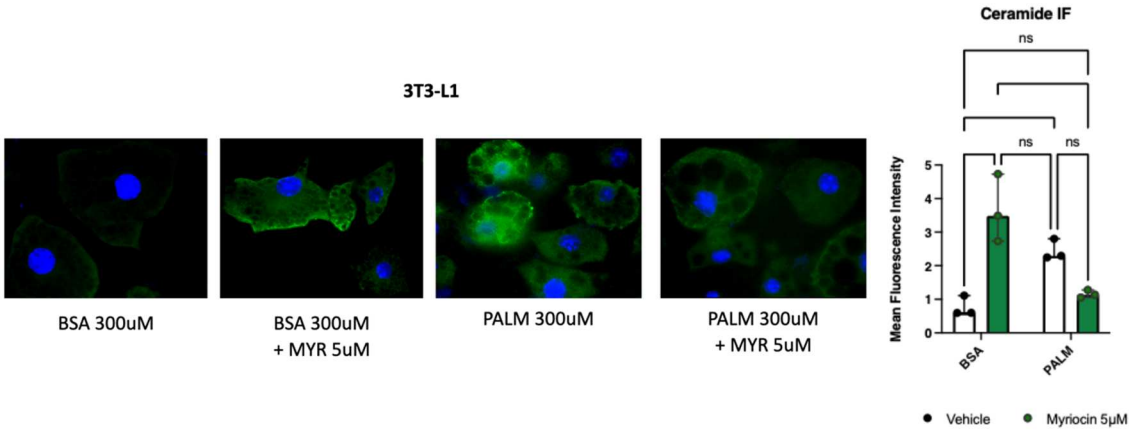

***Figure 12**: Immunofluorescence staining of ceramides (in green) in 3T3-L1cells and quantification, treated with palmitate and myriocin.*

On HEK293 cell lines, we observed that palmitate treatment upregulates the gene transcription of SPTLC1, KDSR, CERS6 and DEGS1, key enzymes involved in ceramide *de novo* synthesis pathway. Myriocin, however, did not significantly affect mRNA levels of any of the above-mentioned enzymes (**Figures 13**). Nevertheless, immunofluorescence analysis indicated that palmitate induced an increase in cellular ceramide content, and a reduction in ceramide signal following myriocin treatment, reinforcing its inhibitory effect on ceramide accumulation (**Figures 14**).

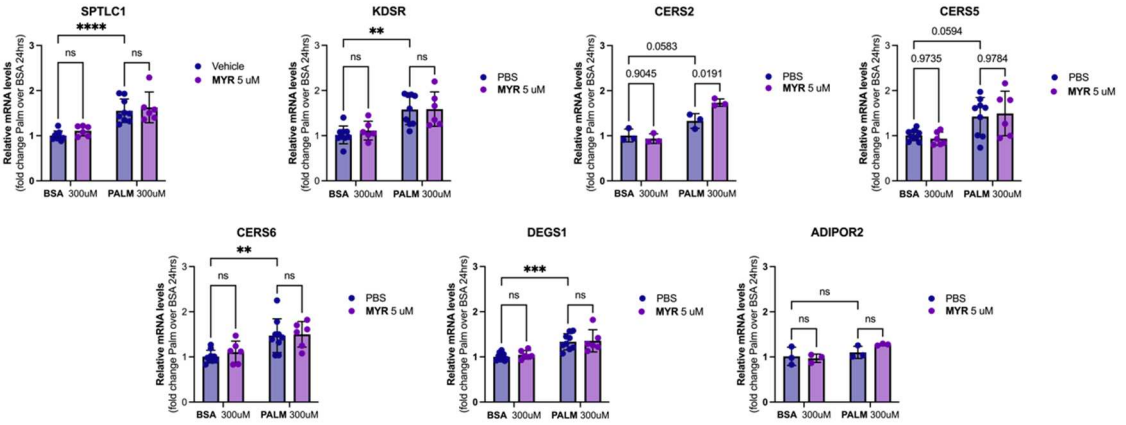

***Figure 13**: Gene expression in HEK293 cells of enzymes involved in ceramide biosynthetic pathways treated with palmitate and myriocin.*

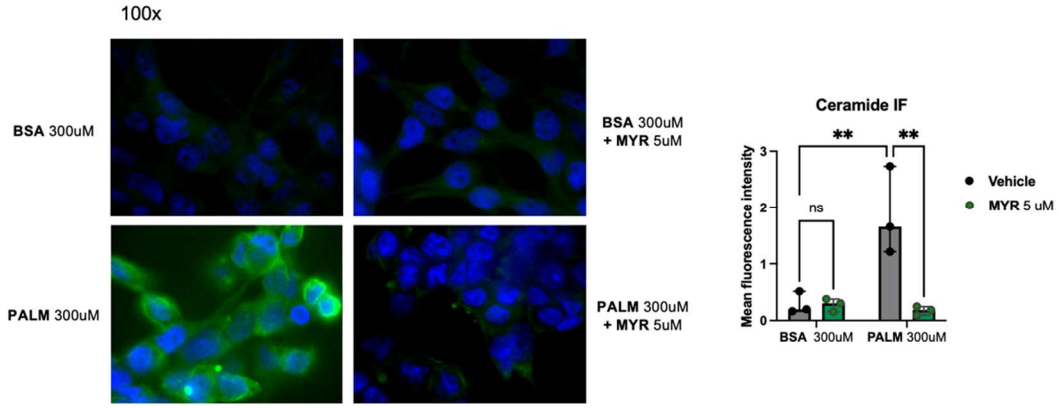

***Figure 14**: Immunofluorescence staining of ceramides (in green) in HEK293 cells and quantification, treated with palmitate and myriocin.*

Our findings validate the use of palmitate-treated HEK293 cells as a model to study ceramide metabolism. The application of myriocin provided insight into the inhibition of ceramide synthesis pre-synthesis, as evidenced by the diminished ceramide signal in immunofluorescence in HEK293 cells only. This model serves as a valuable platform for evaluating ceramide levels.

5.3. The effects of palmitate and JT003 on different cell lines

To investigate ceramide metabolism modulation *in vitro*, we utilized different cell lines including HEK293, AC16, and HepG2, and treated them with palmitate and different doses of JT003. Indeed, considering the lower SPTLC1 levels in the myocardium, and the low levels of *de novo* ceramide synthesis in the heart, reducing ceramide content using SPTLC1 inhibitors like myriocin and acting on a minor pathway before the production of ceramides, is not a feasible option in our HFpEF model. JT003 is an adiponectin receptor agonist. Considering the effects of AdipoR2 activation on ceramide metabolism post-synthesis by activating ceramidase activity, JT003 may represent an alternative approach to reducing ceramide content. Additionally, literature reports have observed recovery in mice from nonalcoholic steatohepatitis and related fibrosis following treatment with this agonist (Xu, H., et al, 2020). However, the effect of JT003 on ceramides levels *in vivo* or *in vitro* has never been investigated before.

We treated HEK293 cells with 100 µM JT003 and 300 µM palmitate. Palmitate, serving as a substrate, elevated the expression of SPTLC1 and KDSR genes involved in ceramide biosynthesis. However, JT003 did not affect the transcription of these *de novo* synthesis enzymes (**Figure 15**). Despite this, immunofluorescence (IF) analysis revealed that JT003 significantly reduced ceramide levels (**Figure 16**). This observation suggests that JT003's mechanism of action does not involve transcriptional regulation but rather post-synthesis modification, likely through enhanced ceramide degradation via ceramidase activity.

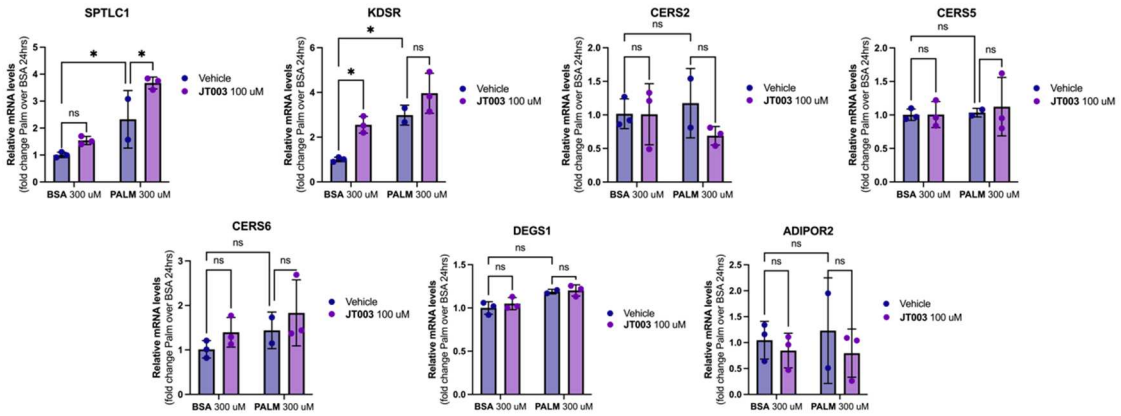

***Figure 15**: Gene expression in HEK293 cells of enzymes involved in ceramide biosynthetic pathways treated with palmitate and JT003.*

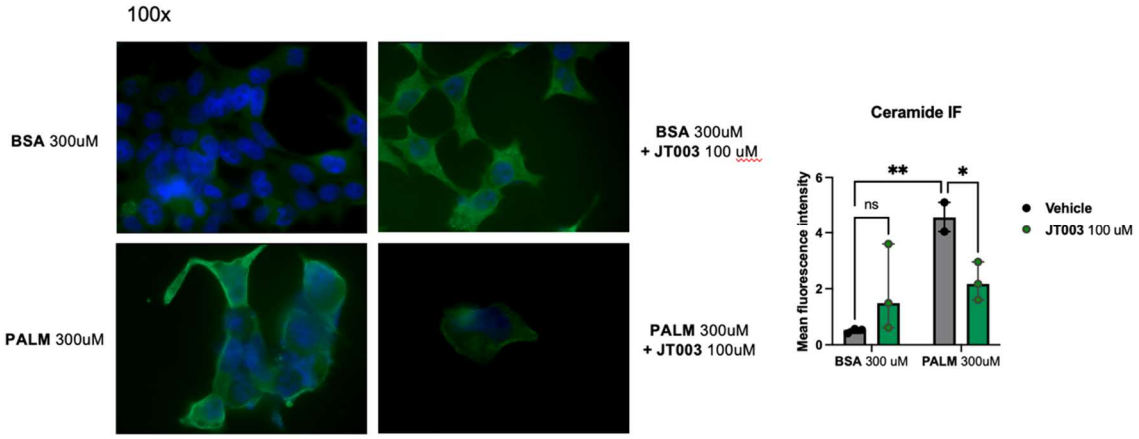

***Figure 16**: Immunofluorescence staining of ceramides (in green) in HEK293 cells and quantification, treated with palmitate and JT003.*

For AC16 cells, an immortalized cell line of human cardiomyocytes, we observed similar results. When treated with 300 µM palmitate and 50 µM JT003, RT-qPCR analysis revealed that JT003 downregulated the expression of KDSR in the absence of palmitate, while CERS2 expression was upregulated by JT003 in the presence of palmitate (**Figure 17**). The expression levels of other genes involved in ceramide biosynthesis were not significantly altered by these treatments. Palmitate treatment increased ceramide levels as detected by IF, but JT003 treatment led to a marked reduction in ceramide signal (**Figure 18**). This indicates that JT003 effectively reduces ceramide accumulation in cardiomyocytes, supporting its potential therapeutic application for heart cells where *de novo* synthesis is not significantly upregulated.

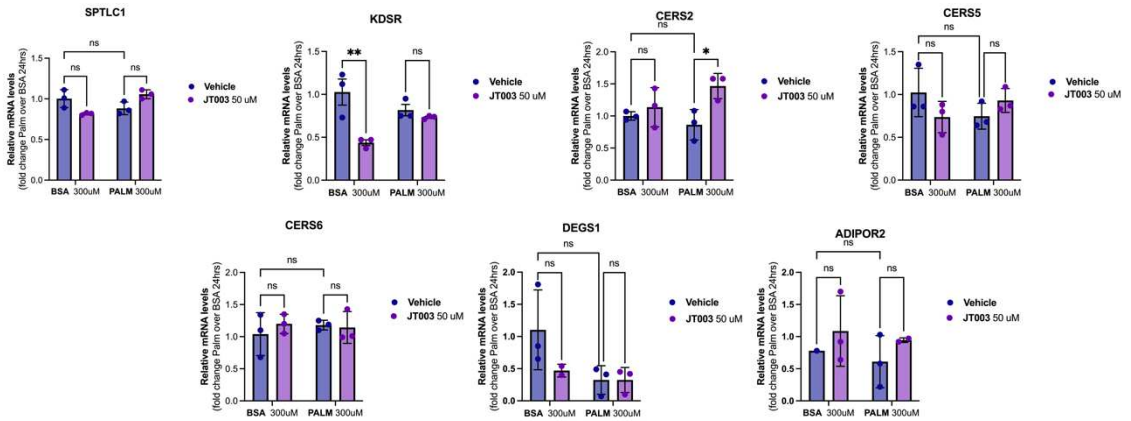

***Figure 17**: Gene expression in AC16 cells of enzymes involved in ceramide biosynthetic pathways treated with palmitate and JT003.*

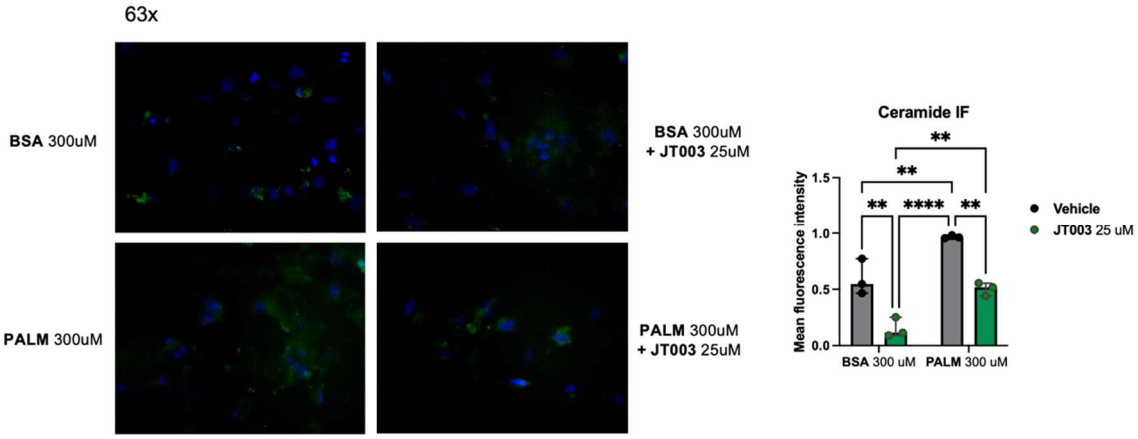

***Figure 18**: Immunofluorescence staining of ceramides (in green) in AC16 cells and quantification, treated with palmitate and JT003.*

In HepG2 cells treated with 300 µM palmitate and 25 µM JT003, the expression of ceramide synthase 2 (CERS2) and ceramide synthase 5 (CERS5) were downregulated by palmitate according to the RTqPCR results (**Figure 19**). For the immunofluorescence (IF) results, they are demonstrating that palmitate enhanced ceramide synthesis. Notably, JT003 treatment reduced the ceramide signal following palmitate treatment (**Figure 20**). This suggests that JT003 effectively modulates ceramide degradation, aligning with its proposed mechanism of action.

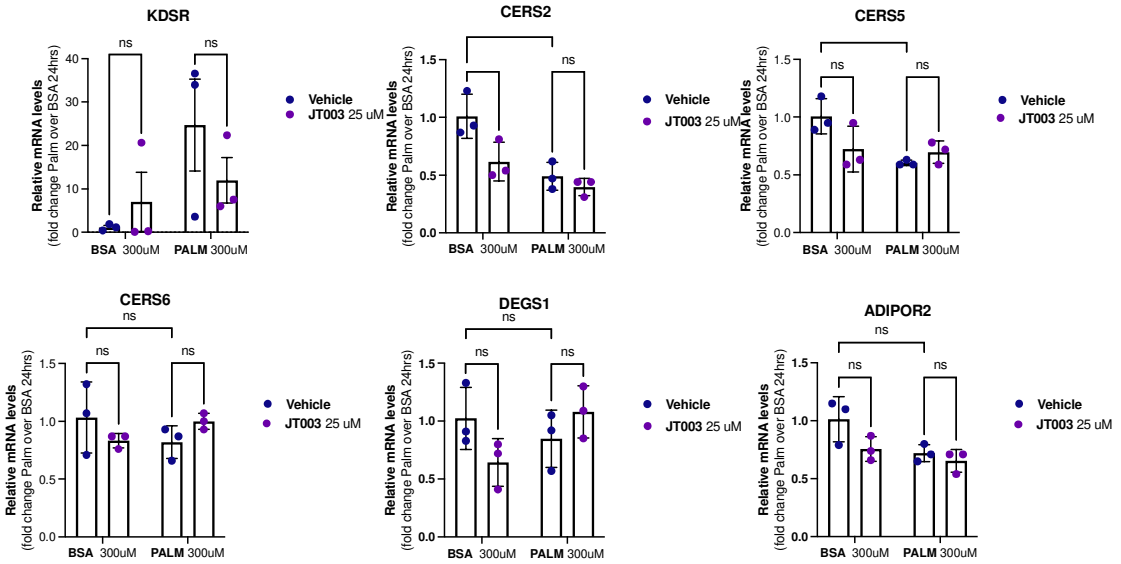

***Figure 19**: Gene expression in HepG2 cells of enzymes involved in ceramide biosynthetic pathways treated with palmitate and JT003.*

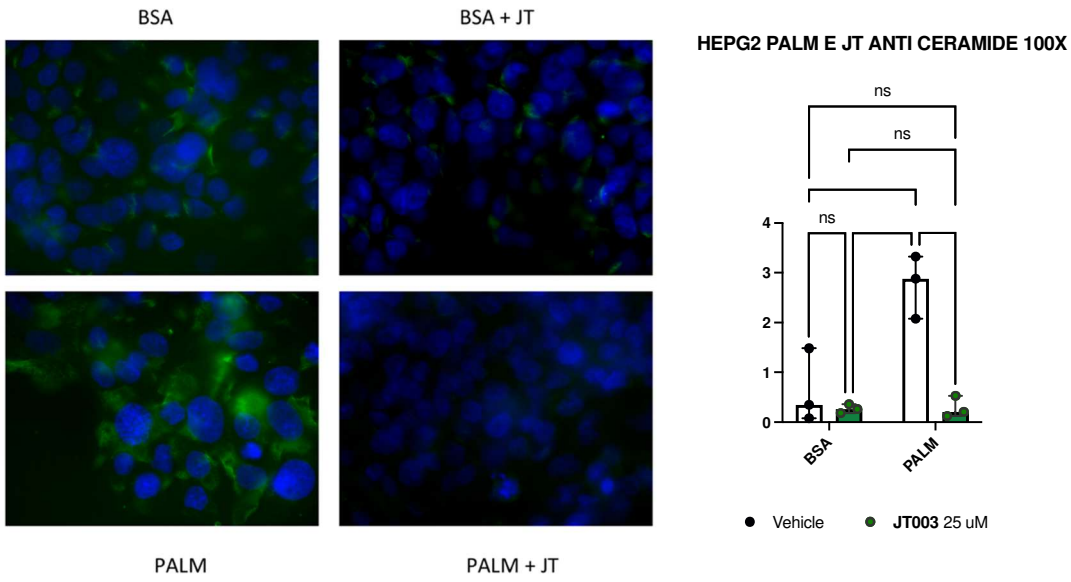

***Figure 20**: Immunofluorescence staining of ceramides (in green) in HepG2 cells and quantification, treated with palmitate and JT003.*

Overall, our findings demonstrate that JT003 effectively reduces ceramide content in different cell lines, including HEK293, AC16, and HepG2. This reduction is achieved without significantly altering the transcription of *de novo* synthesis enzymes, highlighting JT003's role in enhancing ceramide catabolism by activating the adiponectin receptors AdipoR1/AdipoR2. These results suggest that JT003 could be a promising candidate for targeting ceramide accumulation in conditions where *de novo* synthesis is not the primary contributor to ceramide buildup.

6. Discussion

The primary aim of this study was to investigate the role of sphingolipids, particularly ceramides, in multi-organ lipotoxicity associated with metabolic syndrome (MetS). Our findings contribute to the growing body of evidence suggesting that ceramides are crucial mediators of lipotoxicity in conditions such as MASH and HFpEF, both of which are complications of MetS.

We first demonstrated that a high-fat diet (HFD) promotes significant triglyceride and ceramide accumulation in mice myocardial tissue, characteristic of HFpEF-associated lipotoxicity. These findings align with previous studies that have shown lipid overload in the heart contributes to the development of HFpEF by exacerbating myocardial rigidity and systemic inflammation (Leggat J. et al, 2021). Myriocin, a serine palmitoyl transferase (SPTLC1) inhibitor, effectively reduced lipid levels in myocardial tissue of treated mice, underscoring its potential in mitigating lipotoxicity by targeting ceramide *de novo* synthesis. This observation is consistent with the work of Ussher et al. (2012), who demonstrated that inhibition of SPTLC1 reduced cardiac ceramide levels and improved metabolic outcomes, such as increased glycolysis rates, following diet-induced insulin resistance. These parallels highlight the therapeutic relevance of modulating ceramide synthesis in the context of lipid overload and associated cardiac dysfunctions. Systemic inhibition of ceramides biosynthesis, using compounds such as myriocin, is not a feasible option in clinical trials since whole-body inhibition of ceramides content may result in off target side effects. Moreover, cardiac contribution to global ceramide biosynthesis is likely to be low. Exploring strategies to reduce ceramides content increasing ceramides degradation instead of reducing sphingolipids synthesis may therefore worth pursuing.

In our *in vitro* studies using 3T3-L1 and HEK293 cells, we explored ceramide metabolism by inducing ceramide synthesis with palmitate treatment. Although palmitate increased ceramide content in both cell types, it did not significantly alter the gene expression of enzymes involved in ceramide *de novo* synthesis in 3T3-L1 cells, and myriocin failed to reduce ceramide levels in these cells. Although palmitate increased ceramide content in both cell lines, it did not significantly alter the gene expression of enzymes involved in ceramide *de novo* synthesis in 3T3-L1 cells, and myriocin failed to

reduce ceramide levels in these cells. This contrasts with findings from Lee et al. (2012), where myriocin successfully reduced sphingomyelin levels in 3T3-L1 cells. This discrepancy may be attributed to differences in lipid metabolism between cell lines, as sphingomyelin and ceramide are distinct sphingolipids with potentially different effects after myriocin treatment. However, in HEK293 cells, myriocin partially reduced ceramide accumulation. These findings suggest that while myriocin shows potential in reducing ceramide levels in some contexts, its efficacy may be limited, particularly in cell types like 3T3-L1. Nevertheless, the use of immunofluorescence provided valuable insights into ceramide metabolism. These results validate our approach using immunofluorescence staining as a reliable method for evaluating ceramide levels across different cell types.

Given these results and in the context of HFpEF, the therapeutic application of myriocin is complicated by the observed downregulation of SPTLC1 in the LVs (Pérez-Carrillo, L., et al, 2022). This downregulation suggests a reduced role of *de novo* ceramide synthesis in HFpEF, potentially limiting the effectiveness of SPTLC1 inhibitors (such as myriocin) in treating this condition. Moreover, AdipoR2, a receptor with intrinsic ceramidase activity that is enhanced by adiponectin, is also significantly downregulated in MetS and AdipoR agonists have already shown to reduce cardiac lipotoxicity via modulation of ceramide content (Kim, Y., et all, 2022). Considering the established link between HFpEF and MASH, where similar metabolic disruptions are observed, a shift in therapeutic strategy is warranted. Instead of targeting *de novo* ceramide synthesis, we hypothesized that enhancing ceramide degradation through the activation of AdipoRs could provide a more effective approach. This is particularly relevant given that AdipoRs are downregulated in both HFpEF (Tan, W., et al, 2024) and MASH (Matsunami, T., et al, 2011), contributing to ceramide accumulation and exacerbating disease progression.

To test this hypothesis, we employed JT003, an AdipoR agonist, which demonstrated efficacy in reducing ceramide content by enhancing ceramidase activity in vitro (Xu, H., et al, 2020). We tested different cell lines including HEK293, AC16, and HepG2. In our experiments, treatment with palmitate increased the expression of two key genes in ceramide biosynthesis, SPTLC1 and KDSR, key genes in ceramide biosynthesis, in HEK293 cells, yet JT003 did not alter the transcription of these enzymes. Despite this, JT003 significantly reduced ceramide levels visible by immunofluorescence, suggesting its

action is post-translational, likely enhancing ceramidase activity. This observation is consistent with the literature, where interventions targeting ceramide degradation pathways have shown efficacy in reducing ceramide levels without necessarily impacting their synthesis (Senchenkov, A., et al, 2001) (Kim, Y., et all, 2022). Similarly, in AC16 human cardiomyocytes, JT003 modulated ceramide metabolism reducing ceramide levels after JT003 treatment. This finding highlights the potential of adiponectin agonists as therapeutic strategy for reducing ceramide accumulation, particularly in HF where *de novo* synthesis is not the predominant source of ceramides (Pérez-Carrillo, L., et al, 2022). In HepG2 cells, JT003 was effective in reducing ceramide levels following palmitate treatment. This reduction occurred despite the palmitate-induced upregulation of ceramide synthases CERS2 and CERS5, which aligns with the mechanisms outlined by Yu and Wang (2022). Their work discusses how *de novo* ceramide synthesis contributes to MASLD, highlighting the importance of these enzymes in liver disease progression. Our findings support the role of JT003 in enhancing ceramide catabolism and highlight it as a potential treatment against MASH.

7. Conclusion

Overall, our findings for the first time demonstrate that JT003 reduces ceramide content by activating AdipoR1 and AdipoR2, which enhances ceramide degradation in different cell lines including cardiomyocytes and hepatocytes. This mechanism proposes JT003 as a promising therapeutic candidate for conditions like HFpEF and MASH, where reducing ceramide accumulation is critical, but targeting *de novo* synthesis is not effective. However, while our *in vitro* experiments with JT003 shed light on its potential role in modulating ceramide metabolism, some limitations must be addressed for a comprehensive understanding.

Primarily, the number of experimental replicates in our study is limited, which constrains the statistical robustness of our conclusions. Increasing the sample size and repetition frequency would help bolster the reliability of the results and enhance the precision of our findings. Moreover, key experimental parameters, such as the concentration of palmitate and JT003 used, require careful optimization. The current concentrations may not accurately reflect physiological conditions or the range of potential cellular responses, indicating the need for a more nuanced approach to dosing in future experiments. Variability in cell line responses further complicates the interpretation, as different cells may respond uniquely to the same treatment conditions, underscoring the importance of adjusting experimental setups. The leap from *in vitro* models to *in vivo* contexts poses another significant challenge. *In vitro* models, while valuable for understanding cellular mechanisms, cannot fully recapitulate the complexities of an entire organism, including tissue interactions, systemic metabolic responses, and the influence of the immune system. Therefore, translating these findings into potential therapeutic strategies for conditions like HFpEF and MASH necessitates rigorous testing in animal models of these diseases. These models will help validate the efficacy and safety of JT003 and provide insights into its mechanism of action within the living organism.

Understanding the comprehensive role of sphingolipids in multi-organ lipotoxicity linked to MetS is a complex endeavor that remains incompletely elucidated. Despite this, the research field is promising, especially given the escalating global incidence of MetS.

Addressing these challenges could significantly impact public health, highlighting the need for continued and expanded research efforts in this area.

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