

Lipid Remodeling Links Iron and Adiponectin to Ferroptosis, Inflammation, and Metabolic Regulation.

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Abstract

Cardiometabolic diseases (CMD) impose a substantial global health burden marked by chronic metabolic stress, oxidative imbalance, and low-grade inflammation. While disturbances in iron metabolism, lipid metabolism and adipokine signaling are increasingly recognized in CMD, how these processes converge to influence cellular vulnerability and intercellular metabolic regulation remains incompletely understood.

This dissertation investigated the roles of iron-induced ferroptosis and adiponectin-associated extracellular vesicles (EVs) in linking lipid remodeling to cardiometabolic pathology. Study 1 demonstrated that enrichment of ω -6 polyunsaturated fatty acid (PUFA)-containing membrane phospholipids, particularly phosphatidylethanolamines, markedly sensitized cardiomyocytes to iron-induced lipid peroxidation, mitochondrial dysfunction, sterile inflammatory signaling, and ferroptotic cell death. These findings establish membranous lipid composition, rather than lipid abundance alone, as a critical determinant of cellular vulnerability under metabolic stress.

Building on this mechanistic framework, Study 2 extended lipid-mediated cellular vulnerability to the level of intercellular communication. Integrated EV lipidomic and functional analyses in murine models and human metabolic syndrome cohorts identified a conserved PUFA-enriched EV lipid signature associated with adiponectin deficiency. EVs carrying this lipid signature modulated recipient cell phenotypes by promoting oxidative and inflammatory signaling in macrophages, impairing mitochondrial energetics and insulin signaling in skeletal muscle cells, and disrupting glucose-stimulated insulin secretion in pancreatic β -cells. These findings indicate that EV lipid composition can regulate metabolic and inflammatory responses in recipient cells.

Collectively, these findings demonstrate that metabolic stress is associated with PUFA-driven membrane lipid remodeling at the cellular level and with corresponding changes in the lipid composition of EVs. Adiponectin deficiency is linked to EV lipid remodeling and altered metabolic and inflammatory regulation in recipient cells. Together, these observations identify lipid remodeling, iron homeostasis, and EV-associated pathways as interconnected features of CMD biology with implications for biomarker discovery.

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“See the invisible, do the impossible. Why not change the world?”

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“Now faith is confidence in what we hope for and assurance about what we do not see.”

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LIST OF ABBREVIATIONS

Abbreviation	Definition
15-LOX	15-lipoxygenase
AA	Arachidonic acid
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
ACC	Acetyl-CoA carboxylase
ACC1/2	Acetyl-CoA carboxylase 1 / 2
ACSL	Acyl-CoA synthetase long-chain family
ACSL3	Acyl-CoA synthetase long-chain family member 3
ACSL4	Acyl-CoA synthetase long-chain family member 4
AKI	Acute kidney injury
Alix	Programmed cell death 6–interacting protein
ALOX	Arachidonate lipoxygenase
AMPK	AMP-activated protein kinase
ApoA1	Apolipoprotein A1
ATGL (PNPLA2)	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BMDMs	Bone marrow–derived macrophages
BMP6	Bone morphogenetic protein 6
CAD	Coronary artery disease
CD63	Tetraspanin CD63 (EV marker)
CE	Cholesteryl ester
CER	Ceramide
CHF	Congestive heart failure
CISD1–3	CDGSH iron–sulfur domain protein 1–3
CKD	Chronic kidney disease
CMD	Cardiometabolic disease
CoQ	Coenzyme Q10
CoQH2	Reduced coenzyme Q10
CPT	Carnitine palmitoyltransferase
CryoEM	Cryo-electron microscopy

CVD	Cardiovascular disease
DAMP	Damage-associated molecular pattern
DFO	Deferoxamine
DFP	Deferiprone
DG	Diacylglycerol
DGAT	Diacylglycerol O-acyltransferase
DHA	Docosahexaenoic acid
DHODH	Dihydroorotate dehydrogenase
DIA	Data-independent acquisition
DIA-PASEF	Parallel accumulation–serial fragmentation DIA
DMT1 (SLC11A2)	Divalent metal transporter 1
dsDNA	Double-stranded DNA
DXZ	Dexrazoxane
ELOVL	Elongation of very long chain fatty acids
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
ETC	Electron transport chain
EV	Extracellular vesicle
FA	Fatty acid
FADS1/2	Fatty acid desaturase 1 / 2
Fer-1	Ferrostatin-1
FFA	Free fatty acid
Flotillin-1	Lipid raft–associated protein
FOT1	Ferroptosis-related oxidative regulator 1
FPN	Ferroportin
FSP1	Ferroptosis suppressor protein 1
FTH	Ferritin heavy chain
GCH1	GTP cyclohydrolase 1
GLP-1	Glucagon-like peptide-1
GLS	Glutaminase
GO	Gene ontology
GPX4	Glutathione peroxidase 4
GSH	Glutathione
GSIS	Glucose-stimulated insulin secretion

GSR	Glutathione reductase
GSSG	Glutathione disulfide
H9c2	Rat cardiomyoblast cell line
HEPH	Hephaestin
HETE	Hydroxyeicosatetraenoic acid
HF	Heart failure
HFD	High-fat diet
HFpEF	Heart failure with preserved ejection fraction
HG	High glucose
HIF1A	Hypoxia-inducible factor 1- α
HJV	Hemojuvelin
HMOX1	Heme oxygenase 1
HpETE	Hydroperoxyeicosatetraenoic acid
HpETE-PE	Hydroperoxy-eicosatetraenoyl-phosphatidylethanolamine
HSPB1	Heat shock protein beta-1
I/R	Ischemia / reperfusion
IAA	Iodoacetamide
IFN-I	Type I interferon
INS-1	Rat pancreatic β -cell line
iPLA2 β	Ca ²⁺ -independent phospholipase A2 beta
iPSC-CM	Induced pluripotent stem cell–derived cardiomyocyte
IRF	Interferon regulatory factor
IRP	Iron regulatory protein
IS	Internal standard
ISEV	International Society for Extracellular Vesicles
JNK	c-Jun N-terminal kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	Knockout (adiponectin-knockout in chapter3)
L6	Rat skeletal muscle myoblasts
LC/MS	Liquid chromatography–mass spectrometry
LC–MRM–MS	Liquid chromatography–multiple reaction monitoring–mass spectrometry
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
LDH	Lactate dehydrogenase

LOX-1	Lectin-like oxidized LDL receptor 1
LPC	Lysophosphatidylcholine
LPCAT3	Lysophosphatidylcholine acyltransferase 3
LPE	Lysophosphatidylethanolamine
MAPK	Mitogen-activated protein kinase
MASH	Metabolic dysfunction–associated steatohepatitis
MASLD	Metabolic dysfunction–associated steatotic liver disease
MCD	Methionine-choline deficient diet
MEFs	Mouse embryonic fibroblasts
MetS	Metabolic syndrome
MI	Myocardial infarction
MRI-PDFF	Magnetic resonance imaging–proton density fat fraction
MRSI	Magnetic resonance spectroscopy imaging
MS	Mass spectrometry
mtDNA	Mitochondrial DNA
MUFA	Monounsaturated fatty acid
NAC	N-acetylcysteine
NAFLD	Non-alcoholic fatty liver disease
NCF1	Neutrophil cytosolic factor 1
NCOA4	Nuclear receptor coactivator 4
NF- κ B	Nuclear factor kappa B
NLRP3	NOD-like receptor family pyrin domain containing 3
Nrf2	Nuclear factor erythroid 2–related factor 2
NTA	Nanoparticle tracking analysis
OA	Oleic acid
Ox-LDL	Oxidized low-density lipoprotein
OxPC	Oxidized phosphatidylcholine
OxPE	Oxidized phosphatidylethanolamine
OXPPOS	Oxidative phosphorylation
OxPL	Oxidized phospholipid
PA	Palmitic acid
PC	Phosphatidylcholine
PC(O)	Ether-linked phosphatidylcholine
PCA	Principal component analysis

PDSS2	Prenyl diphosphate synthase subunit 2
PE	Phosphatidylethanolamine
PE(P)	Plasmalogen phosphatidylethanolamine
PEBP1	Phosphatidylethanolamine-binding protein 1
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PIP	Phosphoinositide
PK/PD	Pharmacokinetics / pharmacodynamics
PL	Phospholipid
PLD2	Phospholipase D2
PL-PUFA	PUFA-containing phospholipid
PNPLA9	Patatin-like phospholipase domain containing 9
PPAR α	Peroxisome proliferator-activated receptor alpha
PROM2	Prominin-2
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
qPCR	Quantitative polymerase chain reaction
RAW 264.7	Murine macrophage cell line
ROS	Reactive oxygen species
RTA	Radical trapping agent
S1P	Sphingosine-1-phosphate
SCARA1	Scavenger receptor class A member 1
SCARB1	Scavenger receptor class B member 1
SCD1	Stearoyl-CoA desaturase 1
SDS	Sodium dodecyl sulfate
SEC	Size-exclusion chromatography
SFA	Saturated fatty acid
SFXN1	Sideroflexin 1
SGLT2	Sodium-glucose cotransporter-2
SHF	Systolic heart failure
SL	Sphingolipid
SM	Sphingomyelin
STING	Stimulator of interferon genes
T1DM	Type 1 diabetes mellitus

T2D	Type 2 diabetes mellitus
TCEP	Tris(2-carboxyethyl)phosphine
TF	Transferrin
TFRC (TfR1)	Transferrin receptor 1
TG	Triglyceride
TRPV1	Transient receptor potential vanilloid 1
TZD	Thiazolidinedione
UHPLC	Ultra-high-performance liquid chromatography
WT	Wild-type
xCT (SLC7A11)	Cystine/glutamate antiporter
YAP1	Yes-associated protein 1

CHAPTER 1: Literature Review

1.1 Overview of Cardiometabolic Diseases (CMD)

Cardiometabolic diseases (CMD) encompass a spectrum of interrelated conditions, including obesity, type 2 diabetes mellitus (T2D), hypertension, and cardiovascular diseases (CVD), and represent a leading cause of global morbidity and mortality¹⁻⁸. The rising prevalence of CMD is closely linked to sedentary lifestyles, excessive caloric intake, and chronic psychosocial stress, reflecting profound shifts in environmental and behavioral risk factors⁹⁻¹¹. Beyond their individual clinical burdens, these conditions frequently co-occur and share overlapping pathological features such as metabolic dysregulation, chronic low-grade inflammation, and endothelial dysfunction¹². Accordingly, CMD are increasingly recognized not as isolated disorders, but as a complex, multifactorial disease network that accelerates systemic organ dysfunction.

Substantial progress has been made in defining the molecular mechanisms underlying CMD pathogenesis. Emerging evidence highlights dysregulation of iron homeostasis, lipid metabolism, and adipokine signaling—particularly adiponectin—as critical contributors to disease progression¹³⁻¹⁵. These insights have promoted a conceptual shift in therapeutic strategies, moving beyond downstream control of glucose and lipids toward targeting upstream regulators of metabolic, redox, and inflammatory homeostasis¹⁶⁻¹⁸.

Importantly, CMD pathophysiology reflects the convergence of altered nutrient handling, redox imbalance, inflammatory signaling, and inter-organ communication^{8,19}. Within this framework, iron–lipid interactions have emerged as a central axis governing cellular vulnerability under metabolic stress^{13,20}. Excess iron availability combined with remodeling of membrane phospholipids (PLs)—especially enrichment of polyunsaturated fatty acids (PUFAs)—amplifies lipid peroxidation, promoting ferroptotic cell death and sterile inflammation²¹. These processes can propagate across tissues through endocrine and extracellular vesicle (EV)–mediated signaling pathways, thereby contributing to systemic metabolic dysfunction²².

This chapter establishes a conceptual framework that positions CMD as systems-level disorders driven by iron–lipid dysregulation and its downstream effects on redox control, inflammation, and metabolic integrity. The following sections integrate current knowledge on iron metabolism, lipid remodeling, ferroptosis, and inter-organ signaling to provide the foundation for the experimental studies presented in subsequent chapters.

1.1.1 Definition, Classification, and Prevalence of CMD

CMD refer to a spectrum of interrelated metabolic and cardiovascular conditions, including obesity, insulin resistance, T2D, dyslipidemia, hypertension, and atherosclerotic CVD^{1,2}. These conditions frequently co-occur and share common pathophysiological mechanisms, such as chronic low-grade inflammation, oxidative stress, and metabolic inflexibility¹. The term “cardiometabolic” emphasizes the interconnected nature of metabolic dysfunction and cardiovascular complications, highlighting the need for integrated diagnostic and therapeutic approaches²³.

CMD can be classified in several ways depending on the clinical context. A common classification framework includes: (1) primary metabolic disorders, such as T2D, and obesity; (2) primary CVD, such as coronary artery disease, heart failure (HF), and stroke; and (3) mixed or secondary phenotypes, such as metabolic syndrome, which is characterized by a constellation of metabolic abnormalities including central adiposity, hyperglycemia, hypertension, and dyslipidemia²⁴. Various diagnostic criteria have been proposed by organizations including the World Health Organization (WHO), the American Heart Association (AHA), and the International Diabetes Federation (IDF), each emphasizing slightly different metabolic and anthropometric parameters²⁵⁻²⁹.

The global prevalence of CMD has increased dramatically over the past few decades, reflecting shifts in diet, physical activity, and urbanization³⁰. According to the WHO, CVD remain the leading cause of death globally, accounting for approximately 17.9 million deaths annually⁶. Simultaneously, the IDF reports that over 350 million adults worldwide were living with diabetes in 2021, a figure projected to rise to 643 million by 2030⁷. Obesity rates have also reached epidemic proportions, affecting over 650 million adults worldwide⁸. Importantly, CMD disproportionately affects individuals in low- and middle-income countries, where healthcare access and preventive measures are limited³⁰.

This increasing burden underscores the urgent need to understand molecular mechanisms that integrate metabolic and inflammatory dysfunction in CMD¹⁹.

1.1.2 Metabolic Dysfunction and Inflammation in CMD

CMD arise from a failure of metabolic systems to adapt to chronic nutrient excess, resulting in coordinated disturbances in glucose and lipid handling that are tightly coupled to inflammatory activation^{12,31}. A central feature of this maladaptive state is metabolic inflexibility, whereby cells and tissues lose the capacity to appropriately switch between nutrient substrates in response to changing energetic demands^{32,33}. As a consequence, persistent hyperglycemia, dyslipidemia, and ectopic lipid

accumulation develop across insulin-sensitive tissues, reinforcing systemic insulin resistance and metabolic stress^{32,33}.

At the lipid level, CMD are associated with elevated circulating free fatty acids (FFAs), increased triglyceride (TG)-rich lipoproteins, and the accumulation of bioactive lipid intermediates, including diacylglycerols (DGs) and ceramides (CERs)³⁴. These lipid species interfere with insulin signaling through activation of stress-responsive serine kinases such as PKC θ and JNK, leading to inhibitory phosphorylation of insulin receptor substrates and attenuation of downstream signaling cascades^{33,35}. In parallel, mitochondrial dysfunction—characterized by impaired oxidative phosphorylation, reduced metabolic efficiency, and excessive reactive oxygen species (ROS) production—further compromises cellular energy homeostasis and amplifies oxidative stress^{36,37}.

Metabolic dysregulation in CMD is accompanied by a state of chronic, low-grade inflammation, often referred to as “metaflammation”^{12,31,38}. Nutrient overload, lipotoxicity, and oxidative stress converge to activate inflammatory signaling pathways including NF- κ B, JAK/STAT, and MAPK cascades, resulting in sustained expression of pro-inflammatory cytokines such as TNF- α , IL-6, and MCP-1^{12,38,39}. These inflammatory mediators not only exacerbate local tissue injury but also propagate systemic insulin resistance through endocrine and paracrine mechanisms^{12,38,39}.

Adipose tissue is a key contributor to metabolic-immune crosstalk in CMD¹². Excess lipid storage drives adipocyte hypertrophy, local hypoxia, and the recruitment of pro-inflammatory immune cells, including macrophages, neutrophils, and Th1 lymphocytes^{40,41}. This immune infiltration establishes a self-reinforcing inflammatory microenvironment that extends beyond adipose depots into the systemic circulation⁴⁰. In parallel, inflammatory signaling in the liver promotes steatosis and progression toward metabolic dysfunction-associated steatohepatitis (MASH), while pancreatic inflammation impairs β -cell survival and insulin secretory capacity^{42,43}.

Together, these interdependent metabolic and inflammatory disturbances form a vicious cycle that accelerates CMD onset and progression⁴⁴. Increasing evidence suggests that upstream molecular mediators—including iron availability, adipokine signaling (notably adiponectin), and lipid peroxidation-derived signals—serve as integrative nodes linking metabolic stress to inflammatory and redox imbalance^{13,45,46}. These mechanisms are examined in greater detail in the following sections.

1.1.3 Iron, Lipid, and Adiponectin Dysregulation in CMD

Iron is an essential micronutrient that supports oxygen transport, mitochondrial respiration, and redox homeostasis through its incorporation into heme- and iron-sulfur cluster-containing proteins⁴⁷. Because iron directly regulates a broad network of metabolic and redox-sensitive processes, including the expression and activity of enzymes involved in energy metabolism and oxidative defense⁴⁸, tight control of cellular and systemic iron availability is critical for metabolic homeostasis.

Unlike many nutrients, iron lacks a dedicated excretory pathway, rendering systemic iron balance dependent on regulated intestinal absorption and recycling⁴⁹. In a healthy adult male weighing approximately 70 kg, total body iron content ranges from 3,500–4,000 mg, with approximately 65% residing in hemoglobin, 10% distributed among myoglobin and iron-dependent enzymes, and the remaining fraction stored in macrophages, hepatocytes, and bone marrow^{49–51}. Disruption of these regulatory mechanisms can therefore lead to iron imbalance, with important metabolic and pathological consequences.

Perturbations in iron homeostasis are increasingly recognized in CMD⁵². Elevated systemic iron levels, commonly reflected by hyperferritinemia, have been associated with increased risk of insulin resistance, T2D, and CVD⁵². Conversely, iron deficiency is also linked to adverse outcomes, particularly in the context of HF, underscoring the complex and context-dependent role of iron in cardiometabolic pathology⁵³. These paradoxical observations suggest that both iron excess and iron insufficiency can compromise metabolic resilience, highlighting the importance of iron distribution and bioavailability rather than absolute iron burden alone.

At the cellular level, disruption of iron balance intersects closely with lipid metabolism and redox control. Ferroptosis is a regulated form of cell death driven by iron-dependent lipid peroxidation, particularly affecting PUFA-containing membrane PLs^{54–56}. Alterations in iron handling, lipid composition, and antioxidant capacity—features frequently observed in CMD—mirror key biochemical characteristics of ferroptosis^{46,57}. In this context, excess redox-active iron can catalyze lipid peroxidation reactions, amplifying oxidative stress and promoting inflammatory signaling cascades^{58,59}. These observations raise the possibility that ferroptosis-related mechanisms contribute to tissue injury and dysfunction in CMD.

Lipid metabolism is crucial in shaping this vulnerability⁵⁵. Beyond serving as energy substrates, fatty acids (FAs) determine membrane composition and susceptibility to oxidative damage. PLs enriched in PUFAs are essential for membrane fluidity and signaling, yet they are particularly prone to iron-catalyzed peroxidation^{55,56}. Lipid-derived molecules also participate in intra- and intercellular communication,

including through lipoproteins and EVs, thereby linking local metabolic stress to systemic inflammatory and metabolic responses⁶⁰ (Fig. 1.1).

Lipidomic studies have highlighted consistent patterns of lipid remodeling in CMD. Elevated levels of CERs and phosphatidylethanolamines (PEs), together with reduced lysophospholipids and ω -3 FAs, have been associated with obesity and insulin resistance⁶¹. Accumulation of bioactive lipid species such as CERs, diacylglycerols (DGs), and phosphatidylinositols (PIs) correlates with increased cardiometabolic risk, whereas specific sphingomyelin (SM) species appear to exert protective effects⁶¹. These findings emphasize that lipid composition, rather than total lipid burden alone, critically influences metabolic and inflammatory outcomes.

Dietary excess, particularly from saturated fats and refined carbohydrates, further exacerbates lipid dysregulation in CMD^{62,63}. Absorbed dietary lipids are re-esterified into triglycerides (TGs) and transported via chylomicrons to peripheral tissues, where FAs are stored, oxidized, or repackaged into very low-density lipoproteins (VLDLs)⁶⁴. Chronic lipid excess promotes the accumulation of toxic intermediates, including DGs, CERs, and acylcarnitine, which induce lipotoxic stress and impair insulin signaling⁶⁵⁻⁷⁰.

Collectively, iron imbalance, maladaptive lipid remodeling, and redox dysfunction converge to compromise cellular stress tolerance across key metabolic organs, including liver, skeletal muscle, heart, and pancreas⁷¹⁻⁷⁴. Increasing evidence suggests that adipokine signaling—particularly adiponectin—and iron- and lipid-dependent signaling mediators such as EVs may act as upstream modulators of these processes, integrating metabolic stress with inflammatory and redox pathways.

In addition to iron and lipid dysregulation, alterations in adipokine signaling—particularly reduced adiponectin availability—further contribute to metabolic and inflammatory imbalance in CMD⁷⁵⁻⁸¹. Adiponectin is an adipocyte-derived adipokine that supports metabolic homeostasis by enhancing lipid handling, mitochondrial function, and cellular stress resilience across multiple tissues^{78,80,81}. In CMD, hypoadiponectinemia is consistently associated with impaired capacity to buffer lipid overload and oxidative stress, thereby amplifying inflammatory signaling and insulin resistance⁷⁵⁻⁷⁹.

In combination with iron imbalance and PUFA-enriched membrane lipid remodeling, reduced adiponectin signaling may further sensitize tissues to lipid peroxidation and ferroptosis-related injury. This positioning of adiponectin within the iron–lipid–redox framework provides a conceptual basis for its mechanistic and therapeutic relevance in CMD, which is explored in detail in subsequent sections.

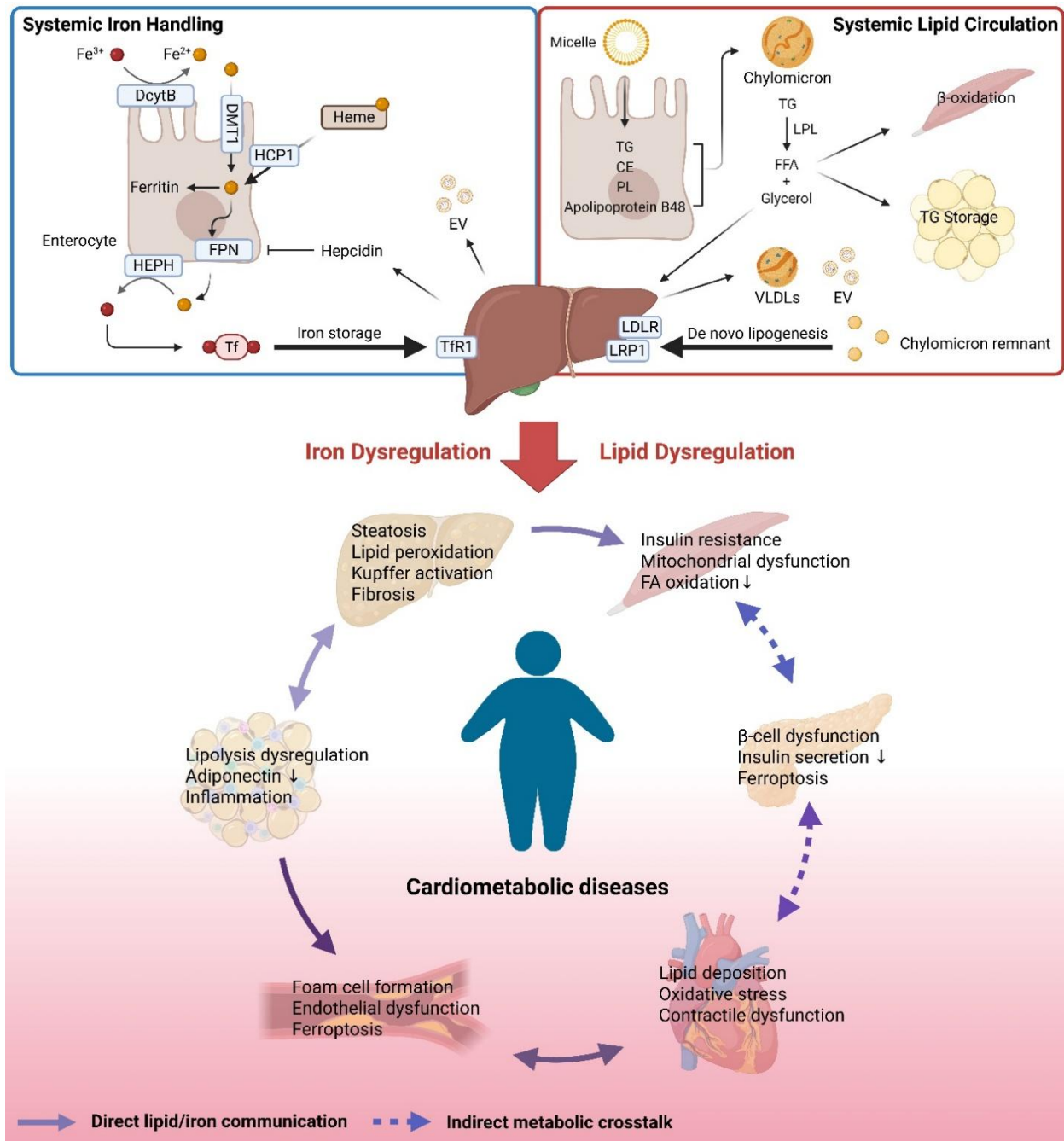


Figure 1.1. Systemic Iron and Lipids and Organ-Level Impact in CMD

This figure depicts the absorption, transport, and regulation of iron and lipids, and the pathological outcomes of their dysregulation in key metabolic organs. Non-heme iron (Fe^{3+}) is reduced by duodenal cytochrome b (DcytB) and absorbed via divalent metal transporter 1 (DMT1); heme iron is internalized through heme carrier protein 1 (HCP1). Exported Fe^{2+} is oxidized by hephaestin (HEPH) or ceruloplasmin

and bound to transferrin (TF) for systemic distribution. Hepcidin modulates iron homeostasis by promoting ferroportin (FPN) degradation.

Dietary lipids are absorbed and assembled into chylomicrons, comprising triglycerides (TG), cholesteryl esters (CE), phospholipids (PL), and apolipoprotein B48 (ApoB48). Lipoprotein lipase (LPL) hydrolyzes TGs to release free fatty acids (FFAs), while remnants are cleared by the liver via low-density lipoprotein receptor (LDLR) and LDLR-related protein 1 (LRP1). The liver also regulates lipid balance through de novo lipogenesis and very low-density lipoprotein (VLDL) secretion.

Iron and lipid dysregulation contributes to CMD by promoting steatosis, oxidative stress, and inflammation in the liver; insulin resistance and mitochondrial dysfunction in skeletal muscle; β -cell failure in the pancreas; lipotoxicity in the heart; vascular endothelial dysfunction; and inflammation and iron overload in adipose tissue.

Images created with BioRender.com.

1.1.4 Current Therapeutic Approaches in CMD

The clinical management of CMD remains a significant global healthcare priority^{3,4}. Current therapeutic strategies primarily target modifiable risk factors such as hyperglycemia, hyperlipidemia, hypertension, and obesity, with the overarching goal of reducing cardiovascular events and organ dysfunction⁴⁴. While pharmacological and lifestyle interventions have yielded measurable clinical benefits, they often fail to address the upstream molecular drivers of CMD, including lipid-induced oxidative stress, inflammation, and iron imbalance.

Pharmacological interventions form the cornerstone of CMD management. For glycemic control, first-line therapy typically involves metformin, which improves insulin sensitivity and reduces hepatic gluconeogenesis⁸². In patients with atherosclerotic CVD or chronic kidney disease, GLP-1 receptor agonists (e.g., liraglutide⁸³, semaglutide^{84,85}) and sodium-glucose cotransporter 2 (SGLT2) inhibitors (e.g., empagliflozin⁸⁶, dapagliflozin⁸⁷) are preferred due to their dual benefits on glucose lowering and cardiovascular/renal protection. Insulin therapy remains essential in later stages of T2D or in the setting of β -cell failure.

Dyslipidemia is primarily managed with statins, which reduce low-density lipoproteins (LDL) cholesterol and cardiovascular risk by inhibiting HMG-CoA reductase^{88,89}. For patients with statin intolerance or severe hypercholesterolemia, PCSK9 inhibitors (e.g., evolocumab⁹⁰ and ezetimibe⁹¹) offer additive lipid-

lowering effects. ω -3 FA, particularly eicosapentaenoic acid (EPA), have shown modest benefit in reducing TGs and cardiovascular events in high-risk populations⁹².

Hypertension, a key component of CMD, is typically managed using renin-angiotensin system (RAS) inhibitors (angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers), calcium channel blockers, and thiazide diuretics⁹³. These agents not only control blood pressure but also mitigate renal and cardiac complications⁹⁴. In patients with metabolic syndrome or T2D, RAS inhibitors are preferred due to their nephroprotective properties⁹⁵.

Lifestyle interventions remain foundational across all stages of CMD. Dietary modification, particularly adopting Mediterranean or dietary approaches to stop hypertension-style diets, combined with regular physical activity and sustained weight loss significantly improves glycemic control, lipid profiles, and blood pressure⁹⁶⁻¹⁰⁰. Structured behavioral interventions, such as intensive lifestyle programs (e.g., Diabetes Prevention Program), have demonstrated long-term benefits in preventing disease progression¹⁰¹.

Despite these established therapies, CMD prevalence and severity continue to rise, underscoring the need for novel strategies that target disease at a molecular level. Iron chelation or ferroptosis inhibition offer potential for mitigating oxidative stress-related organ damage¹⁰²⁻¹⁰⁵. Additionally, adiponectin receptor agonists (e.g., AdipoRON, ALY688) have emerged as promising therapeutics aimed at restoring adiponectin signaling and improving insulin sensitivity, though clinical translation remains limited^{80,106,107}. Advances in proteomics, lipidomics, biomarker discovery, and EV profiling may further refine patient stratification and therapeutic targeting.

In summary, current clinical approaches to CMD focus largely on symptom and risk factor management. While effective in reducing short-term complications, these strategies are insufficient to halt disease progression or reverse underlying pathophysiological processes. Integration of precision medicine approaches that address oxidative stress, immunometabolic dysfunction, and inter-organ communication may pave the way for next-generation therapies in CMD.

1.2 Iron Metabolism and Ferroptosis

Beyond the classical metabolic and inflammatory pathways in the pathogenesis of CMD, emerging evidence highlights the pivotal role of iron dysregulation and ferroptosis, a form of regulated cell death driven by iron-dependent lipid peroxidation^{108,109}. While iron is essential for oxygen transport and mitochondrial energy production, its excess contributes to oxidative stress, membrane damage, and

cellular dysfunction⁴⁶. Recent studies suggest that disrupted iron homeostasis not only impairs systemic metabolic homeostasis but also sensitizes key metabolic tissues, such as the heart, liver, and pancreas, to ferroptotic injury⁴⁶. This section explores the physiological regulation of iron at the systemic and cellular levels, the mechanisms of ferroptosis, and their relevance to cardiometabolic pathology.

1.2.1 Systemic and Cellular Iron Regulation

Iron homeostasis is coordinated through tightly regulated mechanisms at both systemic and cellular levels to ensure adequate availability while preventing toxicity.

At the systemic level, the hepcidin-ferroportin axis is the primary regulator of iron balance¹¹⁰. Hepcidin, a liver-derived peptide hormone, binds to the sole known cellular iron exporter, ferroportin (FPN1), leading to its internalization and degradation¹¹⁰. This suppresses both intestinal iron absorption and the release of stored iron from macrophages and hepatocytes¹¹⁰. Hepcidin expressions are modulated by iron stores, erythropoietic activity, and inflammatory signals. For example, elevated iron levels activate the bone morphogenetic protein 6 (BMP6)-hemojuvelin (HJV) signaling pathway, which promotes hepcidin transcription¹¹¹. Disruption of this regulatory circuit, as observed in hereditary hemochromatosis, results in systemic iron overload¹¹¹.

At the cellular level, iron uptake is primarily mediated through the transferrin (TF)-transferrin receptor 1 (TfR1) system. Ferric iron (Fe^{3+}) bound to TF is internalized via receptor-mediated endocytosis⁴⁹. Within the endosome, Fe^{3+} is reduced to Fe^{2+} by the metalloredutase STEAP3 and subsequently transported into the cytosol via divalent metal transporter 1 (DMT1)⁴⁹. To mitigate iron-induced oxidative stress, cytosolic Fe^{2+} is either safely stored in ferritin or reoxidized by ferroxidases such as ceruloplasmin and hephaestin for loading back onto TF for systemic transport^{112,113}. Ferritin serves as a buffering system that sequesters excess iron while maintaining a readily mobilizable reserve during periods of increased cellular demand¹¹⁴.

Post-transcriptional regulation is mediated by iron regulatory proteins (IRPs), which bind to iron-responsive elements (IREs) located in the untranslated regions of mRNAs encoding key iron-handling proteins¹¹⁵. Under iron-deficient conditions, IRPs stabilizes TfR1 mRNA to enhance iron uptake while repressing ferritin translation to reduce iron storage¹¹⁶. These dynamic regulatory networks allow cells to rapidly adapt to changes in iron availability.

1.2.2 Dysregulated Iron Homeostasis in CMD

Disruptions in systemic or cellular iron metabolism, ranging from deficiency to overload, are frequently observed in CMD and contribute significantly to disease progression through oxidative stress, inflammation, and mitochondrial dysfunction^{13,117}.

Iron deficiency is prevalent in CMD, particularly in the context of chronic inflammation and impaired erythropoiesis, such as T2D^{13,117}. Pro-inflammatory cytokines like interleukin-6 (IL-6) promotes hepcidin upregulation, which suppresses intestinal iron absorption and sequesters iron within macrophages¹¹⁸. This iron-restricted anemia limits oxygen delivery and compromises mitochondrial oxidative capacity, thereby impairing energy metabolism and exacerbating systemic insulin resistance and inflammation¹¹⁹. In patients with T2D, iron deficiency has been implicated in the pathogenesis of HF with preserved ejection fraction (HFpEF), likely via reduced cardiac output and impaired activity of iron-dependent enzymes such as aconitase¹²⁰⁻¹²². These effects exacerbate insulin resistance and inflammation, forming a feed-forward loop that accelerates CMD progression.

In contrast, iron overload is commonly observed in metabolic disorders and CVD^{122,123}. In metabolic dysfunction-associated steatotic liver diseases (MASLD), impaired hepcidin signaling and macrophage dysfunction lead to hepatic iron accumulation¹²⁴. Excess iron generates ROS via the Fenton reaction, resulting in lipid peroxidation and ferroptosis, which in turn worsen liver injury and steatosis¹²⁴. In the cardiovascular system, elevated serum ferritin and transferrin saturation are associated with vascular calcification and endothelial dysfunction^{104,125}. Clinical studies demonstrated that iron chelation therapy improves endothelial function and reduces myocardial infarction (MI), highlighting the role of iron-induced oxidative stress in vascular pathology^{104,125}. Preclinical models further demonstrate that genetic deletion of cardiac ferritin heavy chain results in myocardial iron overload and ferroptosis, which can be mitigated by iron chelation^{126,127}.

Although compensatory changes in iron-handling proteins, such as increased ferritin or altered TfR1, and ceruloplasmin levels, are observed, these responses are often insufficient to re-establish iron balance in chronic CMD. For instance, elevated ferritin levels may reflect both antioxidant defense and systemic inflammation^{128,129}. In MASLD/MASH, ceruloplasmin exhibits compartment-specific regulation: decreased in plasma but elevated in hepatic tissue^{130,131}. This may support intracellular iron processing but inadvertently contribute to elevated circulating iron and oxidative stress.

These findings highlight the complex interplay between systemic and cellular iron regulation and emphasize the need for therapeutic strategies that restore iron balance while minimizing ferroptosis and its pathological consequences.

Table 1.1 summarizes key clinical cohort findings on iron status in CMD, highlighting the multifaceted consequences of both iron deficiency and overload. Differences in systemic versus localized iron regulation offer valuable insight into the complex relationship between iron metabolism and CMD pathophysiology.

Table 1.1. Clinical cohort studies of dysregulated iron metabolism in cardiometabolic diseases.

Disease	Year (Data/Collection)	Location	# of patients	Age (Mean $\pm$ SD)	Sex	Findings	Refs.
MASLD	1988-1994	USA	3,393	22–74	Both	Higher serum iron (SI) and TF saturation (TSAT) are associated with reduced all-cause mortality.	¹³²
Diabetes	1999-2018	USA	5,160	54.51 $\pm$ NA	Both	Moderate ferritin (300–500 ng/mL) and optimal TSAT (25–32%) linked to reduced mortality. Both iron deficiency and overload are associated with poor outcomes.	¹³³
Congestive heart failure	1999-2018	USA	1,905	71 $\pm$ 9	Both	In CHF patients, higher iron and TSAT predicted lower mortality, while elevated sTfR indicated higher risk. Ferritin was not predictive. Derived indices (TRI, TRTS) showed stronger mortality associations.	¹³⁴
Chronic kidney disease	2001-2003 (PREVEND cohort)	The Netherlands	6,544	53 $\pm$ 12	Both	Iron deficiency (low TSAT, high sTfR) and elevated erythropoietin levels were each independently associated with increased all-cause mortality. These associations were substantially mediated by elevated FGF23 levels.	¹³⁵
CMD	2006-2010	Europe	368,406	56.6 $\pm$ 8.1	Both	Higher iron markers associated with reduced CAD risk (7-14%),	¹³⁶

						increased T2D risk, and no effect on insulin sensitivity or heart failure (HF).	
Dyslipidemia, CMD Risk	2009	China	7,540	52.9 ± 9.39	Both	Ferritin is positively associated with apolipoprotein (apoB) and adverse lipid ratios. Transferrin is inversely associated with apoB. Iron overload linked to dyslipidemia.	¹³⁷
Coronary Artery Disease (CAD)	2022	China	1,286 (643 CAD, 643 controls)	58.4 ± 1.84	Both	Ferritin positively associated with CAD in men and women. Serum iron and TIBC protective in women. High ferritin linked to LDL oxidation and oxidative stress.	¹³⁸
Systolic Heart Failure (SHF)	2023	Germany, USA	80 (40 treatment, 40 placebo)	62 ± 12	Both	Empagliflozin increased myocardial iron content despite systemic deficiency; improved LVEF and 6-MWT. Ferritin and hepcidin declined, suggesting mobilization of stored iron.	¹³⁹

1.2.3 Ferroptosis Mechanisms

The convergence of dysregulated iron and lipid metabolism creates conditions that promote ferroptosis in CMD. Ferroptosis is a regulated modality of cell death driven by iron-dependent lipid peroxidation and impaired redox homeostasis, primarily affecting cellular and organellar membranes⁵⁴. A hallmark of CMD is the disruption of iron, lipid and redox balance—features that mirror ferroptosis mechanisms^{46,57}.

Epidemiological studies have linked elevated systemic iron levels, often indicated by hyperferritinaemia, with increased CMD risk⁵². Conversely, iron deficiency is also associated with adverse outcomes such as

HF, highlighting the dual impact of iron imbalance on cardiometabolic health⁵³. Notably, excess iron promotes ROS generation, while redox imbalance and lipid dysregulation further heighten ferroptotic susceptibility^{58,59}. Long-chain CERs and oxidizable PUFA-PLs, frequently elevated in CMD, amplify oxidative stress and membrane vulnerability^{55,56,140}.

The following sections detail the molecular underpinnings of ferroptosis, with emphasis on compartmentalized and disease-relevant mechanisms. Focusing on three axes: iron handling (1.2.3.1), lipid peroxidation (1.2.3.2), and antioxidant defense (1.2.3.3).

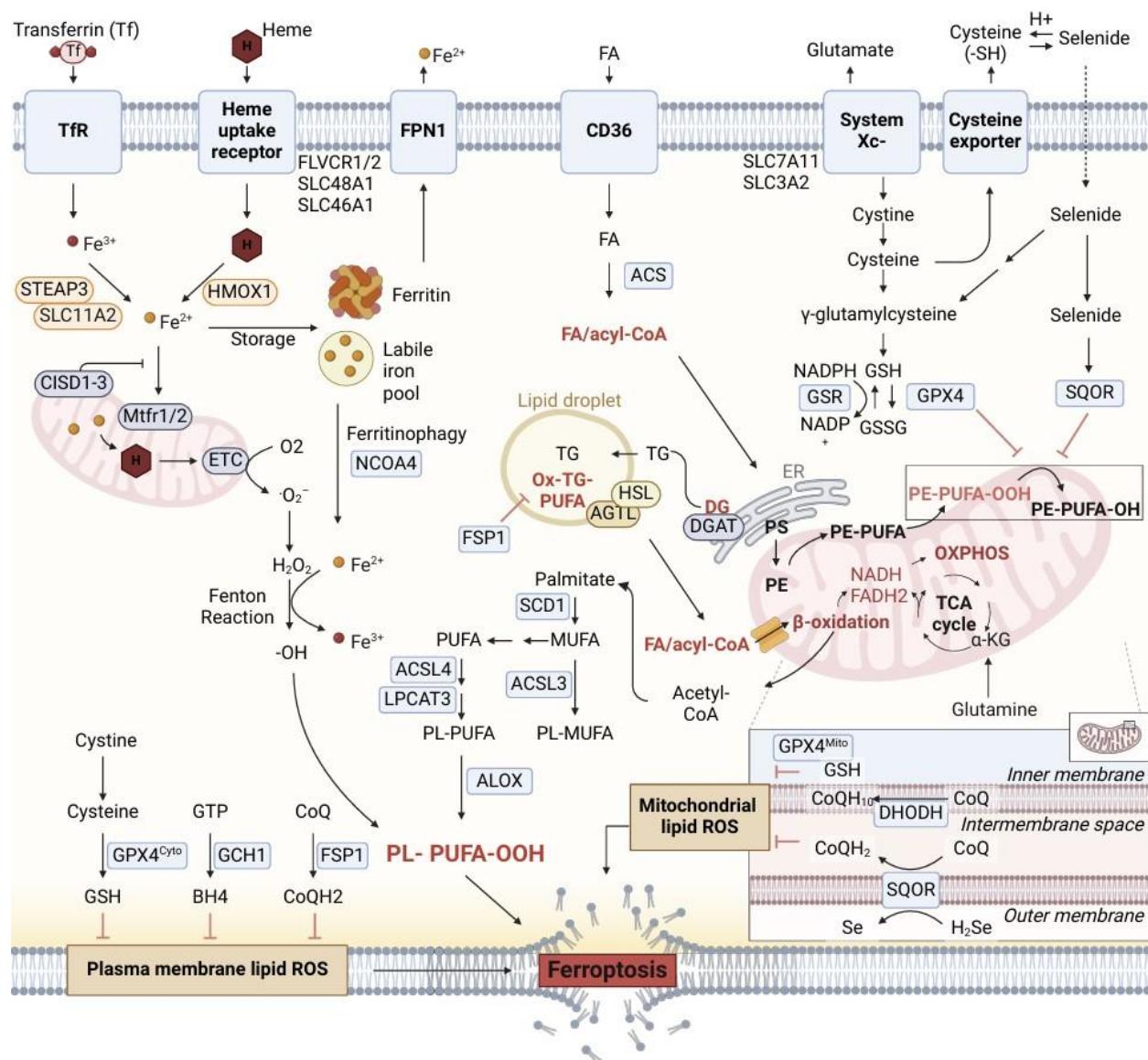


Figure 1.2. Overview of Ferroptosis Mechanisms

Ferroptosis is regulated by iron, lipid, and redox metabolism. Iron enters cells via transferrin receptor (TfR)-mediated endocytosis or heme catabolism through SLC46A1/48A1, and is reduced to Fe²⁺ by STEAP3, SLC11A2, and HMOX1. Fe²⁺ is stored in the labile iron pool or ferritin, and imported into mitochondria by mitoferrin1/2 to support electron transport chain (ETC) activity and ROS production, which is limited by CISD1-3.

The cystine-glutamate antiporter system Xc⁻ (SLC3A2/SLC7A11) imports cystine, enabling glutathione (GSH) synthesis. GSH detoxifies lipid hydroperoxides via GPX4. GSH homeostasis is further supported by the transsulfuration pathway, selenide metabolism, and antioxidant systems including SQOR-CoQH₂, FSP1-CoQH₂ (plasma membrane), and DHODH-CoQH₂ (mitochondria).

Fatty acids (FAs) imported via CD36 are activated by acyl-CoA synthetases (ACSLs) and funneled into lipid droplet synthesis or β -oxidation. De novo lipogenesis generates saturated FAs, later desaturated into MUFAs and PUFAs, PUFA-phospholipids (PL-PUFAs), incorporated by ACSL4 and LPCAT3, are oxidized by iron and ALOXs, initiating ferroptosis. Disruption of these pathways enhances ferroptotic vulnerability.

Abbreviations: ACSL3/4, acyl-CoA synthetase long-chain family member 3/4; ALOXs, arachidonate lipoxygenases; α -KG, α -ketoglutarate; ATGL (PNPLA2), adipose triglyceride lipase; BH4, tetrahydrobiopterin; CD36, cluster of differentiation 36; CISD1–3, CDGSH iron–sulfur domain 1–3; CoQ, coenzyme Q10; CPT, carnitine palmitoyltransferase; DG, diacylglycerol; DGAT, diacylglycerol O-acyltransferase; ER, endoplasmic reticulum; ETC, electron transport chain; FLVCR1/2, FLVCR choline and heme transporter 1/2; GCH1, GTP cyclohydrolase 1; GLS, glutaminase; GSR, glutathione reductase; GSSG, glutathione disulfide; HMOX1, heme oxygenase 1; LPCAT3, lysophosphatidylcholine acyltransferase 3; OXPHOS, oxidative phosphorylation; PE, phosphatidylethanolamine; PS, phosphatidylserine; SCD1, stearoyl-CoA desaturase 1; SLC, solute carrier family; SLC11A2, divalent metal transporter 1 (DMT1); STEAP3, six-transmembrane epithelial antigen of the prostate 3; TF, transferrin; TFRC, transferrin receptor; TG, triglyceride.

Figure created using BioRender.com. Adapted from Dang et al¹⁴¹.

1.2.3.1 Iron Handling and Ferroptosis

Labile intracellular iron fuels ferroptosis by catalyzing hydroxyl radical formation via Fenton chemistry, initiating lipid peroxidation cascades^{54,142,143}. Iron uptake is primarily mediated by TF-TfR1 axis¹⁴⁴. Glutaminolysis further amplifies ROS generation, providing metabolic input for ferroptosis even in iron-rich settings¹⁴⁴. Knockdown of TFRC or chelation suppresses ferroptosis, confirming its central role¹⁴⁴.

TFRC is also a disease-relevant regulator. In viral myocarditis, TFRC upregulation enhances iron uptake and lipid peroxidation in cardiomyocytes¹⁴⁵, while in aged muscle and vascular endothelium, TFRC levels modulate ferroptosis susceptibility and tissue damage^{146,147}.

Subcellular iron localization further defines ferroptotic potential. Ferritinophagy, NCOA4-mediated ferritin degradation, releases stored Fe²⁺ and expands the labile iron pool¹⁴⁸. Mitochondrial iron import via SFXN1 promotes ROS generation and ferroptosis, counteracted by MitoNEET (CISD1), whose loss sensitizes tissues to cardiometabolic injury¹⁴⁹⁻¹⁵⁵. Apelin-13 amplifies this process by inducing both NCOA4 and SFXN1¹⁵⁶.

Lysosomal iron pools also contribute independently to ferroptosis. Upon iron activation, lysosomal lipid peroxidation leads to membrane permeabilization, ER stress, and cell death^{157,158}. This pathway is selectively blocked by lipoxstatin-1, defining lysosomal iron reactivity as a targetable axis¹⁵⁷⁻¹⁶⁰.

Recent suborganellar and redox lipidomics analyses revealed that ER-mitochondria contact sites act as critical hubs for initiating PL peroxidation during ferroptosis, particularly following RSL3 treatment¹⁶¹. These contact sites are enriched in PUFA-PLs, and subsequent accumulation of oxidation products such as OxPE and OxPC in mitochondria underscores a spatially orchestrated lipid peroxidation cascade¹⁶¹.

To suppress ferroptosis, cells engage iron-exporting systems. Ferroxidases like ceruloplasmin and hephaestin oxidize Fe²⁺, allowing extracellular TF loading¹⁶². Loss of these enzymes or treatment with cuprizone, an agent that mobilizes ferritin iron, sensitizes cells to ferroptosis¹⁶³⁻¹⁶⁶. Additional regulators, such as HSPB1 and PROM2, protect cells by limiting TfR1-mediated import or promoting ferritin exocytosis^{167,168}.

Table 1.2 summarizes key molecular regulation of iron metabolism that modulate ferroptotic susceptibility.

Table 1.2. Key modulators of ferroptosis: iron metabolism

Gene	Protein	Function	Effects of targeting it in ferroptosis	Experimental models used	Refs.
<i>BACH1</i>	<i>BTB and CNC homology 1</i>	<i>Downregulating genes involved in antioxidant defense and glutathione (GSH) synthesis</i>	<i>Deletion enhances resistance to ferroptosis.</i>	<i>MI mouse model (Bach1^{-/-}, C57BL/6J); primary mouse embryonic fibroblasts; Hepa-1c1c7 cell line</i>	<i>169,170</i>

<i>CISD1-3</i>	<i>CDGSH</i> iron–sulfur domain- containing protein 1–3	<i>Regulating</i> mitochondrial iron uptake and homeostasis	<i>Deletion promotes</i> ferroptosis.	<i>HepG2/Hep3B</i> (<i>CISD1</i>); 293T (SCSP- 502) & HT1080 (TCHu170) for <i>CISD2</i> ; HL60 for <i>CISD3</i>	150,171, 172
<i>CP</i>	<i>Ceruloplasmin</i>	Converting Fe^{2+} to Fe^{3+}	Knockdown enhances erastin- or RSL3-induced ferroptosis; overexpression suppresses it.	<i>HepG2/Hep3B</i> (<i>CP</i> overexpression [WT & mutant], sh <i>CP</i>)	165
<i>FTH1 & FTL</i>	<i>Ferritin</i> heavy & light chain	Storing cytosolic iron	Knockout or knockdown promotes ferroptosis.	Myocyte/cardiomyocyte -specific KO mice (high-iron diet) for <i>FTH1</i> ; human blood & placentas, Ad-sh <i>FTL</i> KD rats, chorionic villous explants, HTR8/SVneo cells for <i>FTL</i>	126,173
<i>FTMT</i>	<i>Mitochondrial ferritin</i>	Storing mitochondrial iron.	Knockdown promotes ferroptosis.	Primary human macrophages; si <i>FTMT</i> - treated HT1080 cells	174
<i>FXN</i>	<i>Frataxin</i>	Assembling iron-sulfur clusters in mitochondria	Deletion promotes ferroptosis via mitochondrial dysfunction; knock-in suppresses ferroptosis through restoring antioxidant defense.	HT1080 cells with shRNA-mediated <i>FXN</i> knockdown or lentiviral <i>FXN</i> overexpression	175
<i>HAMP</i>	<i>Hepcidin</i>	Inducing <i>FPN</i> internalization, thereby inhibiting iron absorption	Knockout suppresses ferroptosis.	Parabiosis and intracerebral hemorrhage models of	176

				<i>C57BL/6-HAMP^{-/-} mouse model</i>	
<i>HIF1A</i>	<i>Hypoxia-inducible factor 1-α</i>	<i>Regulating cellular adaptation to hypoxia; modulates DMT1 and TfR1 transcription</i>	<i>Knockout or knockdown promotes RSL3 and erastin-induced ferroptosis.</i>	<i>HT1080 & other cancer cell lines ($\pm$ hypoxia, CRISPR); HT1080 CDX/PDX mice $\pm$ drugs</i>	¹⁷⁷
<i>HMOX1</i>	<i>Heme oxygenase 1</i>	<i>Degrading heme into biliverdin, free iron, and carbon monoxide</i>	<i>Overexpression induces ferroptosis; deletion suppresses ferroptosis.</i>	<i>Human carotid plaques; ApoE^{-/-} mice ($\pm$ HFD, $\pm$ AAV-Hmox1); BMDMs $\pm$ oxLDL, hypoxia, haemin, siHmox1, LV-Hmox1</i>	¹⁷⁸
<i>LGMN</i>	<i>Legumain</i>	<i>Lysosomal cysteine protease; degrading proteins and ferritin to modulate intracellular iron release</i>	<i>Deletion attenuates ferroptosis in IR-induced kidney injury.</i>	<i>Lgmn-KO (C57BL/6) mice in IRI and folic acid-induced AKI models; mRTECs with legumain overexpression; HK-2 cells $\pm$ hypoxia</i>	¹⁷⁹
<i>NCOA4</i>	<i>Nuclear receptor coactivator 4</i>	<i>Transferring ferritin to lysosome to degrade and release stored iron</i>	<i>Knockdown suppresses ferroptosis.</i>	<i>PANC1/MEFs (shNCOA4); HeLa & HA22T/VGH (NCOA4 overexpression); HeLa (NCOA4 CRISPR-KO)</i>	^{148,180}
<i>PROM2</i>	<i>Prominin2</i>	<i>Regulating ferritin export</i>	<i>Knockout promotes ferroptosis; inhibition suppresses ferroptosis.</i>	<i>Hs578t xenografts (NOD/SCID, $\pm$ KRIBB11/IKE); 8 human cell lines (MCF10A, Hs578t, etc.)</i>	¹⁸¹
<i>SFXN1</i>	<i>Sideroflexin 1</i>	<i>Regulating mitochondrial iron uptake</i>	<i>Deletion or inhibition suppresses ferroptosis.</i>	<i>H9c2 (siSFXN1); C57BL/6J (apelin-13–induced hypertrophy)</i>	¹⁵⁶

<i>SLC11A2</i>	<i>Divalent metal transporter 1 (DMT1)</i>	<i>Transporting Fe²⁺ into duodenum and within cells</i>	<i>Inhibition reduces erastin-induced ferroptosis.</i>	<i>MCF-7 (sgDMT1); C57BL/6 ± nicotinamide mononucleotide/erastin</i>	<i>182</i>
<i>SCL25A37 & SLC25A28</i>	<i>Mitoferrin 1 & 2</i>	<i>Importing iron into mitochondria for heme and iron-sulfur cluster biosynthesis</i>	<i>Deletion inhibits hepatic ferroptosis.</i>	<i>Bile duct ligation C57BL/6 mice ± shSLC25A37/28 ± erastin; cirrhotic liver; HSC-T6, LX2, primary HSCs ± sh/overexpression-SLC25A37/28</i>	<i>183,184</i>
<i>SLC39A14</i>	<i>Solute carrier family 39 member 14</i>	<i>Transporting non-transferrin binding iron uptake in cells</i>	<i>Deletion or knockdown suppresses hepatic ferroptosis.</i>	<i>Liver tissue (human IHC); Slc39a14-LKO & DKO mice ± CCl₄ ± high-iron diet</i>	<i>185</i>
<i>SLC40A1</i>	<i>Solute carrier family 40 member 1 (Ferroportin)</i>	<i>Exporting iron from the cells to maintain systemic iron homeostasis</i>	<i>Neuronal knockout promotes ferroptosis induction.</i>	<i>FPN cKO (NEX-Cre) mice ± APP/PS1; neurons ± Aβ ± Fer-1/Liproxstatin</i>	<i>186</i>
<i>TF</i>	<i>Transferrin</i>	<i>Transporting iron in the blood by binding Fe³⁺, delivering it to cells via transferrin receptors</i>	<i>Global and hepatocyte-specific knockout sensitizes iron diet-induced hepatic ferroptosis in liver; Knockdown in cancer cells increases ferroptosis sensitivity.</i>	<i>Cirrhosis patient serum/liver biopsies; Trf-KO (CMV-Cre), Trf-LKO (Alb-Cre), Trf/Slc39a14-DKO mice; melanoma CTCs & cell lines (±TF overexpression/shTF); NOD/SCID Il2rg^{-/-} xenografts</i>	<i>185,187</i>

<i>TFRC</i>	<i>Transferrin receptor protein 1</i>	<i>Mediating iron uptake into cells through transferrin-bound Fe³⁺ internalization</i>	<i>Deletion suppresses erastin-induced ferroptosis and amino acid deprivation-induced ferroptosis.</i>	<i>Balb/c mice (CVB3 infection); HeLa ± CVB3, siTfR1</i>	¹⁴⁵
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1.2.3.2 Lipid Peroxidation as a Central Execution Mechanism

Lipid peroxidation occurs through two complementary routes: enzymatic (LOX-mediated) and non-enzymatic (iron-driven autoxidation). Both converge on the accumulation of oxidized PE species (PE-OOH, CL-OOH), the biochemical hallmark of ferroptosis.

PUFAs, including arachidonic and adrenic acid, are highly susceptible to peroxidation and serve as core substrates for ferroptosis^{188,189}. These FAs are esterified into membrane PLs via the coordinated activity of ACSL4 and LPCAT3, embedding oxidizable moieties into lipid bilayers¹⁹⁰. In contrast, monosaturated fatty acids (MUFAs), incorporated via ACSL3, are less prone to peroxidation and confer ferroptosis resistance by limiting the accumulation of lipid peroxides¹⁹¹.

Following incorporation into membrane PLs, PUFAs are oxidized through both enzymatic and non-enzymatic mechanisms. Lipoxygenases (LOXs), particularly ALOX12 and ALOX15, catalyze the insertion of hydroperoxide (-OOH) groups into PUFA chains, producing lipid peroxides that can initiate ferroptotic conditions, but have minimal impact when ferroptosis is induced by direct GPX4 inhibition^{188,189,192}. Notably, several LOX inhibitors function as radical-trapping antioxidants (RTAs), and their protective effects are not strictly dependent on LOX isoform expression¹⁹².

Importantly, ferroptosis driven by GPX4 loss proceeds largely independently of LOX activity. In these contexts, non-enzymatic autoxidation, an iron-amplified radical chain reaction, emerges as the predominant route of lipid peroxide generation. Lethal accumulation of OxPLs, including hydroperoxylated PEs (PE-OOH) and cardiolipins (CL-OOH), has been observed in ferroptotic tissues following genetic or pharmacologic GPX4 inhibition. Among oxidized lipid species, hydroperoxyeicosatetraenoic acids (HpETEs), but not their reduced forms (HETEs), act as potent inducers of ferroptosis¹⁹³.

Spatially, lipid peroxidation is initiated in extramitochondrial compartments, preceding mitochondrial dysfunction and outer membrane rupture¹⁹³. Redox tracing and imaging studies have demonstrated early peroxidation of cytoplasmic membranes, with lipophilic RTAs (e.g., liproxstatin-1) offering greater

protection than mitochondrial-targeted antioxidants¹⁹³. These findings support a model in which membrane-localized lipid peroxidation, rather than mitochondrial ROS, serves as the primary trigger of ferroptosis. Additional oxidoreductases, including cytochrome P450 oxidoreductase (POR) and NADPH oxidases (NOXs), further enhance lipid ROS production at membrane interfaces under metabolic stress^{194,195}. Together, these lipid metabolic enzymes define ferroptotic sensitivity under metabolic stress.

Table 1.3 summarizes the enzymatic mediators and modulators of membrane lipid peroxidation involved in ferroptosis, highlighting their context-dependent contributions to ferroptotic sensitivity.

Table 1.3. Key modulators of ferroptosis: lipid metabolism

Gene	Protein	Function	Effects of targeting it in ferroptosis	Experimental models used	Refs.
<i>ACSL1/3/4</i>	<i>Acyl-CoA synthetase long chain family member 1, 3 and 4</i>	<i>Activating long-chain fatty acids by generating acyl-CoAs; regulating PUFA or MUFA incorporation into PLs</i>	<i>Overexpression of ACSL1 or deletion of ACSL4 prevents ferroptosis; Knockdown of ACSL3 sensitizes to erastin-induced ferroptosis.</i>	<i>OVCA429, ovarian cancer cells (ACSL1 KD/WT/MT); HEK-293 (ACSL3 KO); Gpx4/Acsl4 DKO cells; basal-like breast cancer cells (ACSL4^{high}); ACSL4-inhibited mice; ovarian cancer tissues</i>	^{190,191,196}
<i>AGPAT3 (AGPAT3)</i>	<i>1-acylglycerol-3-phosphate O-acyltransferase 3</i>	<i>Catalyzing the formation of PA, a precursor of PLs</i>	<i>Knockdown reduces PUFA containing phospholipids, suggesting reduced ferroptosis sensitivity.</i>	<i>M4 Sertoli cells (Agpat3/LPAAT3 KD via shRNA); CHO-K1 cells</i>	¹⁹⁷
<i>AGPS</i>	<i>Alkylglycerone phosphate synthase</i>	<i>Synthesizing ether lipids, including plasmalogens that protect against oxidative stress</i>	<i>Deletion promotes ML210-induced ferroptosis by reducing plasmalogen levels and increasing susceptibility to lipid peroxidation.</i>	<i>HT1080 (AGPS KD); other cancer/immortalized cell lines; SH-SY5Y (neuronal diff.); iCell cardiomyocytes/progenitors (± siRNA); human iPSCs</i>	^{198,199}

<i>ALOX12/1</i> 5/5	<i>Arachidonate</i> 12-, 15-, 5- <i>lipoxygenase</i>	<i>Catalyzing the</i> <i>peroxidation of</i> <i>PUFAs, including</i> <i>arachidonic acid, to</i> <i>generate lipid</i> <i>peroxides</i>	<i>Inhibition by</i> <i>ML355 reduces</i> <i>ferroptosis</i> <i>susceptibility in</i> <i>cancer cells and</i> <i>tumor.</i>	<i>HeLa (ALOX12</i> <i>KD/rescue); H1299,</i> <i>786-O (ALOX12 KD);</i> <i>Alox15^{+/−}, WT mice</i> <i>(ischemia ± LA); Wistar</i> <i>rats (HFD); H9C2</i> <i>(ischemia); bladder cell</i> <i>lines (ALOX5 KD);</i> <i>patient bladder tissues</i>	200- 202
<i>ALOXE3</i>	<i>Arachidonate</i> <i>lipoxygenase</i> <i>E3</i>	<i>Catalyzing lipid</i> <i>peroxidation of</i> <i>specific substrates</i>	<i>Overexpression</i> <i>promotes</i> <i>ferroptosis and</i> <i>knockdown inhibits</i> <i>ferroptosis in</i> <i>cancer cells.</i>	<i>LM3 (ALOXE3 KD ±</i> <i>YAPS127A); other liver</i> <i>cancer cells</i> <i>(siALOXE3: Hepa1-6,</i> <i>HuH-7, SK-HEP-1,</i> <i>HepG2); HEK-293T</i>	188,2 03
<i>CHMP1A/</i> 5/6	<i>Charged</i> <i>multivesicular</i> <i>body proteins</i>	<i>Regulating endosomal</i> <i>sorting and exosome</i> <i>formation to affect</i> <i>lipid metabolism and</i> <i>ROS regulation</i>	<i>Knockdown</i> <i>promotes</i> <i>inflammation and</i> <i>lipid peroxidation,</i> <i>resulting in</i> <i>ferroptosis in</i> <i>kidney and cancer</i> <i>cells.</i>	<i>NRK-52E, renal tubule</i> <i>cells (Dpep1/Chmp1a</i> <i>KD/OE); WT, Dpep1^{+/−},</i> <i>Chmp1a^{+/−} mice (renal</i> <i>injury models); PANC1,</i> <i>HepG2 (CHMP5/6 KD)</i>	204,2 05
<i>DECRI</i>	<i>2,4-dienoyl-</i> <i>CoA reductase</i> <i>1</i>	<i>Metabolizing</i> <i>unsaturated fatty</i> <i>acids to prevent lipid</i> <i>peroxidation</i>	<i>Knockdown</i> <i>promotes</i> <i>ferroptosis by</i> <i>increasing ER stress</i> <i>and PUFA</i> <i>peroxidation.</i>	<i>LNCaP series (±DECRI</i> <i>KD/overexpression);</i> <i>CWR22Res, C4-2; 3D</i> <i>organoids; CD1-nude</i> <i>mice (orthograft)</i>	206
<i>EPT1</i>	<i>Ethanolamine</i> <i>phosphotransf</i> <i>erase 1</i>	<i>Synthesizing PE, a key</i> <i>membrane PL</i>	<i>Knockdown</i> <i>stabilizes LPCAT3</i> <i>and PUFA-PLs,</i> <i>resulting in</i> <i>sensitizing to</i> <i>ferroptosis.</i>	<i>Cancer cells (±Ept1</i> <i>KD/overexpression,</i> <i>cystine-free);</i>	207

<i>ELOVL5</i>	<i>Very long chain fatty acid elongase 5</i>	<i>Elongating long-chain PUFAs for incorporation into membrane PLs</i>	<i>Deletion prevents PUFA incorporation into PLs, resulting in alleviating RSL3-induced ferroptosis.</i>	<i>Hs746T, SNU-484, YCC-16 (ELOVL5 KD/KO; $\pm$ RSL3)</i>	²⁰⁸
<i>FABP4</i>	<i>Fatty acid binding protein 4 (FABP4)</i>	<i>Facilitating intracellular transport of fatty acids, including PUFAs, which are substrates for lipid peroxidation</i>	<i>Inhibition activates PPARγ-mediated signaling and prevents ferroptosis in diabetic retinopathy mouse model.</i>	<i>ARPE-19 cells (HG $\pm$ BMS309403); STZ-induced diabetic mice $\pm$ BMS309403</i>	²⁰⁹
<i>FADS1/2</i>	<i>Fatty acid desaturase 1-2</i>	<i>Catalyzing the introduction of a double bond in saturated fatty acids to form PUFAs</i>	<i>Knockout reduces PUFA-PL-mediated lipid peroxidation and prevents ferroptosis.</i>	<i>Hs746T, SNU-484, YCC-16 (FADS1 KD/KO; $\pm$ RSL3); Huh-7.5 (FADS2 KD/KO/overexpression $\pm$ HCV, $\pm$ IKE); uPA-SCID mice (HCV $\pm$ IKE)</i>	^{208,210}
<i>FAR1</i>	<i>Fatty acyl-CoA reductase 1</i>	<i>Catalyzing the reduction of fatty acyl-CoA to fatty alcohols, essential for lipid remodeling and ether lipid synthesis</i>	<i>Deletion increases sensitivity to long-chain fatty acid-induced ferroptosis.</i>	<i>HT1080 & other cancer cells (FAR1 KD/KO/overexpression)</i>	¹⁹⁸
<i>LPCAT3</i>	<i>Lysophosphatidylcholine acyltransferase 3</i>	<i>Incorporating PUFAs into PLs</i>	<i>Deletion or inhibition prevents ferroptosis by inhibiting PUFA incorporation into PL.</i>	<i>Pfal cells (Lpcat3 KO); HEK293T (hLPCAT3 overexpression); other: HT-1080, 786-O, THP-1</i>	^{190,211}
<i>LPIN1</i>	<i>Lipin 1</i>	<i>Converting PA to DG</i>	<i>Overexpression in adipocytes disrupts adiponectin signaling and</i>	<i>Lpin1-Tg mice (ethanol binge model)</i>	²¹²

			<i>promotes hepatic ferroptosis.</i>		
<i>PEBP1</i>	<i>Phosphatidylethanolamine binding protein 1</i>	<i>Binding to PE and suppressing the Raf1-MAPK pathway</i>	<i>Overexpression sensitizes to ferroptosis, and deletion desensitizes to ferroptosis in acute renal failure (AKI) conditions.</i>	<i>AKI urine cells; bronchial epithelial brushings; HAECs; human kidney proximal tubule cells; HT22, HK2, MLE-12; CCI rats; folic acid AKI mice; C57BL/6 lung, kidney, brain (MALDI-MS)</i>	²¹³
<i>PEX2, 3, 6, 16, 19</i>	<i>Peroxisome biogenesis factors</i>	<i>Forming and maintaining peroxisomes, essential for lipid metabolism and ROS regulation</i>	<i>Knockdown reduces sensitivity to ferroptosis.</i>	<i>HT1080 (PEX3/16 KO); other cancer cell lines; U-2OS (peroxisome reporter); HeLa</i>	^{198,214}
<i>PLA2G6 (iPLA2β)</i>	<i>Ca²⁺-independent phospholipase A2β</i>	<i>Hydrolyzing peroxidized PLs</i>	<i>Knockdown increases ferroptosis sensitivity by impairing the hydrolyzing activity toward 15-HpETE-PE.</i>	<i>Parkinson disease patient fibroblasts (PLA2G6 mut); Pnpla9^{R748W} KI mice; rotenone rats; α-synuclein A53T mice</i>	²¹⁵
<i>POR</i>	<i>Cytochrome p450 oxidoreductase</i>	<i>Transferring electrons from NAD phosphate to microsomal cytochrome P450 enzymes</i>	<i>Knockdown suppresses ferroptosis.</i>	<i>HeLa, OVCAR-8 (Cas9, shRNA); HCT-116, HT-1080, MEF, 293T; nu/nu mice (xenograft); C57BL/6 (ConA liver injury)</i>	²¹⁶
<i>PRKAA2 (AMPKα2)</i>	<i>AMP-activated protein kinase α2</i>	<i>Regulating energy metabolism and lipid oxidation</i>	<i>Deletion promotes PUFA-PL levels and induced erastin-induced ferroptosis sensitivity.</i>	<i>MEFs (AMPK WT/DKO); AMPKα1/α2L/L; Rosa26-CreERT2 mice (IRI model); ACHN, Caki-1 (CRISPR KO)</i>	²¹⁷

<i>PRMT6</i>	<i>Protein Arginine Methyltransferase 6</i>	<i>Modulating ferroptosis by activating antioxidant defenses (Nrf2 pathway) and promoting lipid peroxidation (via ACSL1 and PL-PUFA synthesis)</i>	<i>Knockout promotes ferroptosis in diabetic nephropathy (DN) mouse model.</i>	<i>HGECs, HK-2 (PRMT6 KD/inhibition); PRMT6^{-/-} mice (DN model); human DN patients</i>	²¹⁸
<i>SCD1</i>	<i>Stearoyl-CoA desaturase1</i>	<i>Converting SFAs to MUFAs</i>	<i>Overexpression protects cells from ferroptosis, and deletion induces ferroptosis.</i>	<i>Primary FT-t cells & COV362 (SCD1 overexpression); OVCAR4 (SCD1 KD); NSG mice (FT-t xenograft ± erastin/A939572)</i>	²¹⁹
<i>SREBF2 (SREBP2)</i>	<i>Sterol regulatory element-binding protein 2</i>	<i>Regulating cholesterol and lipid metabolism</i>	<i>Deletion promotes ferroptosis by inducing TF transcription.</i>	<i>Melanoma CTC lines; A375; NOD/SCID mice (xenograft); SREBP2 overexpression/shRNA</i>	¹⁸⁷
<i>TFAZZIN (TAZ)</i>	<i>Cardiolipin transacylase</i>	<i>Remodeling cardiolipin, a mitochondrial lipid essential for mitochondrial function</i>	<i>Deletion confers ferroptosis resistance by suppressing EMP1–NOX4 signaling and mitochondria–ER contact.</i>	<i>786O (shTAZ, 3D culture); NOD/SCID mice (786O xenograft ± erastin); RCC PDX (13-789); HEK293T (TAZ KO)</i>	^{195,220}

1.2.3.3 Dysfunction of Redox and Antioxidant Systems

Multiple antioxidant pathways suppress ferroptosis. Central to this defense is GPX4, a selenoenzyme that reduces lipid hydroperoxides (PUFA-PL-OOH) to non-toxic lipid alcohols, maintaining membrane integrity and redox balance^{221–223}. GPX4 activity depends on GSH, which is synthesized from cysteine imported via the cystine/glutamate antiporter system xc- (SLC7A11/SLC3A2)^{222,223}. Notably, excessive SLC7A11 expression may paradoxically increase ferroptotic sensitivity by overwhelming cellular redox capacity²²⁴. GPX4 also requires selenocysteine, whose synthesis relies on mevalonate pathway intermediates^{225,226}.

In addition to GPX4, GPX4-independent antioxidant systems confer ferroptosis resistance. FSP1 reduces ubiquinone (CoQ) to ubiquinol (CoQH₂), preventing plasma membrane lipid peroxidation²²⁷. Within mitochondria, DHODH reduces CoQ to limit lipid ROS accumulation²²⁸. Selenium supports this process via sulfide quinone oxidoreductase activity²²⁶. The GCH1-BH4 axis also mitigates ferroptosis by remodeling membrane PLs to resist peroxidation^{229,230}.

When these systems fail, unchecked redox imbalance drives ferroptosis. NADPH, essential for GSH regeneration and peroxidation function, further supports redox buffering²³¹⁻²³⁴. Its depletion compromises peroxide detoxification, while excess ROS can inactivate antioxidant enzymes via protein glutathionylation²³⁵⁻²³⁷.

Organelle-specific vulnerabilities also exist. Mitochondrial GPX4 loss has been linked to accelerated lipid peroxidation and ferroptosis in metabolically active tissues²³⁸⁻²⁴¹, underscoring the importance of spatial redox regulation.

Together, these antioxidant pathways form a multilayered defense against ferroptosis. Their collapse, through metabolic stress, nutrient deprivation, or iron overload, marks the tipping point toward ferroptotic execution (Table 1.4). These antioxidant defense axes form a multilayered shield against ferroptosis, yet their collapse under metabolic stress provides a mechanistic link to CMD pathology.

Table 1.4. Key modulators of ferroptosis: antioxidant system.

Functional Axis	Key Targets	Mechanism	Impact on Ferroptosis	Refs.
<i>GSH-Dependent System</i>	<i>GPX4, GCLC, CHAC1, GSTZ1</i>	<i>Regulate GSH synthesis, utilization, or degradation; GPX4 reduces lipid peroxides using GSH.</i>	<i>GPX4 is essential for ferroptosis suppression. GCLC deletion and CHAC1 overexpression promote ferroptosis; GSTZ1 modulates response to sorafenib.</i>	<i>222,242-244</i>
<i>GSH-Independent Antioxidant Systems</i>	<i>FSP1, DHODH, PRDX6, PARK7, NADPH-related enzymes (G6PD, IDH1)</i>	<i>Suppress lipid peroxidation independently of GSH; maintain redox homeostasis via CoQ reduction or ROS scavenging.</i>	<i>Overexpression protects against ferroptosis; knockdown sensitizes cells by disrupting redox buffering.</i>	<i>245-248</i>
<i>NRF2 Regulatory Pathway</i>	<i>NFE2L2, KEAP1, PRMT6, SESN2</i>	<i>Control antioxidant gene expression; NRF2 is activated</i>	<i>NRF2 activation suppresses ferroptosis; KEAP1 loss or</i>	<i>249-253</i>

		<i>upon KEAP1 inhibition or PRMT6/SESN2 modulation.</i>	<i>PRMT6/SESN2 activation enhances resistance.</i>	
<i>ROS Production & Oxidative Stress</i>	<i>NOX1/3/4, DUOX1, SAT1, TXNIP, STAT3</i>	<i>Promote ROS production and amplify oxidative stress under metabolic or inflammatory conditions.</i>	<i>Upregulation sensitizes cells to ferroptosis; knockdown/inhibition reduces lipid ROS and ferroptotic death.</i>	<i>233,254-259</i>
<i>Mitochondrial Redox Regulation</i>	<i>SIRT1-3, GPD2, CYB5R1</i>	<i>Control mitochondrial oxidative metabolism and redox enzymes (CoQ, FAD-related pathways).</i>	<i>Overexpression protects from mitochondrial lipid peroxidation; loss promotes ferroptosis in metabolically active tissues.</i>	<i>216,260-264</i>
<i>Stress Response & Protein Quality Control</i>	<i>HSF1, HSPB1</i>	<i>Maintain proteostasis under oxidative stress, stabilize cellular redox machinery.</i>	<i>Overexpression inhibits ferroptosis by buffering redox and maintaining protein integrity.</i>	<i>167,265</i>
<i>Transport & Nutrient Sensing</i>	<i>SLC7A11, ABCC5</i>	<i>Regulate cystine/glutamate exchange (system xc-) and glutathione transport.</i>	<i>SLC7A11 overexpression suppresses ferroptosis; ABCC5 knockdown sensitizes cells to sorafenib-induced ferroptosis.</i>	<i>159,266</i>

1.2.4 Targeting Ferroptosis in CMD: Clinical vs. Preclinical Insights

Given its role across diverse pathologies, ferroptosis has emerged as a compelling therapeutic target across CMD. Prototype ferroptosis inhibitors such as Fer-1 and liproxstatin-1 potently block lipid peroxidation in preclinical models, although none have yet advanced into clinical testing. By contrast, iron chelators such as deferoxamine (DFO) and deferiprone (DFP), already used in iron overload disorders, have shown benefit in CMD by improving endothelial function and cardiac performance²⁶⁷⁻²⁶⁹. Similarly, repurposed metabolic drugs, including SGLT2 inhibitors and PPAR γ agonists, remodel lipid profiles to reduce pro-ferroptotic substrates²⁷⁰⁻²⁷², while antioxidants such as N-acetylcysteine (NAC) and CoQ analogs support redox

buffering^{273,274}. Several such small molecules that modulate ferroptosis pathways are already being tested in CMD-related contexts, including MI, MASH, and diabetic nephropathy (Table 1.5). These studies highlight both the therapeutic promise and the translational gap: while indirect ferroptosis modulation is clinically tractable, dedicated ferroptosis inhibitors remain confined to preclinical development.

Table 1.5. Clinical trials of ferroptosis-targeting small molecules in CMD

Pathway	Agent (Class)	Mechanism	Disease/ Indication	Ferroptosis Role	CMD Relevance	Endpoints	NCT#	Phase	Status
Iron metabolism	Deferoxamine (DFO, chelator)	Iron chelation	Acute renal failure, AKI, stroke	Direct: iron chelation, ROS control	Indirect (vascular/renal complications linked to CMD)	Renal/neur o outcome, adverse events	NCT00870883, NCT04633889, NCT00777140	II	Comple ted
	Deferiprone (DFP, chelator)	Iron chelation	Myocardial infarction	Direct: iron chelation	Direct (post- MI cardiac remodeling)	Cardiac recovery	NCT05604131	II	Recruit ing
	Dexrazoxane (DXZ, chelator/Top2 β inhibitor)	Iron chelation, Top2 β modulation	Doxorubicin cardiotoxicity (HF prevention)	Direct: iron/ROS, ferroptosis block	Direct (cardiac protection)	HF, LVEF, biomarkers , safety	NCT03930680	III	Recruit ing
Lipid metabolism	Pioglitazone (TZD, ACSL4 inhibitor)	ACSL4 inhibition	CKD, MASH	Indirect: lipid peroxidation suppression	Direct (MASH, metabolic overlap)	CKD progressio n, liver function	NCT03471117, NCT05254626	IV	Recruit ing
	Rosiglitazone (TZD, ACSL4 inhibitor)	ACSL4 inhibition	NASH, Ulcerative colitis	Indirect: lipid peroxidation suppression	Direct (MASH, metabolic overlap)	Hepatic, GI endpoints	NCT00492700, NCT00065065	II	Comple ted

	GS-0976 (ACC inhibitor)	ACC inhibition	NASH	Indirect: FA synthesis/lipid peroxidation	Direct (MASH)	MRI-PDFF, enzymes, fibrosis	NCT02856555	I/II	Completed
	MK-4074 (ACC1/2 inhibitor)	ACC inhibition	NAFLD	Indirect: FA synthesis/lipid peroxidation	Direct (MASLD)	Liver TG, safety	NCT01431521	I	Completed
Redox/oxidative stress	Acetaminophen (System Xc ⁻ inhibitor)	SLC7A11 inhibition	Coronary artery disease	Indirect: redox/ferroptosis link	Direct (CAD)	Major cardiac events	NCT04093219	III	Active
	Edaravone (RTA)	Radical trapping agent	MI, stroke	Indirect: lipid ROS suppression	Direct (MI)	Cardiac, neuro outcomes	NCT00265239, NCT00200356	IV	Completed
Redox modulation	N-acetylcysteine (NAC, GSH booster)	GSH synthesis/antioxidant	Diabetic nephropathy, mitochondrial disease	Indirect: antioxidant, GSH support	Direct (diabetic kidney)	Renal, neuro, brain GSH (MRSI)	NCT01265563, NCT05241262	II/I/II	Completed / Recruiting
Multi-pathway	NAC + L-Histidine + L-Serine + L-Carnosine	Antioxidant, GSH, lipid peroxidation modulation	NAFLD, visceral adiposity	Indirect: GSH & lipid ROS boost	Direct (MASLD/obesity)	Visceral adiposity, liver function	NCT06377631	NA	Completed
	NAC + DFO + Ascorbate	Iron chelation, GSH, ROS scavenger	MI reperfusion injury prevention	Direct/indirect : iron/ROS scavenger	Direct (MI)	Safety, PK/PD, oxidative stress biomarkers	NCT05215743	I	Completed

Abbreviations: ACC, acetyl-CoA carboxylase; CAD, coronary artery disease; CKD, chronic kidney disease; GI, gastrointestinal; LVEF, left ventricular ejection fraction; MRI-PDFF, magnetic resonance imaging-proton density fat fraction; MRSI, magnetic resonance spectroscopy imaging; NA, not applicable; PK/PD, pharmacokinetics/pharmacodynamics; RTA, radical trapping agent; TZD, thiazolidinedione

Beyond early clinical trials, landmark preclinical studies demonstrate the therapeutic potential of ferroptosis modulation in CMD: Wang et al.²⁷⁵ demonstrated that ferroptosis inhibition alleviates iron overload cardiomyopathy, while Lavine and Kreisel²⁷⁶ showed that ferroptosis blockade protects against ischemia–reperfusion (I/R) injury. More recent preclinical works extend these findings to models of atherosclerosis, MASH, and diabetic complications (Table 1.6).

Table 1.6. Recent preclinical studies targeting ferroptosis in CMD.

Disease	Models, cells or tissue studied	Key Findings (Ferroptosis-related)	Ref.
Atherosclerosis	HCAEC + Ox-LDL	PDSS2 overexpression activated NRF2 pathway, conferring resistance to oxidative stress and ferroptosis.	²⁷⁷
	ApoE ^{−/−} + HFD mice; VSMCs ± Ox-LDL	Fer-1 improved plaque stability by restoring GLS1 via YAP1, enhancing GSH synthesis and suppressing ferroptosis in VSMCs.	²⁷⁸
	ApoE ^{−/−} + Western diet-fed mice ± carotid ligation	Ionizing radiation induced ferritinophagy and ferroptosis via P38/NCOA4; NCOA4 knockdown alleviated vascular damage.	²⁷⁹
Myocardial infarction	Nrf2 ^{−/−} mice ± LAD; H9c2 cells ± erastin	Fer-1 directly bound Nrf2, enhancing nuclear translocation and upregulating xCT and GPX4, thereby reducing ferroptosis and myocardial injury.	²⁸⁰
	SD rats ± LAD; H9c2 ± OGD	USP7 promoted ferroptosis and oxidative stress; USP7 inhibition activated Keap1-Nrf2-GPX4, reduced Fe ²⁺ and lipid peroxidation, improving outcomes.	²⁸¹
Cardiomyopathy	DOX-treated mice (WT, Ripk3 ^{−/−} , Mlkl ^{−/−} , Fadd ^{−/−} , Nrf2 ^{−/−}); H9c2 cells ± DOX	Mitochondrial iron overload drove DOX-induced ferroptosis. Fer-1 and iron chelation alleviated cardiotoxicity. Nrf2/Hmox1 involved iron overload-induced ferroptosis.	²⁷⁵
	Conditional cardiac Ferritin H KO mice (MCK-Cre, Myh6-Cre); HFD-fed mice	Loss of FTH increased lipid peroxidation and ferroptosis, worsening HF. Fer-1 or SLC7A11 rescued cardiac function.	¹²⁶
Pressure-overload-induced HF	TAC-induced mouse model; NRVMs	ACSL4 overexpression promoted ferroptosis, cardiac hypertrophy, and IL-1β production via NLRP3 activation. ACSL4 KO or Fer-1 mitigated dysfunction and inflammation.	²⁸²

	TAC-induced chronic HF mouse model; HL-1 cells	MLK3 drove ferroptosis and pyroptosis via NF- κ B/NLRP3 (early) and JNK/p53 (late). URM-009 or miR-351 restored function by suppressing MLK3.	283
Cardiac hypertrophy	Aortic-constricted rats; CMVEC + Ang II $\pm$ DHA	DHA activated IRF3/SLC7A11 and suppressed ALOX12, inhibiting ferroptosis.	284
	CISD1 ^{-/-} mice (cardiac-specific); cardiac mitochondria	mitoNEET deficiency caused mitochondrial Fe ²⁺ overload and lipid peroxidation, leading to cardiac dysfunction. ROS scavenging reversed the phenotype.	154
Cardiac I/R injury	Alox15 ^{-/-} mice (cardiac-specific); myocardial I/R model	ALOX15 deletion reduced 15-HpETE production and ferroptosis. ML351 (ALOX15 inhibitor) increased PGC1 α and improved cardiac recovery.	285
	I/R mouse model; NCMs $\pm$ hypoxia/reoxygenation (H/R)	Isoliquiritigenin (ISL) alleviated I/R injury by activating Nrf2, upregulating HO-1, SLC7A11, GPX4, and reducing ACSL4, Fe ²⁺ , and lipid peroxidation.	286
	LAD ligation mouse model $\pm$ YTHDF2 silencing; H9c2 cells $\pm$ H/R	YTHDF2 silencing inhibited ferroptosis by stabilizing SLC7A11 mRNA; overexpression aggravated injury via m6A-mediated degradation.	287
	LAD ligation rat model; H9c2 $\pm$ H/R $\pm$ ACSL4 knockdown or YAP overexpression	YAP suppressed ferroptosis by upregulating NEDD4L, which promoted ACSL4 degradation. YAP loss led to ferroptosis and cardiac dysfunction.	288
	Ex vivo isolated heart $\pm$ I/R; MEFs	Glutaminolysis and TF-mediated iron uptake drove ferroptosis. Inhibiting glutaminolysis (Compound 968) or iron chelation (DFO) rescued heart tissue.	144
	I/R rat model; H9c2 cells $\pm$ oxygen-glucose deprivation/reoxygenation (OGD/R)	Sat1 overexpression promoted ferroptosis via MAPK/ERK signaling. Sat1 inhibition restored GPX4/GSH, reduced ROS and myocardial injury.	289
Obesity & MASLD	CDAHFD & HFHFD-fed mice; GPX4-overexpressing or hepatocyte-specific GPX4 ^{-/-} ; HepG2 $\pm$ HG $\pm$ fatty acids	Hepatic ferroptosis (with decreased GPX4 and FSP1 resulting in increased lipid peroxidation) promoted steatosis. UAMC-3203 reversed these changes. PUFA treatment remodeled cellular membranes, driving ferroptosis in HepG2 cells.	290

		In HepG2 cells, lipid loading, especially with PUFA-enriched membranes, reduced ferroptosis defenses and sustained susceptibility.	
	HFD-fed mice; H9c2 cells and iPSC-derived cardiomyocytes $\pm$ fatty acids $\pm$ FeSO ₄	ω -6 PUFA-containing PLs (PE, PC), increasing susceptibility to iron-induced lipid peroxidation. This led to mtDNA leakage, Caspase-1 activation, sterile inflammation, and cell death. Fer-1 inhibited these effects.	²⁹¹
MASH	MCD & HFD-fed mice; Kupffer cells and hepatocytes	Macrophage NCF1 promoted ferroptosis via OxPL–TLR4-induced hepcidin in hepatocytes. Targeting NCF1 or Fer-1 reduced steatosis, inflammation, and fibrosis.	²⁹²
	HFHC- and MCD- fed mice; primary hepatocytes	FOT1 restored insulin sensitivity, glucose tolerance, and reduced inflammation and lipid peroxidation. FOT1 suppressed c-Myc and ACSL4, halting ferroptosis progression.	²⁹³
Diabetes Mellitus & Complications	STZ-induced diabetic mice $\pm$ Fer-1	Fer-1 preserved β -cell mass and insulin/PDX-1 expression by upregulating Nrf2, GPX4, and xCT, preventing lipid peroxidation–mediated injury.	²⁹⁴
	STZ-induced T1D mice; H9C2 cells $\pm$ HG	Irisin activated SIRT1, suppressed p53 acetylation, restored GPX4/SLC7A11, and inhibited ferroptosis, protecting cardiomyocytes.	²⁹⁵
	Atg5 ^{-/-} and Nrf2 ^{-/-} mice; H9C2 cells $\pm$ glucolipotoxicity	In autophagy-deficient hearts, Nrf2 promoted ferroptosis via ACSL4 upregulation and GPX4/FSP1 downregulation. Nrf2 deletion reversed cardiac injury.	²⁹⁶
	NaAsO ₂ -induced pancreatic injury; MIN6 cells	NaAsO ₂ induced ferroptosis by CHAC1 upregulation and GPX4 downregulation. METTL3 overexpression suppressed ferroptosis via m ⁶ A regulation of CHAC1 mRNA stability.	²⁹⁷
	HFD + STZ mice $\pm$ myocardial infarction; AC16 $\pm$ HG + hypoxia	Capsaicin activated TRPV1 and Nrf2/HO-1, restoring GPX4 and GSH, thereby suppressing ferroptosis and improving post-MI cardiac function.	²⁹⁸

In atherosclerosis, ferroptosis promotes foam cell formation by impairing cholesterol efflux (via ABCA1 and ABCG1 downregulation) and enhancing Ox-LDL uptake through unregulated scavenger receptors (SCARA1, LOX-1, and CD36) under GPX4-deficient conditions²⁹⁹⁻³⁰². In addition, loss of GPX4 correlates with decreased SCARB1 expression, which impairs free cholesterol efflux and exacerbates plaque progression³⁰². Ferroptosis contributes to endothelial dysfunction and plaque instability through lipid peroxidation and ROS accumulation. Pharmacological inhibition using Fer-1 or genetic modulation (e.g., PDSS2 overexpression) alleviates these effects by activating the Nrf2 pathway and restoring antioxidant gene expression^{278,303,304}. AMPK activation is also essential for the protective effects of Fer-1, enhancing expression of GPX4, FTH, and SCARB1; its inhibition abrogates these responses³⁰⁴. Under hypoxic conditions within advanced plaques, heme oxygenase-1 (HMOX1) expression increases substantially, creating a paradoxical situation where this typically protective enzyme promotes ferroptosis through enhanced iron release, ultimately destabilizing atherosclerotic lesions^{277,305,306}.

In MI and I/R injury, ferroptosis is initiated by mitochondrial iron overload and suppression of the SLC7A11-GSH-GPX4 axis³⁰⁷. Ferroptosis inhibitors (Fer-1, DFO, MitoTEMPO), Nrf2 activators (e.g., Sal B, urolithin A), and dietary compounds (e.g., *Salvia miltiorrhiza*) reduced infarct size and preserved cardiac function by restoring antioxidant capacity^{144,275,280,285,286,308-311}. Beyond canonical ferroptosis suppression, several post-transcriptional regulators, including Sat1, USP22, and YTHDF2, modulate ferroptosis sensitivity^{281,287,289}. Sat1 promotes ROS generation by disrupting the MAPK/ERK pathway and depleting GPX4 and GSH; its inhibition rescues redox homeostasis²⁸⁹. USP22 enhances SIRT1 stabilization, suppresses p53 activation and restores SLC7A11 expression³¹². YTHDF2, upregulated during I/R, promotes ferroptosis by degrading SLC7A11 mRNA through m6A modification; silencing it ameliorates myocardial injury²⁸⁷. Combination therapy using deferisirox and cyclosporine A synergistically attenuates lipid peroxidation, ER iron accumulation, and infarct size by preserving mitochondrial permeability transition³¹³. Inhibition of pro-ferroptotic lipid metabolism enzymes such as ALOX15 and ACSL4, and activation of Nrf2 with agents like isoliquiritigenin, further reduce I/R-induced damage^{285,286}.

Cardiomyopathy and Cardiac Hypertrophy

Doxorubicin (DOX)-induced cardiomyopathy involves ferroptosis through HMOX1-mediated iron accumulation and mitochondrial GPX4 downregulation, contributing to oxidative damage and cardiac dysfunction^{241,314}. Restoration of mitochondrial antioxidant capacity via GPX4 overexpression or ferroptosis inhibition (e.g., Fer-1, DFO) significantly reduced lipid peroxidation and preserved cardiac function^{241,275,314}. Mitochondrial iron clearance further attenuated cardiomyocyte injury, highlighting the

role of organelle-specific ferroptosis in DOX cardiotoxicity²⁴¹. Immune-mediated ferroptosis also contributes to pathology; IL-12p40 deficiency mitigates ferroptosis by inhibiting Th17 differentiation and IL-17A production, thereby reducing inflammation and fibrosis³¹⁴.

Chronic systemic iron overload similarly promotes ferroptosis-dependent cardiomyopathy. High-iron diets or cardiac-specific FTH1 deletion elevate labile iron pools, ROS, and lipid peroxidation, resulting in HF and hypertrophy¹²⁶. Ferroptosis suppression via SLC7A11 overexpression or antioxidant treatment alleviated these phenotypes and restored redox balance¹²⁶.

Cardiac hypertrophy, particularly under chronic pressure overload, is exacerbated by mitochondrial iron accumulation and lipid peroxidation, especially in mitoNEET (CISD1)-deficient hearts^{154,311}. Restoration of mitoNEET or ROS scavenging ameliorated mitochondrial dysfunction and pathological remodeling^{103,154,315}. Beyond mitochondrial regulation, systemic iron dysregulation impairs ferritinophagy, leading to iron accumulation and activation of the cGAS-STING pathway, which drives sterile inflammation and hypertrophic remodeling^{103,315}. Ferroptosis intersects with inflammatory (MLK3-NF- κ B-NLRP3) and pyroptotic (JNK-p53) pathways during hypertrophy progression^{282,283,316-319}. Temporal inhibition of these axes, including URM-099 or miR-351 overexpression, suppressed myocardial injury and remodeling²⁸³. Lipid metabolism also modulates susceptibility to ferroptosis: ACSL4-mediated PUFA incorporation enhances lipid peroxidation and inflammation, whereas ACSL4 inhibition confers protection²⁸². Impaired FA oxidation and mitochondrial dynamics further sensitize cardiomyocytes to ferroptotic damage, particularly in TAC-induced models³¹⁷⁻³¹⁹. Restoration of mitochondrial quality control via the USP28-PPAR α -Mfn2 or SIRT1-ATF5 axis reduces oxidative stress and remodeling^{318,319}. Bioactive compounds such as DHA and puerarin also suppressed ferroptosis via IRF3-SLC7A11 and Nrf2-HMOX1-GPX4 signaling, offering therapeutic potential^{284,318-320}.

Metabolic Liver Disease and Obesity

In obesity-associated MASLD and MASH, ferroptosis is triggered by hepatic iron overload, PUFA-enriched PL remodeling, and impaired antioxidant defense due to GPX4 depletion^{290,321,322}. Kupffer cell ferroptosis, driven by NCF1-dependent OxPL production and TLR4-mediated hepcidin signaling, amplifies hepatic iron accumulation and disease progression³²³. Double-negative regulatory T cell (DNTs) also undergo ferroptosis upon Ox-LDL exposure through CD36-mediated uptake, leading to loss of immunoregulatory function³²⁴. This ferroptotic process is mediated by ACSL4 and HIF-1 α signaling and can be reversed by CD36 blockade or ferroptosis inhibition³²⁴.

In obese *ob/ob* mice, hepatocyte ferroptosis is associated with ROS, lipid peroxidation, and macrophage M1 polarization³²¹. Treatment with Fer-1 restored GPX4 levels, reprogrammed liver macrophage toward an anti-inflammatory M2 phenotype, and improved liver histology³²¹. Western diet-induced MASLD models exhibit similar signatures, including iron accumulation, lipid peroxidation, reduced GPX4, and upregulated ACSL4, mimicking human pathology³²⁵. Ferroptosis inhibition by Fer-1 or liproxstatin-1 alleviated hepatic damage and oxidative stress^{321,325}. FerroTerminator1 (FOT1), a novel ferroptosis inhibitor, suppressed c-Myc/ACSL4 signaling, reduced hepatic iron burden, and attenuated inflammation and fibrosis even in Nrf2-deficient mice, highlighting its systemic metabolic benefit²⁹³.

Diabetes and Related Complications

In type 1 diabetes (T1D), ferroptosis contributes to β -cell loss via activation of ACSL4-ALOX15 pathways, and is also implicated in impaired wound healing, and tissue injury in the liver and bone^{294,326,327}. Fer-1 preserved β -cell mass and insulin secretion by activating the Nrf2-GPX4-xCT axis, while GPX4 knockdown led to ATP depletion and enhanced oxidative damage^{294,326,327}. Interestingly, nitric oxide may suppress lipid peroxidation through S-nitrosylating ACSL4, thereby reducing ferroptotic lipid synthesis³²⁶. As a result, cytokine-induced β -cell death may occur partly through ferroptosis-dependent mechanisms³²⁶. In diabetic wound models, GPX4 and SLC7A11 were reduced, while ROS was elevated; Fer-1 restored angiogenesis and redox balance through Nrf2 activation. Arsenic exposure further sensitized β -cells to ferroptosis by depleting GSH and suppressing METTL3; CHAC1 inhibition or METTL3 restoration rescued cell viability²⁹⁷.

Extra-pancreatic complications include liver and bone injury. In diabetic liver, Fer-1 treatment reduced lipid peroxidation, preserved HMGB1 localization, and mitigated hepatic injury³²⁷. In diabetic bone, irisin, a product of FNDC5, restored GPX4, SLC7A11, and ferritin expression by blocking lipid ROS and eIF2 α -ATF4-CHOP signaling, preventing osteopathy³²⁸.

Under hyperglycemic and inflammatory conditions associated with T2D, ferroptosis contributes to β -cell dysfunction via iron overload, GPX4 depletion, and lipid peroxidation. Quercetin, vitamin D, and mesenchymal stem cell-derived extracellular vesicles (EVs) restored β -cell function by enhancing antioxidant defense and activating Nrf2-AKT-ERK pathways³²⁹⁻³³¹. Interestingly, mitochondrial ferritin (FtMT) overexpression in adipose tissue of HFD-fed mice increased local ferroptosis but enhanced insulin secretion via β -cell hyperplasia, suggesting compensatory organ crosstalk³³².

Ferroptosis also underlies diabetic cardiomyopathy (DCM), with key drivers including mitochondrial ROS, iron overload, and lipid peroxidation²⁹⁶. While Nrf2 generally offers cardioprotection, persistent stress or autophagy defects can trigger ferroptosis via ACSL4 upregulation and GPX4 suppression²⁹⁶. Fer-1, sulforaphane, and irisin protect against DCM by activating Nrf2 or SIRT1-p53 signaling^{295,296}. Advanced glycation end-products (AGEs) exacerbate ferroptosis via Ptgs2 induction and SLC7A11 suppression, which can be mitigated by MCC950, TXNIP knockdown, or MitoTEMPO^{298,333,334}. TRPV1 activation with capsaicin boosts Nrf2/HMOX1 signaling, reducing fibrosis and ROS levels²⁹⁸. Finally, the USP28-PPAR α -Mfn2 axis plays a central role in preserving mitochondrial fusion and FA oxidation³¹⁸. Its disruption causes lipid overload, mitochondrial fragmentation, and ferroptosis, reinforcing its potential as a therapeutic target in DCM³¹⁸.

In diabetic nephropathy (DN), renal ferroptosis is associated with mitochondrial ROS and GSH depletion³³⁵⁻³³⁷. Agents like dapagliflozin, naringenin, and curcumin improved redox homeostasis by enhancing SIRT1-FOXO3a-GPX4 signaling³³⁵⁻³³⁷. Combination therapies (e.g., NAC + insulin) activated the SIRT3-Grx3-Trx2 axis and protected renal function³³⁸.

1.3 Lipid Remodeling and Ferroptosis Susceptibility

Lipid metabolism underpins energy balance, membrane structure, and signaling⁵⁵. In CMD, chronic nutrient excess, oxidative imbalance, and inflammation remodel lipid storage, membrane composition, and systemic lipid transport^{339,340}. These processes not only contribute to lipotoxicity and metabolic dysfunction but also amplify ferroptotic vulnerability by expanding the pool of oxidizable substrates³⁴¹. Below, we highlight three interconnected domains—lipid storage, membrane remodeling, and systemic transport—that collectively shape ferroptotic sensitivity across CMD. An overview of lipid classes and their associated molecules is provided in Figure 1 to serve as a reference framework for the following sections.

1.3.1 Lipid Storage and Ferroptosis Susceptibility

Lipid droplets (LDs) are dynamic intracellular organelles that store neutral lipids, primarily TGs and cholesterol esters, and serve as critical regulators of cellular energy balance and lipid homeostasis³⁴². In the context of CMD, dysregulated LD metabolism contributes to lipotoxic stress, ectopic lipid accumulation, and ultimately cellular injury³⁴³⁻³⁴⁵. For instance, excessive LD deposition in pancreatic β -cells impairs insulin secretion and promotes β -cell dysfunction in T2D³⁴⁶, while lipid accumulation in cardiomyocytes is associated with cardiac contractile dysfunction³⁴⁷.

Under metabolic stress conditions, such as ER stress, oxidative stress, and iron overload, cells activate adaptive reprogramming of lipid metabolic pathways to mitigate damage^{344,348,349}. These responses include enhanced *de novo* lipogenesis, increased phospholipase activity, and elevated rates of lipophagy^{344,350,351}. While initially protective, excessive lipid turnover may paradoxically exacerbate lipotoxicity by increasing the flux of FFAs and lipid intermediates to sensitive organelles such as the ER and mitochondria^{344,351}.

LD biogenesis is initiated at the ER through the accumulation and condensation of neutral lipids, forming a buffering system that sequesters excess or toxic lipids, preserves membrane integrity^{344,348,352}. Recent studies suggest that LDs also influence ferroptosis sensitivity. Particularly, loss of FSP1 disrupts CoQ10 recycling, which promotes peroxidation of neutral lipids, such as TGs and cholesteryl esters, stored in LDs, thereby triggering ferroptosis³⁵³.

LD catabolism occurs via lipolysis, lipophagy and exocytosis, releasing FFAs for mitochondrial and peroxisomal β -oxidation³⁵⁴⁻³⁵⁶. Notably, enhanced lipophagy has been shown to alleviate hepatic steatosis and improve metabolic outcomes in models of MASH³⁵⁰. However, excessive lipophagy may also increase PUFA availability and incorporation into membrane PLs, sensitizing cells to lipid peroxidation and ferroptosis³⁵⁷.

LD-associated enzymes and proteins critically regulate ferroptosis sensitivity. Diacylglycerol O-acyltransferase 1/2 (DGAT1/2), which catalyze TG synthesis, promote PUFA sequestration into LDs and reduce their incorporation into cellular membranes, thereby protecting against ferroptosis³⁵⁸. Conversely, pharmacological or genetic inhibition of DGAT enzymes increases PUFA-containing PLs and ferroptotic vulnerability³⁵⁸. Members of the perilipin (PLIN) protein family also modulate LD dynamics and ferroptosis susceptibility: PLIN1 mutations are linked to lipodystrophy, PLIN5 regulates cardiac lipid oxidation, and PLIN4 promotes stress-induced LD formation and has been implicated in ferroptotic sensitivity³⁵⁹⁻³⁶². In addition, acyl-CoA thioesterase 1 (ACOT1) plays a context-dependent role in CMD: while its deficiency enhances FA flux to mitochondria and attenuates high-fat diet (HFD)-induced obesity³⁶³, its downregulation in doxorubicin (DOX)-induced cardiomyopathy exacerbates lipid peroxidation and cardiac dysfunction³⁶⁴.

In summary, metabolic stress-associated dysregulation of lipid storage and turnover not only contributes to lipotoxicity but also shapes ferroptosis susceptibility through complex mechanisms involving LD dynamics, FA trafficking, and lipid remodeling enzymes. Key molecules implicated in these processes are summarized in Table 1.3.

1.3.2 Membrane Lipid Remodeling as a Molecular Switch

Membrane PL composition is a decisive determinant of ferroptotic vulnerability^{339,365-367}. Enzymes such as ACSL4 and LPCAT3 enrich PLs with arachidonic and adrenic acids, while lipoxygenases (e.g., ALOX15) catalyze their peroxidation, generating oxidized PL species that destabilize membranes^{55,368,369}. Across CMD subtypes—atherosclerosis plaques, diabetic myocardium, steatotic hepatocytes—lipidomic studies consistently reveal PUFA enrichment that increases susceptibility to oxidative membrane rupture³⁷⁰⁻³⁷³. This convergence highlights membrane remodeling as a unifying ferroptotic axis in CMD, rather than a disease-specific phenomenon.

Protective remodeling mechanisms counterbalance these pro-oxidant changes. Ether lipids (plasmalogens) act as endogenous antioxidants, and their depletion exacerbates CMD progression^{374,375}. Monounsaturated fatty acid (MUFA) incorporation via ACSL3 competes with PUFAs for membrane incorporation, lowering ferroptotic risk¹⁹¹. Therapeutically, agents such as pioglitazone and SGLT2 inhibitors, already in clinical use for T2D, have been shown to reprogram lipid remodeling, reducing pro-ferroptotic lipid pools and restoring membrane resilience²⁷⁰⁻²⁷².

1.3.2.1 Emerging Roles of Specific Lipids

Beyond neutral lipids, various PL subclasses also contribute to ferroptosis regulation and CMD pathogenesis. Plasmalogens, a unique class ether-linked PLs enriched in PUFAs, serve both as reservoirs for PUFAs and as endogenous antioxidants that mitigate oxidative stress³⁷⁴. In obesity, elevated levels of arachidonic acid-containing plasmalogens may reflect compensatory membrane remodeling^{376,377}. Therapeutically, exogenous plasmalogen enrichment using batyl alcohol has been shown to attenuate atherosclerosis in HFD-fed mice, highlighting their protective role³⁷⁸.

CERs and their precursors, such as dihydroceramide (DHC), are key regulators of lipid signaling, remodeling, and cellular homeostasis^{379,380}. DHC has been implicated in inhibiting adipogenesis and promoting adipocyte death, suggesting a pathogenic role in adipose tissue dysfunction^{379,380}. CER accumulation in subcutaneous and visceral fat (SAT and VAT) is commonly observed in obesity, but their precise role in ferroptosis remains under investigation³⁷⁹.

1.3.2.2 Therapeutic Interventions Targeting Lipid Remodeling

Targeting lipid remodeling has emerged as a promising therapeutic avenue to mitigate CMD progression and ferroptotic injury. Pioglitazone, a PPAR γ agonist used in T2D treatment, improves insulin resistance by promoting lipid redistribution from visceral to subcutaneous adipose depots²⁷¹. Shotgun lipidomic

analysis have showed that pioglitazone alters membrane lipid composition, increasing saturated PE species while reducing arachidonic acid-containing PLs and cardiolipin, collectively decreasing ferroptosis susceptibility²⁷¹.

Similarly, SGLT2 inhibitors, such as empagliflozin, have reported to induce tissue-specific lipid remodeling, elevating oxidized FAs in gonadal WAT while reducing lysophospholipids in SAT²⁷⁰. These effects may contribute to the observed cardiometabolic benefits beyond glucose lowering.

PLs, particularly PC and PE, are critical to membrane structure and signal transduction³⁸¹. Alterations in the saturation level and acyl-chain composition of PLs significantly affect membrane fluidity, redox balance, and receptor signaling dynamics³⁸¹. Elevated levels of PC and PE have been associated with insulin resistance and mitochondrial dysfunction^{368,369}. Furthermore, oxidized PLs (OxPLs), including oxidized PE and PS, function as pro-inflammatory mediators that exacerbate tissue injury in conditions such as atherosclerosis and non-alcoholic fatty liver disease (NAFLD)⁵⁵.

Taken together, modulating membrane lipid composition and lipid remodeling enzymes offers a promising strategy for reducing ferroptosis sensitivity and mitigating CMD progression.

1.3.3 Systemic Lipid Transport and Inter-organ Crosstalk

Systemic lipid distribution is coordinated by lipoproteins and EVs, both of which serve as a carrier of ferroptosis-prone lipids^{60,382,383}. Lipoproteins transport TGs, cholesterol, and PLs; their remodeling in CMD shifts the lipid milieu toward pro-oxidant species⁶⁰. Elevated LDL and VLDL foster atherosclerosis³⁸², while oxidized LDL (Ox-LDL) accelerates endothelial dysfunction and inflammation^{384,385}. HDL, normally protective, becomes dysfunctional in CMD, losing its antioxidant capacity and contributing to systemic oxidative stress^{386,387}.

EVs complement lipoproteins by transferring complex lipid cargo between tissues³⁸⁸. Their lipid composition reflects the metabolic state of donor cells and actively shapes recipient cell responses³⁸⁸. In obesity and diabetes, EV lipidomes shift toward pro-inflammatory and peroxidation-prone species, including PUFA-PLs and CERS³⁸⁹⁻³⁹⁴. Such cargo not only mirrors cellular stress but also propagates ferroptotic vulnerability across tissues, linking adipose tissue liver, vasculature, and skeletal muscle in a maladaptive network of lipid exchange²².

Together, these systemic carriers amplify CMD pathology by redistributing ferroptosis-prone lipids beyond individual organs. This inter-organ crosstalk provides a conceptual framework for understanding how

localized lipid dysregulation escalates into systemic ferroptotic stress, underscoring the therapeutic potential of targeting lipoprotein and EV lipid composition to mitigate CMD progression.

1.4 Extracellular Vesicles (EVs)

EVs, including exosomes and microvesicles, are emerging as key mediators of intercellular communication in CMD⁶⁰. EVs are secreted by nearly all cell types and carry diverse bioactive cargo, including proteins, lipids, and nucleic acids, that reflect the physiological or pathological state of the originating cell³⁹⁵⁻³⁹⁸. In the context of CMD, EVs contribute to systemic metabolic dysfunction, immune activation and inter-organ crosstalk by modulating signaling pathways involved in insulin sensitivity, inflammation, and lipid metabolism^{398,399}. Notably, considering recent EV-based drug trials under FDA highlighted the potential of modulated EVs as both biomarkers and potential therapeutic strategies⁴⁰⁰.

1.4.1 EV Biogenesis and Function

EVs are lipid bilayer-enclosed vesicles that are secreted through either endosomal (exosome) or plasma membrane (microvesicles) pathways³⁸⁸. Their formation is tightly regulated by lipid sorting machinery, including CER-dependent budding and cholesterol-rich raft domains that influence curvature and cargo selection^{388,401-403}. The lipid composition of EV membranes, enriched in PLs, SLs, and cholesterol, ensures vesicle stability and bioactivity³⁸⁸. In particular, CERs and phosphoinositides (PIPs) promote intraluminal vesicle (ILV) formation within multivesicular bodies (MVBs), while bis(monoacylglycerol)phosphate (BMP) facilitates vesicle maturation and trafficking^{388,404}.

Functionally, EVs deliver their cargo and affect to recipient cells and via membrane fusion, endocytosis, or receptor-ligand interactions, thereby regulating gene expression, metabolic activity, and immune responses^{388,405} (Fig. 1.3). Through these mechanisms, EVs influence diverse cardiometabolic processes, including endothelial function, insulin sensitivity, and lipid storage⁴⁰⁵.

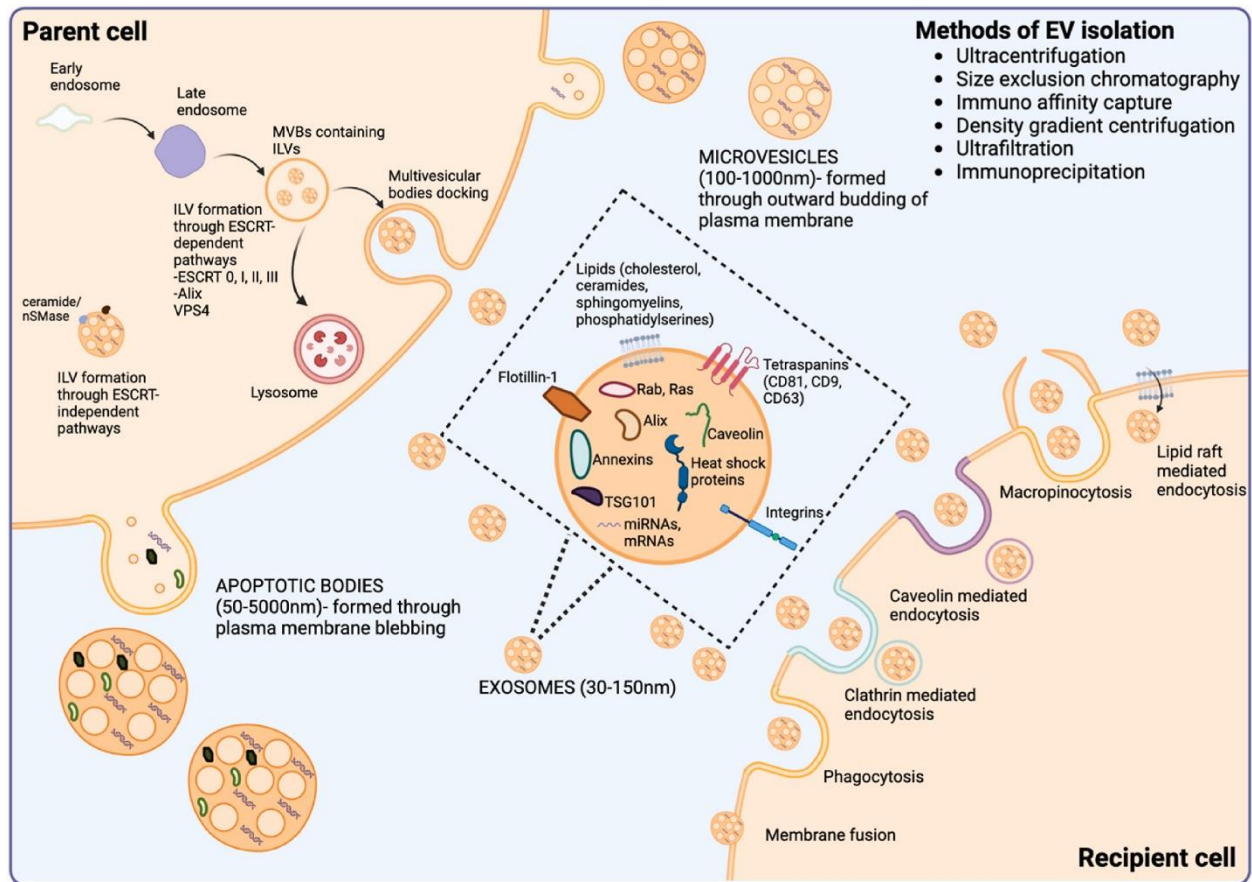


Figure 1.3. Summary of parent cell EV biogenesis, classes of EVs, methods of EV isolations and recipient cell EV uptake. The different classes of EVs are generated through various biogenesis pathways including the ESCRT-dependent and -independent pathways. EVs generated from the ESCRT-dependent pathway are derived from multivesicular bodies, containing intraluminal vesicles that fuse with the plasma membrane to release into the extracellular space as EVs. Microvesicles are generated from budding of the plasma membrane and apoptotic bodies are blebbed from the plasma membrane. EV internalization in recipient cells is mediated primarily by clathrin-mediated endocytosis, but also include caveolin-mediated endocytosis, phagocytosis, micropinocytosis, lipid raft-mediated endocytosis and direct membrane fusion. A wide variety of EV isolation and purification techniques are used, these generally fall into the categories of ultracentrifugation, size exclusion chromatography, immunoaffinity capture, density gradient ultracentrifugation, ultrafiltration, and immunoprecipitation.

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1.4.2 EV Cargo Alterations in CMD

EVs are known to transport a diverse range of cargo molecules, including nucleic acids, proteins, and lipids⁴⁰⁶. In the context of CMD, alterations in the molecular composition of EV cargo have been increasingly reported, with such changes being implicated in modulating cellular response in recipient tissues^{405,407}.

EV miRNA Cargo in CMD

Among EV cargos, microRNAs (miRNAs) have been extensively studied due to their established roles in post-transcriptional gene regulation. miRNAs (~22 nucleotides, noncoding) bind to the 3' untranslated region (3'UTR) of target mRNAs, leading to altered mRNA stability and translation^{408,409}. These molecules are selectively packaged into EVs and released into circulation, enabling intercellular communication across organs⁴¹⁰.

A growing body of evidence indicates that EV-miRNA profiles are dysregulated in obesity and associated comorbidities such as T2D, CVD, and NAFLD^{411,412}. For example, a large-scale human study identified circulating EV-miRNAs, including miR-92a-3p, miR-21-5p, and miR-486-5p, that correlate with BMI, dyslipidemia, and insulin resistance⁴¹².

Adipose tissue is a key source of circulating EV-miRNAs. Adipocyte-derived EVs deliver miRNAs such as miR-27a, miR-222, and Let-7d, which collectively impair insulin signaling in muscle or inhibit myogenic differentiation via HMGA2 repression⁴¹³⁻⁴¹⁵. Similarly, hepatic inflammation and insulin resistance are exacerbated by miRNAs such as miR-130b and miR-103, while ASC-derived EVs carrying miR-144/486 promote hepatocyte proliferation via Txnip targeting^{416,417}.

In endocrine pancreas, macrophage- or adipocyte-derived EVs enriched in miR-155 or miR-27a-5p impair insulin secretion and β -cell function^{418,419}. Additionally, EV-miRNAs such as miR-9-3p and miR-132/212 affect CNS or vascular function, and others like miR-802-5p and miR-221-3p contribute to cardiac insulin resistance and vascular remodeling^{420,421}.

BAT-derived EVs exhibit protective metabolic functions, delivering miRNAs (e.g., miR-99b, miR-378a-3p, miR-125b-5p) that regulate hepatic FGF21 or protect cardiomyocytes from MAPK-driven apoptosis^{420,422}. Liver- and muscle-derived EVs also contribute to inter-organ communication via miRNAs such as miR-122 or miR-29b^{423,424}.

Taken together, these findings support the notion that EV-miRNAs are dynamically regulated by metabolic state and play pivotal roles in mediating systemic metabolic dysfunction and inflammation in CMD.

EV Protein Cargo in CMD

Another major component of EVs is protein. EV-associated proteins include membrane markers, enzymes, signaling molecules, and inflammatory mediators that can modulate immune, metabolic, and vascular functions in recipient cells^{425,426}.

In human studies, altered profiles of EV protein markers have been linked to metabolic dysfunction. For instance, CD36⁺ and CD14⁺ plasma EVs, originating from metabolic and immune cells, are elevated in obese individuals with insulin resistance and visceral adiposity, and correlate with glucose and lipid abnormalities^{427,428}. Similarly, CD62E⁺ endothelial-derived EVs are increased in subjects with metabolic syndrome, indicating endothelial activation and dysfunction⁴²⁹.

Functionally, protein cargos in EVs can act as direct modulators of target tissue physiology. Adipocyte-derived EVs have been shown to deliver RBP4, a molecule associated with insulin resistance, to macrophages and endothelial cells, triggering inflammation and vascular dysfunction⁴³⁰. These findings underscore the role of EV proteins not just as biomarkers but as active participants in disease progression.

Notably, recent studies have identified mitochondrial metabolic enzymes (e.g., ACADVL, HADH, CPT2) in adipocyte-derived EVs, along with free fatty acids such as palmitate and oleate³⁹⁵. These cargos are taken up by hepatocytes and skeletal muscle cells, enhancing mitochondrial β -oxidation and oxygen consumption³⁹⁵. This suggests that EVs function not only as signal carriers but also as metabolic shuttles that deliver enzymatic machinery and substrates. Such activity may be perturbed under metabolic stress, contributing to metabolic imbalance in CMD.

Overall, the proteomic composition of EVs reflects their tissue of origin and metabolic context. Under CMD conditions, EV proteins may amplify inflammation, impair insulin sensitivity, or reprogram energy metabolism in distant organs.

EV Lipid Cargo in CMD

Lipid composition of EVs is also affected by CMD^{389,431}. In obesity, EVs are enriched in pro-inflammatory lipids such as CERs and may be depleted of anti-inflammatory lipids³⁸⁹. Depot-specific

differences have been reported: for example, VAT-derived EVs are enriched in PG and PI, correlating with a more inflammatory phenotype than SAT-derived EVs³⁹⁰. EVs from obese gonadal fat exhibit lower CER levels compared to lean controls, reflecting complex tissue and condition-specific remodeling³⁹⁰.

Additionally, EVs carry TGs, particularly under conditions of impaired lipolysis, such as obesity, which may provide an alternative pathway for lipid mobilization³⁹¹. Inflammatory stimuli like TNF α and palmitate increase EV secretion and modulate their lipid cargo, whereas anti-inflammatory agents like eicosapentaenoic acid (EPA) have minimal effect³⁹¹. Increased numbers of plasma EVs, possibly indicating higher lipid cargo content, correlate with BMI, plasma triglyceride levels, and markers of systemic inflammation^{390,432-434}. Importantly, EVs enriched in PUFA-containing PLs may increase vulnerability to lipid peroxidation and ferroptosis, linking EV lipid remodeling to redox imbalance and tissue injury in CMD.

Collectively, alterations in EV cargo, including miRNAs, proteins, and lipids, reflect and propagate the metabolic and inflammatory perturbations observed in CMD. These findings support the growing view of EVs as active mediators of inter-organ communication and systemic disease progression, with implications for biomarker discovery and therapeutic targeting.

1.4.3 Therapeutic Strategies of Using EVs in CMD

EVs have emerged as both potential therapeutic tools and actionable targets in CMD^{400,405}. Their endogenous origin, capacity to carry diverse bioactive cargo, and ability to interact with recipient cells via membrane fusion or receptor engagement make them attractive candidates for precision medicine approaches⁴⁰⁰.

Engineered EVs for Therapeutic Delivery

Advances in bioengineering have enabled the generation of customized EVs loaded with defined cargo, such as miRNAs, peptides, proteins, or small molecules, designed to modulate specific pathological pathways⁴³⁵. Engineered EVs have been developed to enhance insulin sensitivity, reduce chronic inflammation, or promote mitochondrial function in target tissues^{436,437}. For instance, EVs loaded with anti-inflammatory miRNAs (e.g., miR-146a) or antioxidant enzymes such as catalase have demonstrated vascular and metabolic benefits in preclinical models of atherosclerosis and diabetes⁴³⁸⁻⁴⁴². In addition, adiponectin-enriched EVs, either isolated from adipose tissue or synthetically reconstituted, have been

shown to exert insulin-sensitizing and anti-inflammatory effects in target tissues, highlighting their therapeutic promise in CMD⁴⁴³.

Pharmacological Modulation of EV Biogenesis and Cargo

Pharmacological agents can influence both the release and composition of EVs. Several studies have shown that commonly used drugs, including metformin and statins, can modulate EV secretion and cargo content under metabolic stress^{444,445}. For example, metformin has been shown to modulate the microbial composition of plasma-derived EVs in PCOS-IR patients, potentially contributing to improved insulin resistance by enriching beneficial taxa such as *Streptococcus salivarius*⁴⁴⁴. Similarly, polyphenols such as quercetin have been shown to modulate the content of mesenchymal stem cell (MSC)-derived EVs, enriching them with anti-inflammatory miRNAs and enhancing their therapeutic efficacy in models of cartilage degeneration and liver fibrosis⁴⁴⁶⁻⁴⁴⁸. Similarly, quercetin has been reported to enhance the therapeutic potential of MSC-derived EVs by modulating their cargo^{447,448}. In osteoarthritis models, quercetin-modified MSC-EVs exhibit anti-inflammatory and cartilage-protective effects, partly through activation of PI3K-AKT signaling and glutathione metabolism pathways⁴⁴⁷. In the context of chronic liver disease (CLD), quercetin pretreatment enhances the efficacy of bone marrow-derived MSC-EVs by enriching them with miR-136-5p, which targets and suppresses GNAS, a key regulator of fibrosis and inflammation⁴⁴⁷. Transcriptomic and pathway analyses further revealed that these miR-136-5p-enriched EVs modulate signaling cascades involved in hepatic repair⁴⁴⁷. These findings suggest that EV modulation may represent an additional mechanism through which existing CMD therapies exert beneficial effects.

Lifestyle and Dietary Interventions

Lifestyle modifications, including physical activity and dietary strategies, are increasingly recognized as modulators of EV biogenesis and cargo composition^{431,449,450}. For instance, caloric restriction and weight loss have been associated with decreased levels of circulating EVs enriched in pro-inflammatory proteins and lipids, suggesting a potential role for lifestyle interventions in mitigating EV-mediated inflammatory signaling^{451,452}.

Beyond macronutrient restriction, specific dietary components can influence the composition and functional properties of EVs. For example, human milk-derived small EVs have been shown to carry bioactive ω -3 fatty acid-derived oxylipins such as 14-HDHA, 17-HDHA, and 19,20-DiHDPA, which

exert anti-inflammatory and epithelial-protective effects in models of intestinal injury⁴⁵³. These findings highlight the broader concept that diet-origin bioactive molecules can shape EV cargo and function, with implications for gut health and immune homeostasis.

In parallel, emerging evidence suggests that dietary modulation of the gut microbiota, particularly through probiotic supplementation, can reprogram the microbial EV secretome^{454,455}. These microbiota-derived EVs act as key mediators of host-microbe communication, influencing metabolic regulation, immune tone, and epithelial barrier integrity⁴⁵⁴. Notably, probiotics influence host immunity not only via microbial metabolites but also through EVs enriched in bioactive molecules such as lipoproteins and small RNAs^{454,455}. These vesicles can engage pattern recognition receptors (e.g., TLR2) on macrophages and T cells, driving cytokine production and enhancing IgA secretion⁴⁵⁶⁻⁴⁵⁸. Specific EV components, such as GAPDH and lipoteichoic acid, have been identified as immune-activating ligands⁴⁵⁹⁻⁴⁶¹. Functionally, probiotic EVs have demonstrated beneficial effects in preclinical models of colitis, atopic dermatitis, and intestinal injury, supporting their potential as postbiotic immunomodulators⁴⁵⁴.

Regular physical exercise represents another potent non-pharmacological intervention that influences EV dynamics^{462,463}. Muscle-derived EVs released during exercise can cross the blood-brain barrier and deliver neuroactive cargo that enhances synaptic plasticity, suppresses neuroinflammation, and promotes hippocampal neurogenesis in models of neurodegenerative and metabolic diseases⁴⁶³. These findings support the concept of an EV-mediated muscle-brain axis contributing to cognitive and metabolic resilience.

While compelling, most evidence to date stems from preclinical studies. The extent to which lifestyle and dietary interventions reprogram EV cargo and signaling in humans remains an open question, one that warrants focused translational investigation.

Collectively, these findings underscore the therapeutic potential of EVs in CMD, not only as delivery vehicles for engineered cargo or targets of pharmacological and lifestyle-based modulation, but also as dynamic mediators of inter-tissue communication. As EV biogenesis and cargo composition are responsive to metabolic stress and external interventions, harnessing this plasticity may enable precision strategies to restore immune-metabolic homeostasis. While the therapeutic promise of EVs continues to expand, future work should also refine our understanding of their role as reflective indicators of tissue status and treatment response, thereby bridging therapeutic and diagnostic applications in a unified framework⁴⁶⁴.

1.5 Adiponectin in CMD Pathophysiology

Adiponectin is a key adipokine with pleiotropic roles in regulating glucose and lipid metabolism, inflammation, and oxidative stress responses^{76,465-468}. Circulating adiponectin levels are inversely correlated with BMI and positively associated with insulin sensitivity, mitochondrial function, and cardiovascular health⁴⁶⁹. Hypoadiponectinemia is a hallmark of obesity, T2D, and metabolic syndrome, and is associated with increased risk of CVD and adverse clinical outcomes⁴⁶⁹.

The biological actions of adiponectin are primarily mediated by its receptors, AdipoR1 and AdipoR2, which are widely expressed in metabolic organs such as skeletal muscle, liver, heart, and adipose tissue⁴⁷⁰. Additionally, T-cadherin, a glycosylphosphatidylinositol (GPI)-anchored membrane protein, binds high-molecular-weight (HMW) adiponectin and facilitates its retention and function at the vascular endothelium, despite lacking an intracellular signaling domain^{81,471}. Recently, adiponectin has also been detected within EVs, suggesting a novel mechanism by which adiponectin may endocrine-like effects through intracellular communication⁴⁴³.

1.5.1 Mechanism of Action of Adiponectin

Adiponectin (also known as Acrp30, AdipoQ, or apM1) is a ~30 kDa protein originally identified as adipocyte-derived, but now known to be expressed in other cell types including liver, skeletal muscle cells, cardiomyocytes, and macrophages^{76,472-475}. It circulates in various oligomeric forms (trimeric, hexameric, and HMW complexes) with the HMW isoform generally regarded as the most biologically active in metabolic regulation⁷⁶ (Fig. 1.4).

Upon binding to AdipoR1 or AdipoR2, adiponectin activates several signaling pathways, including AMP-activated protein kinase (AMPK), PPAR α , and ceramidase activity⁷⁶. These downstream effects enhance FA oxidation, reduce CER accumulation, improve insulin sensitivity, and suppress inflammatory signaling⁷⁶.

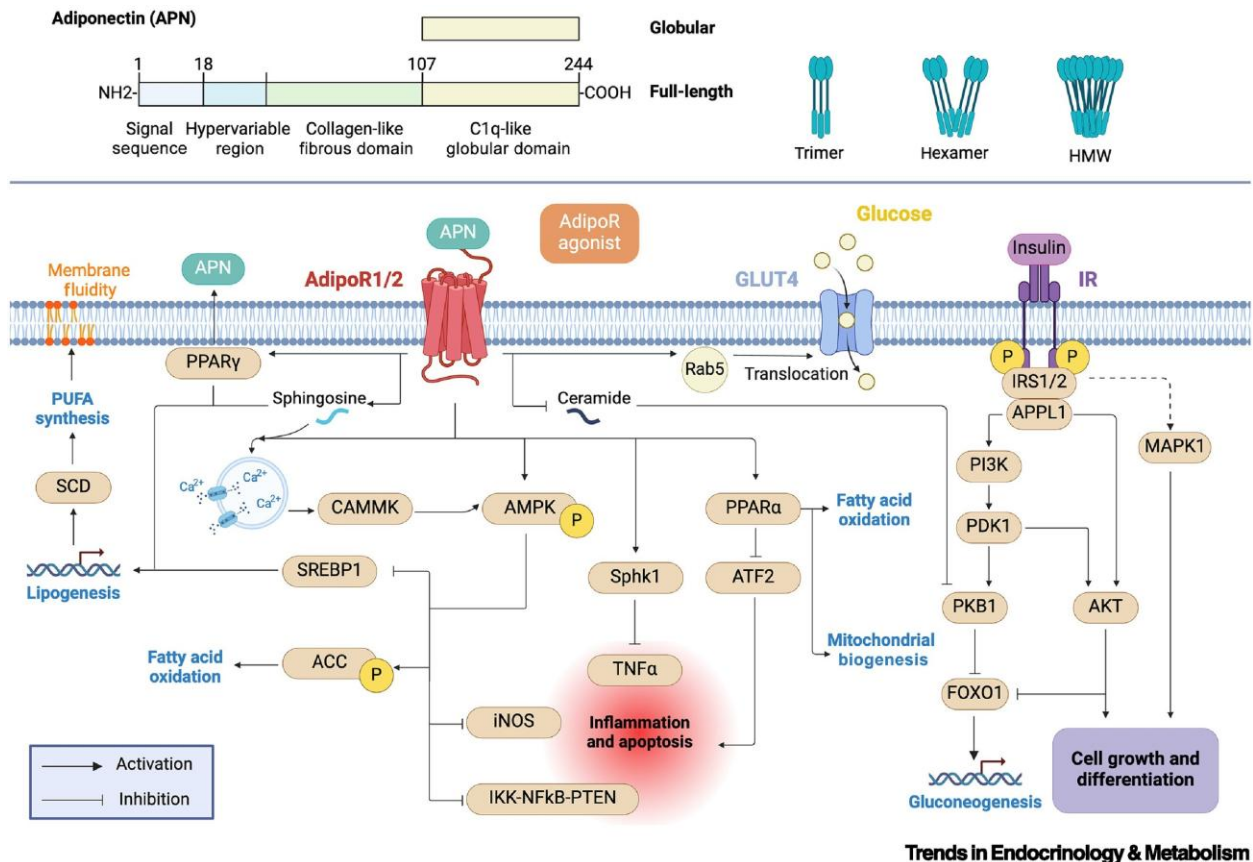


Figure 1.4. Adiponectin gene regulatory region, structure, and signaling pathways. Adiponectin (also known as ACRP30, AdipoQ, GBP28, and APM1) is a major adipokines secreted by adipocytes in multimeric forms. It binds to its receptors, which are widely expressed across various tissues. Cellular adiponectin (APN) expressions can be regulated by peroxisome proliferator-activated receptor γ (PPAR γ) activity and insulin levels, while its circulating levels are influenced by factors such as sex, body mass index (BMI), age, and other physiological conditions.

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1.5.2 Roles of Adiponectin in Lipid Metabolism, Inflammation, and Insulin Sensitivity

Adiponectin plays a central role in maintaining systemic metabolic homeostasis. It promotes FA oxidation in muscle and liver, reduces hepatic lipid accumulation, and enhances insulin-stimulated glucose uptake by activating AMPK and PPAR α pathways⁷⁶. In addition, adiponectin modulates LD dynamics, preserves mitochondrial integrity, and limits ER and oxidative stress⁴⁷⁶⁻⁴⁷⁸.

Anti-inflammatory effects of adiponectin are also well documented. It inhibits activation of NF- κ B and the NLRP3 inflammasome, thereby reducing the production of cytokines such as TNF- α , IL-6, and IL-1 β ^{476,479-482}. In the inflamed adipose tissue of individuals with CMD, adiponectin supports macrophage polarization from pro-inflammatory M1-like to anti-inflammatory M2-like phenotypes, promoting tissue remodeling and metabolic recovery⁴⁸².

This immunometabolic regulation is particularly relevant in CMD, where sterile inflammation is often driven by damage-associated molecular patterns (DAMPs), including oxidized lipids and misfolded proteins^{31,483}.

1.5.3 Therapeutic Interventions Utilizing Adiponectin

Considering adiponectin as a pleiotropic adipokine with anti-inflammatory, insulin-sensitizing, and cardioprotective effects, adiponectin is a compelling therapeutic target in CMD and related conditions. Numerous preclinical studies have demonstrated that native adiponectin, adiponectin-derived peptides, or synthetic AdipoR agonists (e.g., AdipoRon, ALY688, AdipoRaMab) exert protective effects across a wide range of metabolic and cardiovascular pathologies.

Several representative studies highlight the therapeutic potential of adiponectin and its receptor agonists in CMDs. For example, in MI models, genetic deletion or pharmacological inhibition of IP6K1 increased circulating high-molecular-weight (HMW) adiponectin and activated AMPK signaling, leading to reduced infarct size and cardiomyocyte apoptosis⁴⁸⁴. Similarly, administration of globular adiponectin alleviated MI-induced cardiac dysfunction⁴⁸⁵. These cardioprotective effects were abolished in adiponectin-deficient mice, underscoring the essential role of adiponectin in AMPK-mediated protection.

In pressure overload-induced cardiac hypertrophy and diet-induced MASH, the adiponectin receptor agonist ALY688 significantly attenuated disease progression^{80,486}. In diabetic cardiomyopathy models, AdipoRon improved cardiac lipid metabolism by reducing CER accumulation and enhancing acid ceramidase activity⁴⁸⁷. These effects were accompanied by AMPK-PPAR α -PGC-1 α activation and improvements in oxidative stress, apoptosis, inflammation, and insulin sensitivity in both *db/db* mice and human cardiomyocytes⁴⁸⁷.

In MASH, AdipoRon mitigated hepatic steatosis fibrosis, and ER stress in Western diet-fed and STZ-treated mice and reduced palmitate-induced lipid accumulation and inflammation in hepatocytes¹⁰⁷. The protective effects were reversed by the ER stress activator cinchonine, suggesting that suppression of ER stress is central to its mechanism¹⁰⁷.

Adiponectin-based therapies have also shown promise in Duchenne muscular dystrophy (DMD)-associated cardiomyopathy⁴⁸⁸. In early-stage D2.mdx mice, ALY688 prevented cardiac fibrosis and inflammation in a chamber-specific manner, especially in the right ventricle and left atrium, and reduced mitochondrial stress, indicating its potential to mitigate cardiomyopathy in DMD⁴⁸⁸.

Finally, in carbon tetrachloride (CCL₄)-induced hepatic fibrosis, both AdipoRon and the adiponectin-derived peptide ADP355 significantly reduced fibrotic markers such as α -SMA and TGF- β 1, along with serum transaminases and histological fibrosis^{489,490}. These effects were associated with AMPK and eNOS activation and inhibition of hepatic stellate cell (HSC) activation, highlighting the anti-fibrotic potential of adiponectin pathway modulators^{489,490}.

Table 1.7 summarizes the key therapeutic applications of adiponectin and its receptor agonists, across various models of cardiovascular, metabolic, and CMD-related conditions. These interventions have demonstrated efficacy in improving metabolic parameters, reversing inflammation and fibrosis, and restoring mitochondrial and lipid homeostasis in a tissue-specific manner.

Table 1.7. Adiponectin and AdipoR Agonists: Therapeutic Potential Across Cardiometabolic and Associated Diseases.

Adiponectin Modality	Disease Category	Specific Disease	Model	Key Findings	Ref.
Adiponectin (native or KO)	Cardiovascular Disease	Myocardial infarction (MI)	Whole-body IP6K1 ^{-/-} ; Adipocyte-specific IP6K1 ^{-/-} ; Adiponectin ^{-/-} ; Pharmacological inhibitor of 5-InsP7 (TNP)	IP6K1 KO or 5-InsP7 inhibition increased plasma HMW adiponectin, activated AMPK signaling (heart, MEFs), reduced infarct size and apoptosis; cardioprotection was abolished in adiponectin KO mice, indicating adiponectin-dependence.	484
Adiponectin (native or KO)	Cardiovascular Disease	Myocardial infarction (MI)	Ischemic preconditioning (IPC) + MI rat models; APN/APN receptor siRNA; gAd injection	IPC increased cardiac and plasma adiponectin (peak at 12–24 h), improved cardiac function and reduced infarct size; effects were reversed by APN knockdown and restored	485

				by globular Adiponectin; protection mediated via AMPK phosphorylation.	
Adiponectin (native or KO)	Metabolic disease	Diabetic cardiomyopathy	STZ-induced T1D rat models $\pm$ adiponectin adenovirus.	Adiponectin protects against diabetic cardiomyopathy by inducing HMOX-1 through Nrf2–Brg1 signaling.	⁴⁹¹
AdipoRaMab	Metabolic disease	T2D / MASH	HFD–fed C57BL/6J mice; STZ + HFD-induced NASH mice; AdipoR1/R2 double-knockout (DKO) mice; HEK293 cells overexpressing human AdipoR1 or AdipoR2	AdipoRaMab, a long-acting agonistic monoclonal antibody targeting AdipoR1/R2, activated AMPK signaling, improved glucose intolerance in HFD-fed mice, and reduced inflammation and fibrosis in a MASH model. Its efficacy was abolished in AdipoR1/R2 DKO mice.	⁴⁹²
AdipoRon	Cardiovascular Disease	Atherosclerosis / Endothelial Dysfunction	Human Umbilical Vein Endothelial Cells (HUVECs) $\pm$ TNF- α	TNF- α induced endothelial inflammation ($\uparrow$ ICAM1/VCAM1, ROS, glycolysis; $\downarrow$ FA oxidation, mitochondrial ATP; $\uparrow$ intracellular CERs/S1P, $\downarrow$ extracellular S1P). AdipoRon reversed these effects ($\downarrow$ inflammation, $\uparrow$ NO, FA oxidation, ATP; normalized CER/S1P levels). SKI-I reduced S1P but lacked protection, causing $\uparrow$ CER and mitochondrial dysfunction.	¹⁰⁶
AdipoRon	CMD and related conditions	Duchenne Muscular Dystrophy (DMD)-	Aged DMD mouse model	AdipoRon improved cardiac dysfunction and hypertrophy in aged DMD mice by reducing inflammation, oxidative stress, and fibrosis, while enhancing	⁴⁹³

		associated cardiomyopathy		mitochondrial biogenesis via AdipoR1–CAMKK2–AMPK signaling.	
AdipoRon	Cardiovascular Disease	Cardiac hypertrophy	Isoprenaline (ISO) or l-thyroxine-induced cardiac hypertrophy in Sprague–Dawley (SD) rats	AdipoRon alleviated ISO- or l-thyroxine-induced cardiac hypertrophy in SD rats by restoring energy metabolism proteins (PGC-1 α , ERR α , pAMPK/AMPK).	494
AdipoRon	Cardiovascular Disease	Cardiac hypertrophy	Pressure overload-induced cardiac hypertrophy mouse model (aortic banding); AMPK α 2 ^{-/-} mice; neonatal rat cardiac fibroblasts; adult mouse cardiac fibroblasts $\pm$ AngII $\pm$ AdipoRon; human heart tissues (dilated cardiomyopathy patients)	AdipoRon attenuated pressure-overload-induced cardiac hypertrophy and fibrosis by activating AMPK α and suppressing TGF- β 1 expression and myofibroblast differentiation; these protective effects were lost in AMPK α 2 ^{-/-} mice, highlighting AMPK dependence.	495
AdipoRon	Cardiovascular Disease	Heart failure with preserved ejection fraction (HFpEF)	Two-hit HFpEF mouse model (60% HFD along with 0.5 g/L L-NAME in drinking water)	HFpEF mice exhibited reduced AdipoR expression, disrupted FA metabolism, and cardiac lipid overload. AdipoRon improved HFpEF features by limiting lipid accumulation and fibrosis through AdipoR1–AMPK α –TGF- β and AdipoR2–PPAR α signaling.	496
AdipoRon	CMD and related conditions	Diabetic cardiomyopathy	C57BLKS/J <i>db/m</i> and <i>db/db</i> mice $\pm$ AdipoRon; human cardiomyocytes $\pm$	In <i>db/db</i> mice and human cardiomyocytes, AdipoRon improved diabetic cardiomyopathy by reducing	487

			high glucose $\pm$ palmitate $\pm$ AdipoRon	lipotoxicity, CER accumulation, inflammation, oxidative stress, and apoptosis, while enhancing AdipoR expression, AMPK–PPAR α –PGC-1 α signaling, acid ceramidase activity, and lipid metabolism.	
AdipoRon	CMD and related conditions	T2D-associated periodontitis	Adiponectin ^{-/-} mouse; diet-induced obese mouse models	In diabetic mice with experimental periodontitis, AdipoRon reduced blood glucose, osteoclast activity, inflammation, and alveolar bone loss, while promoting bone regeneration via osteogenic differentiation and AdipoR activation. Similar effects were observed in Adiponectin ^{-/-} mice.	497
AdipoRon	CMD and related conditions	Diabetic cardiomyopathy	<i>db/db</i> or C57BLKS/J mice	In <i>db/db</i> mice, AdipoRon shifted cardiac substrate utilization toward glucose oxidation by reducing circulating TGs, but failed to improve cardiac output, efficiency, or mitochondrial respiration, indicating that metabolic reprogramming alone was insufficient to reverse diabetic cardiomyopathy.	498
AdipoRon	Metabolic disease	MASLD/MASH	Western diet or MCD diet-fed mouse model; AML12 hepatocytes $\pm$ Palmitate	AdipoRon reduced PA-induced lipid accumulation and inflammation in hepatocytes and alleviated hepatic injury, steatosis, inflammation, and fibrosis in diet-induced MASH mice. Mechanistically, it suppressed ER stress, and its	107

				protective effects were reversed by the ER stress activator cinchonine, supporting its therapeutic potential in MASH.	
AdipoRon	Metabolic disease	Obesity	HFD-fed rat models	In HFD-induced obese rats, AdipoRon or SG combined with exercise (SG+Ex) improved skeletal muscle atrophy and reduced peri-muscular adipose tissue (PMAT), associated with increased adiponectin and MyoD expression. SG or exercise alone, or pair-feeding, did not replicate these effects.	499
AdipoRon	CMD-related conditions	Chronic ethanol-induced cardiotoxicity	Lieber-DeCarli ethanol diet-fed mouse model $\pm$ AdipoRon; H9c2 cells $\pm$ ethanol $\pm$ C6 CER	In chronic ethanol-fed mice, AdipoRon improved cardiac function and suppressed necroptosis by reducing CER accumulation via PPAR α -mediated lipid metabolism and limiting mitochondrial ROS through the SIRT3–SOD2 pathway, highlighting its therapeutic potential against ethanol-induced cardiotoxicity.	500
AdipoRon	Metabolic disease	Hepatic Fibrosis	C57BL/6J mice $\pm$ CCl $_4$ $\pm$ AdipoRon; Primary mouse hepatic satellite cells; LX-2 cells $\pm$ TGF- β $\pm$ AdipoRon	In CCl $_4$ -induced liver fibrosis and LX-2 cells, AdipoRon suppressed ATF4-mediated ER stress and PSAT1-dependent serine/glycine biosynthesis in hepatic stellate cells, reducing collagen production and highlighting its anti-fibrotic potential.	501
AdipoRon	Metabolic disease	T2D	Diet-induced obesity (DIO) mouse model;	AdipoAI, a novel adiponectin receptor agonist, enhanced AdipoR1/R2, AMPK, and	502

			C2C12 myotubes ± AdipoAI	PPAR α signaling in C2C12 cells and DIO mice, improving glucose tolerance, insulin sensitivity, and reducing adiposity, inflammation, and hepatic injury, comparable to AdipoRon, supporting its therapeutic potential for obesity and T2D.	
AdipoRon	CMD and related conditions	T2D-associated periodontitis	DIO mouse model; RAW 264.7 cells differentiated into osteoclasts by RANKL treatment	In DIO mice with experimental periodontitis, AdipoAI reduced fasting glucose, gingival inflammation, and alveolar bone loss by inhibiting osteoclastogenesis via autophagy activation ($\uparrow$ LC3A/B), showing greater potency than AdipoRon without cytotoxicity, and highlighting its therapeutic potential for T2D-associated periodontitis.	503
AdipoRon	CMD and related conditions	Obesity-associated impairment of lung regeneration	Lep ^{ob} mice; Adiponectin ^{-/-} mice; Human lung microvascular endothelial cells (HLMVECs)	In Lep ^{ob} obese and adiponectin ^{-/-} mice, post-pneumonectomy lung regeneration was impaired due to reduced VEGF/VEGFR2 signaling and angiogenesis. AdipoRon restored alveolar and vascular regeneration by promoting adiponectin–Twist1–mediated endothelial function, highlighting its therapeutic potential for obesity-impaired lung repair.	504
AdipoRon	CMD and related conditions	T2D with associated impaired dental	STZ + HFD-fed mice	In diabetic C57BL/6 mice, high-dose AdipoRon (50 mg/kg/day) significantly enhanced dental implant osseointegration, as	505

		implant osseointegration		shown by improved bone integration and histological parameters. AdipoRon also modulated salivary microbiota composition, suggesting a potential microbiome-mediated mechanism.	
AdipoRon	Metabolic disease	MASLD	Aged C57BL/6J mice (up to 62 weeks) $\pm$ HFD	Long-term oral AdipoRon treatment in HFD-fed middle-aged mice enhanced exercise endurance, reduced hepatic steatosis, and reversed myosteatorosis by improving mitochondrial function and lipid oxidation, promoting healthier skeletal muscle and liver aging despite persistent obesity.	506
AdipoRon	Metabolic disease	Fatty liver disease	Primary bovine hepatocytes $\pm$ NEFA	In bovine hepatocytes, AdipoRon alleviated NEFA-induced lipid accumulation and mitochondrial dysfunction by improving lipid metabolism, enhancing autophagy, and restoring mitochondrial markers, suggesting its therapeutic potential for preventing fatty liver in dairy cows during the transition period.	507
AdipoRon	Metabolic disease	Diet-induced fatty liver disease	HFD-fed Huoyan goslings	In HFD-fed goslings, AdipoRon alleviated hepatic lipid accumulation and oxidative stress, regulated apoptosis and autophagy, and reduced inflammation by modulating AMPK and p38 MAPK signaling pathways,	508

				suggesting its protective role against fatty liver injury.	
AdipoRon	Metabolic disease	Acute liver injury	D-galactosamine (D-GalN)-induced liver injury mouse model; L02 hepatocytes;	AdipoRon ameliorates D-galactosamine-induced acute liver injury by activating AMPK, reducing oxidative stress and inflammatory cytokines, and promoting hepatocyte protection, suggesting its therapeutic potential in acute hepatic damage.	509
AdipoRon	Metabolic disease	MASLD	Human iPSC-derived hepatocyte-like cells (HLCs)	iPSC-HLCs from 4 human donors with varying NAFLD stages were stimulated with oleic acid to model steatosis; AdipoRon treatment partially reversed lipid accumulation and modulated gene expression related to metabolism, immune response, and cellular stress.	510
AdipoRon	Metabolic disease	T2D	HFD-fed C57B/6 mice; STZ-injected adiponectin ^{-/-} mice; INS-1 pancreatic β -cells	AdipoRonPEG5, a PEGylated analog of AdipoRon, demonstrated enhanced solubility, bioavailability, and therapeutic efficacy in reducing lipotoxicity and CER levels in INS-1 β -cells and HFD- or STZ-induced diabetic mice, outperforming AdipoRon by improving insulin sensitivity and activating ceramidase activity, suggesting potential for further drug optimization targeting diabetes and lipotoxicity.	511

AdipoRon	Metabolic disease	Liver fibrosis	C57BL/6 mice $\pm$ CCL4	AdipoRon ameliorated CCL ₄ -induced acute hepatitis and liver fibrosis in mice by reducing oxidative stress, restraining inflammation, and deactivating hepatic stellate cells (HSCs).	489
ADP355	Metabolic disease	Acute and chronic liver injury	Adiponectin ^{-/-} mice $\pm$ Thioacetamide (TAA)	ADP355, an adiponectin-derived peptide, attenuated TAA-induced acute and chronic liver injury in mice by reducing necroinflammation and fibrosis, inhibiting TGF- β 1/SMAD2 signaling, and activating AMPK and STAT3 pathways, suggesting its therapeutic potential in chronic liver disease.	512
ADP355	Metabolic disease	Liver Fibrosis	C57BL/6 mice $\pm$ CCL4	ADP355 significantly attenuates CCL ₄ -induced liver fibrosis in mice by reducing fibrotic markers (α -SMA, TGF- β 1, CTGF, TIMP1) and serum liver enzymes, while activating eNOS and AMPK signaling, supporting its potential as an anti-fibrotic therapy.	490
ALY688	Cardiovascular Disease	Heart failure with reduced ejection fraction (HFrEF)	Pressure overload-induced cardiac hypertrophy mouse model (aortic banding)	AdipoR agonist ALY688 attenuated pressure-overload-induced heart failure, reducing cardiac hypertrophy, inflammation, and fibrosis, while enhancing FA mobilization and oxidation.	486
ALY688	Metabolic disease	MASLD/MASH	C57BL/6J mice $\pm$ CDAHFD; Human iPSC-derived hepatocytes	In CDAHFD-induced MASH mice, the adiponectin-derived peptide ALY688-SR reduced hepatic steatosis, inflammation,	80

				and fibrosis without affecting body weight. Mechanistically, it activated AMPK and p38 MAPK signaling, enhanced fatty acid oxidation, and suppressed pro-inflammatory and pro-fibrotic gene expression, mimicking adiponectin activity.	
ALY688	CMD and related conditions	DMD-associated cardiomyopathy	D2.mdx mice	In early-stage D2.mdx mice (DMD model), cardiac fibrosis was confined to the right ventricle and left atrium and associated with pro-inflammatory macrophage infiltration and mitochondrial dysfunction. Short-term ALY688 treatment prevented fibrosis, inflammation, and mitochondrial stress in a chamber-specific manner, supporting its therapeutic potential in DMD-related cardiomyopathy.	488
Tyr-Pro	Metabolic disease	T2D	Spontaneously Diabetic Torii (SDT) rats	Oral Tyr-Pro (1 mg/kg/day), a natural AdipoR1 agonist, prevented diabetes onset in spontaneously diabetic Torii (SDT) rats by improving glucose intolerance, enhancing insulin secretion, and activating AdipoR1–AMPK signaling in skeletal muscle, while reducing inhibitory p-IRS-1(Ser) signaling.	513

1.5.4 Adiponectin-Mediated EV Regulation: Current Understanding and Future Directions

Emerging evidence suggests that adiponectin influences not only cellular signaling but also EV biogenesis and function. In endothelial cells, adiponectin promotes EV production and secretion through interactions with T-cadherin, highlighting a noncanonical role in EV modulation⁴⁷¹. Furthermore, adiponectin itself can be packaged within EVs and secreted into circulation, enabling endocrine-like activity that affects distant tissues⁴⁴³.

Studies have shown that EVs containing adiponectin can modulate insulin sensitivity, lipid metabolism, and inflammatory responses in recipient cells^{392,397,443}. For instance, in obesity and T2D, circulating EVs are often enriched in CERs and saturated lipids that promote insulin resistance and inflammation^{430,514-517}. In contrast, EVs from adiponectin-sufficient or lean models exhibit anti-inflammatory and metabolically protective lipid profiles^{430,514-517}.

However, it remains unclear whether adiponectin actively shapes the cargo composition of EVs or whether its incorporation is a passive reflection of cellular state. The precise mechanisms by which adiponectin-modulated EVs affect metabolic signaling, and how they interact with ferroptosis sensitivity or organ crosstalk in CMD, are still largely unknown.

Further investigation is needed to: (1) elucidate how adiponectin regulates EV biogenesis and cargo loading; (2) determine how EV-associated adiponectin contributes to inter-tissue communication; and (3) assess the diagnostic and therapeutic potential of EV-adiponectin signatures in CMD. Unraveling these mechanisms may reveal novel biomarkers and therapeutic strategies for CMD based on the modulation of adiponectin-EV pathways.

1.6 Hypothesis and Research Aims

CMD are characterized by chronic metabolic stress, lipid dysregulation, and low-grade inflammation, which collectively drive tissue injury and disease progression. Increasing evidence implicates ferroptosis as a key contributor to CMD pathogenesis; however, the mechanisms linking lipid remodeling to ferroptosis susceptibility and inflammatory signaling in metabolically stressed tissues remain incompletely defined. In parallel, hypoadiponectinemia—a hallmark feature of obesity and metabolic syndrome—has emerged as an important modifier of cardiometabolic risk. Despite this, how adiponectin deficiency reshapes EV cargo and whether such alterations mediate intercellular propagation of metabolic dysfunction and inflammation remain poorly understood.

Central Hypothesis

This dissertation posits that lipid remodeling under metabolic stress sensitizes cells to iron-dependent ferroptosis and inflammation, and that hypoadiponectinemia exacerbates this vulnerability by reshaping EV cargo to propagate metabolic and inflammatory stress.

Overall Objective

The overarching goal of my PhD research is to elucidate how lipid remodeling contributes to ferroptosis susceptibility and inflammation in cardiomyocytes, and to determine how adiponectin deficiency, as a disease-relevant metabolic state, alters EV lipid composition and function, thereby influencing metabolic and inflammatory outcomes in recipient cells.

Specific Aims

Aim1: To investigate mechanisms linking lipid remodeling to ferroptosis susceptibility and inflammatory signaling in cardiomyocytes under metabolic stress, with a particular focus on ω -6 PUFA-enriched PL composition and iron-dependent lipid peroxidation.

Aim2: To characterize how adiponectin deficiency shapes the lipid composition of circulating EVs and to determine the functional consequences of these EVs on metabolic dysfunction in recipient cells.

Together, these aims seek to define a mechanistic framework in which lipid remodeling and hypoadiponectinemia converge to promote ferroptosis, metabolic inflammation, and intercellular propagation of cardiometabolic dysfunction.

CHAPTER 2: Study 1

ω -6 PUFA-enriched membrane phospholipid composition of cardiomyocytes increases the susceptibility to iron-induced ferroptosis and inflammation

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2.1 Abstract

Ferroptosis is an attractive therapeutic target in cardiometabolic diseases (CMD); however, its contribution to myocardial damage requires further elucidation. This study was designed to examine whether altered phospholipid composition in cardiomyocytes enhanced ferroptosis susceptibility, and the underlying mechanisms. Human iPSC-derived cardiomyocytes and H9c2 cells were used to study iron-induced lipid peroxidation, cell death, and inflammation after exposure to different types of fatty acids. Lipidomic analysis was performed using LC/MS to assess changes in phospholipid composition, with a focus on ω -6 PUFA-containing phospholipids. Cellular and mitochondrial lipid peroxidation, sterile inflammation, and cell death were evaluated. Additionally, the release of damage-associated molecular patterns (DAMPs) and macrophage responses, including STING and type I interferon (IFN-I) signaling, were investigated. LC/MS lipidomic analysis indicated that treating cells with arachidonic acid (AA) elevated ω -6 PUFA-containing phospholipids, particularly phosphatidylethanolamines (PE) and phosphatidylcholines (PC). This significantly increased susceptibility to iron-induced total cellular as well as mitochondrial lipid peroxidation. Subsequently, increased release of mitochondrial DNA to cytosol was detected, resulting in both sterile inflammation and subsequent cell death. Furthermore, iron-induced release of one or more damage associated molecular patterns (DAMP) from AA-treated cells that induced crosstalk with macrophages eliciting a STING and type I interferon (IFN-I) response. These results indicate that cardiomyocytes enriched with ω -6 PUFA-containing phospholipids are more susceptible to lipid peroxidation, underscoring ferroptosis as a critical factor in myocardial damage associated with CMD.

Keywords: ferroptosis/ lipid peroxidation/ mitochondrial DNA/ PUFA-containing phospholipids/ sterile inflammation

2.2 Introduction

Cardiometabolic diseases (CMD) are major health concern worldwide, driven by factors such as insulin resistance, impaired glucose metabolism, dyslipidemia and hypertension⁵¹⁸. There are many ways which CMD can impact morbidity and mortality⁵¹⁹. An important factor contributing to CMD is disrupted iron homeostasis⁵²⁰. Epidemiological studies suggest a positive association between conditions of elevated iron levels, such as detected by hyperferritinemia, and the prevalence of CMD^{52,521-526}. Of note, heart failure also occurs due to iron deficiency^{53,527}. This emphasizes the mechanistic connection between iron metabolism and CMD pathology. Given this, understanding how iron dysregulation contributes to detrimental outcomes relevant to CMD becomes important.

Ferroptosis, known as iron-dependent cell death, is characterized by impaired antioxidative enzyme activity and excessive membrane lipid peroxidation^{54,144,528,529}. In particular, phospholipids containing polyunsaturated fatty acids (PL-PUFA) appear to be more vulnerable to peroxidation, and this contributes to enhanced cellular and organellar susceptibility to damage^{188,189,530}. Meanwhile, exogenous monounsaturated fatty acids (MUFAs) provide resistance against such damages¹⁹¹. Consequently, key enzymes such as ACSL4 and LPCAT3 which catalyze the incorporation of PUFAs into PLs, are indicative markers for enhanced susceptibility to ferroptosis¹⁹⁰. Lipid peroxidation and ferroptosis are also involved in promoting sterile inflammation, for example by releasing damage-associated molecular patterns (DAMPs), such as mitochondrial DNA (mtDNA)⁵³¹⁻⁵³³. The release of mtDNA activates the cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) pathway or caspase-1 (Cas-1) via NOD-like receptor 3 (NLRP3) and absent in melanoma 2 (AIM2) inflammasomes, forming a complex cascade of events leading to ferroptosis-induced inflammation⁵³⁴⁻⁵³⁶. Notably, inhibiting ferroptosis has been demonstrated to mitigate inflammation, resulting in improved outcomes of metabolic diseases and heart failure^{109,537,538}. This emphasizes the interconnected contributors to ferroptosis, spanning iron metabolism, oxidative stress, lipid metabolism, peroxidation and inflammation.

Despite growing evidence on the contribution of ferroptosis to CMD pathology, the precise mechanisms by which iron overload promotes myocardial damage under metabolic stresses remain poorly understood^{531,539}. To address this gap, my study aims to elucidate the role of iron in CMD pathology, with a specific focus on how altered PL composition in cardiomyocytes contributes to their enhanced susceptibility to peroxidation and subsequent cellular damage. My research revealed that AA-treated cardiomyocytes were enriched in ω -6 PUFA-containing PLs, becoming more susceptible to iron-induced lipid peroxidation. This resulted in increased cytosolic mtDNA levels, promotion of sterile inflammation and subsequent cell death. Consequently, my findings indicate that cardiomyocytes enriched with membranous ω -6 PUFA-containing PLs exhibit higher susceptibility to ferroptosis. These insights highlight the potential of targeted therapeutic strategies to mitigate myocardial damage in patients with metabolic diseases.

2.3 Methods and Materials

Cell culture and Treatment

H9c2 rat embryonic cardiac myoblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. Once the cells reached approximately 90% confluency, they were treated with 100 μ M fatty acids (arachidonic acid, linoleic acid, oleic acid, or

palmitate) in DMEM containing 2% bovine serum albumin (BSA), 2% FBS, and 1% P/S for 24 hours. Following fatty acid treatments, cells were exposed to FeSO₄ (0–100 µM) in DMEM with 0.5% FBS for 8 hours as a stimulus.

For arachidonic acid (AA) metabolite inhibition, cells were pretreated with 1 µM aspirin, 50 µM Zileuton, or 500 µM 1-aminobenzotriazole for 30 minutes before AA treatment. To test the effects of ferroptosis and apoptosis inhibitors, cells were pretreated with 1 µM Ferrostatin-1, 50 µM Z-VAD-FMK, or 50 µM AC-YVAD-CHO before FeSO₄ treatment in DMEM with 0.5% FBS.

RAW-Dual Cell Culture

RAW-Dual cells (InvivoGen, San Diego, CA), stably expressing reporter genes for SEAP (secreted embryonic alkaline phosphatase) and Lucia luciferase to detect NF-κB and IRF activation, respectively, were cultured in DMEM with 4.5 g/L D-glucose, 10% heat-inactivated FBS, and 1% P/S. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

Differentiation of Human Induced Pluripotent Stem Cells (hiPSCs) into Cardiomyocytes

hiPSCs from the Personal Genome Project Canada (PGPC) 14 iPSC cell line, generously provided by Dr. James Ellis (The Hospital for Sick Children, Toronto), were used in this study. hiPSCs were passaged using ReLeSR reagent and maintained in mTeSR medium. For differentiation, cells were seeded onto BD hESC-qualified Matrigel-coated 6-well plates (1:50 dilution in DMEM/F12) prepared one day prior to seeding. The medium was replaced daily, and cells were cultured for 5–7 days until reaching 90% confluency.

At >95% confluency, cells were transferred to 12-well plates and differentiated into cardiomyocytes using the STEMdiff Ventricular Cardiomyocyte Differentiation Kit following the manufacturer's instructions. Cardiomyocytes were treated with 200 µM AA in culture media for 24 hours, followed by FeSO₄ treatment (25, 50, or 100 µM) for 8 hours.

Animal studies

Male C57BL/6N mice were purchased from Charles River and used under a protocol approved by the Animal Care Committee at York University. Mice were kept in temperature and humidity control rooms (21 ± 2°C, 35–40%) with a daily 12:12-h light-dark cycle. Mice were fed 60% fat diet (Research diets, D12492) for 3 weeks beginning at the age of 8 weeks, and heart tissue and serum were collected for further analysis.

Lipidomics analysis

The lipids from H9c2 cells and whole heart tissues were extracted in (1:10 w:v mg:uL) extraction solvent Butanol:MeOH (1:1) containing 1 ug/mL Equisplash internal standard mix. The samples were homogenized, sonicated, and centrifuged 13000 x g for 10 minutes at room temperature (23°C). ~100 µL of each sample supernatants were used for the LC-MS analysis. Leftover samples were transferred to -20°C storage. Pooled samples for each group were made by combining 5µL of each sample (Total). LC-MRM-MS analyses were performed using Agilent 1290 LC with 16 minutes gradient (A): 0.1% formate in 5:3:2 water: acetonitrile: 2-propanol. (B): 0.1% formate in 1:9:90 water: acetonitrile: 2-propanol at 400 µL/min flow rate on Zorbax eclipse plus C18 RRHD 100x2.1 mm 1.8u (RP-LC-MS). 1 µL injection volumes were used for each run. MRMs were acquired using Agilent 6495A spectrometer in positive and negative modes in 2 separate runs. 519 and 768 lipid species were detected in H9c2 cells and heart tissues, respectively. Data for each LC/acquisition type were analyzed using MassHunter (Agilent). Statistical analysis was performed using MetaboAnalyst.

Lipid Peroxidation assays

Lipid peroxidation was detected by Image-iT™ lipid peroxidation kit. In brief, treated cells with the conditions of interest were washed with PBS. After wash, cells were stained with 10 µM of Image-iT Lipid Peroxidation Sensor (BODIPY 581/591 C11 reagent) with DAPI for 30 minutes at 37°C in a cell culture incubator. The shift from red to green fluorescence upon oxidation is shown in the ratio of red to green. The fluorescence of BODIPY was detected and analyzed using Cx7 high-content screening. To measure mitochondrial lipid peroxidation, MitoPeDPP probe, which is a fluorescence probe that specifically reacts with lipid peroxide in the mitochondrial inner membrane, was used with DAPI. After fixation for 15 minutes at room temperature, cells on glass coverslip were mounted onto microscope slide in Prolong Gold Antifade Mountant. The stained cells were observed with a Nikon A1R confocal laser scanning microscope system.

TBARS assay

After treatments, H9c2 cells were harvested into 1.5 mL Eppendorf microfuge tubes and lysed in ice-cold RIPA buffer supplemented with a cocktail of protease and phosphatase inhibitors. The lysates were used without dilution for subsequent assays. Malondialdehyde (MDA) concentration was measured using the TBARS Assay Kit according to the manufacturer's protocol. The MDA levels were normalized to protein concentrations, which were determined using the BCA assay.

Cell death assays

Cytotoxicity and cell death were quantitatively evaluated by measuring lactate dehydrogenase (LDH) release using the CytoScan™ LDH Cytotoxicity Assay. H9c2 cells were treated with 100 µM fatty acids

for 24 hours, followed by treatment with FeSO₄ at concentrations ranging from 0 to 100 μM for 8 hours. For additional experiments, H9c2 cells were treated with 100 μM arachidonic acid (AA) for 24 hours, followed by FeSO₄ (0–100 μM) for 8 hours, with or without 1 μM Ferrostatin-1 or 50 μM Z-VAD-FMK.

For the LDH assay, 50 μL of cell culture medium was transferred to a 96-well plate and mixed with 50 μL of CytoScan™ substrate solution. The plate was incubated for 30 minutes in a cell culture incubator (37°C, 5% CO₂), protected from light. Absorbance was measured at 490 nm, and background absorbance at 680 nm was subtracted. Results were expressed as relative values compared to the control condition.

Cell death was also assessed using the ReadyProbes Cell Viability Imaging Kit after AA and iron treatments. Cells were stained by adding 2 drops of NucBlue® Live reagent and 2 drops of NucGreen® Dead reagent per 1 mL of cell growth medium, as described previously. Staining was carried out for 30 minutes at 37°C in a 5% CO₂ incubator. Images were captured using an EVOS microscope and analyzed with Celleste 6 Image Analysis software.

Detecting mitochondrial DNA (mtDNA)

Cytosolic mtDNA in H9c2 cells was quantified by qPCR after cytosolic fractionation. Briefly, H9c2 cells were lysed for 10 minutes on a rotator at 4°C in digitonin buffer (25 μg/mL digitonin, 150 mM NaCl, 50 mM HEPES, and Halt protease inhibitor). The lysate was centrifuged at 1,000 × g for 10 minutes at 4°C, and the supernatant was transferred to a new tube and centrifuged again at 10,000×g for 30 minutes at 4°C to isolate the cytosolic fraction in the supernatant. DNA was purified from the cytosolic fractions using the DNeasy Blood & Tissue Kit. Whole cell extract DNA was prepared under the same conditions for normalization. For qPCR, 40 ng of DNA was used per reaction. The mitochondrial DNA copy number was calculated using the ΔΔCt method, with the median ΔCt from nuclear DNA (*Gapdh* gene) used as the reference and mitochondrial DNA quantified via the *Cytochrome b* gene⁵⁴⁰.

Immunofluorescent double-label staining was performed to visualize mtDNA release into the cytoplasm. After treatments, H9c2 cells and iPSC-derived cardiomyocytes were washed twice with PBS. Quant-iT™ PicoGreen™ dsDNA Reagent was diluted 200-fold per the manufacturer's instructions. Cells were stained with the PicoGreen reagent for 1 hour at 37°C in the dark, then washed three times with PBS.

MitoTracker Red stock solution (1 mM) was diluted in growth media without FBS to a final working concentration of 500 nM. Prewarmed staining solution was added to cells in a 4-well chamber and incubated for 30 minutes under standard growth conditions in the dark. After staining, cells were washed with PBS and incubated with fresh prewarmed phenol red-free medium.

Cells were imaged using a Nikon A1R confocal laser scanning microscope. The approximate excitation/emission peaks were 579/599 nm for MitoTracker Red and 502/523 nm for PicoGreen. Quantification of cytosolic mtDNA release involved determining the percentage of PicoGreen-positive areas in the cytoplasm, excluding regions colocalized with mitochondria. This was achieved using the JACoP colocalization macro, an image analysis plug-in for ImageJ software. Specifically, the cytosolic PicoGreen-only area was calculated by subtracting the colocalized area of PicoGreen and MitoTracker Red from the total cytosolic PicoGreen-positive area. Outliers were identified and excluded using the GraphPad ROUT test ($Q = 1\%$).

Measurement of GSH/GSSG ratio

The GSH/GSSG ratio was measured using the GSH/GSSG-Glo™ Assay Kit, following the manufacturer's instructions.

Flow cytometry-based analysis of ROS and caspase activity

Post-treatment, H9c2 cells were incubated with MitoSOX™ Red (100 nM final concentration; Thermo Fisher Scientific) or FLICA™ 660 Caspase-1 Assay Kit (diluted as 1:300 as per manufacturer's protocol; ImmunoChemistry Technologies) in serum-free DMEM. A total staining volume of 500 μ L was used per sample, and cells were incubated for 30 minutes at 37°C in the cell incubator. Following staining, cells were harvested and washed three times with PBS (centrifuged at 250 x g, 3 minutes), then resuspended in 300 μ L of FACS buffer (PBS supplemented with 2% FBS). Cells were kept on ice and protected from light until acquisition.

Flow cytometry was performed on a Cytex Aurora spectral flow cytometer. MitoSOX Red was detected using the channel YG3, and FLICA 660 was detected using the channel R3. Unmixing controls (unstained, MitoSOX-only, and FLICA-only stained samples) were included to set gates and correct spectral overlap or positive/negative signals. Data were analyzed using FlowJO v10.8 (BD Biosciences). Doublets and debris were excluded via FSC-A vs. FSC-H gating, followed by SSC-A vs. SSC-H gating, and live singlet cells were gated for analysis. Mitochondrial superoxide levels and caspase-1 activity were quantified based on either the percentage of positive cells or median fluorescence intensity (MFI), depending on the experiment.

Western blotting

H9c2 cells were harvested and lysed in RIPA buffer supplemented with a protease and phosphatase inhibitor cocktail. Total protein concentration was determined using the BCA assay to standardize the lysates. The

samples were denatured in Laemmli buffer at 90°C for 10 minutes and resolved by SDS-PAGE. Proteins were then transferred onto a PVDF membrane.

Membranes were blocked with 3% BSA for 1 hour at room temperature, followed by overnight incubation at 4°C with primary antibodies. After washing, membranes were incubated with the appropriate horseradish peroxidase (HRP)-linked secondary antibody (anti-mouse IgG, HRP-linked, 1:5000; anti-rabbit IgG, HRP-linked, 1:5000) for 1 hour at room temperature.

The antibody-antigen complexes were visualized using enhanced chemiluminescence reagents, and signals were detected on autoradiography films. Band intensities were quantified using ImageJ software.

Table 2.1 Antibody information.

Antibody	Company	Cat#
cGAS	Abcam	ab252416
TMEM173/STING	Proteintech	19851-1-AP
GPX4	Abcam	ab125066
Cytochrome C	Abcam	ab133504
Histone H3	Abcam	ab1791
GAPDH	CST	14C10
Anti-mouse IgG, HRP-linked Antibody	Cell Signaling	7076
Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling	7074

Quantitative PCR analysis

Total RNA from tissues and H9c2 cells was extracted using Trizol, following the manufacturer's protocol. Briefly, mouse heart tissues were lysed in Trizol using a bead homogenizer, mixed with chloroform at a 1:5 ratio, and centrifuged at 2,000 rpm for 15 minutes at 4°C. The upper aqueous phase was collected and processed to isolate RNA using the PureLink™ RNA Mini Kit.

The extracted RNA was quantified using the Varioscan-based µDrop™ platform. Approximately 30 ng of total RNA per sample was used for reverse transcription, performed with the RevertAid RT Reverse Transcription Kit and random hexamer primers to synthesize total cDNA.

qPCR reactions were prepared with cDNA, SsoAdvanced™ SYBR® Green Supermix, and specific primers (Dataset#). Reactions were run on a QuantStudio™ 3 Real-Time PCR System with the following thermal

cycling conditions: 95°C for enzyme activation, 95°C for denaturation, 60°C for annealing, 72°C for extension, followed by plate reading.

Quantification was performed using the $\Delta\Delta C_t$ method, with results normalized to 18S rRNA.

The primer information is provided in Table 2.2.

Table 2.2 Primer sequence used for qPCR

Primer	Species	Sequence (Forward)	Sequence (Reverse)
SCD	Rat	CCCAGGGCAAACAGTGGTAT	TCTGGATTGGGCTACAGGGA
Acs13	Rat	CAATTACAGAAGTGTGGGACT	CACCTTCCTCCCAGTTCTTT
Acs14	Rat	TATGGGCTGACAGAATCATG	CAACTCTTCCAGTAGTGTAG
Lpcat3	Rat	TTGCTTCCAGATGGCCTAC	ATGAGGGGACACCCAGTATG
Fads1	Rat	GAGGGCATTTCATGCACAGA	AGGCAGACATGGTCACACC
Fads2	Rat	AGAGAAGCCGCTGCTGAG	TGCTTCATTTGTGGAGGTAGG
Cytochrome b	Rat	CGGCTGACTAATCCGATACC	TGGGAGTACATAGCCCATGA
Gapdh	Rat	AACTGTTTATCTTCCATGCTGGC	CAGAGCAGAGCTTAATCGGA
Primer	Species	Sequence (Forward)	Sequence (Reverse)
Acs13	Mouse	TTCCTTCCCCTGCTCCAGT	CGATTCTGAAAAAGTCTCCTGTTG
Acs14	Mouse	GGGCAAATAAAATGCGGCT	GGCTTAAACTCTAGAACTAAAAACC ATCA
Lpcat3	Mouse	CCCCACATCACAGACGACTATC	TCTCACGGTCCCATTTTCATC
Gapdh	Mouse	CAGGGCTGCCTTCTCTTGTG	ACTGTGGTCATGAGCCCTT

Statistics

All statistical analyses were performed using GraphPad Prism 8.0 software. One-way or two-way analysis of variance (ANOVA) with Tukey's post-hoc test was used for group comparisons. Data are presented as mean $\pm$ SEM, except for line and bar graphs, which are presented as mean $\pm$ SD. Statistical significance was defined as follows: $P < 0.05$: significant differences compared to the control group (no treatment, 0 μ M FeSO₄). # $P < 0.05$: significant differences compared to AA + FeSO₄ conditions.

MetaboAnalyst R version 5.0 was used to compute Principal Component Analysis (PCA) and generate heatmaps showing the top 50 different lipid species among groups.

2.4 Results

Short-term HFD feeding remodelled lipid composition toward PUFA-enrichment in mouse heart.

Because ferroptosis is tightly linked to lipid composition, we hypothesized that altered cardiac lipid profiles predispose ferroptosis-induced cardiomyopathy. To test this, we used a 3-week high-fat diet (HFD) model to capture early lipidomic remodeling before overt heart failure. We observed the expected HFD-induced increase in body weight (Fig. 2.1A), and lipid-remodeling related genes expressions: upregulated *LPCAT3* and downregulated *ACSL3* gene expression by qPCR (Fig. 2.1B). This suggested the possible cardiac lipid profile alteration toward PUFA-enriched phospholipids (PUFA-PLs), and away from MUFA-enriched PLs. We confirmed this with heart-tissue lipidomics. Principal component analysis (PCA) revealed a remarkably distinct lipid profile between normal chow (NC) and HFD groups (Fig. 2.1C). Among detected over 750 lipid species, the proportion of PUFA-containing lipids increased by 2.2%, while MUFA-containing species decreased by 1% in the hearts of HFD group (Fig. 2.1D). The heatmap analysis in Figure 2.1E showed increased PUFAs and decreased MUFAs in the hearts of the HFD group. At the species level, HFD-fed hearts exhibited enrichment of arachidonic acid (AA; 20:4)- and adrenic acid (22:4)-containing PLs, such as PE(16:0_20:4) and PE(18:0_20:4), suggesting increased susceptibility to lipid peroxidation and ferroptosis (Fig. 2.1E&F)⁵⁴¹. Overall, short-term HFD feeding remodeled cardiac membrane lipid composition toward PUFA enrichment, potentially predisposing the heart to ferroptotic stress.

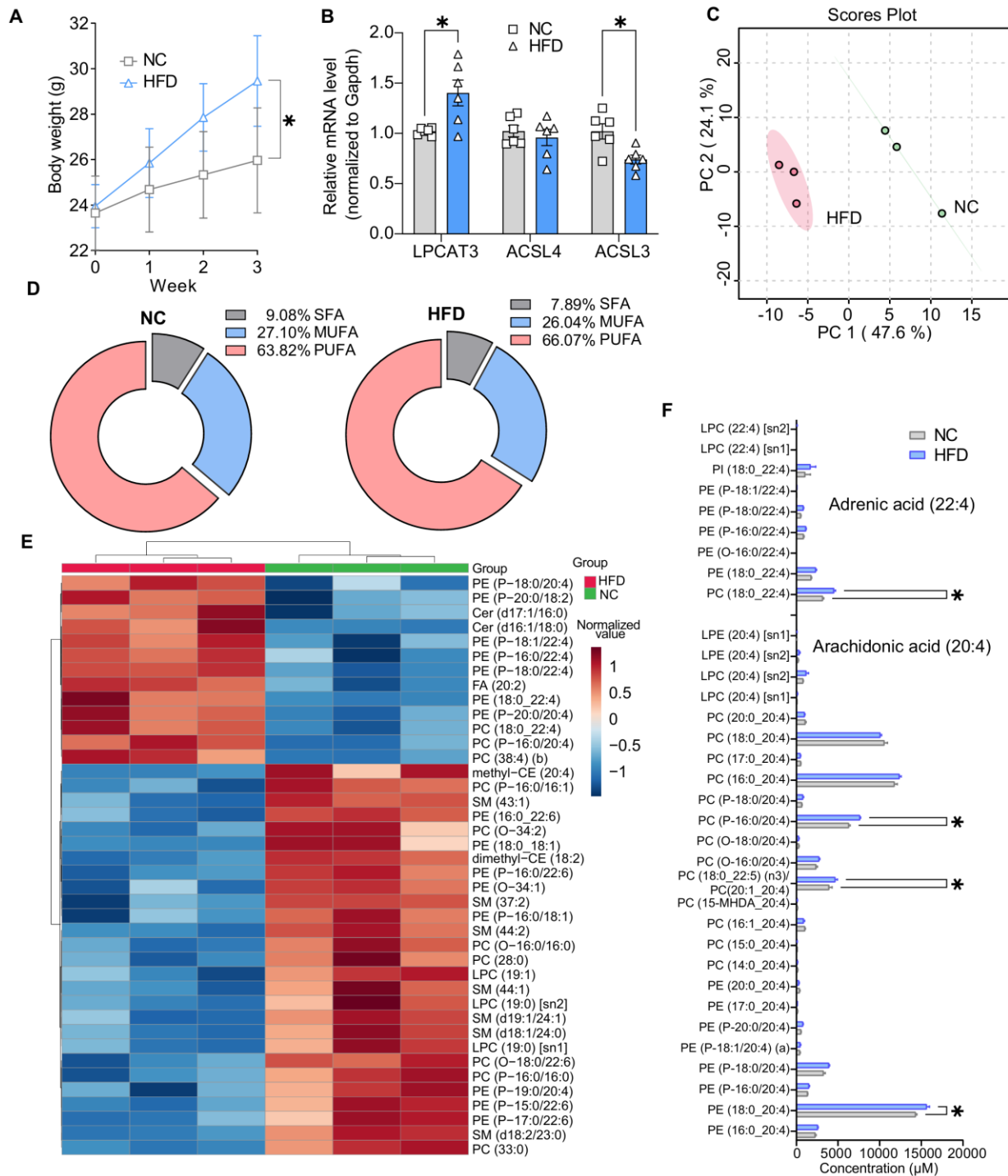


Figure 2.1. Three weeks HFD-fed mice showed increased PUFA-contained phospholipids in the heart.

(A) Body weight of the mice ($n = 8$ per group). (B) qPCR measurements of indicated genes involved in lipid metabolism. ($n = 6$). (C-E) (C) Principal Component Analysis (PCA) plot of total lipids, (D) pie chart of fatty acyl tail type proportions in each group, and (E) heatmap from lipidomics with top 50 lipid species

from the heart. ($n = 3$ per group). (F) The estimated concentration of phospholipids containing ω -6 PUFA (arachidonic acid, 20:4 and adrenic acid, 22:4) ($n = 3$ per group). Cer, ceramide; FA, fatty acid; HFD, LPC, lysophosphatidylcholine; high-fat diet; NC, normal chow; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin. Data are presented as mean $\pm$ SEM for panel B, and mean $\pm$ SD for the rest. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (Two-way ANOVA)

Exogenous fatty acid treatment and 3 weeks of HFD feeding reshapes the lipid profile in H9c2 cells and mouse heart, respectively.

To examine the cellular lipid remodeling and iron-induced downstream effects in the context of CMD, we treated H9c2 cells with exogenous fatty acids: palmitate (PA), a saturated fatty acid (SFA); oleic acid (OA), a MUFA; and AA, an ω -6 PUFA. The cellular lipidome was then analyzed using LC/MRM-MS (Fig. 2.2). PCA revealed clearly distinct lipid profiles after treatments of each fatty acid (100 μ M, 24 h) (Fig. 2.2A). The heatmap (Fig. 2.2B) showed distinct enrichment of PL species in response to each fatty acid, along with notable changes in sphingomyelin (SM) and triglycerides (TG). Of particular interest, AA treatment led to PL remodeling characterized by increased PE and PC species containing polyunsaturated acyl chains (Fig. 2.2C&D). In contrast, ω -3 PUFAs, particularly docosahexaenoic acid (DHA), remained unchanged or decreased (Fig. 2.2E). Notably, AA-treated cells also exhibited upregulation of key lipid-metabolizing enzymes previously linked ferroptosis susceptibility⁵⁶, including *ACSL4*, *LPCAT3*, *FADS1*, and *FADS2* (Fig. 2.2F). Collectively, these results demonstrated that exogenous fatty acid exposure altered the cellular lipidome, and that AA-treated cells displayed an ω -6 PUFA (AA and adrenic acid)-enriched PL composition, suggesting heightened sensitivity to ferroptotic stress.

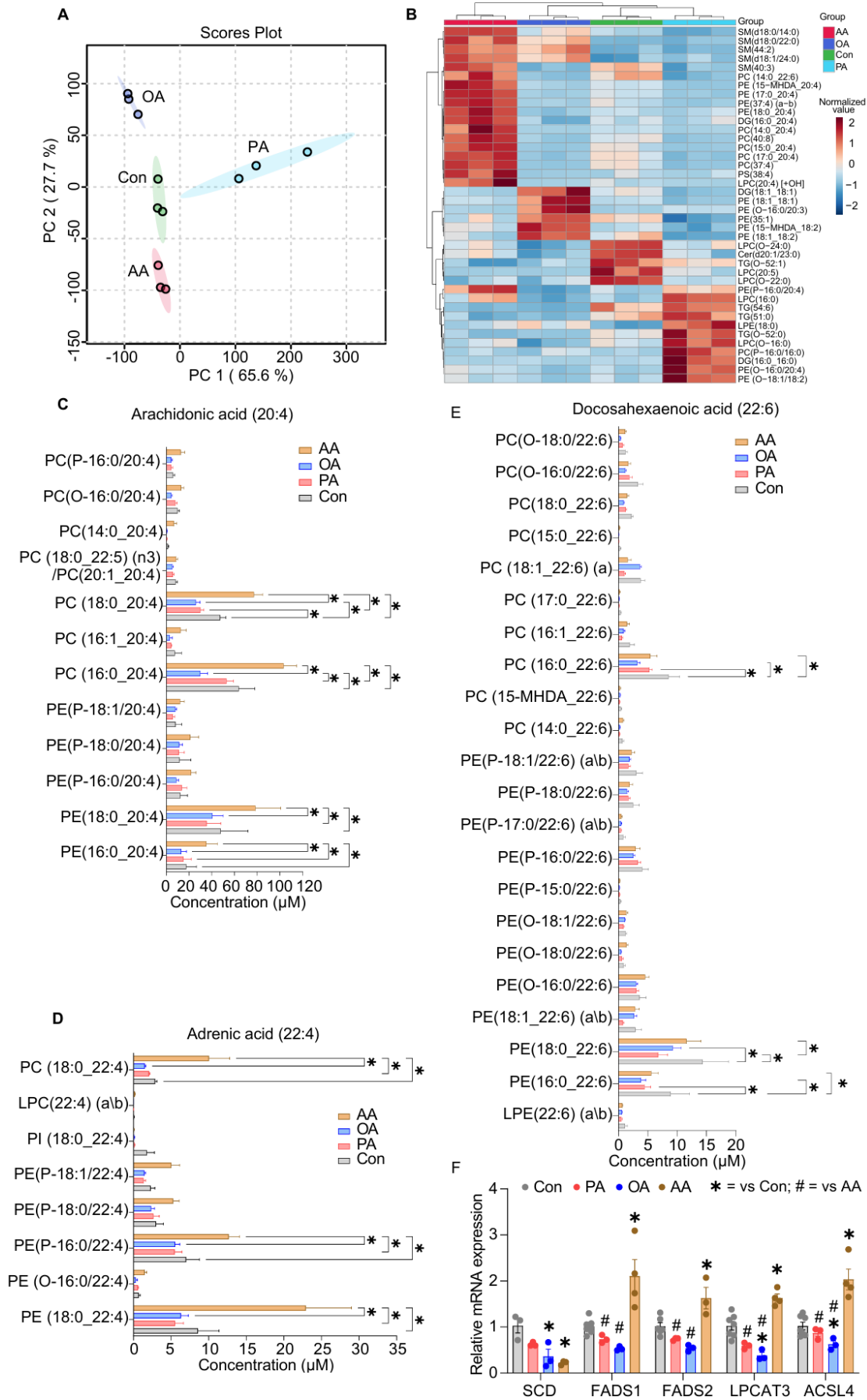


Figure 2.2. Exogenous fatty acid treatment reshaped cellular lipid composition in H9c2 cells.

*(A) PCA plot of total lipids from H9c2 cells after fatty acid treatment (100 μ M of PA, OA and AA for 24h) ($n = 3$ / condition). (B) Heatmap from lipidomics with top 50 lipid species ($n = 3$ / condition). (C-E) Estimated concentration of the PLs containing ω -6 PUFA. (C) arachidonic acid, 20:4, (D) adrenic acid, 22:4, and (E) docosahexaenoic acid, 22:6. ($n = 3$ / condition) F. qPCR measurements of indicated genes involved in lipid metabolism ($n = 3\sim7$). PA, OA and AA were treated to cells for 24 hours ($n = 3$ / condition). Data are presented as mean $\pm$ SD, * $P < 0.05$ (Two-way ANOVA).*

Arachidonic acid pretreatment increased cellular susceptibility to iron-induced cell death.

To investigate the subsequent effects of altered lipid profiles on iron-induced cell death, I treated H9c2 cells with varying concentrations of FeSO_4 (0 to 100 μ M) for 8 hours following fatty acid treatments as described in Fig. 2.3A. Lactate dehydrogenase (LDH) assay indicated that only cells pre-treated with AA exhibited increased cell death upon exposure to iron across this range of concentrations (Fig. 2.3B). This effect was consistent with the treatment of another ω -6 PUFA, linoleic acid (LA) (Fig. 2.4A). To elucidate the mechanisms underlying this cell death, I first examined the effect of various inhibitors of AA-metabolizing enzymes, such as aspirin, zileuton, and 1-aminobenzotriazole (1-ABT). These inhibitors did not alter the iron-induced cell death (Fig. 2.3C), suggesting that AA metabolites were not directly responsible for the observed effect. In contrast, the inhibitor of lipid peroxidation, ferrostatin-1 (Fer-1) and the pan-caspase inhibitor Z-VAD-FMK, significantly attenuated iron-induced cell death in AA-pretreated cells measured by LDH release (Fig. 2.3D). Notably, Fer-1 was more effective than Z-VAD-FMK, suggesting that lipid peroxidation was the primary cause of iron-induced cell death in these cells. The effect of Fer-1 was further verified using fluorescent probe-based tool to assess cell death (Fig. 2.3E&F). To further validate the cellular significance of AA treatment increasing susceptibility to iron-induced cell death, I used human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs). The AA-treated iPSC-CMs exhibited increased cell death upon iron treatment, which was also prevented by Fer-1 (Fig. 2.3G & Fig. 2.4B). These findings suggest that cardiomyocytes treated with ω -6 PUFA, such as AA or LA, became more susceptible to iron-induced cell death, with lipid peroxidation serving as a central mediator.

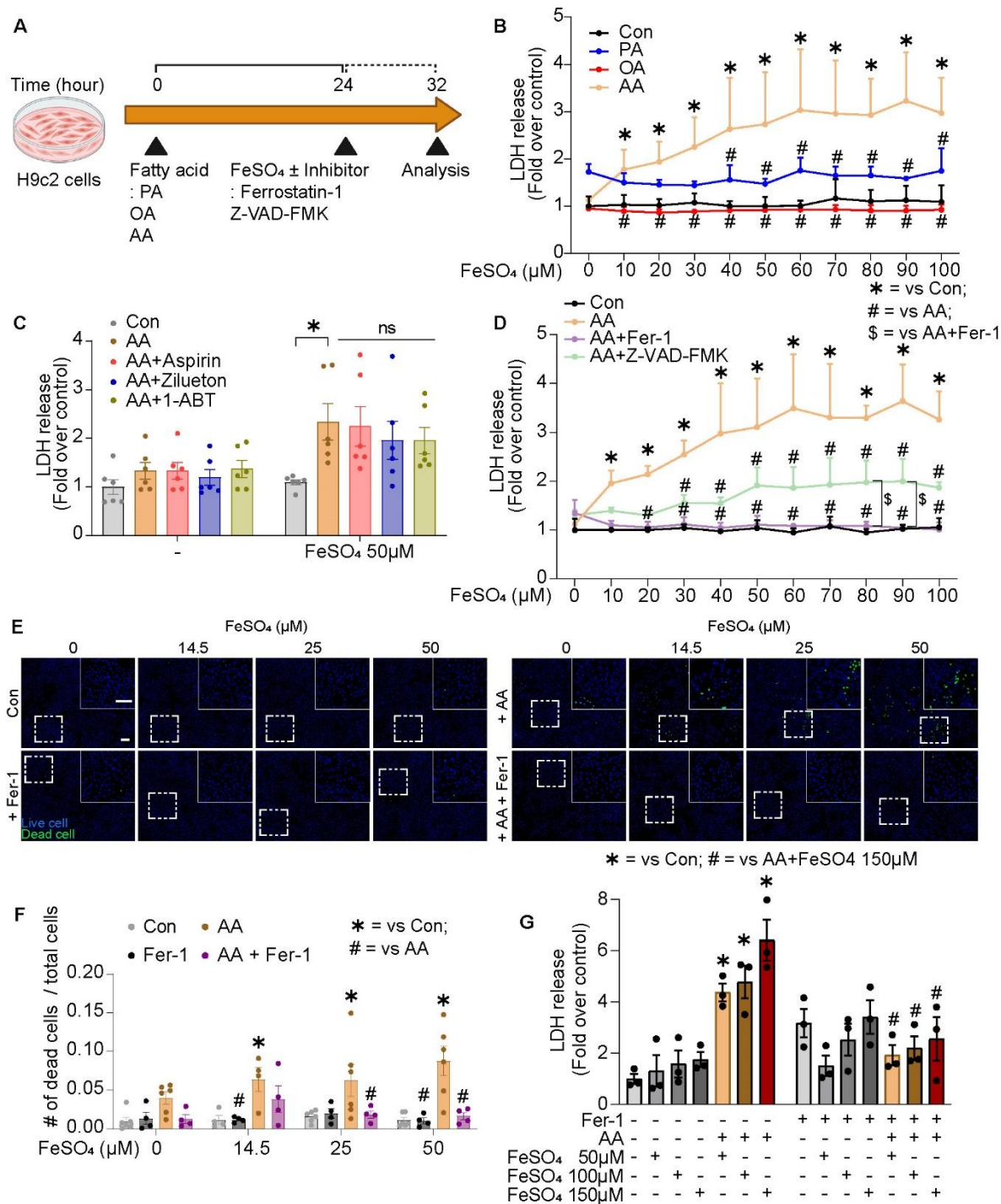


Figure 2.3 Arachidonic acid increased iron-induced cell death in H9c2 cells and iPSC-CMs.

(A) Schematic diagram of in vitro experiments. (B) LDH assay from H9c2 cells after treatment of 100 μM of PA, OA and AA for 24h followed by FeSO₄ treatment (0 to 100 μM) for 8h. (C) LDH assay from H9c2 cells following treatment with 50 μM FeSO₄ for 8h. Inhibitors of AA metabolizing enzymes (1 μM aspirin,

50 μ M Zileuton, 500 μ M I-ABT) were pretreated 30 minutes prior to AA treatment. ($n = 5$). (D) LDH assay from 24h AA-treated H9c2 cells followed by FeSO_4 treatment (0 to 100 μ M) for 8h $\pm$ inhibitors (Fer-1 1 μ M, Z-VAD-FMK 50 μ M) (E-F) Representative images and quantitation of cell viability using ReadyProbe Cell viability Imaging kit. Blue = DAPI, Green = Dead cell. ($n = 4\sim 6$, size bar = 750 μ m). (G) LDH assay from human iPSC-derived cardiomyocytes after treatment of 200 μ M of AA for 24h followed by FeSO_4 treatment (50, 100 and 150 μ M) for 8h. Data for line graphs are presented as mean $\pm$ SD, while other data are presented as mean $\pm$ SEM. Statistical significance is indicated as follows: * $P < 0.05$ compared to the control group (no treatment, 0 μ M FeSO_4). # $P < 0.05$ compared to the AA + FeSO_4 condition. \$ $P < 0.05$ for comparisons between AA + Fer-1 and AA + Z-VAD conditions (Two-way ANOVA).

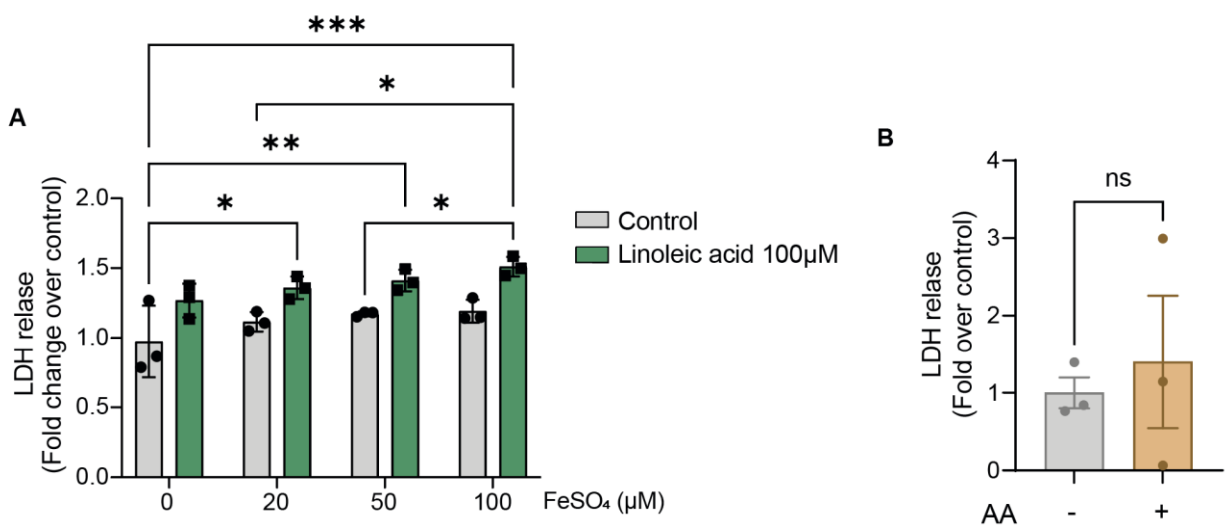


Figure 2.4 The effect of ω -6 PUFA on iron-induced cell death in H9c2 cells and iPSC-CMs

(A) LDH release assay in the culture medium of H9c2 cells after 24-hour treatment with 100 μ M linoleic acid (LA), followed by 8-hour treatment with FeSO_4 (24, 50 and 100 μ M) ($n = 3$). (B) LDH release assay in the culture medium of iPSC-derived cardiomyocytes after 24-hour treatment with 200 μ M arachidonic acid (AA) ($n=3$). This panel is part of Fig. 2.3G. Data are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Two-way ANOVA & T-test).

Arachidonic acid pretreatment increased iron-induced lipid peroxidation and mitochondrial lipid peroxidation.

To confirm whether iron induced more lipid peroxidation in AA-pretreated cells, I employed two methods: the BODIPY 581/591 C11 reagent (Fig. 2.5A&B) and the TBARS assay (Fig. 2.5C). Both assays demonstrated increased total cellular lipid peroxidation after iron treatment in AA-treated cells versus

control cells (Fig. 2.5A-C). In addition, I examined the protein level of glutathione peroxidase 4 (GPX4), a critical regulator of ferroptosis, and found no changes (Fig. 2.6A&B). However, the significantly reduced ratio of reduced to oxidized glutathione (GSH/GSSG) highlights a compromised antioxidant system due to iron-induced oxidative stress in AA-pretreated cells (Fig. 2.5D). This may also suggest a potential impairment in GPX4 activity or capacity. Importantly, I utilized a mass spectrometry protocol to detect oxidized lipids, particularly targeting PLs, and this revealed a dramatic increase in concentrations of iron-induced oxidized PL species in AA-pretreated H9c2 cells. In particular, there was a notable increase in oxidized PUFA-PLs (Fig. 2.7A). Specifically, I observed elevated levels of oxidized PE, SM and phosphatidylinositol (PI) (Fig. 2.5E&F, Fig. 2.7B&C). Some oxidized PC were found under basal conditions and showed only minimal changes after treatments (Fig. 2.5G).

Having established alterations in total cellular lipid profiles and oxidation, I next focused on mitochondrial lipid peroxidation, previously established to be of importance in ferroptosis⁵⁴². To do so, I used mitochondria-targeted probes; MitoSOX to assess mitochondrial superoxide production and MitoPeDPP to measure mitochondrial lipid peroxidation. I found that AA-pretreated cells exhibited significantly increased levels of iron-induced mitochondrial reactive oxygen species (ROS; Fig. 2.5H&I). Iron treatment significantly increased mitochondrial lipid peroxidation in AA-treated cells, which was significantly inhibited by Fer-1 (Fig. 2.5J&K). These findings collectively indicated that iron-induced lipid peroxidation, including mitochondrial lipid peroxidation, is the primary cause of ferroptosis in AA-treated cardiomyocytes.

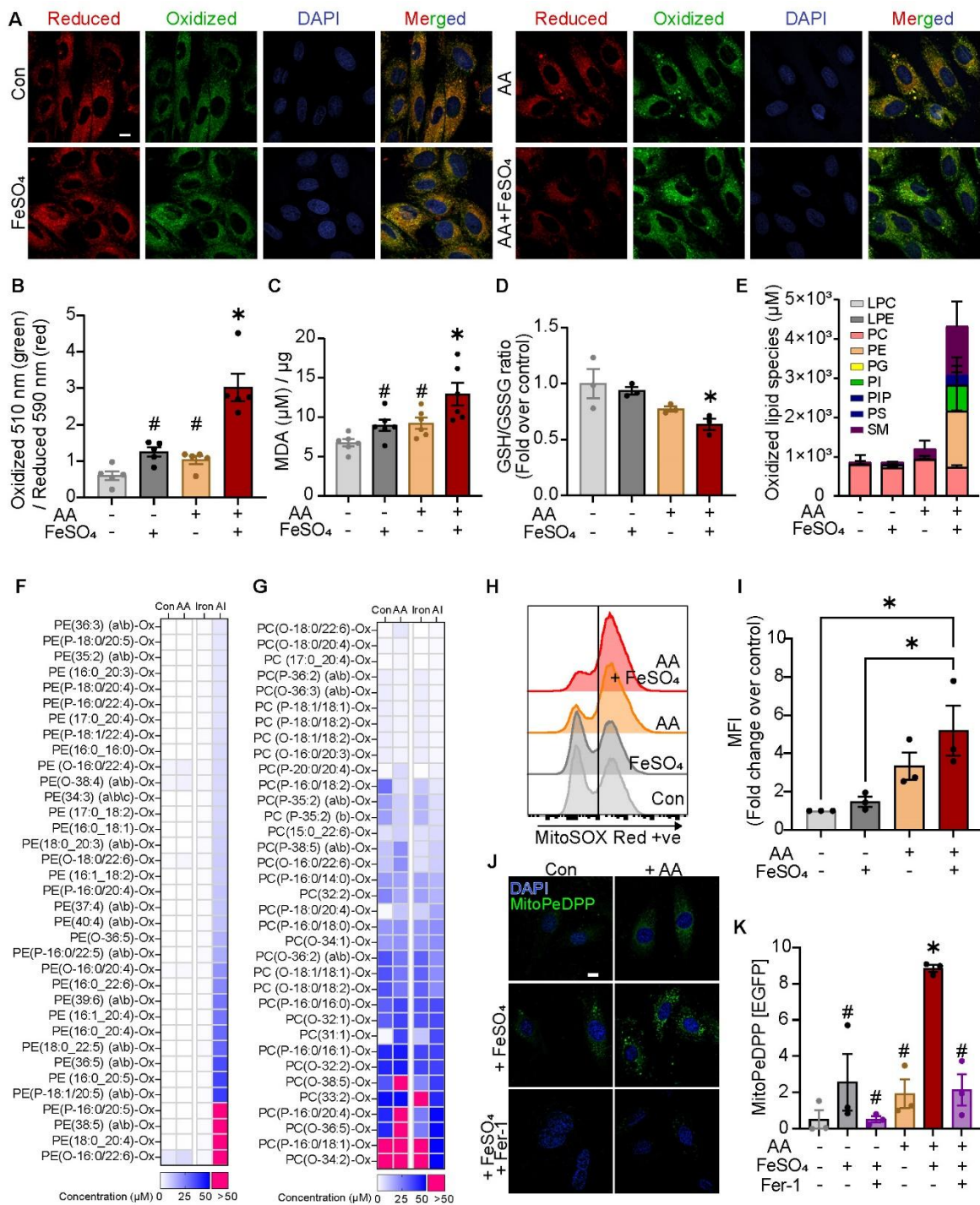


Figure 2.5 Arachidonic acid increased iron-induced lipid peroxidation and mitochondrial lipid peroxidation in H9c2 cells.

(A-B) (A) Representative images showing the red-to-green shift corresponding to lipid peroxidation. Nuclei of live cells were stained with DAPI (blue). Images were taken using Nikon confocal microscope and (B)

its quantification. (AA, 100 μ M; FeSO₄, 50 μ M) (n = 3, size bar = 100 μ m). (C) TBARS normalized by protein concentration. (AA, 100 μ M; FeSO₄, 50 μ M) (n = 6). (D) The relative reduced (GSH) and oxidized (GSSG) ratio of glutathione in H9c2 cells. (AA, 100 μ M; FeSO₄, 50 μ M) (n = 3). (E) The concentrations of oxidized lipid species in each PL classes, measured by using oxidized lipidomic analysis. (AA, 100 μ M; FeSO₄, 50 μ M) (n = 3 per group). (F-G) Heatmap showing the mean of oxidized lipids in the (F) PE and (G) PC classes, quantified by oxidized lipidomics analysis (n =3 per condition). (H-I) (H) Representative histograms of MitoSOX Red-positive cells. (I) Mean fluorescence intensity (MFI), expressed as fold change over control, of MitoSOX-positive cells measured by flow cytometry. (AA, 100 μ M; FeSO₄, 50 μ M; Fer-1, 1 μ M; n = 3). J. Representative images showing GFP intensity of MitoPeDPP probe in H9c2 cells. K. Quantitation of mitochondrial lipid peroxidation using GFP intensity. (AA, 100 μ M; FeSO₄, 50 μ M; Fer-1, 1 μ M; n = 3; scale bar = 100 μ m). Data are presented as mean $\pm$ SEM. Statistical significance: * P < 0.05 compared to the control group (no treatment, 0 μ M FeSO₄). # P < 0.05 compared to the AA + FeSO₄ condition (One-way ANOVA).

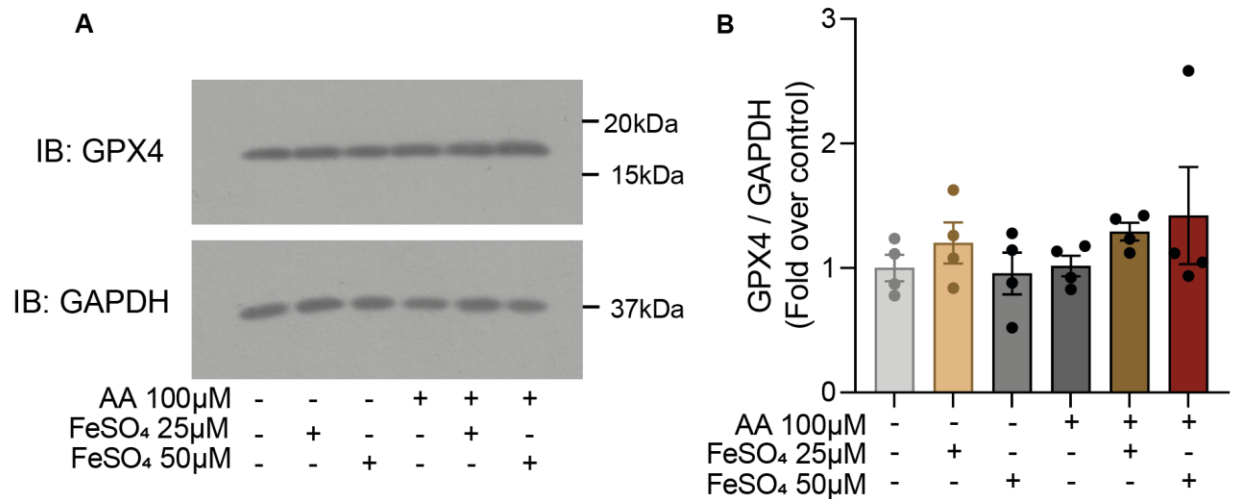


Figure 2.6 The effect of arachidonic acid on iron-induced lipid peroxidation in H9c2 cells.

(A-B) (A) Western blot against GPX4 and GAPDH antibodies using 20 μ g of proteins extracted from H9c2 cells, and (B) its quantification. Data are presented as mean $\pm$ SEM.

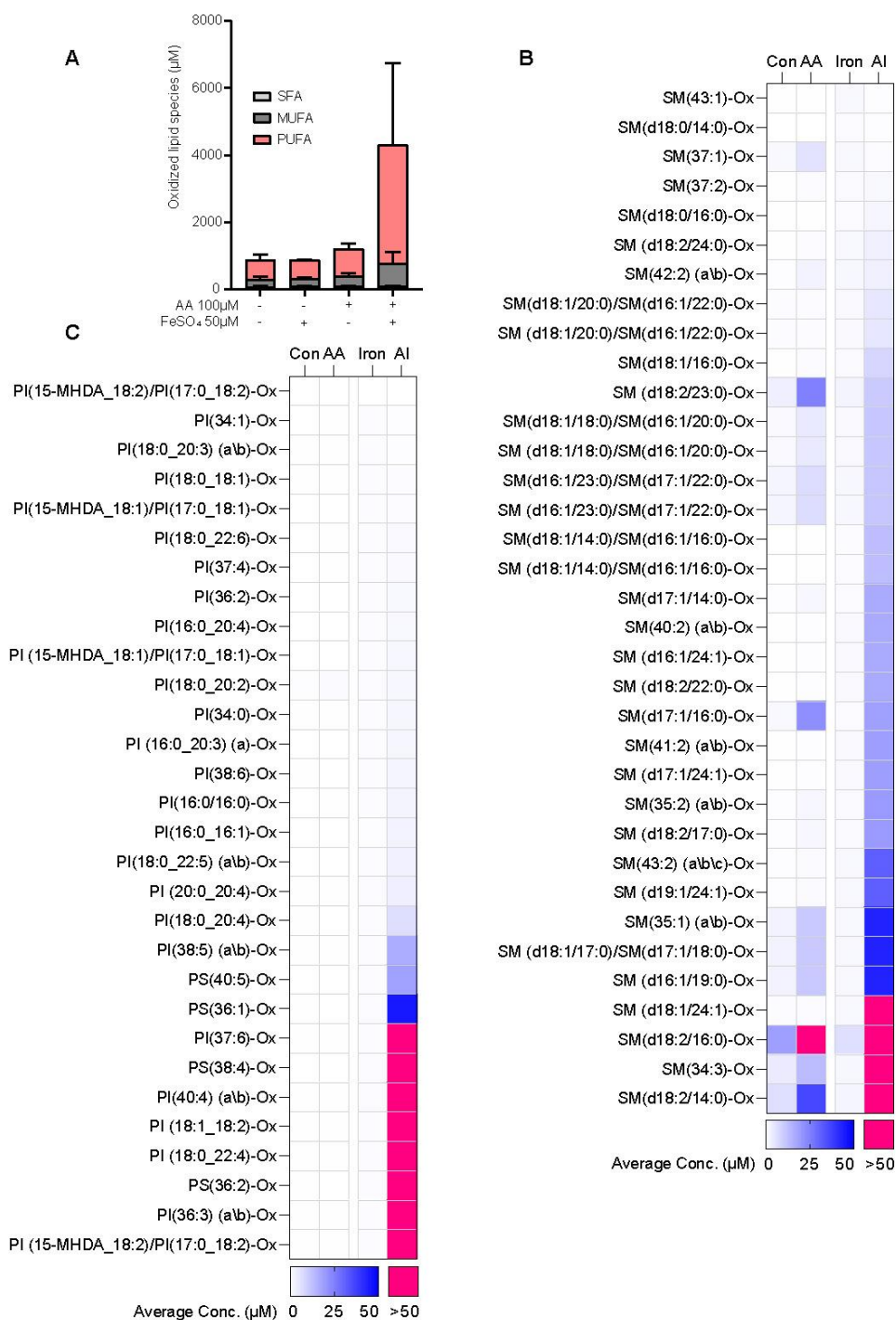


Figure 2.7 Oxidized lipid species concentration in phospholipid classes of H9c2 cells.

(A) Concentrations (μM) of oxidized lipid species in H9c2 cells, categorized by tail type: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). (n = 3 per

group). (B-C) Heatmap showing the mean of oxidized lipids in the (F) SM and (G) PS, and PI classes, quantified by oxidized lipidomics analysis ($n=3$ per condition). Data are presented as mean $\pm$ SEM.

Arachidonic acid promoted iron-induced increases in cytosolic mtDNA and mtDNA-driven inflammation.

Emerging evidence suggested a bidirectional relationship between ferroptosis and inflammation, and mitochondria play a central role in this interplay^{535,543}. Therefore, I investigated mtDNA-induced sterile inflammation in my experimental model^{532,533}. I first verified mtDNA release into the cytosol by isolating cytosolic fraction and quantifying cytosolic mtDNA using RT-qPCR (Fig. 2.9A). Data showed a higher cytosolic mtDNA copy number in cells with AA+iron treatment that was attenuated by Fer-1, with no significant changes in the total mitochondrial content (Fig. 2.8A&B). In addition, I observed an iron-induced increase in cytosolic double-stranded DNA (dsDNA) outside of mitochondria in AA-pretreated H9c2 cells, as detected by dual PicoGreen and MitoTracker staining (Fig. 2.8C&D). Importantly, this increase was prevented by Fer-1 (Fig. 2.8C&D). This observation was also seen in iPSC-CM (Fig. 2.9B&C). To examine the subsequent functional impact of mtDNA release into the cytosol, I examined cGAS-STING and Cas-1. Although cGAS protein levels remained unchanged, STING protein level was significantly increased following iron treatment in AA-treated cells (Fig. 2.9D-F). In addition, iron treatment in AA-pretreated cells showed significantly elevated the level of active Cas-1, measured using flow cytometry (Fig. 2.8E&F). Notably, using the Cas-1 inhibitor YVAD-FMK-CHO prevented iron-induced lipid peroxidation and cell death (Fig. 2.8G; Fig. 2.9G). This suggested an interaction between mitochondrial lipid peroxidation, mtDNA release and inflammation.

I next investigated the potential paracrine effects of mtDNA release by using conditioned media from H9c2 cells to treat macrophage RAW 264.7 dual-reporter cells. These were engineered to express the Lucia luciferase reporter gene, reflecting the activation of the STING and type I interferon (IFN-I) pathways⁵⁴⁴ (Fig. 2.8H). Using the conditioned media from AA + iron treated H9c2 cells, I observed increased luminescence in RAW 264.7 cells, reflecting IFN-I response (Fig. 2.8I).

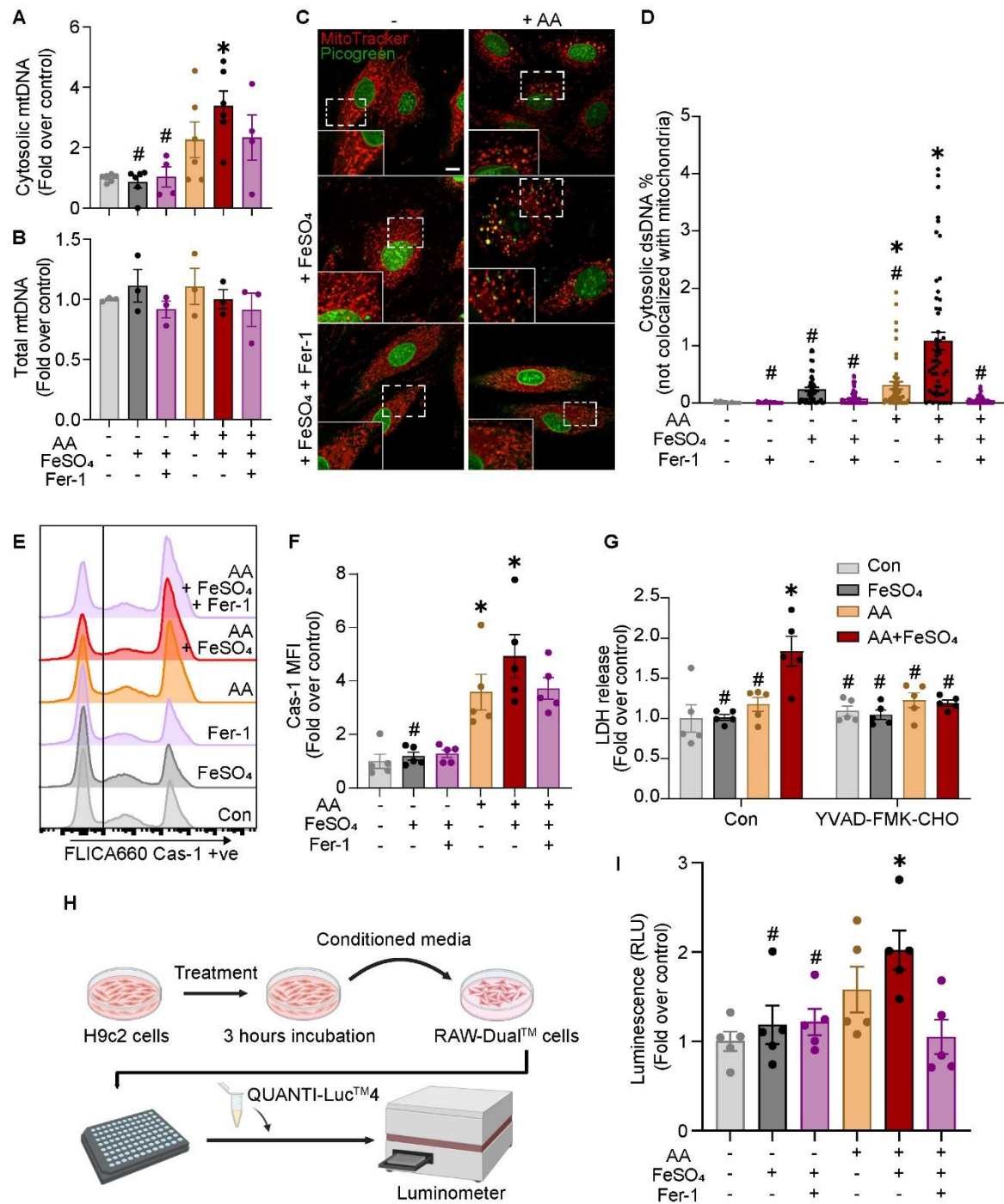


Figure 2.8 Arachidonic acid promoted iron-induced cytosolic mtDNA leakage and mtDNA-driven inflammation in H9c2 cells.

(A-B) (A) mtDNA copy number in the cytosolic fraction and (B) whole cells, measured by qPCR. Nuclear DNA was quantified using the *Gapdh* gene, and mtDNA was quantified using *Cytochrome b* gene (AA, 100

μM ; FeSO_4 , 50 μM ; Fer-I , 1 μM ; $n = 4-5$). (C) Representative images of cytosolic mtDNA, identified by PicoGreen staining (cytosolic PicoGreen-positive areas excluding MitoTracker Red-positive regions). (D) Quantification of the cytosolic mtDNA area. Each dot represents an individual cell from 4–7 biological replicates per group. (AA, 100 μM ; FeSO_4 , 50 μM ; Fer-I , 1 μM ; scale bar = 100 μm). (E) Representative histograms of FLICA™ 660 Caspase-1-positive cells. (F) Mean fluorescence intensity (MFI) of FLICA™ 660 Caspase-1-positive cells, expressed as fold change over control, measured by flow cytometry in H9c2 cells. (AA, 100 μM ; FeSO_4 , 50 μM ; Fer-I , 1 μM ; $n = 4-5$). (G) LDH release from H9c2 cells treated with AA (100 μM) for 24 hours, followed by FeSO_4 (50 μM) treatment for 8 hours $\pm$ YVAD-FMK-CHO inhibitor (50 μM). ($n = 3$). (H) Schematic diagram of co-culture experiment set-up and (I) Relative luminescence (RLU) measured in dual-reporter cells incubated for 3 hours with conditioned media. (AA, 100 μM ; FeSO_4 , 50 μM ; Fer-I , 1 μM ; $n = 5$). Data are presented as mean $\pm$ SEM. Statistical significance: * $P < 0.05$ compared to the control group (no treatment, 0 μM FeSO_4). # $P < 0.05$ compared to the AA + FeSO_4 condition (One-way ANOVA; Two-way ANOVA for panel G).

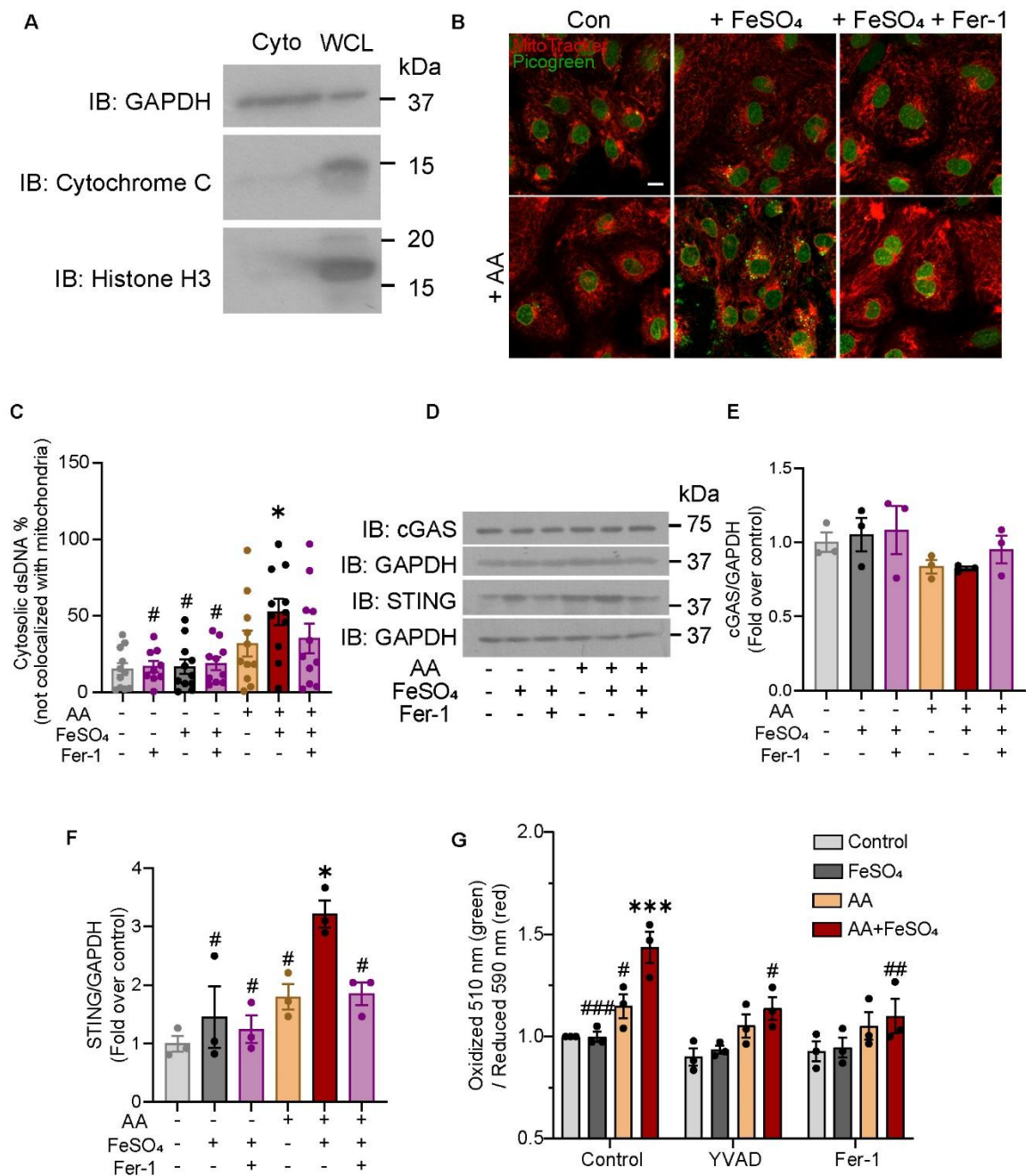


Figure 2.9 Arachidonic acid followed by iron treatment increased cytosolic dsDNA in iPSC-CM and activated STING-Cas-1 pathways in H9c2 cells.

(A) Western blot of proteins extracted from H9c2 cells after cytosolic fractionation (Cyto = cytosolic fraction, WCL = whole cell lysates). (B-C) (B) Representative images of cytosolic mtDNA detected using

*PicoGreen (cytosolic dsDNA) and MitoTracker Red. (C) Quantification of cytosolic mtDNA. Each dot represents an individual cell from three biological replicates per group. Scale bar = 100 μ m. (D-F) (D) Western blot of proteins (20 μ g per sample) extracted from H9c2 cells probed with cGAS, STING, and GAPDH antibodies. (E, F) Quantification of Western blot bands ($n = 3$). (G) Quantification of the red-to-green fluorescence ratio of BODIPY 581/591 C11 reagent in H9c2 cells. Cells were treated with AA for 24 hours, followed by FeSO_4 (25 and 50 μ M) for 8 hours $\pm$ inhibitors (YVAD-FMK-CHO, 50 μ M; Fer-1, 1 μ M). Quantification was conducted using the Cx7 high-content screening system. ($n = 3$). Data are presented as mean $\pm$ SEM. Statistical significance: * $P < 0.05$ compared to the control group (no treatment, 0 μ M FeSO_4). # $P < 0.05$ compared to the AA + FeSO_4 condition (One-way ANOVA; Two-way ANOVA for panel G).*

Results were summarized in the schematic diagram in Figure 2.10. This included the significant impact of fatty acid treatment and HFD feeding on reshaping cellular and cardiac lipid profiles, and increased susceptibility of AA-pretreated cells to iron-induced lipid peroxidation and subsequent cell death. My findings also demonstrated the role of mitochondrial lipid peroxidation and mtDNA release in cell death and inflammation.

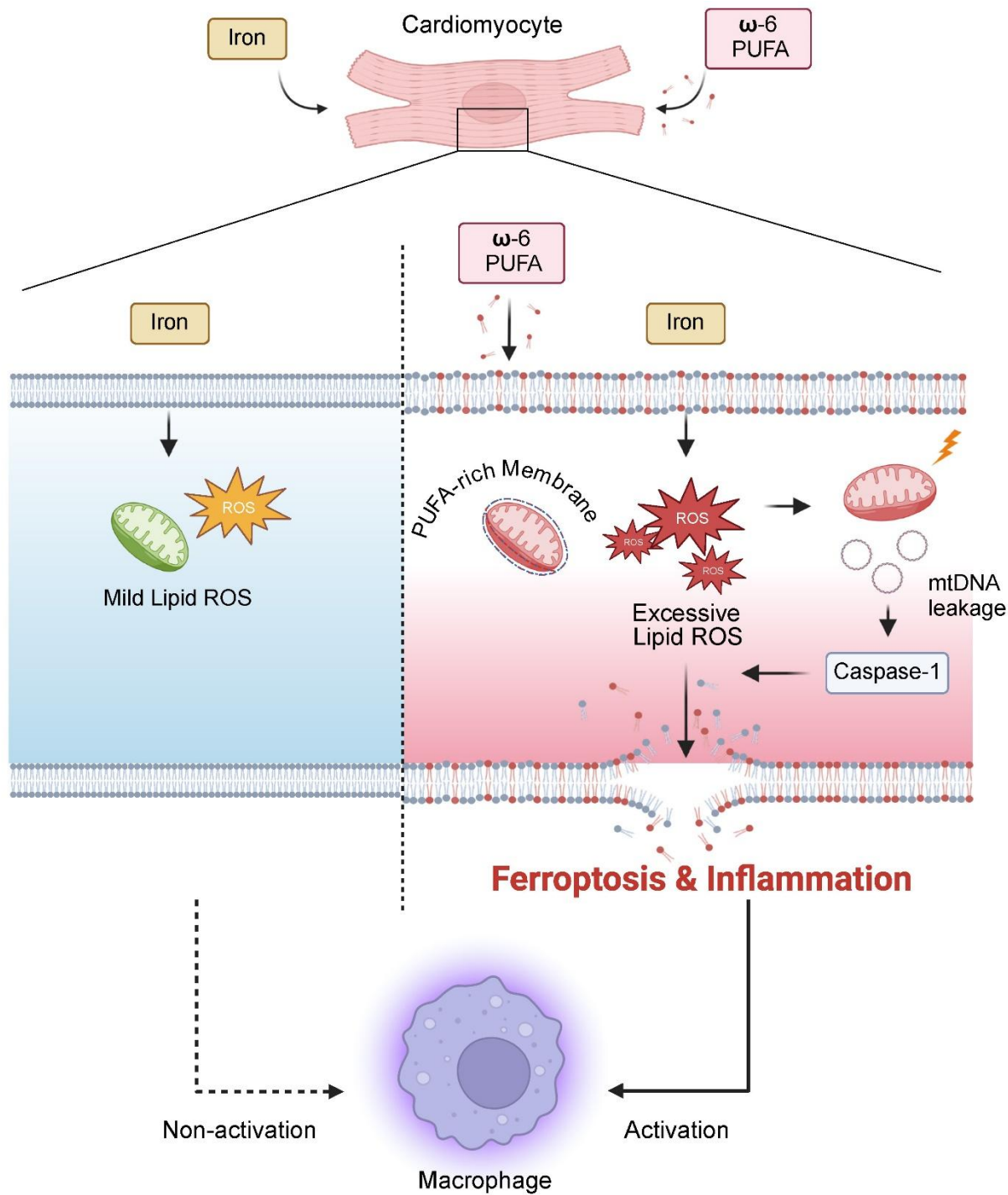


Figure 2.10 Schematic summary of findings

Membranes enriched with ω -6 PUFA-containing phospholipids are more susceptible to iron-induced lipid peroxidation. This susceptibility leads to mitochondrial DNA (mtDNA) leakage, activation of caspase-1, and ferroptotic cell death. The release of damage-associated molecular patterns (DAMPs), particularly

mtDNA, exerts paracrine effects. Created with BioRender.com. Abbreviations: DAMP, damage-associated molecular pattern; PUFA, polyunsaturated fatty acid; mtDNA, mitochondrial DNA; ROS, reactive oxygen species.

2.5 Discussion

CMD is a term encompassing a range of conditions such as obesity, diabetes, metabolic dysfunction-associated steatohepatitis (MASH), atherosclerosis and heart failure which collectively have a major impact on human health^{119,333,545-547}. Multimorbidity and mortality as a consequence of CMD mean that more effective therapies are required^{119,333,545-547}. Ferroptosis has emerged as an important cellular target as it links disruption of lipid metabolism, altered iron homeostasis and inflammation^{119,333,538,546-549}.

Ferroptosis is distinguished by features such as excessive accumulation of labile iron, impaired antioxidative systems and excessive lipid peroxidation^{54,528}. In this study I designed experiments to investigate the impact of a cellular milieu rich in ω -6 PUFA containing PE on susceptibility to lipid peroxidation and subsequent cellular consequences. One of the established intersections between iron and lipid metabolism is the role of iron-induced ROS in driving lipid peroxidation and I used excess iron treatment of cells to induce ROS in my study^{151,341,550-552}. I also compared the effects of AA, PA and OA on the cellular lipidomic profile with an emphasis on PL changes. As expected, only AA treatment produced a profile of ω -6 PUFA containing PL. I also showed that a similar lipid signature was replicated in hearts of mice fed a HFD for 3 weeks. Many studies have shown that PL metabolism significantly influences the occurrence of ferroptosis³⁴¹. Importantly, certain types of membranous PLs, especially those containing two polyunsaturated fatty acyl tails, ω -6 PUFAs like AA (20:4) and adrenic acid (22:4), along with ether lipids, were reported to promote ferroptosis due to their enhanced susceptibility to oxidation^{189,530,553}. I found that while ω -3 PUFA, specifically docosahexaenoic acid (DHA), remained unchanged or decreased under all conditions, AA-treated cells showed a significant increase in PLs containing ω -6 PUFA (AA and adrenic acid). These lipid species were found to be easily oxidized under conditions promoting oxidative stress, such as the absence of GPX4-mediated antioxidative system¹⁸⁹. Indeed, enzymes involved in PUFA metabolism, such as ACSL4 and LPCAT3, correlate with ferroptosis¹⁹⁰. In this study I found that AA increased gene expressions of *Acs14* and *Lpcat3*.

PE composition has become the focus of many studies on ferroptosis susceptibility, making associated mechanisms bona fide therapeutic targets, and my data strongly support this contention⁵⁶. Of note, a key oxidation product, 15-hydroperoxy-arachidonoyl-PE (15-HpETE-PE), is generated by the activity of 15-LOX in complex with the scaffold protein PE-binding protein 1 (PEBP1)²¹⁵. Notably, the Ca^{2+} -independent

phospholipase A2 β (iPLA2 β , encoded by the *PNPLA9* gene) preferentially hydrolyzes peroxidized PLs like 15-HpETE-PE, effectively eliminating the ferroptotic death signal^{215,554}. This is relevant to numerous lipid species detected in my study. Conversely, genetic or pharmacological inactivation of iPLA2 β sensitizes cells to ferroptosis²¹⁵. Thus, targeting peroxidized PEs, such as 15-HpETE-PE, is an attractive strategy to inhibit ferroptosis independent of the GPX4 pathway⁵⁵⁵. In this study I validated that Fer-1, which acts via binding to the 15-LOX/PEBP1 complex resulting in suppressed generation of peroxidized ETE-PE, protected against ferroptosis⁵⁵⁶. These findings further emphasize the critical role of PEs, especially oxidized PEs, in triggering ferroptosis.

Translational studies in animal models showed that targeting ferroptosis can ameliorate cardiomyopathy, and that elevated PE content exacerbated angiotensin II-induced ferroptosis in mice^{275,557}. Excessive oxidation of PEs has been observed in the liver of mice with metabolic dysfunction-associated steatotic liver disease (MASLD) and kidney of *Gpx4*^{-/-} mice^{189,193,558}. Indeed, targeting ferroptosis for intervention by inhibiting iron-mediated ROS production or inducing GPX4-dependent antioxidant capacity also reduced non-alcoholic fatty liver diseases^{559,560}. Suppressing ferroptosis also mitigated diabetes in rodent models^{102,561,562}. Clinically, heart failure patients exhibited altered serum lipid profile with increased PE levels⁵⁶³. In cardiovascular disease patients with insulin resistance, the PC/PE molar ratio in circulation was inversely correlated with NLRP3 levels, which were directly associated with increased left ventricle remodeling⁵⁶⁴. Circulating PE levels were also associated with MASLD progression in obese adults⁵⁶⁵. Interestingly, I found that the cardiac lipid profile of 3 weeks HFD-fed mice also revealed a similar lipid composition to AA-treated cardiomyocytes, with increased PUFA and decreased MUFA PL composition. The general observations made in my study also have wide ranging implications beyond CMD. For example, children with cystic fibrosis showed significantly altered PE metabolism⁵⁶⁶. Moreover, the activity of iPLA2 β via metabolizing oxidized PLs such as HpETE-PE into LPE mitigated placental ferroptosis⁵⁶⁷.

Given the lipid profile alteration described above, as expected I saw that cell death was significantly increased by only 10 μ M iron after AA treatment, versus a ten times higher concentration in control cells. This order of magnitude changes in sensitivity correlated with consequent plasma membrane damage shown by LDH release. This was expected to result in elevated ferroptotic cell death and I confirmed this by showing that it was inhibited by Fer-1. I also proposed that lipid peroxidation in membrane of cellular organelles would also be of functional significance and investigated the impact on mitochondria. Mitochondrial damage, such as fragmentation and permeabilization resulting in release of DAMPs such as mtDNA are known to elicit sterile inflammation^{568,569}. This has been implicated in the cascade of events associated with lipid toxicity and ferroptosis^{54,538,568,569}. Importantly, considering the established close association between sterile inflammation and ferroptosis in CMD, inhibiting ferroptosis presents a potential

approach for managing inflammation linked to lipid toxicity, and conversely, addressing inflammation may also mitigate ferroptotic processes^{39,532,533,542}. My data supported that iron caused mtROS production and enhanced mitochondrial membrane lipid peroxidation in AA-treated cells. Accordingly, I observed a significantly increased level of cytosolic mtDNA in AA-treated cells with iron treatment, without change in the total mtDNA level. As a consequence, downstream signaling pathways related to mtDNA-mediated inflammation, such as STING and caspase-1, were activated. Pharmacological inhibition of caspase-1 activity using YVAD-FMK-CHO attenuated gasdermin-D cleavage and cell death induced by iron in AA-treated cells, indicating closely shared mechanisms of ferroptotic and pyroptotic cell death.

Having demonstrated release of mtDNA to cytosol and damaged cell membrane integrity, it is also likely that mtDNA or other DAMPs will be released from the cell with potential to elicit paracrine inflammatory responses⁵⁷⁰. I tested this using RAW macrophages treated with conditioned media from H9c2 cells. Indeed, my data indicated that media from cells treated with AA+iron induced a type I interferon (IFN-I) response. This is likely to be of most significance for elevated myocardial sterile inflammation *in vivo* as the magnitude of macrophage driven inflammation resulting from paracrine cardiomyocyte-derived stimuli is more impactful than cardiomyocyte inflammation⁵⁷¹.

I acknowledge several limitations associated with the study. Firstly, although H9c2 cells are a well-established model, their use is limited due to differences from mature adult cardiomyocytes⁵⁷². To address this limitation, I employed human iPSC-CMs to validate key findings in this study. Although iPSC-CMs do not fully recapitulate the phenotype of mature cardiomyocytes, they are widely used in cardiomyocyte-based research as a more physiologically relevant model system⁵⁷³. Importantly, I found a significant increase in cell death in AA-treated iPSC-CMs following iron treatment, accompanied by increased leakage of mtDNA into the cytosol, all in agreement with data observed in H9c2 cells.

Secondly, it would be of great interest to translate these observations to a long-term HFD-fed animal model to study the chronic disease context, yet I believe this is beyond the scope of this manuscript and will be a future study.

In this study, I demonstrated that a membrane lipid profile enriched in ω -6 PUFA-containing PE increases the susceptibility of cardiomyocytes to iron-induced lipid peroxidation. This led to mitochondrial and plasma-membrane damage, leakage of mitochondrial DNA into the cytosol, activation of sterile inflammatory signaling, and eventual cell death. The release of cardiomyocyte-derived DAMPs further elicited an IFN-I response in macrophages, indicating intercellular propagation of ferroptotic stress.

Importantly, short-term HFD feeding recapitulated this lipid-remodeling signature *in vivo*, shifting cardiac phospholipid composition toward ω -6 PUFA-enriched species such as PE(16:0_20:4) and PE(18:0_20:4),

while reducing MUFA-containing phospholipids. This early remodeling occurred before overt cardiac dysfunction, suggesting that metabolic stress primes the heart for ferroptotic injury.

Together, these findings link diet-induced lipidomic remodeling to heightened ferroptotic vulnerability, providing a mechanistic framework that connects systemic metabolic stress and myocardial damage. My work expands current understanding of how concurrent elevations in PUFAs and iron exacerbate CMD and may inform the development of targeted antioxidant or lipid-modifying therapies to mitigate ferroptosis-driven cardiac injury.

2.6 Experimental Contributions

I performed the majority of the experimental work in this study, including animal experiments, cell death assays, lipid peroxidation assays (including BODIPY-C11, and TBARS assay), cytosolic mitochondrial DNA quantification, GSH/GSSG ratio measurements, flow cytometry-based analyses, Western blotting, qPCR, lipidomics data analysis, microscopy image acquisition and analysis, and co-culture experiments using H9c2 cells and RAW 264.7 cells. Eddie Tam and Khang Nguyen performed hiPSC-CM differentiation and generated data using this model. Yubin Lei assisted me with the validation of LDH assays in H9c2 cells. Carine Fillebeen assisted me with the animal experiments. Hye Kyoung Sung performed confocal microscopy imaging in cellular models. Lipidomics data acquisition was conducted by the Segal Cancer Proteomics Centre at the Lady Davis Institute for Medical Research, Jewish General Hospital, McGill University.

Chapter 3: Study 2

Adiponectin deficiency drives a conserved PUFA-enriched extracellular vesicle lipid signature that promotes oxidative, inflammatory, and metabolic dysfunction.

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Hye Kyoung Sung: Conceptualization, Writing-Review & Editing, Project administration

Khang Nguyen: Investigation

Yubin Lei: Investigation

Sivaporn Wannaiampiku: Resources

Brian Lee: Resources

Eddie Tam: Investigation

Yihan Luo: Investigation

Evgeniy V. Petrotchenko: Methodology, Formal analysis, Investigation, Resources

Christoph H. Borchers: Resources

Kacey Prentice: Conceptualization, Project administration, Writing-Review & Editing

Scott A. Summers: Conceptualization, Writing-Review & Editing

Dylan Burger: Conceptualization, Writing-Review & Editing

Gary Sweeney: Conceptualization, Resources, Writing-Review & Editing, Supervision, Funding acquisition

*Equal contribution.

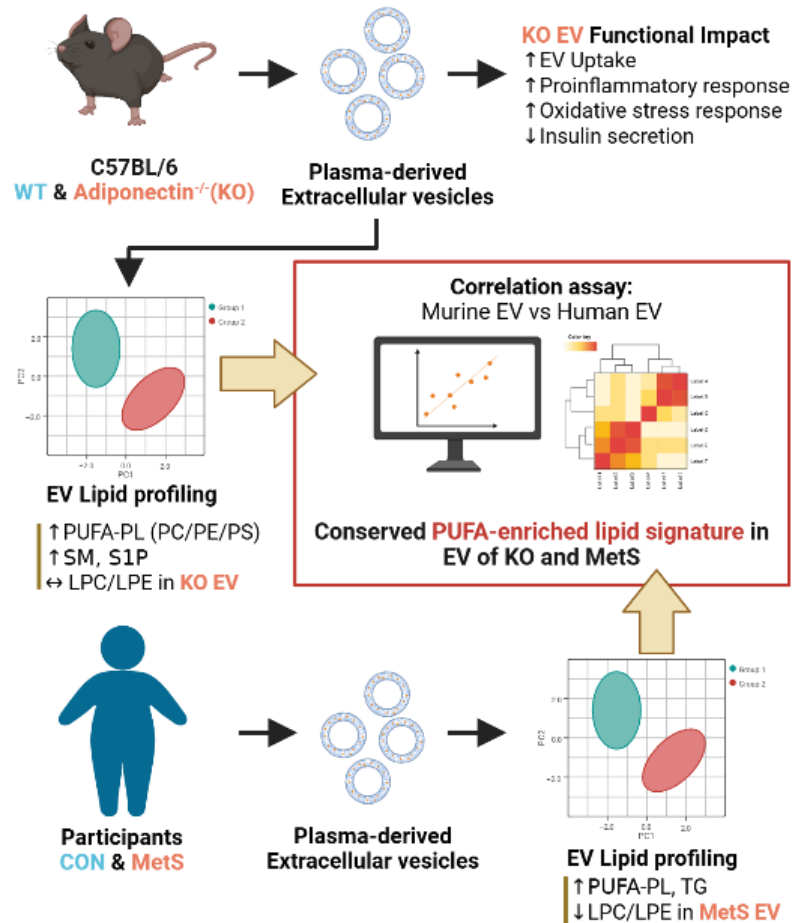
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Lipidomic analysis of plasma extracellular vesicles from adiponectin deficient mice or metabolic syndrome patients reveals pro-oxidative and pro-inflammatory lipid signatures correlating with metabolic dysfunction. *Journal of Extracellular Vesicles*, 2026, 2026 Feb;15(2):e70229..

3.1 Abstract

Extracellular vesicles (EV) are emerging regulators of metabolic homeostasis through their bioactive cargo. This study first investigated the lipidomic profile and functional effects of plasma EV derived from adiponectin-knockout (KO) mice to identify EV-associated lipid signatures linked to metabolic dysfunction. Lipidomic profiling revealed that KO EV were enriched in sphingolipids and polyunsaturated phospholipids compared to wild-type (WT) EV. To evaluate functional consequences, recipient cell assays were conducted using macrophages, skeletal muscle cells, and pancreatic beta cells. KO EV showed an increased uptake in RAW 264.7 macrophages and induced elevated reactive oxygen species and activation of NF- κ B and IRF inflammatory pathways. In L6 skeletal muscle cells, WT EV increased ATP production, while KO EV failed to elicit this effect. Furthermore, KO EV impaired glucose-stimulated insulin secretion in INS-1 pancreatic beta cells. These findings suggested that altered lipid composition in EV from KO mice contributes to oxidative stress, inflammation, and impaired metabolic regulation in recipient cells. Next, translational relevance was established by documenting that plasma EV from patients with metabolic syndrome exhibited lipidomic remodeling features in parallel to the murine KO phenotype, in particular enriched PUFA-containing lipids. Together, these findings identify a conserved adiponectin-EV lipid composition axis regulating oxidative stress, inflammation, and impaired metabolic regulation. The new knowledge presented in this study has implications for biomarker discovery and therapeutic targeting in metabolic disease. These data reveal a conserved PUFA-enriched phospholipid EV signature associated with adiponectin deficiency and metabolic dysfunction in both murine and human systems.

Keywords: adiponectin; lipidomic; polyunsaturated fatty acids; extracellular vesicles; oxidative stress; inflammation.



Graphic Abstract

Adiponectin deficiency remodels the lipid composition of plasma extracellular vesicles (EV), enriching polyunsaturated phospholipids and reducing lysophospholipids. These remodeled EV impact oxidative, inflammatory and metabolic function in recipient cells. These observations reveal a conserved adiponectin–EV–lipid axis linked to cellular stress and metabolic dysfunction.

3.2 Introduction

Extracellular vesicles (EVs) are increasingly recognized as contributors to metabolic diseases, with their quantity correlating with parameters such as body mass index (BMI), blood pressures, and insulin resistance⁵⁷⁴⁻⁵⁷⁷. Among their diverse cargo, lipids and lipid metabolism-regulating proteins are emerging as key mediators of EV function and biomarkers of metabolic states^{402,578,579}.

Lipidomic analyses have revealed that EV lipid profiles reflect the metabolic condition of their origin. For example, EVs derived from adipose tissue of obese individuals exhibit obesity-associated lipid fingerprints, while urinary EVs from prostate cancer patients present distinct lipidomic profiles^{390,580}. Beyond serving as biomarkers, EV lipids actively influence disease pathophysiology. Macrophage-derived EVs, enriched in arachidonic acid precursors and enzymes, modulate the tumor microenvironment by promoting cancer signaling and anti-tumoral immune responses⁵⁸¹. In addition, cholesterol-enriched EVs from repair-associated macrophages have been shown to enhance oligodendrocyte remyelination⁵⁸². Similarly, adipocyte-derived EV exposed to TNF α -induced inflammation show altered lipid profiles enriched in polyunsaturated fatty acid (PUFA)-containing phospholipids and sphingolipids⁵⁸³, and can drive metabolic remodeling in melanoma cells by enhancing fatty acid oxidation³⁹⁵. Despite these advances, the role of EV lipids in metabolic diseases and their downstream effects remain incompletely understood.

Adiponectin, an adipokine primarily secreted by adipocytes, plays a central role in regulating lipid metabolism, insulin sensitivity, and inflammation, with circulating levels inversely correlated with metabolic disease severity^{78,465,467,584}. Although adiponectin has been detected in circulating EVs and shown to mediate interorgan communication^{392,397,443}, it remains unclear how adiponectin influences EV lipid composition and whether such effects are reflected in metabolic disease states.

In this study, I tested the hypothesis that adiponectin deficiency remodels the lipid composition of circulating EVs toward a PUFA-enriched phospholipid signature, thereby altering EV uptake and including oxidative, inflammatory, and metabolic dysfunction in recipient cells. Using adiponectin-knockout (KO) mice, I first defined how loss of adiponectin affects EV biogenesis, lipid composition, and biophysical properties. I then assessed the functional consequences of these remodeled EVs in macrophages, skeletal muscle cells, and pancreatic β -cells, focusing on oxidative stress, insulin signaling, and glucose-stimulated insulin secretion (GSIS). Finally, I integrated lipidomic profiling of plasma EVs from patients with metabolic syndrome (MetS) to determine whether adiponectin-linked EV lipid remodeling represents a conserved metabolic signature across species and relates to clinical indices of insulin resistance and glycemic control.

3.3 Materials and Methods

Animal model

Mice were kept in a temperature and humidity control room ($21 \pm 2^\circ\text{C}$, 35–40%) with a daily 12h light-dark cycle in the animal care facility of York University in accordance with the guidelines of the Canadian Council on Animal Care. All procedures performed here were approved by the Animal Care Committee of York University. In-house Adiponectin knockout (KO) mice⁵⁸⁵ and wild type littermates were maintained with ad libitum access to water and regulate chow diet (LabDiet 5001) until 11 weeks of age when they were humanely euthanized and blood and tissues were harvested. Blood was collected by cardiac puncture, which was further separated to plasma using EDTA-coated tubes. Tissues were kept at -80°C until used in downstream analyses.

Cell culture

L6 rat skeletal muscle cells were cultured in alpha-minimum essential medium (α -MEM; Wisent, #210-010) supplemented with 10% fetal bovine serum (FBS; Gibco, #12483020) and 1% antibiotic-antimycotic (Gibco, #15240062) at 37°C in a humidified incubator with 5% CO_2 . RAW 264.7 murine macrophage cells and RAW-DualTM reporter cells (InvivoGEN) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher, #11995-065) containing 4.5 g/L D-glucose, 10% FBS, and 1% penicillin-streptomycin, at 37°C and 5% CO_2 . INS-1 pancreatic β -cells were grown in RPMI-1640 medium supplemented with 2 mM L-glutamine (TMS-002-C), 1mM sodium pyruvate (TMS-005-B), 10mM HEPES (TMS-003-C), 0.05 mM β -mercaptoethanol (ES-007-E), and 10% FBS (ES-009-B), at 37°C and 5% CO_2 . For all EV treatment experiments, media were prepared using EV-depleted FBS, which was generated by ultracentrifugation at $100,000 \times g$ for 4 hours at 4°C .

Samples from metabolic syndrome cohort

Plasma samples were obtained from female participants enrolled at the Outpatient Department, Division of Internal Medicine, HRH Princess Maha Chakri Sirindhorn Medical Center, and the Srinakharinwirot University (SWU) Clinic (Ethics approval no. SWUEC 161/2563F). Clinical and biochemical data from fasting venous blood samples ($n = 8$ per group) are presented in Table 3.4. All biochemical assays were performed on an automated clinical analyzer (Abbot CI 8200, USA).

Anthropometric assessments were conducted using standardized procedures. Body weight was recorded with participants wearing light clothing and no footwear, using a calibrated body composition scale. Height was measured to the nearest 0.1 cm with a stadiometer, and body mass index (BMI) was computed as weight (kg) divided by height squared (m²). Waist circumference (WC) was determined midway between the lowest rib margin and the iliac crest using a non-stretchable measuring tape. Blood pressure was assessed on the left arm in a seated position after a 10-minute rest period using an automated sphygmomanometer (Omron VP-100, Japan).

The Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated as:

$$\text{HOMA-IR} = \frac{\text{Fasting plasma insulin } (\mu\text{U/mL}) \times \text{Fasting plasma glucose (mg/dL)}}{405}$$

Clinical categorizations were determined following established international and regional guidelines. BMI was classified according to the Asia-Pacific criteria, defining normal weight as <23.0 kg/m², overweight as 23.0-24.9 kg/m², and obesity as ≥25.0 kg/m²⁵⁸⁶. Central adiposity was defined using Asian-specific cutoffs, with WC (≥80 cm) and waist-to-hip ratio (WHR ≥0.80) serving as diagnostic thresholds^{587,588}.

Glycemic control was assessed based on the American Diabetes Association recommendations, where fasting blood glucose (>130 mg/dL) and HbA_{1c} ≥7.0% denote poor glycemic regulation⁵⁸⁹. Insulin resistance was evaluated by the homeostatic model assessment (HOMA-IR), using Thai population-specific thresholds of ≥1.56 for females^{590,591}.

Albuminuria was classified using the urine albumin-to-creatinine ratio (UACR): <30 mg/g Cr (normal), 30-299 mg/g Cr (microalbuminuria), and ≥300mg/g Cr (macroalbuminuria). Serum lipid levels were interpreted according to conventional clinical reference ranges—total cholesterol (TC ≥200 mg/dL), triglycerides (TG ≥150 mg/dL), low-density lipoprotein cholesterol (LDL-C ≥100 mg/dL), and high-density lipoprotein cholesterol (HDL-C <50 mg/dL) for females. Systemic inflammation and cardiovascular risk were estimated using high-sensitivity C-reactive protein (hs-CRP ≥2.0 mg/dL)⁵⁹².

Extracellular vesicle (EV) isolation

EV from plasma samples were isolated through size-exclusion chromatography (SEC) approach using qEV single 70nm columns (IZON, Cambridge, MA) according to the manufacturer's instructions. In all experiments, fractions 3-6 for mouse plasma, and fractions 4-7 for human plasma were collected and used for subsequent experiments

Plasma adiponectin level measurement

Plasma adiponectin levels were measured using mouse or human adiponectin ELISA kits (ImmunoDiagnostics Limited, 32010 and 31010), following the manufacturer's instructions.

Western blot

Isolated EV were lysed in SDS lysis buffer containing proteinase and phosphatase inhibitors (Thermo Fisher, A32963). Protein lysates were denatured at 95°C for 5 minutes and 10 µg of protein lysates per sample were loaded onto SDS-PAGE gels and separated by electrophoresis. Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane using a Trans-Blot Turbo Transfer system (Bio RAD, 17001718). The membrane was blocked with 3% BSA in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 hour at room temperature. The membrane was incubated with primary antibodies (Table 1) overnight at 4°C. After three 10-minute washes with TBST, the membrane was incubated with secondary antibodies for 1 hour at room temperature. Clarity Western ECL Substrate was used to visualize the protein bands, and the results were captured using either CL XPosure Film (Thermo Fisher, 34090) or the Bio-Rad ChemiDoc Imaging System. Antibody information is in Table 3.1.

Table 3.1 Antibody information.

1st Antibody	Company	Cat#	Dilutions
Alix	Cell Signaling	92880	1:1000
CD63	Thermo Fisher	PA5-92370	1:1000
Flotillin-1	Abcam	133497	1:1000
Adiponectin	ImmunoDiagnostics	12010	1:1000
Anti-human Adiponectin	ImmunoDiagnostics	11010-S10	1:1000
Calnexin	Abcam	ab22595	1:1000
β-actin	Cell Signaling	4970	1:1000
ApoA1	Thermo Fisher	PA5-29557	1:1000
2nd Antibody	Company	Cat#	Dilutions
Anti-mouse IgG, HRP-linked Antibody	Cell Signaling	7076	1:5000

Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling	7074	1:5000
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Cryo-EM Sample Preparation and Imaging

For cryogenic electron microscopy (cryo-EM), EV were applied to glow-discharged Quantifoil R 1.2/1.3 holey carbon grids (Electron Microscopy Sciences). 3 μ L of EV suspension was deposited onto the grid, blotted for 2–3 seconds under controlled humidity (~95%) and temperature (4°C) using a FEI Vitrobot Mark IV (Thermo Fisher Scientific), and plunge-frozen in liquid ethane. Cryo-EM imaging was performed using a Talos L120C transmission electron microscope (Thermo Fisher Scientific)

Nano tracking analysis (NTA)

The number of particles and their size distribution in isolated EV samples was measured using NanoSight 3000 (NanoSight Ltd, Salisbury, UK). 30-second videos of EV (pooled EV from 6 mice per group, 5 independent experiments at 22°C) were collected, averaged, and analyzed using the equipped software. For size calibration, 100 nm polystyrene beads (Malvern Instruments, Saint-Laurent, Canada) were used as previously described⁵⁹³.

Proteomic analysis of EV cargo

Isolated EV were lysed in 5% SDS (Fisher Chemical), 200 mM TRIS (Fisher BioReagents™) pH 8.5 with 10 mM TCEP (60C x 25 minutes). Free disulfides were alkylated with 40 mM IAA (RT, dark, 30 minutes) prior to proteolytic digestion by S-TRAP micro (see attached for the SOP from the vendor that we use). Peptide containing eluates were lyophilized and rehydrated at 10 ng/ μ L prior to loading on EvoTips and analysis by library free DIA using an Evosep One system coupled to a Bruker timsTOF HT MS operated in DIA-PASEF mode. Samples were separated on an EV1137 column @ 30SPD. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE⁵⁹⁴ partner repository with the dataset identifier PXD070917.

Lipidomics analysis

Targeted lipidomics analysis was performed as described in Agilent application note⁵⁹⁵. For each sample, 100 μ g of total EV protein (quantified by BCA assay) was extracted using 10 volumes (1:10 v/v) of

extraction solvent consisting of butanol : methanol (1:1, v/v) containing 0.1% (v/v) EquiSPLASH internal standard mix (Sigma, 330731). Samples were sonicated for 5 min and centrifuged at $21,000 \times g$ for 1 min at room temperature (23 °C). The supernatant (80 μ L) was collected for subsequent liquid chromatography–mass spectrometry (LC–MS) analysis.

LC–MS/MS with multiple reaction monitoring (LC–MRM–MS) was performed using an Agilent 1290 UHPLC system with a 16-min gradient: 1) Solvent A: 0.1% formic acid, 10 mM ammonium formate in water : acetonitrile : 2-propanol = 5 : 3 : 2 (v/v/v). 2) Solvent B: 0.1% formic acid, 10 mM ammonium formate in water : acetonitrile : 2-propanol = 1 : 9 : 90 (v/v/v).

Chromatographic separation was carried out at a flow rate of 400 μ L/min using a ZORBAX Eclipse Plus C18 Rapid Resolution HD column (100 $\times$ 2.1 mm, 1.8 μ m; Agilent) maintained at 45 °C, with 1 μ L injection volume per run. Data were acquired on an Agilent 6495A Triple Quadrupole Mass Spectrometer and processed using MassHunter Quantitative Analysis software (Agilent).

Raw LC–MRM–MS peak areas were normalized to the internal standard corresponding to each lipid class and converted to absolute concentrations (nM) using the known quantities of internal standards. Missing values (<5% of total measurements) were imputed as 50% of the minimum detected value for that lipid species. Lipid species showing signals comparable to blank levels or yielding negative values were excluded. Blank (solvent-only) and extraction blank samples (EV-depleted PBS processed identically to samples) were analyzed every ten injections to assess analytical stability and background contamination. My lipidomic analyses operate with absolute concentration estimates, as we use class-specific ISs (Table 3.2).

Absolute concentrations were used to calculate fold changes and generate volcano plots. Heatmaps were constructed using Z-score–scaled values within each lipid species row to visualize relative differences across samples.

Table 3.2 Information of Internal Standards of EV Lipidomic Analysis.

Lipid Component	Catalog #	Chemical Formula	Exact Mass ([M+H]⁺)	Conc. (μM)
18:1(d7) MAG	791646	C21H33D7O4	364.3366	2.50
18:1(d7) Lyso PE	791644	C23H39D7NO7P	487.3451	1.87
18:1(d7) Lyso PC	791643	C26H46D7NO7P	529.3994	1.72
C15 Ceramide-d7	860681	C33H58D7NO3	531.5477	1.71
15:0-18:1(d7) DAG	791647	C36H61D7O5	588.5579	1.55
18:1(d7) Chol Ester	791645	C45H71D7O2	658.6514	1.38

15:0-18:1(d7) PE	791638	C38H67D7NO8P	711.5664	1.28
d18:1-18:1(d9) SM	791649	C41H73D9N2O6P	739.6543	1.23
15:0-18:1(d7) PC	791637	C41H74D7NO8P	754.6207	1.21
15:0-18:1(d7) PG (Na Salt)	791640	C39H68D7O10P	742.5610	1.19
15:0-18:1(d7) PS (Na Salt)	791639	C39H67D7NO10P	755.5563	1.17
15:0-18:1(d7)-15:0 TAG	791648	C51H89D7O6	812.7721	1.12
15:0-18:1(d7) PI (NH4 Salt)	791641	C42H72D7O13P	830.5771	1.07

EV uptake in recipient cells

Isolated EV were labeled with 1,1'-Diocadecyl-3,3',3'-Tetramethylindotricarbocyanine Iodide (DiR) dye, which integrates into lipid membranes, for 1 hour at room temperature. Labeled EV were subsequently purified according to methods described previously⁵⁹⁶. For all uptake assays, 5×10^7 particles of EV as determined by NTA were used in each condition. After 6 and 18 hours of treatment with DiR-labeled EV, recipient cells were washed three times with FACS buffer (2% EV-free FBS in sterile PBS) and analyzed using a Cytex spectral flow cytometer. DiR-positive cells were quantified using FlowJo software. For imaging, recipient cells were stained with cell membrane staining (Dojindo, P504-10; 1:500 dilution) for 5 minutes. After replacing the EV-free FBS growth media, DiR-labeled EV were added to the cells for 6 and 18 hours. Following three washes with sterile PBS, images were acquired in phenol red-free DMEM media using a Nikon A1R confocal laser scanning microscope system (Nikon Corporation).

Oxidative stress assessment

To assess EV-induced oxidative stress in RAW 264.7 cells and L6 cells, several probes were used in high-content screening platform or flow cytometry method. Lipid peroxidation was assessed using BODIPY C-11 (Thermo Fisher, C10445). After EV treatment recipient cells (RAW 264.7 cells and L6 cells) were stained with 5 μ M of BODIPY C-11 probe and Hoechst for 30 minutes followed by 2 washes with filtered PBS. Media was changed to phenol free-growth media, and images for quantification were acquired using Cx7 high-content screening platform (Thermo Fisher, HCSDCX7LZRPRO). Cellular oxidative stress was measured using CellROX deep red probe (Thermo Fisher, C10422). Mitochondrial oxidative stress, particularly oxidation by superoxide, was measured by MitoSox Red (Thermo Fisher, M36008).

RAW-dual reporter cell experiment

RAW-dual reporter cells (InvivoGen, rawd-ismip) were seeded in 96-well plates and grown to 60-70% confluency, then treated with EV for 4-24hours. The absorbance and luminescence, which indicates the activation of NF- κ B dependent pathway and IRF3-dependent pathway, respectively, were quantified using QUANTI-Blue™ Solution and QUANTI-Luc™ 4 Lucia/Gaussia following manufacturer's protocol.

Quantitative PCR (qPCR)

RNA in tissues and cells was extracted with Trizol (Invitrogen, 15596026) following the manufacturer's instructions. Briefly, primary mouse tissues and cells were lysed in Trizol with bead homogenizer (Fisherbrand™, 15340163). After adding chloroform in 1:5 (v/v) to Trizol and vortexing, the samples were centrifuged at 12000rpm for 20 minutes at 4°C. The upper aqueous phase was collected and RNA was purified using the PureLink™ RNA Mini Kit (Invitrogen, 12183018A). The extracted RNA was quantified using the plate reader-based μ Drop™ Plate (Thermo Fisher, VLBLATGD0) and approximately 1 μ g of total RNA per sample was used for reverse transcription using the Revert Aid RT reverse transcription Kit (Thermo Fisher, K1691) to obtain cDNA. The qPCR was performed using cDNA, SYBR Green Supermix (Bio-Rad, 1725274), and primers listed in Table 3.2, following the manufacturer's protocol. Reactions were run on a QuantStudio 3 system (Thermo Fisher, A28567). Data was quantified and analyzed using the delta-delta Ct method normalized over the *Rpl0* or *18s rRNA*.

Table 3.3 qPCR Primer List

Primer	Species	Sequence (Forward)	Sequence (Reverse)
18s rRNA	Mouse	AGTGAAACTGCGAATGGCTCA	CGAGCCACCAAAGGAACCA
Acs11	Mouse	TGCAGCGAGTGTGGGAAAG	TGGTAAGACCCCGTGGAC
Acs13	Mouse	TTCCTTCCCCTGCTCCAGT	CGATTCTGAAAAAGTCTCCTGTTG
Acs14	Mouse	GGGCAAATAAAATGCGGCT	GGCTTAAACTCTAGAACTAAAAACCATCA

AdipoR1	Mouse	GAGGCTCGAGGTTTCTGAGG	CCCGTCTCACTGCTGGTATG
AdipoR2	Mouse	CATAGGGCAGATAGGCTGGC	AAGAAGCGCTCAGGGATACG
Cept1	Mouse	TTCTGGAAC TTCACGATTCTGT	AATATGGTCCCTGCCACAGT
Chpt1	Mouse	GCCTGCAAATTTCAAGACACCT	TTTGAAGAAAGAACTTGAACCTGT
Fads1	Mouse	CCAGCTTTGAACCCACCAA	CATGAGGCCCATTCGCTCTA
Fads2	Mouse	TCAAAACCAACCACCTGTTCTTC	GATGAACCAGGCAAGGCTTTC
Gpx4	Mouse	GCCTGGATAAGTACAGGGGTT	CATGCAGATCGACTAGCTGAG
Ifn-a	Mouse	CCTGAGAGAGAAGAAACACAGCC	GGCTCTCCAGACTTCTGCTCTG
Ifn-b	Mouse	TCCAAGAAAGGACGAACATTCG	TGAGGACATCTCCACGTCAA
Il-1 beta	Mouse	GCTTCAGGCAGGCAGTATCA	AAGGTCCACGGGAAAGACAC
Il-6	Mouse	ACAGAAGGAGTGGCTAAG	AGAGAACAACATAAGTCAGATAC
Lpcat1	Mouse	TGGTGCTACGCTACCCAAAC	CACAGGATTTTCAACGCTCCA
Lpcat2	Mouse	AACTTGCCTACTCAACCTACCA	CTTTGACCTTAACCAGGAACCC
Lpcat3	Mouse	CCCCACATCACAGACGACTATC	TCTCACGGTCCCATTTTCATC
Lpcat4	Mouse	CTCATCCGATACCCCAACAGT	GGGAGGAACTCTACATCCACG
Mboat1	Mouse	ACATCCTGGTAAAGCCAGCC	AGAGATGTACAGCATACCAACCA
Mboat2	Mouse	TTGGCTTACGAAATTCACGACG	ATGCGCCTCACAGCTAATCC
Nox1	Mouse	CCTGATTCTGTGTGTCGAAA	TTGGCTTCTTCTGTAGCGTTC
Nox2	Mouse	AGTGCGTGTGCTCGACAA	GCGGTGTGCAGTGCTATCAT
Pisd	Mouse	CATACTGCTCCTGTCCGATCC	TTCCGTTCCCTGTACTTCTCATA

Pla2g2a	Mouse	TTCGCCCCGGAACAAGAAAAC	ATTTGGGCTTCTTCCCTTTGC
Pla2g4a	Mouse	CAGCACATTATAGTGGAACACCA	GTCCAGCATATCGCCAAAGGT
Pla2g4c	Mouse	ATGGCAGTGGGGAACTGTTT	CAGATGGAGAAGCTCTCGGC
Ptdss1	Mouse	TACTTCGCCTTCACCAGGGAT	GGAATGCTAACACGCTGATGA
Ptdss2	Mouse	GCCTACAACACCAAGAGAGGT	AGGCCCTCTGGTCTGGATGA
Rpl0	Mouse	TTGGAGTGACATCGTCTT	ATCTTGAGGAAGTAGTTGGA
Scd1	Mouse	GAGAAGGGCGGAAAACCTGGA	TGAAGCACATCAGCAGGAGG
Sgms1	Mouse	TCAACTGTTCTCCGAAGCTCT	GGATAAGCCACCTCCAGCAA
Sgms2	Mouse	CGGGATGGTATTGGTTGGGT	GCGCCCCACTATTGACTTGT
Smpd1	Mouse	TCAACCTTAACCCTGGCTACC	CCAGGACCACATGAGAGCTT
Smpd2	Mouse	TGTCAGAGAAGTGCCTGGCT	TTTCCTGGATGAAGGGTGCC
Sod1	Mouse	AACCAGTTGTGTTGTCAGGAC	CCACCATGTTTCTTAGAGTGAGG
Sod2	Mouse	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
Tnf-a	Mouse	AGAATGAGGCTGGATAAGAT	GAGGCAACAAGGTAGAGA

ATP measurement

To measure intracellular ATP levels following 24 hours of EV treatment in high-glucose (25 mM) condition, L6 cells were trypsinized and gently washed three times with filtered PBS (0.45 nm filter). ATP levels were assessed using the ATP Assay Kit (Abcam, ab83355) following the manufacturer's instructions. Prior to the reaction, the samples were deproteinized using the Deproteinizing Sample Preparation Kit (Abcam, ab204708). The optical density was measured at 570 nm using a plate reader (Thermo Fisher, VLBLATGD0).

Mitochondrial network measurement

L6 cells were cultured to 70% confluency on chambered coverslips (ibidi, 80426) and treated with total 1 µg of EVs in EV-free 0.5% FBS AMEM media for 24 hours. After treatment, cells were stained for 30 minutes with 100 nM MitoTracker Green (Thermo Fisher, M7514) and Hoechst (Thermo Fisher, 62249). Cells were washed, media was replaced with phenol-free media, and imaging was performed using a Nikon A1 confocal microscope. (Nikon Instruments)

Mitochondrial length, network connectivity, and density were quantified using the Mitochondrial Network Analysis (MiNA) toolset in ImageJ software⁵⁹⁷. For 3D imaging, Z-stacks were acquired with 0.2 µm planes of focus and processed using IMARIS software (Oxford Instruments) with the surface's algorithm.

Kinetics and dose response of insulin sensitivity measurement using Akt biosensor cells

FoxO1-transfected L6 cells were used to monitor insulin sensitivity in real-time⁵⁹⁸. Cells were grown on a µ-slide 4-well chambered polymer cover slip till 80% confluency. Cells were treated with plasma-EVs diluted in 0% AMEM media at the concentration of 10 µg/ mL for 24 h. After treatment, cells were starved for 90 min, followed by 10 nM insulin stimulation. Cells were imaged at 20x objective (Nikon A1 confocal microscope) in a 5% CO₂ live-cell chamber, over a span of 20 min to observe fluorescence translocation. For the dose-response study, cells were stimulated with different serial dilutions of insulin, starting from 100 nM. Nuclear fluorescence signal was measured after 20 min of insulin treatment for dose response study and after every 2 min for kinetics study. Data quantitation was performed using ImageJ software.

Glucose-stimulated insulin secretion (GSIS) assay

96-well plates were coated with Poly-D-Lysine at a concentration of 0.1% (w/v) according to manufacturer's instructions (Sigma P6403). INS1 832/3 cells (Sigma SCC208) were then seeded in 96-well plates at a density of 80,000 cells/well and incubated overnight in standard growth media. Cells were incubated with 1 ml of 1 µg/100 µL EVs in growth media prepared with EV-free FBS for 4, 24 and 48 hours prior to glucose stimulation. GSIS assays were performed by treating the cells using Krebs Ringer Bicarbonate (KRB) buffer (131 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂*2H₂O, 25 mM HEPES, 1,2 mM KH₂PO₄, 1.2 mM MgSO₄*7H₂O with 0.5% RIA grade, low insulin BSA) containing 2.0 mM low glucose (LG) or 16.7 mM high glucose (HG), respectively, for 1 hour. Supernatant was collected for quantification of insulin secretion by HTRF ultra-sensitive insulin detection kit according to manufacturer's instructions

(Revvity 62IN2PEG). At the conclusion of the GSIS experiment, cells were lysed in 100 μ L ultrapure water and total DNA quantified by Nanodrop 2000 (Thermo Fisher) for normalization to cell number.

Statistics

All statistical analyses were performed using GraphPad Prism 9.0 software, using a student t-test, one- or two-way analysis of variance (ANOVA) with Tukey's post-hoc test. The data are presented as mean $\pm$ SD except for line graphs, which were given as mean $\pm$ SEM. Differences were considered significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Metaboanalyst 6.0 was utilized to compute PCA and heatmaps. Lipica and BioPAN software was used for conducting KEGG analysis and lipid network analysis.

3.4 Results

Adiponectin deficiency was associated with impaired glucose homeostasis.

Prior to examining the impact of adiponectin deficiency on plasma extracellular vesicles (EVs), I first confirmed the loss of circulating adiponectin in the adiponectin-knockout (KO) mouse model (Fig. 3.1A). Plasma adiponectin was undetectable in KO mice whereas robust levels were observed in wild-type (WT) controls (Fig. 3.1B).

Body weight and tissue weight normalized to body weight were comparable between genotypes (Fig. 3.1C-D). However, KO mice displayed evidence of altered glucose handling, as reflected by elevated fasting blood glucose levels and impaired glucose clearance during intraperitoneal glucose tolerance testing (Fig. 3.1E-G).

While these assessments do not constitute comprehensive metabolic phenotyping, they indicated a metabolically altered context associated with adiponectin deficiency, providing a relevant physiological background for subsequent analyses of circulating EVs.

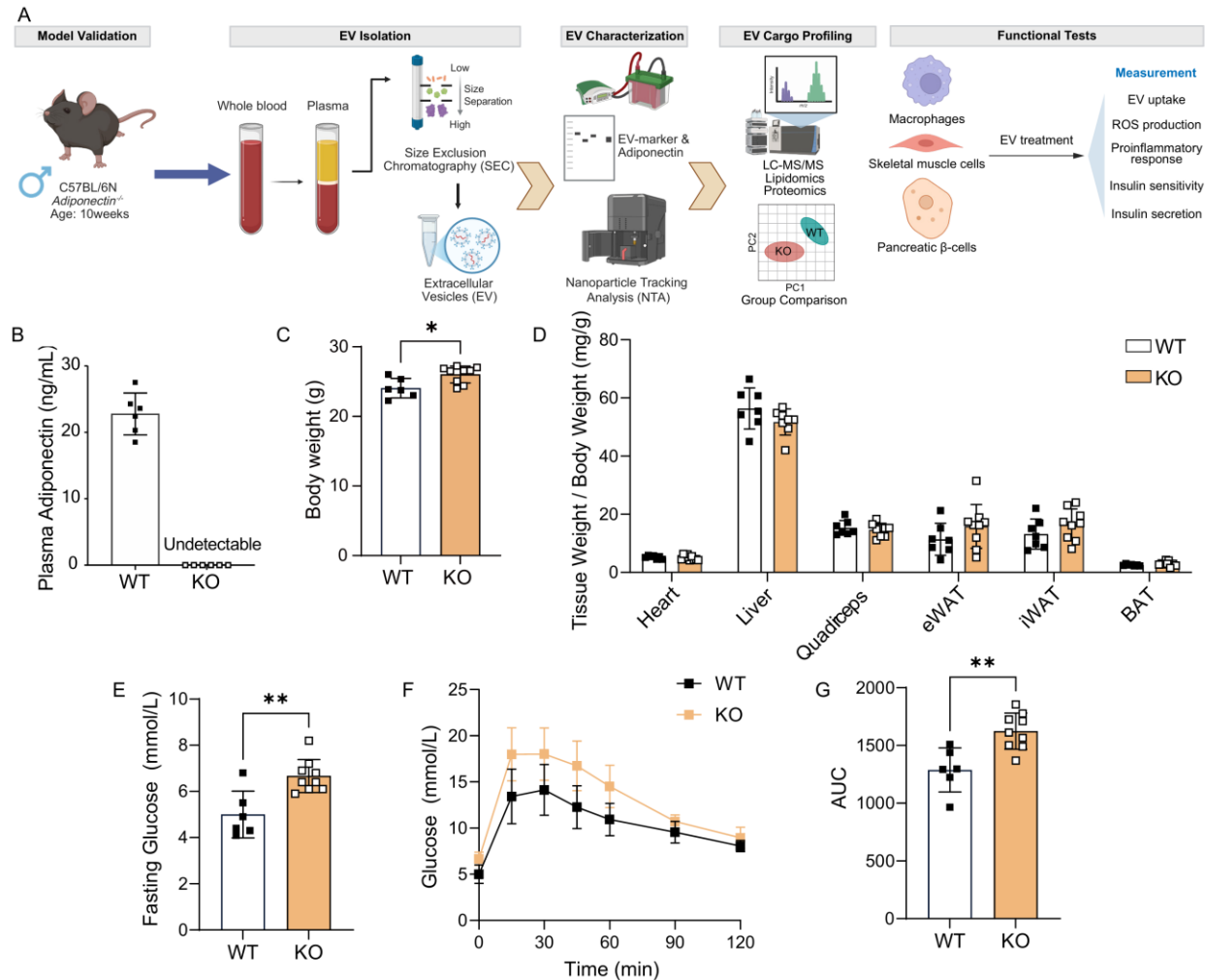


Figure 3.1 Validation of the adiponectin-knockout mouse model.

(A) Schematic diagram of the study workflow. (B) Quantification of plasma adiponectin levels (ng/mL; $n=6$ per group). (C) Body weight ($n=7$ for WT, $n=9$ for KO). (D) Tissue weight/body weight comparison between WT and KO mice. ($n=7-9$ per group). (E-G) Glucose tolerance test (GTT): (E) Fasting blood glucose level (mmol/L), (F) time and (G) area under the curve (AUC).

Data are presented as mean $\pm$ SD. Statistical significance: * $P < 0.05$, ** $P < 0.01$ (unpaired t -test).

Adiponectin deficiency altered tissue expression of lipid metabolism-related genes in a depot-dependent manner.

To determine whether adiponectin deficiency is associated with transcriptional changes relevant to lipid remodeling, I assessed the expression of genes involved in phospholipid (PL) and sphingolipid (SL)

metabolism across key metabolic tissues (Fig. 3.2). Gene expression was quantified in inguinal white adipose tissue (iWAT), epididymal white adipose tissue (eWAT), quadriceps muscle, and liver.

Adipose tissue exhibited the most pronounced transcriptional alterations. In iWAT and eWAT from KO mice, multiple genes involved in PL biosynthesis and acyl-chain remodeling—including *Ptdss1*, *Ptdss2*, *Pisd*, *Lpcat1-3*, and *Chpt1*—were significantly upregulated relative to WT controls (Fig. 3.2A). Genes associated with SL metabolism, such as *Sgms1*, *Cert1*, and *Smpd1*, were also elevated in adipose depots, indicating enhanced capacity for membrane lipid remodeling in the absence of adiponectin (Fig. 3.2A-B).

In quadriceps muscle, a subset of lipid metabolism-related genes, including *Acs11*, *Acs14*, *Scd1*, and *Fads1/2*, showed altered expression, consistent with changes in fatty-acyl activation and desaturation pathways (Fig. 3.2C). In contrast, liver tissue exhibited comparatively modest transcriptional changes (Fig. 3.2D), suggesting that adiponectin deficiency exerts tissue-selective effects on lipid metabolic gene expression.

Collectively, these tissue-specific transcriptional patterns indicated that adiponectin deficiency was associated with enhanced expression of genes involved in PL and SL remodeling, particularly in adipose tissue. Such changes provided a plausible upstream context for the altered lipid composition observed in circulating EVs described below.

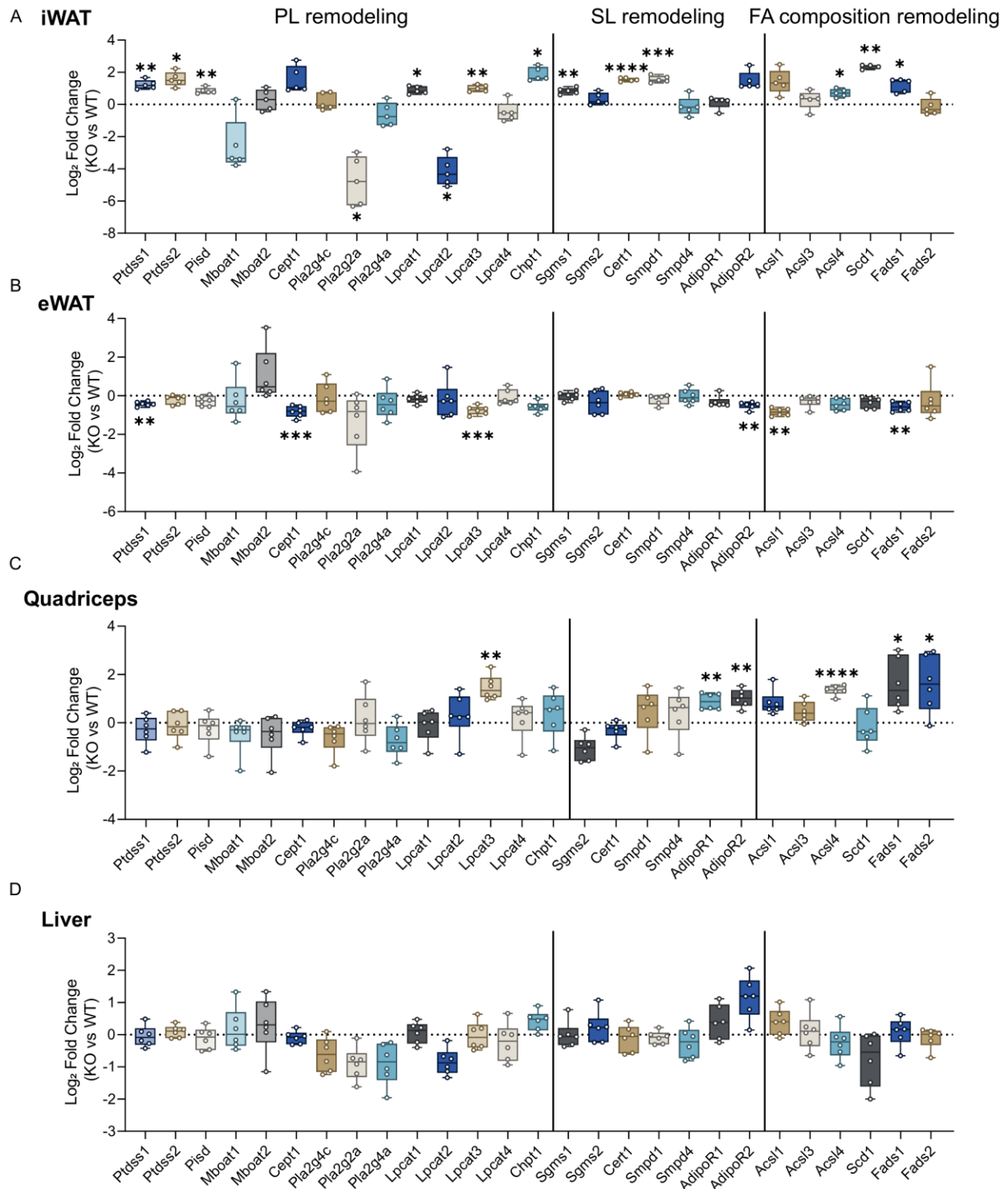


Figure 3.2. Tissue-specific remodeling of lipid metabolic gene expression in adiponectin-deficient mice.

qPCR analysis of gene expression related to remodeling of phospholipid (PL), sphingolipid (SL), and fatty acid composition, presented as log₂ fold change (KO vs WT; *n*=5 per group). (A) Gene expression in

inguinal white adipose tissue (iWAT). (B) Gene expression in epididymal white adipose tissue (eWAT). (C) Gene expression in quadriceps muscle. (D) Gene expression in liver tissue.

*Data are presented as a box plot with the box representing the interquartile range, the horizontal line within the box indicating the median, and the whiskers extending to the minimum and maximum values. Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (unpaired t test).*

Plasma EVs from adiponectin-deficient mice exhibited increased abundance and altered biophysical properties.

Plasma EVs were isolated using size-exclusion chromatography (SEC) and characterized in accordance with International Society for Extracellular Vesicles (ISEV) guidelines⁵⁹⁹. Western blot analysis across SEC fractions revealed enrichment of canonical EV markers, including Alix and Flotillin-1, within fractions 4-6, which were subsequently pooled for downstream analyses (Fig. 3.3A-B).

The pooled fractions contained EV-specific markers CD63, Flotillin-1, and Alix, while non-EV contaminants such as calnexin, β -actin, and ApoA1 were undetectable, confirming the purity of the EV preparations (Fig. 3.3C-D). Consistent with whole-plasma measurements, adiponectin protein was absent in EVs derived from KO mice, further validating knockout efficiency at the vesicular level (Fig. 3.3C, E).

Cryo-electron microscopy (CryoEM) demonstrated intact, membrane-bound vesicles with characteristic EV morphology in both genotypes (Fig. 3.3F). Nanoparticle tracking analysis (NTA) revealed a significant increase in EV particle concentration in plasma from KO mice compared with WT controls, accompanied by a shift toward larger vesicle size distributions (mean size: WT, 95.9 ± 2.3 nm; KO, 120.0 ± 8.0 nm; Fig. 3.3F-H).

Together, these data indicated that adiponectin deficiency was associated with increased circulating EV abundance and altered EV biophysical properties, establishing a robust foundation for subsequent analyses of EV lipid composition and functional effects.

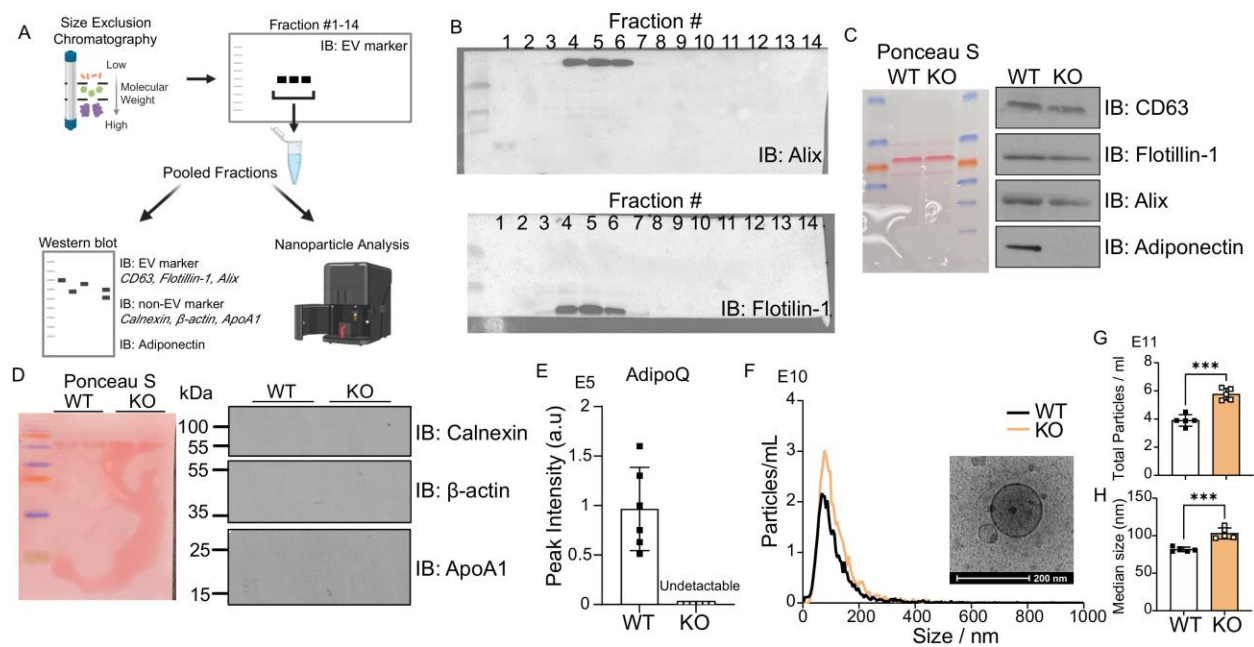


Figure 3.3 Biophysical and molecular characterization of plasma EV from WT and KO mice.

(A) Schematic workflow summary. (B) Western blot analysis of all SEC fractions for EV markers (Alix and Flotillin-1). (C-D) Ponceau S staining and Western blot analysis for (C) EV markers (CD63, flotillin-1, Alix) and adiponectin, and (D) non-EV markers (Calnexin, β -actin, and ApoA1). (E) Peak intensity of EV AdipoQ from proteomic analysis. (F-H) Nano Tracking Analysis (NTA) and representative Cryo-EM image of EV: EV were isolated from six mice per group, pooled, and analyzed for (F) size distribution, (G) concentration (particles/mL), and (H) median particle size (nm) ($n=5$ per group).

Data are presented as mean $\pm$ SD. Statistical significance: *** $P < 0.001$ (unpaired t -test).

Adiponectin deficiency remodeled the lipid composition of plasma-derived EV.

To determine whether the altered abundance and size of EVs observed in adiponectin-deficient mice were accompanied by changes in lipid cargo, I performed lipidomic profiling of plasma-derived EVs using LC-MS/MS with multiple reaction monitoring (MRM) (Fig. 3.4). Equal amounts of EV protein (100 μ g) were analyzed, and lipid abundances were normalized to class-specific internal standards (Table 3.2).

Principal component analysis revealed clear separation between WT and KO EV lipidomes, indicating a global remodeling of EV lipid composition associated with adiponectin deficiency (Fig. 3.4A). While total EV lipid content was increased in KO mice, this difference was driven predominantly by changes in specific lipid classes rather than uniform expansion across the lipidome (Fig. 3.4B-C).

At the class level, KO EVs exhibited significant enrichment of multiple PL subclasses—including phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylglycerol (PG)—as well as SLs and sterol lipids (Fig. 3.4D). In contrast, lysophospholipid species and phosphoinositides (PIP) showed relatively higher abundance in WT EV (Fig. 3.4D-E). Together, these findings indicated a shift toward a PL- and SL-enriched EV phenotype in the absence of adiponectin.

Species-level analyses further highlighted distinct lipid signatures between genotypes. Heatmap analysis identified enrichment of PUFA-containing PLs and selected SL species in KO EVs, whereas WT EVs were characterized by relatively higher levels of lysophospholipids and ether-linked species (Fig. 3.4E).

Collectively, these results demonstrated that adiponectin deficiency induced qualitative remodeling of EV lipid cargo, favoring membrane lipid species with established roles in vesicle structure, cellular uptake, and downstream signaling.

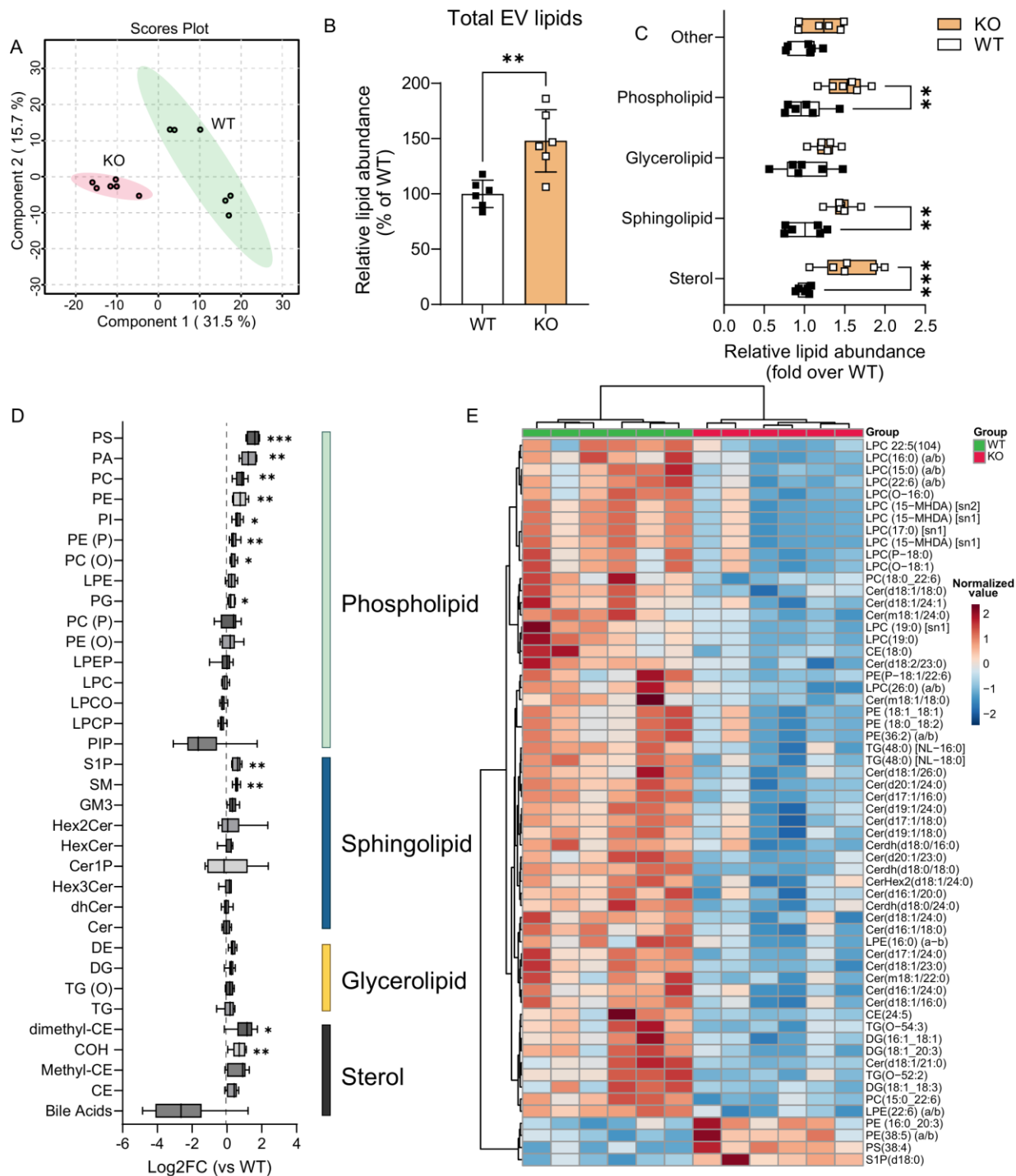


Figure 3.4 Adiponectin deficiency remodels the lipid composition of plasma-derived EV.

(A) Principal component analysis (PCA) of EV lipid profiles. (B) Total lipid concentration (% of WT) in EV. (C) Relative abundance across five lipid classes (fold over WT). (D) Individual lipid class concentrations

in EV, presented as log2 fold change (KO vs. WT). (E) Heatmap of the top 50 differentially abundant lipid species.

Data are presented as mean $\pm$ SD. Statistical significance: * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ (unpaired t -test; $n=6$ per group).

Pathway and network analyses revealed coordinated PL and SL remodeling in KO EVs.

To assess whether the lipid compositional changes observed in KO EVs reflect coordinated alterations in lipid metabolic pathways, I performed pathway enrichment and lipid network analyses based on the EV lipidomic dataset.

KEGG pathway analysis identified significant enrichment of glycerophospholipid metabolism, either lipid metabolism, and SL metabolism in KO EVs relative to WT controls (Fig. 3.5A). These pathways collectively encompass key reactions involved in membrane lipid biosynthesis, acyl-chain remodeling, and SL turnover, consistent with the class-level enrichment of PLs and SLs observed in KO EVs. Several of the lipid classes enriched in KO EVs have been implicated in redox-sensitive and membrane remodeling processes in other contexts, which were explored further in the Discussion.

Complementary lipid network analysis further supported these findings by revealing increased biosynthetic flux toward PS, PE, and PC within the PL network, as well as enhanced sphingomyelin-related pathways within the SL network (Fig. 3.5B). Notably, these network-level changes indicated preferential activation of lipid synthetic and remodeling routes rather than isolated accumulation of individual lipid species.

Together, these pathway-level analyses demonstrated that adiponectin deficiency was associated with coordinated remodeling of EV lipid metabolic networks, reinforcing the notion that the observed lipidomic changes represent a structured and biologically relevant EV lipid phenotype.

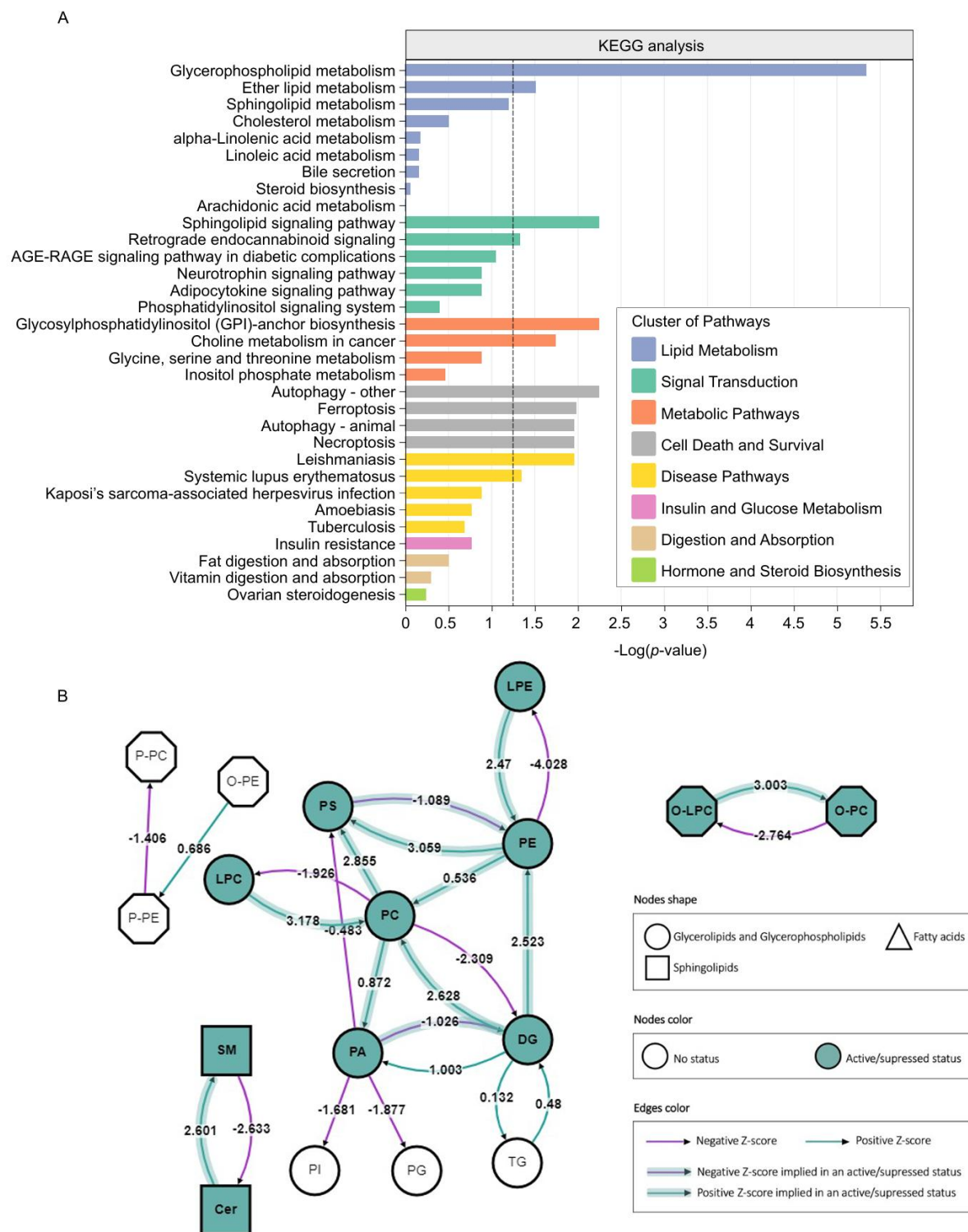


Figure 3.5 Lipid pathway remodeling in EV from KO mice.

(A) KEGG pathway analysis of EV lipidomic data. (B) Lipid network comparison of KO vs WT generated using BioPAN software. Green nodes represent active lipids; shaded arrows indicate predicted lipid

pathways. Significant changes ($P < 0.05$, $Z\text{-score} > 1.645$) are color-coded: green arrows indicate pathways activated in KO EV (positive $Z\text{-score}$), while purple arrows indicate pathways activated in WT EV (negative $Z\text{-score}$).

Adiponectin deficiency shifted EV lipid composition toward unsaturated fatty-acid-rich species.

To further characterize the qualitative nature of EV lipid remodeling, lipid species were stratified based on their fatty-acyl tail composition into saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acid (PUFA)-containing categories.

Across the EV lipidome, KO EVs displayed a significant enrichment of MUFA- and PUFA-containing species relative to WT controls, indicating a global shift toward unsaturated fatty-acid incorporation (Fig. 3.6A). This pattern was particularly pronounced within PL classes, PC, PE, and PS species in KO EVs were dominated by PUFA-containing molecular species, whereas WT EVs exhibited a more balanced distribution across SFA-, MUFA-, and PUFA-containing lipids (Fig. 3.6B-E).

In contrast, SL species, including sphingomyelin (SM) and sphingosine-1-phosphate (S1P), were elevated in KO EVs largely as a result of increased class abundance rather than selective enrichment of unsaturated acyl chains (Fig. 3.6F-G). Given that both PUFA-enriched PLs and S1P have been implicated in oxidative stress and inflammatory signaling^{600,601}, the altered lipid composition of KO EVs may underlie distinct biological activities relative to WT EVs. This distinction suggests that PL and SL remodeling under adiponectin deficiency is governed by partially divergent mechanisms.

Collectively, these findings demonstrated that adiponectin deficiency promoted a MUFA- and PUFA-enriched PL EV phenotype, highlighting unsaturated fatty-acid incorporation as a defining feature of EV lipid remodeling in this model.

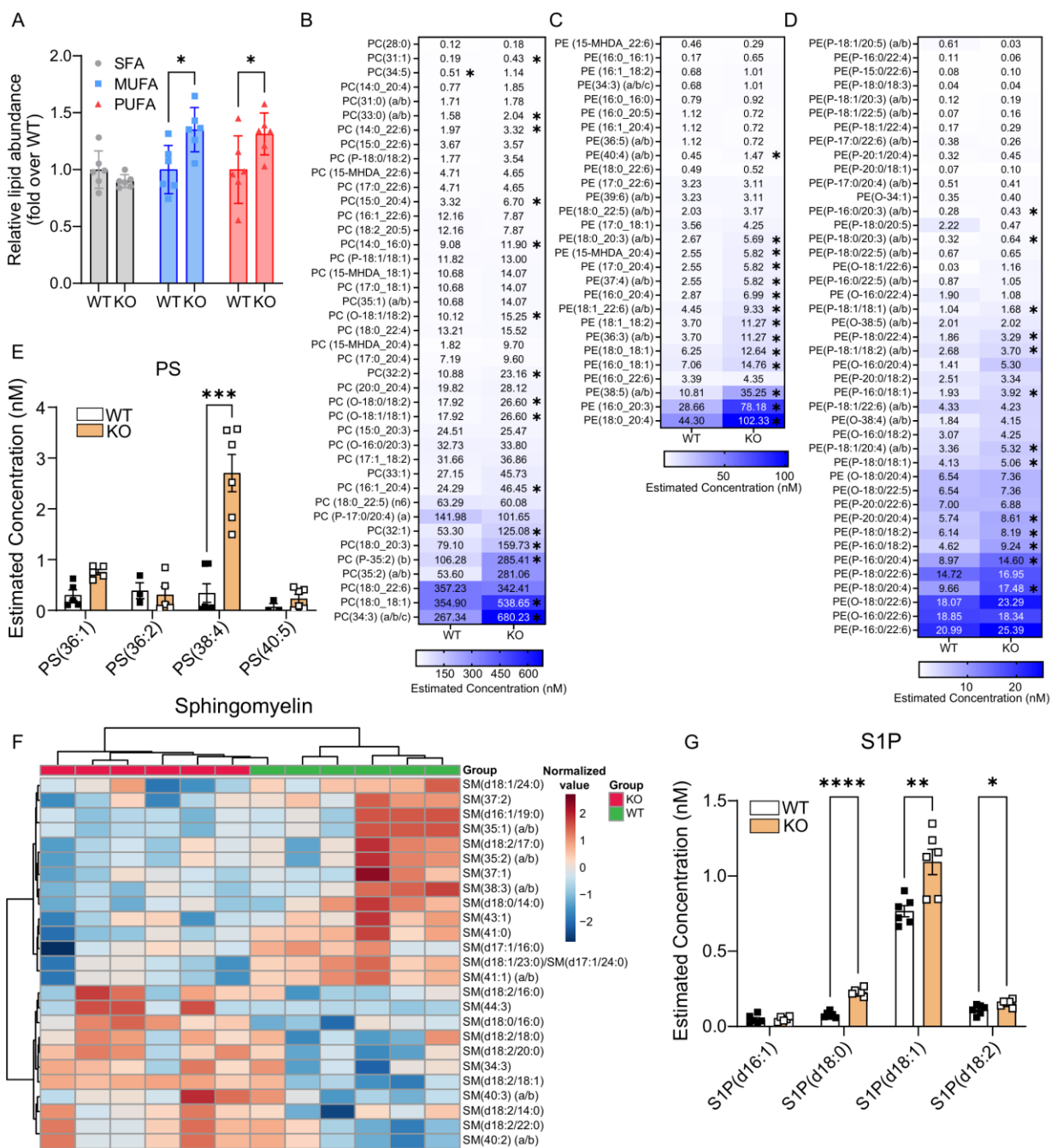


Figure 3.6 Unsaturated fatty acid-containing lipids are enriched in EV from KO mice.

(A) Relative abundance of lipids categorized by the type of fatty acid tails they contain (fold over WT). (B-D) Mean estimated concentrations (nM) of lipid species in PC, PC (O), PE, PE (O), and PE (P) classes. (E) Estimated concentration of PS class lipid species (nM). (F) Heatmap of top 25 differentially abundant lipid species in SM class. (G) Estimated concentration of S1P class lipids (nM). Data are presented as mean

$\pm$ SD. Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (unpaired t -test; $n=6$ per group).

KO-derived EV exhibited enhanced uptake and elicited oxidative and inflammatory responses in macrophages.

Macrophages are key cellular targets of circulating EVs and play central roles in metabolic inflammation⁶⁰². To assess whether EV lipid remodeling under adiponectin deficiency alters macrophage interactions, plasma-derived EVs from WT and KO mice were labeled with DiR and incubated with RAW 264.7 macrophages. Confocal microscopy confirmed internalization of EVs at both 6- and 18-hours following treatment (Fig. 3.7A). Quantitative flow cytometry analysis revealed a significantly higher proportion of DiR-positive macrophages following exposure to KO EVs compared with WT EVs at both time points, suggesting the enhanced EV uptake under adiponectin deficiency-induced EV lipid remodeling, particularly the enrichment in PLs, SL, and unsaturated fatty-acyl chains⁴³¹.

In parallel, KO EV treatment induced a modest but significant increase in oxidative stress in RAW 264.7 cells. Lipid peroxidation, assessed by BODIPY C11 oxidation, and total intracellular ROS levels, measured using CellROX, were elevated following KO EV exposure relative to WT EV (Fig. 3.7E-G). These changes occurred without significant alterations in the expression of antioxidant enzymes (Sod1, Sod2, Gpx4) or ROS-generating enzymes (Nox1, Nox2), suggesting that EV-induced oxidative stress was not driven by transcriptional reprogramming of redox-related genes (Fig. 3.7H).

To evaluate inflammatory signaling, RAW 264.7 dual-reporter cells were used to monitor activation of NF- κ B- and IRF-dependent pathways (Fig. 3.7I). KO EV treatment resulted in significantly increased reporter activity for both pathways compared with WT EV (Fig. 3.7J-K). Consistently, expression of downstream pro-inflammatory cytokines (Il1 β , Il6, Tnf α) and interferon-related genes (Ifn α , Ifn β) was elevated following KO EV exposure (Fig. 3.7L).

Together, these data demonstrated that EVs derived from adiponectin-deficient mice displayed enhanced, uptake by macrophages and were associated with increased oxidative stress and activation of pro-inflammatory signaling pathways.

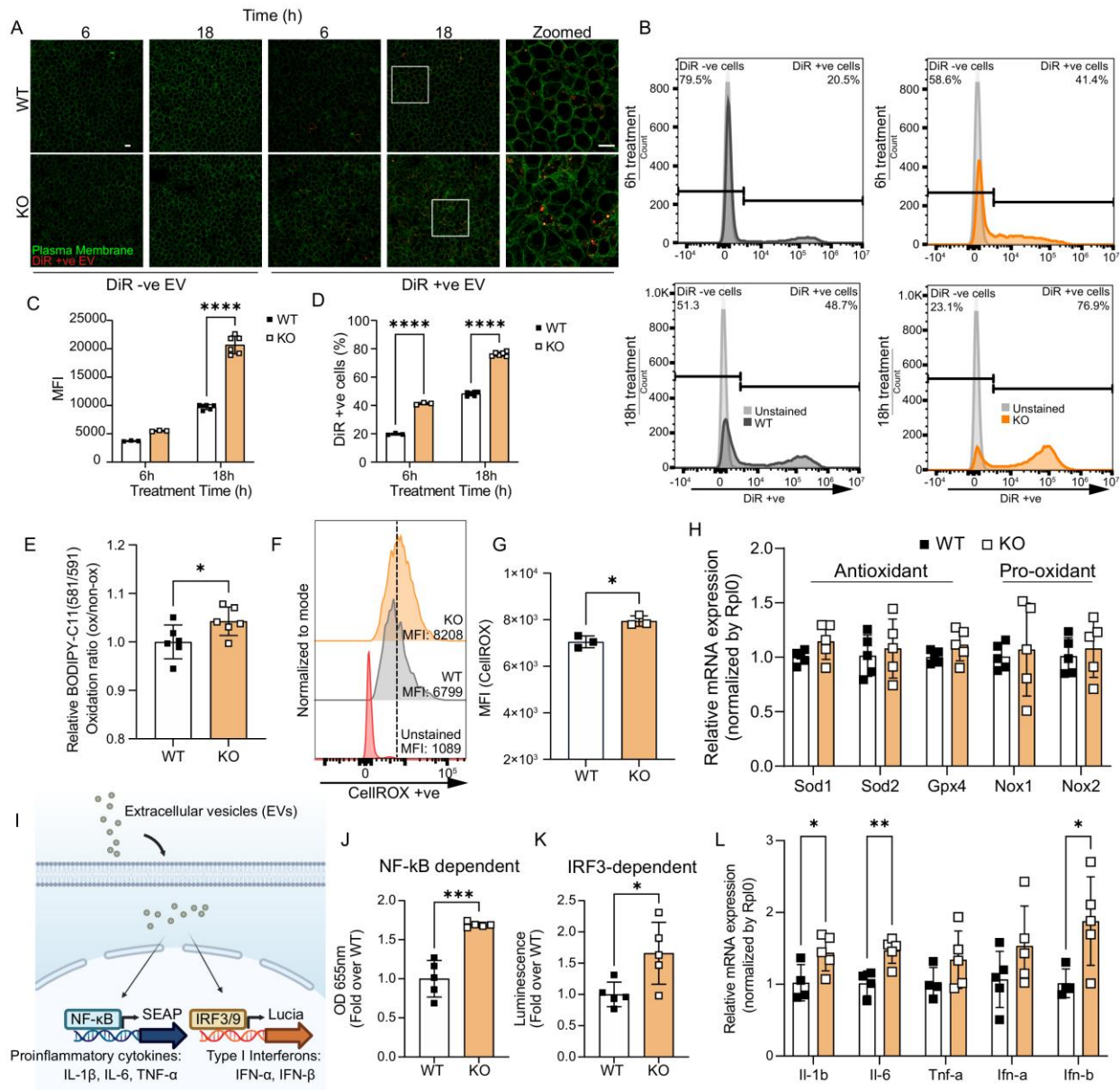


Figure 3.7 Enhanced uptake of adiponectin-deficient EV promotes oxidative and pro-inflammatory responses in RAW 264.7 cells.

(A) Representative images of RAW 264.7 cells stained for plasma membrane (green) after 6 and 18 hours of treatment with DiR-labeled EV (red) from WT and KO mice. Scale bar = 10 μ m. (B-D) Flow cytometry analysis: representative histograms showing mean fluorescence intensity (MFI) and percentage of DiR-positive cells. (n=3 for 6h, n=6 for 18h). (E) Relative BODIPY C11 oxidation ratio (oxidation/non-oxidized) compared to WT EV treatment (n=6 per group). (F) Representative CellROX histogram normalized by mode; (G) mean fluorescence intensity (MFI) of CellROX signal (n=3 per group). (H) Relative gene expression of Sod1, Sod2, and Gpx4 in RAW 264.7 cells by qPCR (n=5 per group). (I) Schematic diagram of dual-reporter

cell assay. (J-K) Dual-reporter cell readouts: (J) optical density (OD) and (K) luminescence (relative light units, RLU) after EV treatment ($n=5$ per group). (L) qPCR analysis of NF- κ B and IRF3/9 signaling genes in RAW 264.7 cells after 12-hour EV treatment ($n=4-5$ per group).

Data are presented as mean $\pm$ SD. Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (Two-way ANOVA for panel C-D; unpaired t -test for all other comparisons).

KO-derived EV induced oxidative stress and impaired mitochondrial function in skeletal muscle cells.

To evaluate the impact of adiponectin-deficient EVs on a metabolically active tissue relevant to insulin sensitivity, I examined EV uptake and downstream responses in L6 skeletal muscle cells. Similar to macrophages, L6 cells internalized plasma-derived EVs, with KO EVs exhibiting moderately higher uptake compared with WT EVs following 18 hours of exposure (Fig. 3.8A–C). However, the magnitude of uptake differences in L6 cells was less pronounced than that observed in RAW 264.7 macrophages.

Despite these modest uptake differences, KO EV treatment significantly increased oxidative stress in L6 cells. Lipid peroxidation, assessed by BODIPY C11 oxidation, as well as total intracellular ROS and mitochondrial superoxide levels, were elevated following KO EV exposure relative to WT EV (Fig. 3.8D–J).

Functionally, KO EV-treated cells exhibited reduced intracellular ATP content compared to WT control (Fig. 3.8K). Consistent with compromised energy metabolism, mitochondrial network analysis revealed shorter branch length and reduced network connectivity in KO EV-treated cells, findings that were further supported by three-dimensional mitochondrial reconstructions showing loss of elongated mitochondrial structures (Fig. 3.8L–O).

Together, these data indicated that EVs derived from KO mice induce oxidative stress and compromise mitochondrial integrity and energy production in skeletal muscle cells, highlighting a cell-type-specific metabolic vulnerability to EV lipid remodeling.

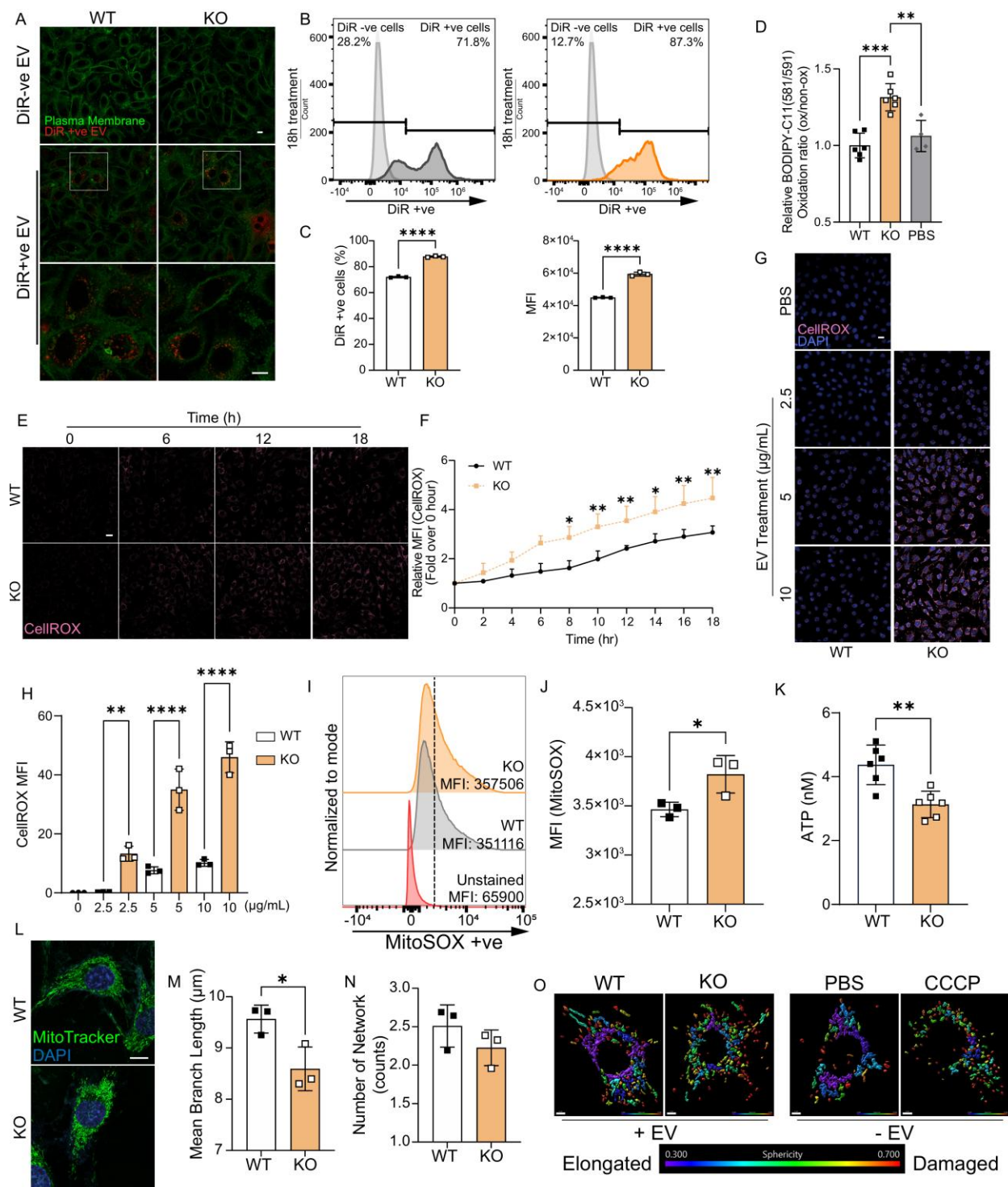


Figure 3.8 KO-derived EV induce mild ROS and impair energy metabolism in L6 skeletal muscle cells.

(A) Representative images of L6 cells stained with a plasma membrane marker (green) after 18 hours of DiR-labeled EV treatment (red) (scale bar = 10 μ m). (B-C) Flow cytometry: representative histograms showing the mean fluorescence intensity (MFI) and percentage of DiR-positive L6 cells (n=3 per group).

(D) Relative BODIPY C11 oxidation ratio (oxidized/non-oxidized) compared to WT EV-treated cells (n=6 per group). (E-F) Time-course analysis of ROS production: representative images of CellROX-stained L6 cells treated with EV over 0-18 hours and mean fluorescence intensity (MFI) quantification (scale bar = 20 μ m). (G-H) Representative images of L6 cells stained with CellROX probe after 18 hours of EV treatment at the indicated dose (scale bar = 20 μ m) and corresponding quantification of MFI. (I-J) MitoSOX flow cytometry histograms and MFI quantification after 24-hour EV treatment (n=3 per group). (K) Intracellular ATP levels (nM) in EV-treated cells under high glucose environment (n=6 per group). (L-N) Representative MitoTracker Green-stained images and mitochondrial network analysis (scale bar = 10 μ m, n=3 per group). (O) 3D visualization of mitochondria using IMARIS imaging (scale bar = 4 μ m).

Data are presented as mean $\pm$ SD. Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (Unpaired t-test except for panel D, which was compared using One-way ANOVA).

EV from adiponectin-deficient mice attenuated insulin signaling in skeletal muscle cells.

To further assess the functional consequences of EV on insulin signaling, I used L6 cells expressing a GFP-tagged FoxO1, which enables real-time monitoring of insulin-stimulated Akt activation via nuclear export of FoxO1⁵⁹⁸.

Following 24-hour EV treatment, live-cell imaging revealed distinct differences in insulin signaling dynamics between groups. WT EV-treated cells exhibited rapid insulin-induced FoxO1 nuclear exclusion within 8 minutes, consistent with efficient Akt activation (Fig. 3.9A-C). In contrast, KO EV-treated cells displayed delayed and blunted FoxO1 nuclear export in response to insulin stimulation, indicating impaired activation kinetics of the Akt pathway (Fig. 3.9A-C).

Consistent with these imaging-based observations, Western blot analysis confirmed a reduction in insulin-stimulated Akt phosphorylation at Ser473 in KO EV-treated cells compared with WT EV-treated cells, providing biochemical validation of impaired insulin signaling (Fig. 3.9D-E).

Together, these findings demonstrated that EV derived from adiponectin-deficient mice impaired insulin signaling dynamics in skeletal muscle cells.

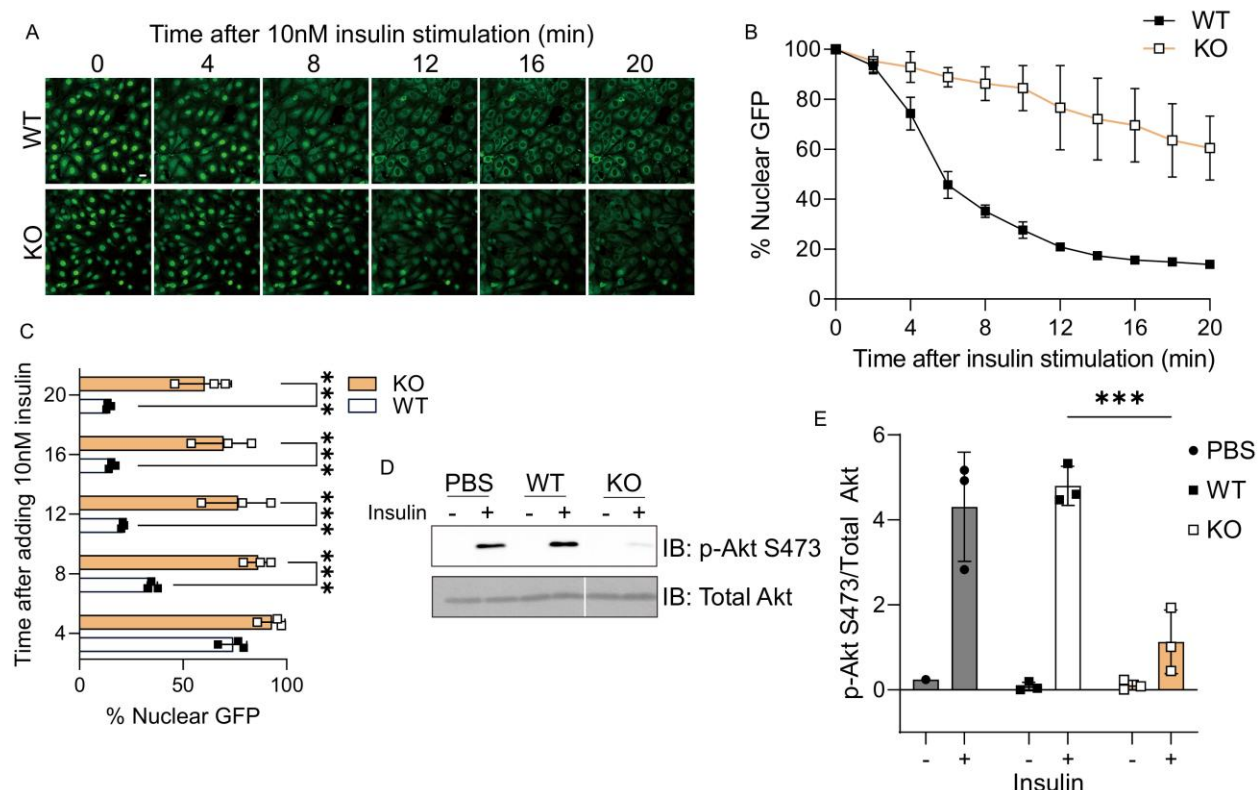


Figure 3.9 KO-derived EV reduce insulin sensitivity in L6 cells.

(A) Representative fluorescence images and (B&C) Nuclear GFP intensity (%) at 20 min post-insulin stimulation (10 nM; $n=3$, scale bar = 20 μm). (D&E) (D) Representative Western blot and (E) its quantification analysis of phosphor-Akt (S473) $\pm$ insulin (100 nM, 10 min) after 24h EV treatment ($n=3$; $n=1$ for no EV/no insulin control).

Data are presented as mean $\pm$ SD. Statistical significance: *** $P<0.001$ (Two-way ANOVA).

Prolonged exposure to KO-derived EV impaired glucose-stimulated insulin secretion in β -cells.

To determine whether EV derived from adiponectin-deficient mice also affect insulin secretory function, I assessed glucose-stimulated insulin secretion (GSIS) in INS-1 pancreatic β -cells following exposure to plasma-derived EVs. Cells were treated with WT or KO EV for 4, 24, or 48 hours prior to stimulation with low (2.0 mM) or high glucose (16.7 mM) concentrations (Fig. 3.10).

Short-term EV exposure (4 and 24 hours) did not significantly alter insulin secretion in response to high glucose, although a trend toward reduced GSIS was observed in KO EV-treated cells (Fig. 3.10A-B). In

contrast, prolonged exposure to KO EV for 48 hours resulted in a significant attenuation of GSIS compared with WT EV-treated cells (Fig. 3.10C).

These findings indicated that sustained exposure to EV from adiponectin-deficient mice compromised β -cell glucose responsiveness, suggesting that EV-mediated effects extend beyond insulin target tissues to include insulin-producing cells.

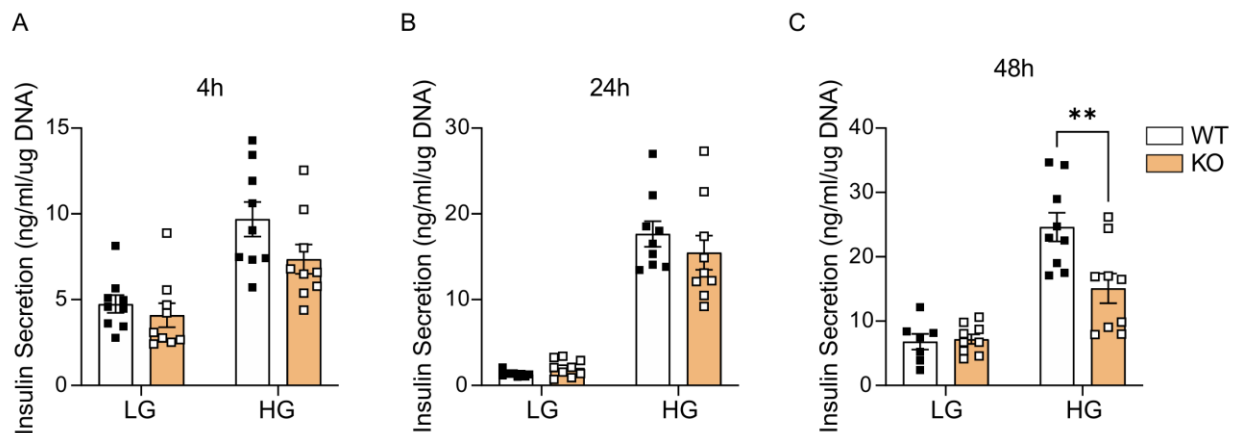


Figure 3.10 Prolonged exposure to KO-derived EV impair glucose-stimulated insulin secretion (GSIS) in INS-1 cells.

Insulin secretion from INS-1 cells was measured after (A) 4 hours, (B) 24 hours, and (C) 48 hours of high glucose stimulation. Insulin levels were normalized to cellular DNA content. ($n=7-9$ per group). Data are presented as mean $\pm$ SD. Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Two-way ANOVA).

Circulating and EV-associated adiponectin levels were reduced in patients with metabolic syndrome.

To evaluate the translational relevance of the adiponectin-deficient EV phenotype observed in mice, I analyzed plasma-derived EV from patients with metabolic syndrome (MetS) and metabolically healthy control (CON) subjects (Fig. 3.11A). Clinical and metabolic characteristics of the study cohorts are summarized in Table 3.4.

Table 3.4. Clinical parameters of the CON and MetS patients

	CON	MetS	<i>P</i> -value (<i>t</i> -test)	
AGE	45.3 $\pm$ 1.68	54.5 $\pm$ 3.58	0.042	*
SBP (mmHg)	106.6 $\pm$ 2.82	136.5 $\pm$ 4.24	<0.0001	****
DBP (mmHg)	64.8 $\pm$ 3.34	83.4 $\pm$ 1.91	<0.001	***

Height (cm)	159.3 ± 0.73	155.6 ± 2.82	0.249	ns
Weight (kg)	51.3 ± 0.86	79.5 ± 3.78	<0.001	***
BMI (kg/m³)	20.2 ± 0.32	33.5 ± 1.26	<0.0001	****
WC (cm)	72.4 ± 1.97	100.3 ± 3.65	<0.0001	****
Hip (cm)	83.3 ± 6.81	116.6 ± 3.56	0.001	**
WHR	0.8 ± 0.02	0.9 ± 0.01	0.046	*
FPG (mg/dL)	86.4 ± 1.92	142.4 ± 17.44	0.015	*
HbA1c (%)	5.1 ± 0.07	7.0 ± 0.49	0.007	**
URIC (mg/dL)	4.8 ± 0.23	5.9 ± 0.71	0.197	ns
INS (uU/mL)	3.6 ± 0.50	9.6 ± 1.28	0.002	**
HOMA-IR	0.8 ± 0.11	3.3 ± 0.55	0.002	**
TC (mg/dL)	213.6 ± 9.17	177.4 ± 14.84	0.060	ns
TG (mg/dL)	68.5 ± 3.33	153.1 ± 19.52	0.003	**
HDL (mg/dL)	73.1 ± 2.17	51.4 ± 2.99	<0.0001	****
LDL-C (mg/dL)	130.1 ± 7.88	102.0 ± 14.10	0.110	ns
hs-CRP (mg/L)	0.5 ± 0.11	9.8 ± 4.84	0.096	ns
sCr (mg/dL)	0.8 ± 0.02	0.8 ± 0.07	0.605	ns
eGFR (mL/min per 1.73 m²)	107.5 ± 5.87	85.8 ± 8.12	0.049	*
UACR (mg/gCr)	3.9 ± 0.58	232.6 ± 176.15	0.235	ns
AST (U/L)	20.8 ± 1.66	36.5 ± 10.36	0.175	ns
ALT (U/L)	14.1 ± 1.89	37.125 ± 12.79	0.117	ns
GGT (U/L)	14.8 ± 2.17	76.375 ± 39.22	0.160	ns

Abbreviations: AST, Aspartate aminotransferase; ALT, Alanine transaminase; GGT, Gamma-Glutamyl Transferase; WC, waist circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; URIC, uric acid; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein cholesterol; LDL-C, Low-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; sCr, serum creatinine; eGFR, estimated glomerular filtration rate; UACR, urine albumin-creatinine ratio.

Consistent with the metabolic disease context, plasma adiponectin levels were significantly lower in MetS patients compared with controls (Fig. 3.11B). SEC of human plasma samples revealed enrichment of the EV marker CD63 within fractions 4-7, which were subsequently pooled for downstream analyses (Fig. 3.11C).

Western blot analysis confirmed the presence of canonical EV markers (CD63, Flotillin-1, and Alix) and the absence of non-EV contaminants in pooled EV fractions from both groups (Fig. 3.11G). Notably, EV-associated adiponectin abundance was markedly reduced in MetS-derived EV compared with CON EV, paralleling the adiponectin-deficient Ev phenotype observed in mice (Fig. 3.11G-H).

NTA revealed a modest, non-significant trend toward increased EV particle number and a slight reduction in median EV size in MetS patients relative to CON (Fig. 3.11D-F). While these biophysical differences did not reach statistical significance, they indicate that MetS is associated with subtle alterations in EV characteristics alongside reduced adiponectin content.

Together, these findings demonstrated that MetS was associated with reduced adiponectin levels in both plasma and circulating EV, providing a direct human counterpart to the adiponectin-linked EV alterations identified in the murine model.

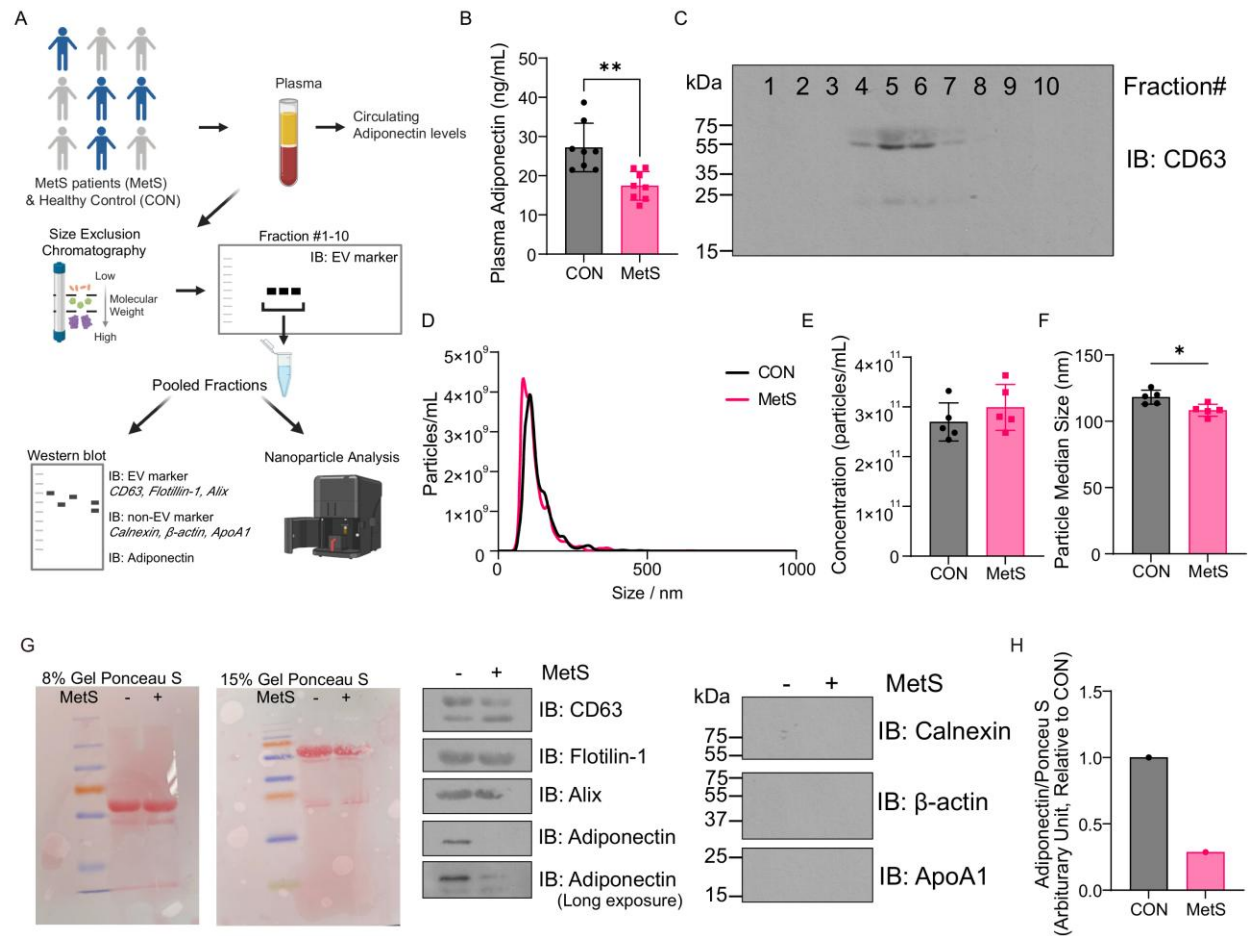


Figure 3.11 Circulating and EV-associated adiponectin levels were reduced in patients with MetS.

(A) Schematic summary of EV characterization workflow. (B) Plasma adiponectin levels (ng/mL). (C) Western blot of all SEC fractions for EV marker CD63. (D-F) NTA of EV isolated from 8 participants per group, pooled, and analyzed for (D) size distribution, (E) particle concentration (particles/mL), and (F) median particle size (nm) ($n=5$ per group). (G&H) Western blot analysis of EV: (G) Ponceau S-stained PVDF membrane and representative blots for EV markers, adiponectin and non-EV markers in pooled samples; (H) quantification of adiponectin abundance normalized to Ponceau S staining.

Data are presented as mean $\pm$ SD. Statistical significance: * $P < 0.05$ (unpaired t -test).

Plasma EVs from patients with MetS exhibited distinct lipidomic remodeling.

To determine whether lipid compositional changes are present in circulating EVs from individuals with MetS, I performed lipidomic profiling of plasma-derived EVs from MetS patients and CON.

Partial least squares–discriminant analysis (PLS-DA) revealed clear separation between EV lipidomes derived from MetS patients and CON, indicating global remodeling of EV lipid composition in the disease state (Fig. 3.12A). In contrast, total EV lipid content was comparable between groups, suggesting that disease-associated differences reflect qualitative rather than quantitative changes in lipid cargo (Fig. 3.12B).

At the class level, MetS-derived EVs displayed significant enrichment of PL and glycerolipid fractions, with notable increases in PC, PE, PG, and triglyceride (TG) species relative to CON EVs (Fig. 3.12C-D). This was accompanied by an approximately 13% higher proportion of glycerolipids in MetS-derived EVs compared with CON (Fig. 3.13A). Conversely, lysophospholipid species were relatively enriched in CON EVs, consistent with altered membrane lipid turnover in the MetS context (Fig. 3.12E; Fig. 3.13B).

Together, these results demonstrated that MetS was associated with coordinated remodeling of the plasma EV lipidome at both lipid class and individual species levels.

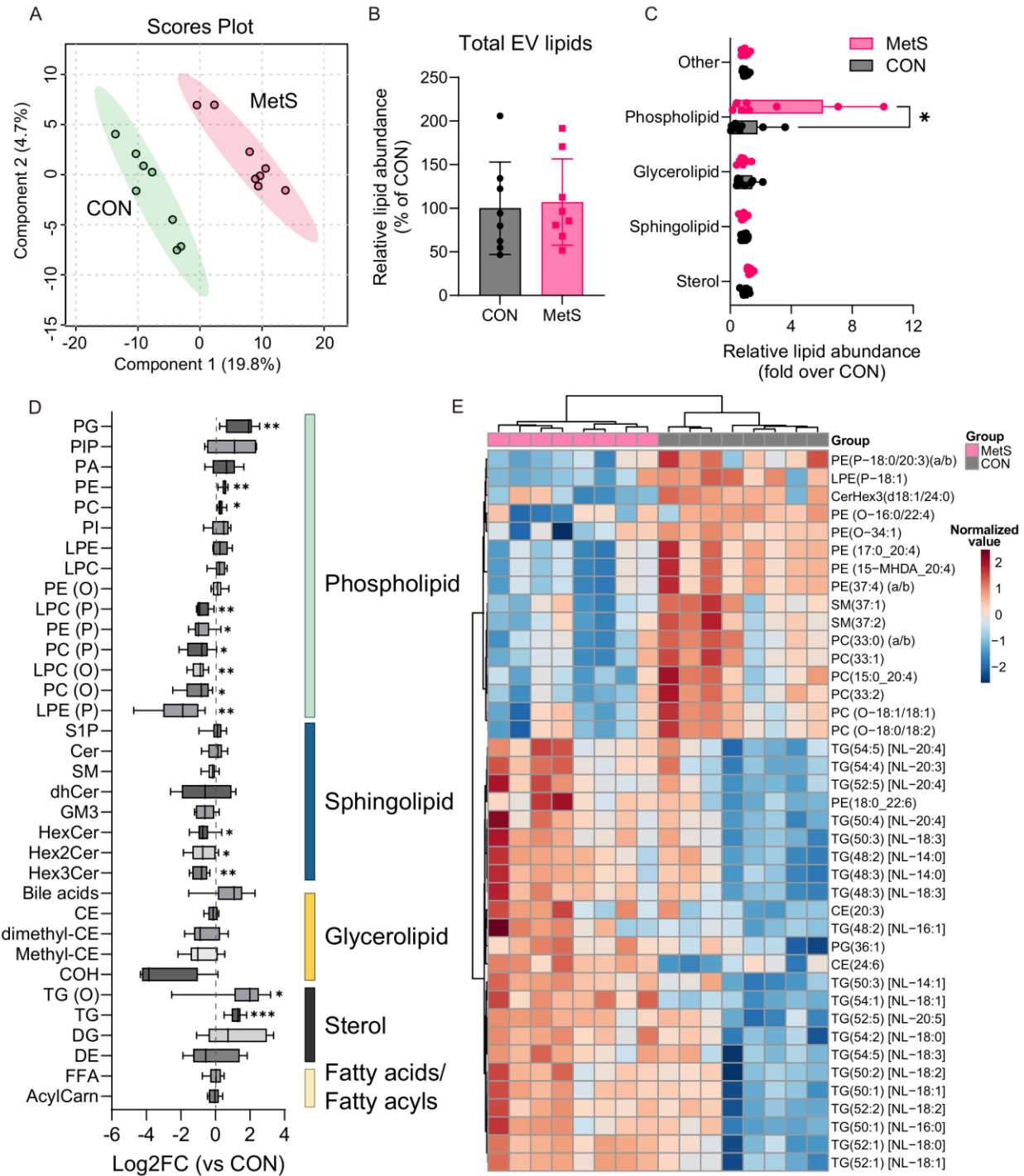


Figure 3.12 Plasma EVs from patients with MetS exhibit lipidomic remodeling.

(A) PLS-DA of EV lipid profiles. (B) Total lipid concentration (% of CON). (C) Relative abundance across five lipid classes in EVs (fold change vs. CON). (D) Individual lipid class abundance in EVs, presented as log2 fold change (MetS vs. CON). (E) Heatmap of the top 40 differentially abundant lipid species in EVs. Data are presented as mean $\pm$ SD. Statistical significance: * $P < 0.05$ (unpaired t -test; $n=8$ per group).

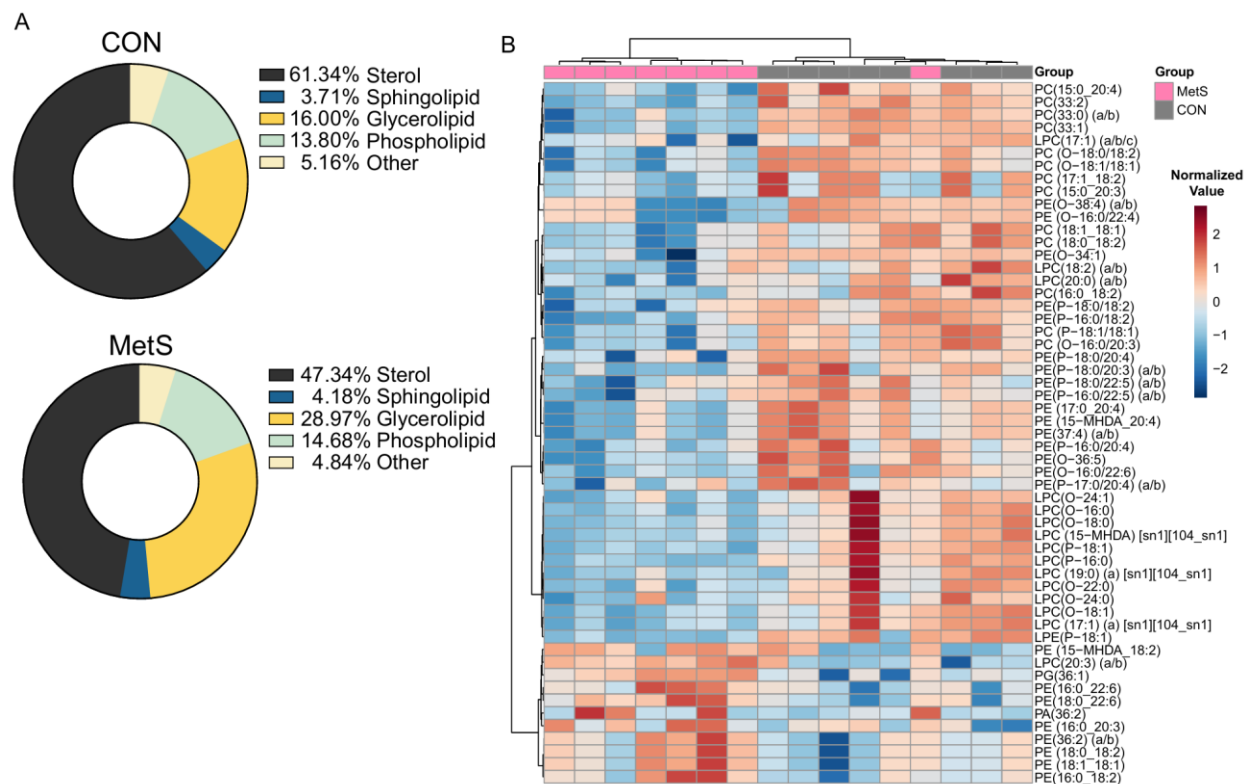


Figure 3.13 Class- and species-level lipidomic remodeling of human plasma EVs in MetS.

(A) Pie charts showing the percentage distribution of lipid abundance across five lipid classes in EVs. (B) Heatmap of the top 55 differentially abundant phospholipid species.

EVs derived from patients with MetS attenuated insulin signaling in skeletal muscle cells.

To assess whether EVs derived from patients with MetS exert functional effects on insulin signaling similar to those observed with adiponectin-deficient mouse EVs, L6 cells expressing GFP-tagged FoxO1 were treated with EVs isolated from MetS patients or CON (Fig. 3.14A-B).

Following 24 hours of EV treatment, insulin signaling dynamics were assessed by monitoring insulin-stimulated FoxO1 translocation over a 10-minute period. EVs derived from MetS patients exhibited delayed and blunted FoxO1 nuclear exclusion compared with CON EV-treated cells, with significantly attenuated responses evident from 4 minutes after insulin stimulation (Fig. 3.14A-B).

To further validate these findings in a human-relevant muscle model, insulin signaling was also assessed in iPSC-derived myoblasts. Western blot analysis confirmed that treatment with MetS-derived EVs

resulted in a clear reduction in insulin-stimulated Akt phosphorylation at Ser473 compared with CON EV-treated cells (Fig. 3.14C-D).

Together, these findings demonstrated that EVs derived from individuals with MetS can attenuate insulin signaling responses in skeletal muscle cells, providing translational support for the functional relevance of EV-associated lipid remodeling observed in the murine model.

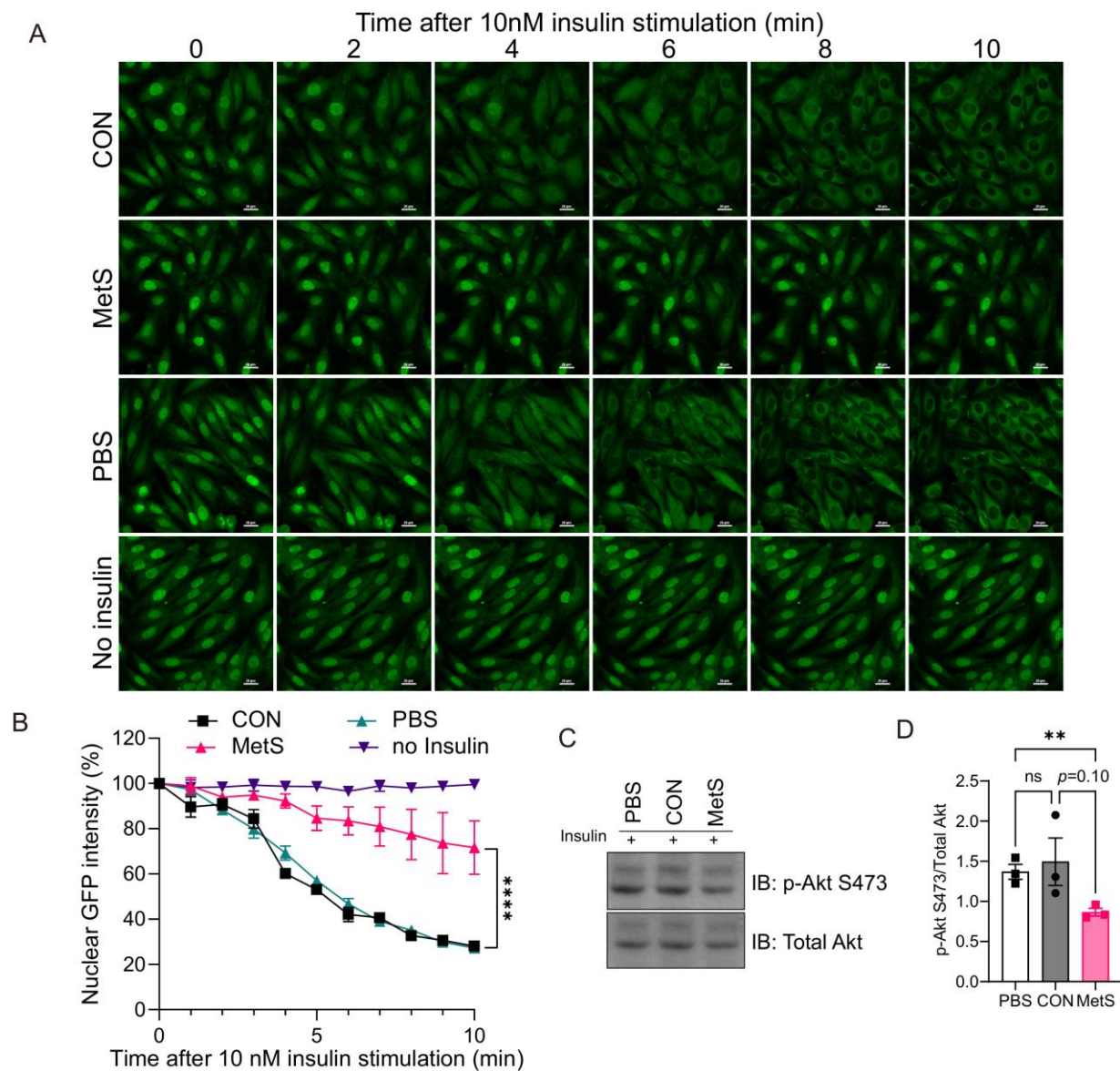


Figure 3.14 MetS-derived EV impair insulin signaling in skeletal muscle cells.

(A) Representative images of L6 Akt biosensor cells post-insulin stimulation following EV treatment. (B) Line graph for nuclear GFP intensity (% relative to the 0-time point of the non-insulin treated condition,

scale bar = 20 μ m, n=3). (C&D) Western blot analysis of p-Akt (S473) / total Akt in iPSC-derived myoblast cells treated with EV for 24 hours post-insulin 100 nM stimulation. (n=3)

Data are presented as mean $\pm$ SEM. Statistical significance: ** $P < 0.01$, **** $P < 0.0001$ (Two-way ANOVA for panel B, unpaired t-test for panel D).

EV lipid species associated with clinical metabolic indices in MetS.

To evaluate the clinical relevance of EV lipid remodeling observed in MetS, correlation analyses were performed between EV lipid features and key metabolic indices, including circulating adiponectin levels, HbA1c, HOMA-IR, and TG:HDL ratio (Fig. 3.15A).

At the lipid class level, partial-correlation analysis adjusted for age and body mass index (BMI) revealed that glycerolipid and sterol lipid classes (TG, diacylglycerol [DG], cholesterol [COH], acyl carnitines [AC]) exhibited positive correlations with indices of insulin resistance and glycemic dysregulation (HOMA-IR and HbA1c). In contrast, several PL and lysophospholipid classes (LPC, LPE, PE, PG, PI, and PA) showed inverse associations with these metabolic parameters (Fig. 3.15A). Notably, circulating adiponectin levels were positively correlated with lysophospholipid abundance and negatively correlated with glycerolipid enrichment (Fig. 3.15A).

Species-level Kendall's τ correlation analyses further revealed directional patterns based on lipid features (Fig. 3.15B-D). Most TG and cholesteryl-ester (CE) species (e.g., CE 24:6) exhibited positive τ values with HOMA-IR and HbA1c ($p < 0.05$), whereas ether- or plasmalogen-type LPC/LPE and short-chain SMs (SM 37:1, SM 37:2) were negatively correlated with these metabolic indices (Fig. 3.15B).

When profiling lipid species that were significantly correlated in quadrants Q1 and Q3 (highlighted as colored dots in the scatter plots), EV lipid species containing PUFAs were predominantly positively associated with HOMA-IR and HbA1c, whereas negatively correlated species displayed a more balanced fatty-acyl composition (Fig. 3.15C-D). At the lipid subclass level, CERs and sphingosine-1-phosphate (S1P) were negatively correlated with metabolic indices, whereas hexosylceramides showed positive associations, suggesting differential regulation of SL subclasses in the MetS context⁶⁰³ (Fig. 3.15C-D).

Collectively, these correlation analyses demonstrated that EV lipid remodeling in MetS was systemically associated with clinical metabolic indices at both the lipid class and molecular species levels, supporting the relevance of EV lipid signatures as indicators of metabolic disease state.

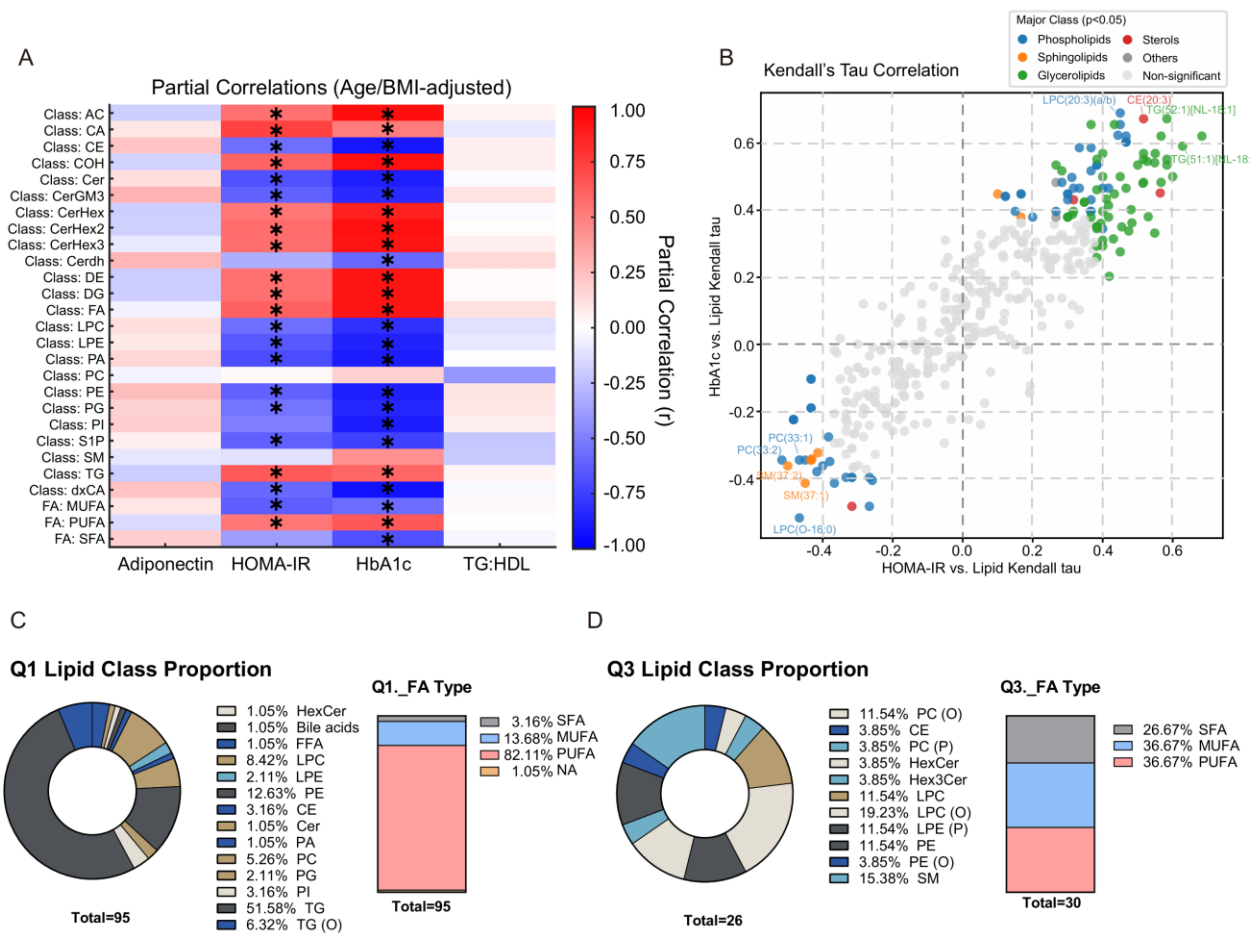


Figure 3.15 Association between human EV lipid features and metabolic indices.

(A) Partial-correlation heatmap showing associations between human EV lipid features (class-level and fatty acid types) and metabolic indices (plasma adiponectin, HOMA-IR, HbA1c, and TG:HDL ratio). Values denote partial r adjusted for age and BMI; positive correlations are shown in red, negative in blue, and significant ones (Benjamini-Hochberg FDR < 0.05) are indicated by asterisks. (B) Kendall's τ correlation scatter plot illustrating rank-based associations between individual EV lipid species and clinical indices. All species are shown in light gray, and those with significant associations ($p < 0.05$) are highlighted and color-coded by lipid class. (C&D) Pie charts and bar graphs summarizing the lipid class composition (%) and fatty acid tail type distribution (%) of EV lipids showing significant ($p < 0.05$) positive (C) or negative (D) correlations with both plasma HbA1c and HOMA-IR levels.

Adiponectin-associated EV lipid species differed by class and fatty-acyl composition.

To further delineate adiponectin-associated EV lipid features in MetS, we examined the directional associations between individual EV lipid species and circulating adiponectin levels using Kendall's τ correlation analysis (Fig. 3.16).

Volcano plot analysis highlighted a subset of EV lipid species that were either positively or negatively correlated with plasma adiponectin levels ($p < 0.05$), revealing asymmetric distribution of lipid species across correlation direction (Fig. 3.16A).

Lipid species negatively correlated with adiponectin were enriched in glycerolipid classes and long-chain polyunsaturated fatty-acyl tails (Fig. 3.16B), whereas lipid species positively correlated with adiponectin were dominated by PL and lysophospholipid classes with more balanced saturated and monounsaturated fatty-acyl composition (Fig. 3.16C).

These class- and fatty-acyl-specific patterns indicated that adiponectin-associated EV lipid species differ systematically in their structural composition, supporting a link between reduced adiponectin levels and enrichment of PUFA-containing EV lipids.

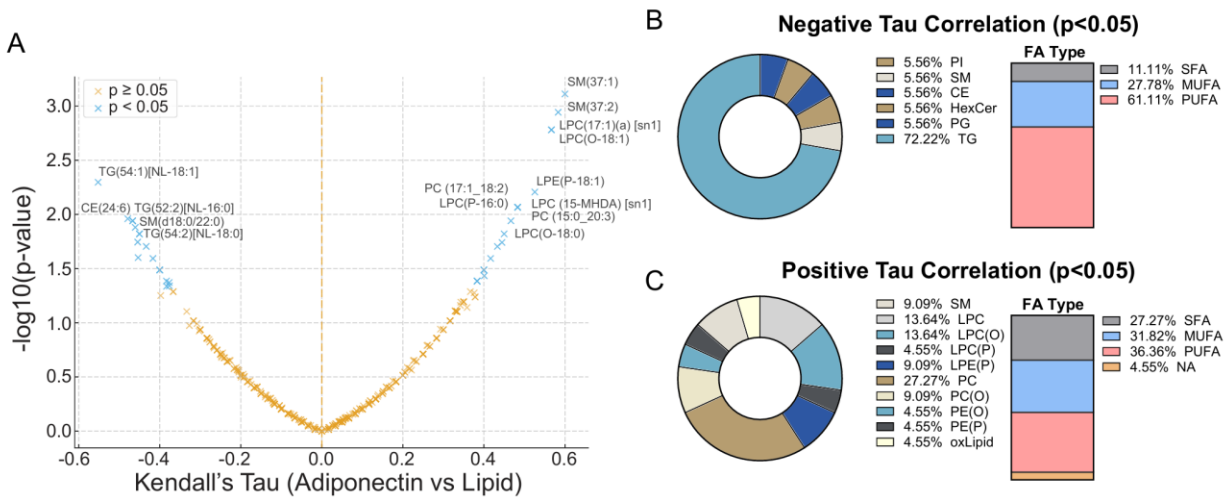


Figure 3.16 Adiponectin-associated EV lipid species differ by class and fatty-acyl composition.

(A) Volcano plot depicting Kendall's τ correlation coefficients (x-axis) versus $-\log_{10}(p\text{-value})$ (y-axis) for individual EV lipid species. The dashed vertical line marks $\tau = 0$. Lipids with $p < 0.05$ are highlighted in blue, and the top 15 species by absolute τ are labeled. (B&C) Pie charts and bar graphs summarizing the lipid class composition (%) and fatty acid tail type distribution (%) of EV lipids showing significant ($p < 0.05$) negative (B) or positive (C) correlations with plasma adiponectin levels.

Cross-species integration identified a conserved PUFA-enriched EV lipid signature.

To determine whether adiponectin-associated EV lipid features observed in humans are conserved across species, I performed cross-species integration of EV lipidomic datasets derived from adiponectin-deficient mice and patients with MetS. Lipid species were aligned across datasets based on lipid class, acyl-chain composition, and directionality of regulation relative to metabolic dysfunction (Fig. 3.17).

Intersection analysis revealed a subset of EV lipid species that were consistently altered in both adiponectin-deficient mouse EVs and human MetS EVs, exhibiting concordant directional changes across species (Fig. 3.17A). Scatter comparison of matched lipid species demonstrated directional concordance between murine KO (KO vs. WT) and human MetS (MetS vs. CON) datasets. Lipid species increased in both datasets (quadrant 1; Q1) were predominantly PUFA-containing lipids, including PE (16:0_20:3), PC (35:2)(a/b), PE (38:5)(a/b) and DG (18:2_22:4) (Fig. 3.17A, Table 3.5). In contrast, lipid species abundant in WT and CON (quadrant 3; Q3) were enriched in lysophospholipids, such as LPC (18:0) and LPE (18:1), and short-chain SMs (Fig. 3.17A; Table 3.5).

Table 3.5 Intersection analysis of EV lipid species showing concordant directional changes between adiponectin-deficient mice and patients with MetS.

Lipid	Class	FA Type	log2FC_KO vs WT	log2FC_MetS vs CON	Quadrant
DG(18:0_20:4)	DG	PUFA	0.45831	1.6064	Q1 (up/up)
DG(16:0_18:1)	DG	MUFA	0.59709	1.2023	Q1 (up/up)
DG(18:1_18:1)	DG	MUFA	1.3087	1.6571	Q1 (up/up)
PA(34:1)	PA	MUFA	1.1265	0.36579	Q1 (up/up)
PC (16:0_20:3) (a)	PC	PUFA	0.40438	1.1002	Q1 (up/up)
PC (18:1_18:2)	PC	PUFA	0.62726	1.1002	Q1 (up/up)
PC (15-MHDA_18:2)	PC	PUFA	1.864	0.53015	Q1 (up/up)
PC(35:2) (a/b)	PC	PUFA	1.864	0.53015	Q1 (up/up)
PC (16:1_20:4)	PC	PUFA	0.35698	0.55012	Q1 (up/up)
PE(18:1_22:6) (a/b)	PE	PUFA	0.30279	1.1216	Q1 (up/up)
PE(16:0_20:4)	PE	PUFA	0.45093	0.86522	Q1 (up/up)
PE (16:0_20:3)	PE	PUFA	0.72989	3.4396	Q1 (up/up)
PE (18:1_18:2)	PE	PUFA	0.75945	1.3728	Q1 (up/up)
PE(36:3) (a/b)	PE	PUFA	0.75945	1.3728	Q1 (up/up)
PE(38:5) (a/b)	PE	PUFA	1.0381	0.87323	Q1 (up/up)
PE(16:0_22:6)	PE	PUFA	1.6667	1.0649	Q1 (up/up)

PI(18:1_18:2)	PI	PUFA	1.031	1.7882	Q1 (up/up)
TG(O-52:2) [NL-16:0]	TG(O)	PUFA	0.93208	2.1262	Q1 (up/up)
CE(18:0)	CE	SFA	-0.97442	-0.43518	Q3 (down/down)
CE(17:0)	CE	SFA	-0.86731	-0.58967	Q3 (down/down)
CE(20:0)	CE	SFA	-0.64943	-1.3941	Q3 (down/down)
CE(22:0)	CE	SFA	-0.52828	-2.2349	Q3 (down/down)
CE(22:1)	CE	MUFA	-0.44285	-1.3481	Q3 (down/down)
CE(24:4)	CE	PUFA	-0.40489	-0.45117	Q3 (down/down)
CE(20:1)	CE	MUFA	-0.36981	-0.85943	Q3 (down/down)
CER(d18:1/24:0)	CER	MUFA	-0.70114	-0.41071	Q3 (down/down)
DG(18:2_22:6)	DG	PUFA	-2.3732	-3.4471	Q3 (down/down)
DG(18:1_18:2)	DG	PUFA	-0.38384	-1.4812	Q3 (down/down)
CerHex(d18:1/24:0)	HexCer	MUFA	-0.70775	-0.67667	Q3 (down/down)
CerHex(d18:1/16:0)	HexCer	MUFA	-0.69539	-0.70635	Q3 (down/down)
LPC (15-MHDA) [sn1] [104_sn1]	LPC	NA	-0.78406	-1.1854	Q3 (down/down)
LPC (19:0) (a) [sn1] [104_sn1]	LPC	SFA	-0.73746	-1.3007	Q3 (down/down)
LPC (17:1) (a) [sn1] [104_sn1]	LPC	MUFA	-0.59479	-0.94744	Q3 (down/down)
LPC(18:2) (a/b)	LPC	PUFA	-0.51438	-0.65724	Q3 (down/down)
LPC(O-16:0)	LPC(O)	SFA	-0.8004	-1.175	Q3 (down/down)

LPC(O-18:1)	LPC(O)	MUFA	-0.77534	-0.94744	Q3 (down/down)
LPC(O-18:0)	LPC(O)	SFA	-0.58636	-1.1399	Q3 (down/down)
LPC(O-20:0)	LPC(O)	SFA	-0.50056	-1.6719	Q3 (down/down)
LPC(P-18:0)	LPC(P)	SFA	-0.77534	-0.84139	Q3 (down/down)
LPC(P-16:0)	LPC(P)	SFA	-0.65404	-0.8849	Q3 (down/down)
PC(18:0_22:6)	PC	PUFA	-0.69709	-1.0222	Q3 (down/down)
PC (15-MHDA_22:6)	PC	PUFA	-0.60282	-0.45667	Q3 (down/down)
PC (17:0_22:6)	PC	PUFA	-0.60282	-0.45667	Q3 (down/down)
PC (P-18:1/18:1)	PC	MUFA	-0.40109	-1.0902	Q3 (down/down)
PC (15:0_20:3)	PC	PUFA	-0.36347	-1.3544	Q3 (down/down)
PC (O-16:0/20:3)	PC(O)	PUFA	-0.43223	-1.0776	Q3 (down/down)
PC (P-17:0/20:4) (a)	PC(P)	PUFA	-1.6889	-0.78248	Q3 (down/down)
PE (18:0_22:4)	PE	PUFA	-0.56394	-1.8708	Q3 (down/down)
PE (O-16:0/22:4)	PE(O)	PUFA	-0.94828	-1.9006	Q3 (down/down)
PE (O-16:0/20:3)	PE(O)	PUFA	-0.65189	-0.985	Q3 (down/down)
PE (O-18:1/18:2)	PE(O)	PUFA	-0.65189	-0.71721	Q3 (down/down)
PE(O-36:3) (a/b)	PE(O)	PUFA	-0.65189	-0.985	Q3 (down/down)
PE(O-16:0/22:6)	PE(O)	PUFA	-0.60084	-0.81507	Q3 (down/down)

PE(P-18:1/22:6) (a/b)	PE(P)	PUFA	-0.6057	-0.81286	Q3 (down/down)
SM(38:3) (a/b)	SM	PUFA	-0.4956	-0.97867	Q3 (down/down)
SM(43:1)	SM	MUFA	-0.40222	-0.50374	Q3 (down/down)
SM(d18:1/24:0)	SM	MUFA	-0.32751	-0.43602	Q3 (down/down)
SM(37:1)	SM	MUFA	-0.30569	-1.0776	Q3 (down/down)

Consistent with these trends, quadrant-based analysis revealed that the majority of overlapping lipid species were PLs enriched in long-chain PUFAs, particularly within PE and PS subclasses (Fig. 3.17B). Most FDR-significant species clustered within disease-directional quadrants, indicating a shared enriched of PUFA-PL species under low-adiponectin or metabolic stress conditions (Fig. 3.17A-B; Table 3.4). Analysis of lipid composition across quadrants further demonstrated consistent enrichment of PUFA fractions within quadrants representing disease-associated directional changes (Q1, Q2, and Q4) compared to Q3, which showed more balanced distribution of fatty-acyl composition (Fig. 3.17B).

Integration of cross-species log₂ fold-change comparisons with Kendall's τ correlation analyses identified a subset of lipid species in Figure 3.15B that overlapped between datasets and exhibited consistent directional associations with metabolic indices (Fig. 3.17C–D). Shared lipid species were primarily distributed within Q1 and Q3 (13 and 18 species, respectively), with PUFA-enriched PLs and TGs predominating in Q1 (Fig. 3.17C), whereas Q3 was enriched in ether- or plasmalogen-type PC and lysophospholipid species with evenly balanced fatty-acyl composition (Fig. 3.17D).

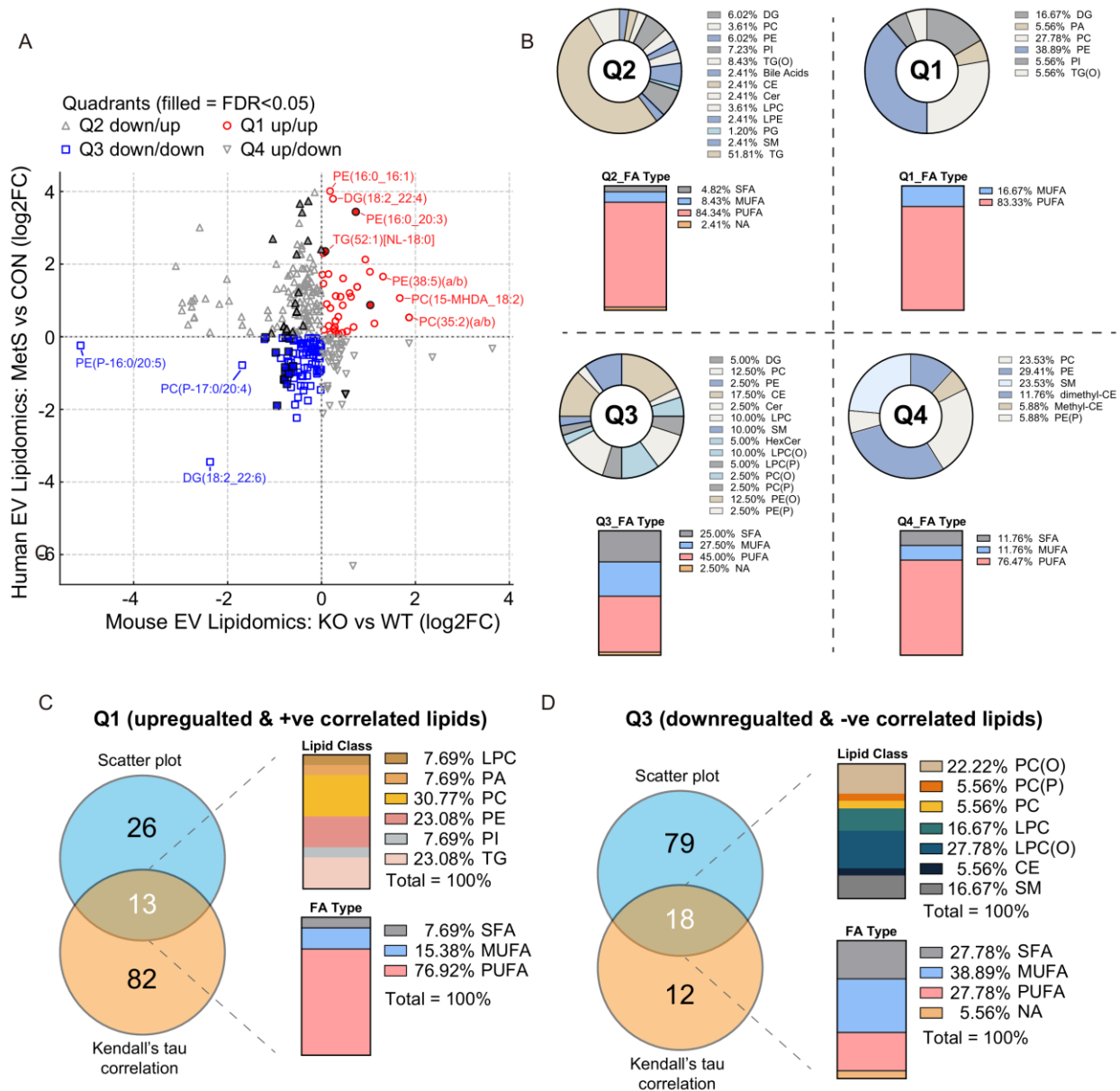


Figure 3.17 Conserved EV lipid remodeling between adiponectin-deficient mice and MetS patients.

(A) Scatter plot comparing log₂ fold changes of matched lipid species between mouse (KO vs. WT; x-axis) and human (MetS vs. CON; y-axis) EV lipidomes. Each dot represents a common lipid species (red = increased in both, blue = decreased in both, gray = discordant). Lipids significant in either dataset (FDR < 0.05) are shown as filled circles. (B) Pie charts showing the proportion (%) of lipid classes and bar graphs indicating the distribution of fatty acid tail types (%) among lipid species in each quadrant (log₂FC > 0.3). Q1–Q4 denote quadrants 1–4. (C&D) Venn diagrams summarizing lipid species significantly regulated in both analyses—those overlapping between the log₂FC comparison (panel A) and Kendall's τ correlations (Fig. 3.15B). Overlaps represent lipids increased or decreased in both mouse and human

datasets and positively or negatively correlated with HOMA-IR or HbA1c. Adjacent bar graphs display the proportional distribution of lipid classes (%) and fatty acid tail types (%) among the overlapping lipids. Full lipid lists and statistical details are provided in Table 3.5.

Collectively, these analyses identified a conserved, PUFA-enriched EV lipid signature shared between adiponectin-deficient mice and patients with metabolic syndrome, demonstrating common EV lipid remodeling features associated with metabolic stress across species (Fig. 3.18).

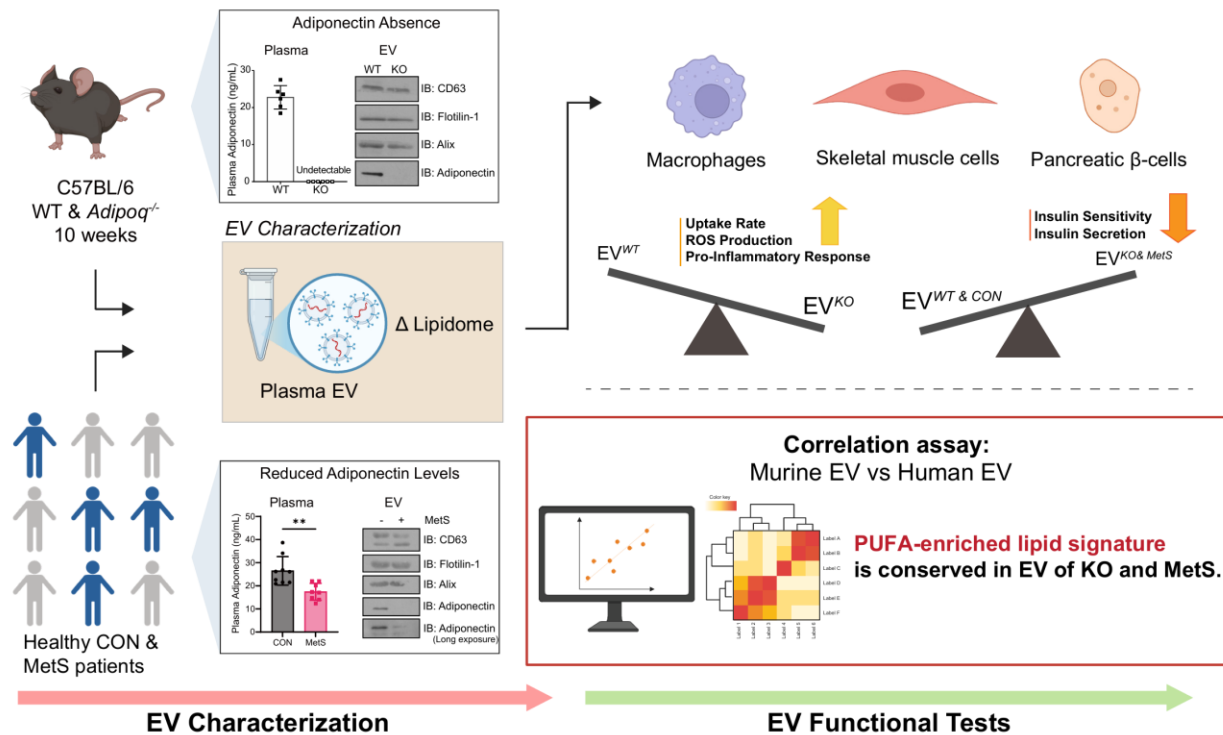


Figure 3.18 Summary schematic of findings

Adiponectin deficiency drives conserved PUFA-enriched EV lipid remodeling and metabolic dysfunction.

3.5 Discussion

This study demonstrated that adiponectin deficiency drives systemic remodeling of the plasma EV lipidome, with functional consequences for metabolic target cells. Using integrated EV lipidomic profiling and cell-based function assays, my study showed that loss of adiponectin alters EV lipid composition in mice and promotes oxidative stress, inflammatory activation, and impaired metabolic signaling in recipient macrophages, skeletal muscle cells, and pancreatic β -cells. Importantly, cross-species integration of murine

and human datasets identified a conserved, PUFA-enriched EV lipid signature associated with metabolic stress, linking adiponectin status to EV-mediated metabolic dysfunction. Together, these findings established adiponectin as a key regulator of EV lipid composition and function, and highlight EV lipid remodeling as a systemic mechanism contributing to metabolic disease.

EV lipid composition is a critical determinant of EV biophysical properties and interactions with recipient cells. The asymmetric enrichment of PS and COH on EV membranes enhances interactions with cell-surface receptors, facilitating EV internalization through endocytic pathways such as clathrin-mediated endocytosis, micropinocytosis, or direct membrane fusion⁶⁰⁴⁻⁶⁰⁶. In parallel, alterations in COH and PUFA-containing PLs are expected to modify membrane fluidity and rigidity, thereby influencing EV uptake efficiency and intracellular delivery^{372,604,606}.

Beyond uptake, these lipid features may also shape inflammatory signaling. SM-enriched membrane domains are integral components of detergent-resistant membranes and participate in cytokine receptor clustering and signal transduction^{607,608}. In addition, PS and COH in EV membrane may also potentiate inflammatory responses^{401,402}. Accordingly, enrichment of SM, PS, and COH in EVs derived from adiponectin-deficient mice provides a plausible mechanism by which EV exposure enhances macrophage inflammatory activation.

Although antioxidant enzyme expression (SOD1, SOD2, and GPX4) remained unchanged in recipient cells treated with KO EVs, the altered lipid composition of these EVs is likely sufficient to drive oxidative stress and mitochondrial dysfunction. Lipid species enriched in KO EVs, including PUFA-containing PLs, SLs, and COH, are prone to peroxidation and can perturb mitochondrial membrane integrity, thereby disrupting cellular redox balance. Such lipid-driven oxidative stress is expected to have pronounced effects in metabolically active tissues such as skeletal muscle, pancreatic islets, and adipose tissue.

In addition, KO EVs exhibited elevated COH and reduced bile acid levels, suggesting impaired COH catabolism⁶⁰⁹ and potential dysregulation of nuclear receptor signaling pathways such as LXR or FXR⁶⁰⁹. Disruption of these pathways could compromise hepatic COH clearance and systemic metabolic homeostasis, with secondary effects on insulin sensitivity through FXR-PPAR crosstalk⁶⁰⁹⁻⁶¹². Although sterol metabolism-related genes were not directly assessed at the tissue level in this study, future work integrating COH and bile acid metabolic pathways will be important to link EV-associated sterol signatures to tissue-specific metabolic regulation.

Because my *in vitro* assay used macrophages, skeletal muscle cells, and pancreatic β -cells—major metabolic cell types likely to encounter circulating EVs—my results are intended to model systemic exposure rather than tissue-restricted effects. Plasma-derived EVs represent a composite population from

multiple sources, including adipose tissue, endothelial cells, and immune cells^{613,614}; this systemic perspective underlies my experimental design.

While direct analysis of EVs derived from specific tissues or *in vivo* assessment of tissue-resident cells in adiponectin-deficient mice could reveal cell-type-specific alterations, such approaches would not fully capture the integrated EV phenotype present in circulation. Future studies combining cell-type-specific EV isolation strategies with *in vivo* EV administration and biodistribution analyses will be important to dissect tissue-specific contributions while preserving systemic context.

Phosphoinositides (PIPs) were relatively enriched in EVs derived from WT mice, potentially reflecting more active membrane trafficking and signaling states in donor cells⁶¹⁵. In recipient cells, PIPs are central regulators of insulin signaling through the PI3K-AKT pathway, which governs glucose uptake, energy metabolism, and cell survival^{615,616}. Accordingly, EVs enriched in PIPs may enhance AKT activation and promote insulin responsiveness in target tissues.

In contrast, reduced PIP abundance in EVs from adiponectin-deficient mice could compromise PI3K-AKT signaling efficiency, providing a plausible mechanistic link between altered EV lipid composition and the impaired insulin signaling observed in skeletal muscle cells. These findings suggest that adiponectin-dependent modulation of EV PIP content may influence insulin sensitivity by shaping the lipid signaling landscape delivered to recipient cells.

Bioactive lipid species enriched in EVs derived from adiponectin-deficient mice are well-established mediators of inflammatory signaling and cellular stress responses. Lipids such as S1P and LPC are known to activate immune signaling pathways^{617,618}, and their enrichment in KO EVs provided a plausible mechanism for the enhanced inflammatory activation observed in RAW 264.7 macrophages. These findings link adiponectin deficiency to EV-mediated amplification of metabolic inflammation.

In pancreatic β -cells, prolonged exposure to KO EVs impaired GSIS, consistent with β -cell dysfunction observed in adiponectin-deficient mice⁴⁸¹. β -cells are particularly vulnerable to lipid-induced stress due to their low intrinsic antioxidant capacity⁶¹⁹. Accordingly, chronic exposure to altered EV lipid species—especially SMs, CERs, and PUFA-enriched PLs—may promote oxidative stress and mitochondrial dysfunction, disrupting stimulus-secretion coupling and insulin granule trafficking⁶²⁰. Together, these observations suggested that EV lipid remodeling under adiponectin deficiency exerts deleterious effects on both immune and endocrine components of metabolic regulation.

The mechanisms by which adiponectin modulates EV lipid composition are likely multifaceted and involve coordinated regulation of SL and PL metabolism. Adiponectin receptors (AdipoR1 and AdipoR2) possess

intrinsic ceramidase activity, enabling direct modulation of CER levels and downstream SL balance^{621,622}. Moreover, S1P receptor-mediated signaling influences membrane fluidity and lipid remodeling^{623,624}. In adiponectin-deficient mice, reduced levels of long-chain CER—known AdipoR substrates implicated in insulin resistance and oxidative stress—were accompanied by elevated S1P, consistent with altered CER-to-S1P flux. Concurrent increases in SMs and upregulation of SL metabolic genes such as *Sgml*, *Cert1*, and *Smpd1* further suggest compensatory remodeling of SL pathways under adiponectin-deficient conditions.

In parallel, adiponectin deficiency was associated with selective upregulation of genes involved in PL synthesis (*Ptdss1*, *Ptdss2*, *Cept1*) and acyl-chain remodeling (*Lpcat3*) in iWAT, indicating depot-specific lipid remodeling that may influence EV lipid output. Such alterations could favor enrichment of PUFA-containing PLs within EV membranes.

Finally, phospholipase D2 (PLD2), which regulates PA generation and EV biogenesis in adipocytes⁶²⁵, may represent an additional node through which adiponectin signaling influences EV lipid packaging. Collectively, these pathways suggest that altered adiponectin signaling shifts the balance between membrane lipid remodeling and vesicle release, thereby modulating the composition and functional impact of circulating EVs.

Pathway-level analyses further support a link between EV lipid remodeling and redox-sensitive cellular stress responses. KEGG enrichment analysis identified significant overrepresentation of autophagy- and ferroptosis-related pathways among lipid species altered under adiponectin-deficient conditions. PUFA-PLs, particularly PUFA-PE species, which were enriched in KO EVs, are well-established substrates for lipid peroxidation and critical initiators of ferroptotic cell death^{199,291}. Accordingly, enrichment of these lipid species suggested that EVs derived from adiponectin-deficient mice may sensitize recipient cells to ferroptotic stress.

In parallel, several EV lipid classes enriched under adiponectin deficiency—including PE, PS, and PI—play essential roles in autophagosome formation and membrane expansion⁶²⁶. SMs and S1P can further modulate autophagy through mTOR-dependent or -independent mechanisms⁶²⁷. Together, these lipid alterations suggested that EV-mediated delivery of redox-active and membrane-remodeling lipids may promote coordinated activation of autophagy and ferroptotic vulnerability, linking adiponectin deficiency to disrupted redox and organelle homeostasis in recipient cells.

Integrated correlation and rank-based analyses reveal a unified pattern of EV lipid remodeling associated with metabolic stress. Across both lipid class- and molecular species-level analyses, insulin resistance and hyperglycemia were consistently associated with enrichment of PUFA-containing PLs and TGs, alongside

depletion of remodeling-associated lysophospholipids. This pattern was evident across multiple analytical frameworks, indicating a robust directional shift in EV lipid composition under metabolic stress.

Stratification by circulating adiponectin levels further highlighted an inverse compositional gradient. Higher adiponectin levels were associated with enrichment of ether- and plasmalogen-type LPC/LPE species and short-chain SMs—lipid features linked to membrane flexibility and antioxidant capacity—whereas lower adiponectin levels aligned with accumulation of PUFA-enriched TG and CE species, which are more susceptible to peroxidation.

Together, these analyses defined a coordinated EV lipid remodeling axis in which adiponectin status aligns with opposing lipid compositional states, providing a systems-level framework that integrates lipidomic alterations with metabolic disease severity.

Collectively, my findings further supported a conserved pattern of EV lipid remodeling linked to adiponectin status and metabolic health across species. In adiponectin-deficient mice, EV lipid remodeling was characterized predominantly by enrichment of PUFA-containing PLs with relatively limited accumulation of neutral lipids, suggesting that adiponectin primarily modulates membrane lipid unsaturation and remodeling.

In contrast, EVs derived from patients with MetS exhibited additional enrichment of TGs and CEs, features likely reflecting secondary dyslipidemia and insulin resistance rather than direct adiponectin loss alone. These observations indicated that while PUFA-enriched PLs constituted a core, adiponectin-dependent EV lipid signature conserved across species, accumulation of neutral lipids represents a context-specific overlay associated with advanced metabolic disease.

This distinction highlighted a hierarchical model of EV lipid remodeling in which adiponectin-dependent membrane lipid alterations form the foundation upon which disease-specific lipid accumulation was layered, integrating adiponectin status with broader metabolic dysfunction.

Several limitations of this study should be acknowledged. First, although inclusion of a human MetS cohort enhanced the translational relevance of my findings, the relatively small sample size ($n = 8$ per group) and inclusion of only female participants may limit generalizability. Nonetheless, the strong concordance between human and murine EV lipidomic patterns supported the robustness of the adiponectin-linked EV lipid signature across species. Second, many functional insights were derived from murine and *in vitro* models, which may not fully recapitulate human physiology. Direct *in vivo* administration of adiponectin-deficient EVs into WT recipients, coupled with EV biodistribution analyses, would provide important validation of systemic metabolic effects. Third, while my data revealed strong association between EV lipid

composition and cellular phenotypes, causal relationships for individual lipid species were not directly tested. Fourth, EV isolation methodology can influence lipidomic profiles. To minimize such effects, I employed SEC, a widely accepted approach for preserving EV integrity⁶²⁸. Nevertheless, alternative isolation strategies (e.g., ultracentrifugation or immunoaffinity capture) may enrich distinct EV subpopulations and warrant comparative evaluation⁴⁰². Finally, although my study focused on EV lipid cargo, other EV constituents—including proteins, nucleic acids, and metabolites—likely contribute to biological effects and represent important avenues for future investigation.

In summary, my study identified adiponectin as a key regulator of EV lipid composition and function across species. Adiponectin deficiency drove systemic remodeling of plasma EV lipidome toward enrichment of PUFA-containing PLs and altered SL balance, promoting oxidative stress, inflammatory activation, and impaired metabolic signaling in recipient macrophages, skeletal muscle cells, and pancreatic β -cells. Through cross-species lipidomic integration, I demonstrated that enrichment of PUFA-enriched lipids constituted a conserved, adiponectin-dependent EV lipid signature shared between adiponectin-deficient mice and patients with MetS, whereas additional TG accumulation represented a context-specific feature of advanced metabolic disease. Together, these findings established an adiponectin-EV-lipid axis that links systemic metabolic stress to EV-mediated membrane remodeling and redox vulnerability, providing a conceptual framework for understanding how adiponectin deficiency contributes to metabolic dysfunction and identifying EV lipid composition as a potential biomarker and therapeutic target in CMD.

3.6 Experimental Contributions

I performed all animal experiments, EV isolation, plasma adiponectin measurements, Western blotting, NTA, proteomic and lipidomic data analysis, EV uptake assays (flow cytometry and confocal imaging), oxidative stress assessments, RAW-dual reporter cell experiments, qPCR, intracellular ATP measurements, and mitochondrial network analyses. Hye Kyoung Sung performed EV Cryo-EM imaging. Yubin Lei assisted me with the animal experiments (including GTT, body weight, and tissue weight measurements) and EV uptake assays (EV DiR labeling, flow cytometry and confocal imaging). Eddie Tam performed hiPSC-MP differentiation and IMARIS-based 3D mitochondrial imaging analysis. Khang Nguyen assisted me with EV isolation, and performed Western blotting, oxidative stress assessments, and kinetics and dose–response analyses of insulin sensitivity using L6 Akt biosensor cells. Yihan Luo performed GSIS. Sivaporn Wannaiampiku and Brian Lee collected and provided clinical samples. EV lipidomics and proteomics data acquisition was conducted at the Segal Cancer Proteomics Centre, Lady Davis Institute for Medical Research, Jewish General Hospital, McGill University.

CHAPTER 4: General Discussion and Conclusions

4.1 Research Summary and Integrative Discussion

CMD impose sustained metabolic and oxidative stress on multiple tissues, leading to impaired cellular stress adaptation and progressive metabolic dysfunction⁸. Although iron dysregulation, lipid remodeling, and altered adipokine signaling have independently been linked to CMD pathophysiology^{13,14}, how these axes converge to shape membrane vulnerability, inflammatory signaling, and intercellular communication remains poorly resolved. A unifying mechanistic framework is therefore needed to connect lipid-centered metabolic remodeling with iron-dependent stress responses and systemic metabolic regulation.

This dissertation examined how lipid remodeling integrated iron stress and adipokine deficiency to shape cellular vulnerability, inflammatory signaling, and metabolic regulation in the context of CMD. By combining mechanistic analyses of iron-induced ferroptosis with lipidomic and functional studies of adiponectin-associated EVs, the work advanced a unified framework in which membrane lipid composition emerged as a critical determinant of stress responsiveness across both cellular and intercellular contexts. Rather than viewing ferroptosis, adiponectin signaling, and EV biology as independent or sequential processes, the findings suggested that these pathways could converge at the level of lipid remodeling, providing a coherent lens through which CMD pathophysiology could be reinterpreted.

At the cellular level, I demonstrated that the qualitative composition of membrane PLs was a key determinant of susceptibility to iron-induced stress. Enrichment of ω -6 PUFA-containing PLs, particularly PEs, markedly sensitized cardiomyocytes to iron-dependent lipid peroxidation, mitochondrial dysfunction, sterile inflammatory signaling, and ferroptotic cell death. These effects occurred independently of total lipid abundance, underscoring the importance of membrane lipid quality rather than lipid load per se. In this context, ferroptosis served as a biologically informative readout of lipid-iron imbalance, reflecting a state in which PUFA-enriched membranes lowered the threshold for oxidative injury and inflammatory activation under metabolic stress. Importantly, these findings positioned lipid remodeling upstream of ferroptotic execution, indicating that altered membrane architecture preceded and amplified iron-induced cellular injury rather than simply accompanying it.

Building on this cellular framework, I extended the concept of lipid-mediated vulnerability to the level of intercellular communication. Integrated EV lipidomic analyses in murine and human MetS cohorts identified a conserved PUFA-enriched EV lipid signature associated with adiponectin deficiency. This signature recapitulated key features of cellular lipid remodeling, suggesting that EV lipid composition reflected the metabolic state of the parent tissue. Functional studies further demonstrated that EVs

carrying this lipid signature modulated recipient cell responses across multiple cell types, promoting oxidative and inflammatory signaling in macrophages, impairing mitochondrial energetics and insulin signaling in skeletal muscle cells, and disrupting GSIS in β -cells. These observations support the conclusion that EV lipid composition can influence metabolic and inflammatory regulation in recipient cells.

Within this intercellular context, EVs were best viewed as modulators and reporters of metabolic stress rather than autonomous drivers of pathology⁴⁰⁵. The functional consequences of EV exposure appeared to arise from the interaction between EV lipid cargo and the intrinsic metabolic state of recipient cells, highlighting the importance of cellular context in shaping EV responsiveness^{407,578,579,605}. Although peripheral to the primary focus of this dissertation, complementary analyses presented in Appendix B revealed that HFD-induced EV proteome remodeling was distinct under adiponectin-deficient conditions and was accompanied by corresponding alterations in cellular proteomic profiles, particularly within pathways related to catabolic metabolism. These observations suggest that not only the direct effects of adiponectin deficiency, but also secondary adaptations to diet-induced metabolic stress, differentially shape EV cargo composition and its functional impact on recipient cells. This perspective aligned with the broader framework of CMD as a condition characterized by altered stress adaptation, in which lipid remodeling serves as a shared signal across cellular, intercellular, and systemic scales⁴³¹.

Taken together, the findings from Studies 1 and 2 supported lipid remodeling as an integrative axis linking iron-dependent cellular stress responses and adipokine-associated metabolic regulation. While iron-induced ferroptotic vulnerability and adiponectin-associated EV lipid remodeling represented distinct biological processes, both converged on PUFA-enriched membrane architecture as a common feature of metabolic stress. Within this framework, iron availability functioned as a proximal amplifier of lipid peroxidation and cellular injury, whereas adiponectin deficiency shaped the systemic metabolic milieu in which lipid remodeling occurred. Rather than operating through a single linear mechanism, these factors modulated a shared lipid-centered stress landscape that influenced both cellular resilience and intercellular signaling capacity.

Collectively, this integrative perspective reframed CMD not solely as a consequence of nutrient excess or hormonal imbalance, but as a disorder of impaired lipid-centered stress adaptation. Membrane lipid composition emerged as a biologically meaningful layer of regulation that linked intracellular stress pathways with extracellular signaling mechanisms. Iron homeostasis and adipokine signaling were thus positioned as contextual modifiers of lipid remodeling, rather than isolated drivers of pathology. By situating ferroptotic vulnerability and EV-associated metabolic modulation within a common lipid remodeling framework, these works provided a conceptual foundation for interpreting CMD biology and

for considering lipid-based signatures as indicators of metabolic stress states with potential relevance for biomarker discovery and future therapeutic strategies.

4.2 Limitations and Future Directions

While I established an integrative framework linking lipid remodeling, iron stress, and adipokine deficiency across cellular and intercellular contexts in CMD, several conceptual and translational limitations remain. These limitations primarily reflect gaps in resolving causal connectivity across biological scales, incomplete integration of systemic regulatory axes, and the current distance between mechanistic insight and therapeutic implementation. Addressing these challenges will be essential for refining the proposed framework and enabling its translational application.

Limitation 1. Translational gap between acute mechanistic models and chronic CMD physiology

Integrative limitation

Key mechanistic insights into ferroptotic vulnerability and lipid remodeling were derived primarily from acute *in vitro* perturbation models, which may not fully capture the chronic, adaptive, and heterogeneous nature of CMD. The temporal stability of lipid-centered vulnerabilities and their interaction with long-term metabolic remodeling, aging, and environmental exposures remain insufficiently characterized.

Future directions

Longitudinal *in vivo* models incorporating chronic dietary stress, progressive insulin resistance, and aging will be necessary to validate whether lipid-centered stress susceptibility persists and evolves over disease trajectories. Deployment of *in vivo* ferroptosis reporters and redox-sensitive imaging platforms may enable dynamic assessment of lipid–iron stress in physiological contexts.

Therapeutic relevance

Demonstrating durable ferroptotic vulnerability in chronic disease models will be critical for evaluating the feasibility and safety of long-term interventions targeting iron handling, lipid peroxidation, or antioxidant capacity.

Limitation 2. Causal attribution of individual lipid species in EV-mediated effects

Integrative limitation

Although EV lipidomic profiling identified a conserved PUFA-enriched lipid signature associated with adiponectin deficiency, the causal contribution of individual lipid species to recipient cell phenotypes was not directly resolved. Functional effects observed following EV treatment therefore likely reflect the combined influence of complex lipid cargo interacting with the intrinsic metabolic state of recipient cells.

Future directions

Mechanistic studies combining genetic or pharmacological manipulation of adiponectin signaling with controlled perturbation of iron homeostasis will be required to establish causal connectivity across this axis. Cell type-specific models interrogating iron transporters, lipid remodeling enzymes, and adiponectin receptors within the same experimental systems may further clarify hierarchical regulation and feedback relationships.

Therapeutic relevance

Defining causal relationships between adipokine signaling and iron-lipid stress pathways could identify combinatorial intervention strategies, such as coordinated modulation of iron availability and adiponectin signaling to mitigate ferroptotic vulnerability and metabolic dysfunction.

Limitation 3. Incomplete resolution of EV-mediated signal specificity and systemic propagation

Integrative limitation.

While EV lipid signatures were conserved across murine and human models and were shown to modulate recipient cell phenotypes *in vitro*, the specificity, directionality, and physiological magnitude of EV-mediated lipid signaling *in vivo* remain incompletely defined. The framework does not yet resolve how EV lipid cargo is selectively packaged, how tissue targeting is regulated, or how EV-mediated signaling integrates with parallel endocrine and neural communication pathways in CMD.

Future directions.

In vivo EV-tracking approaches, tissue-specific EV labeling strategies, and conditional perturbation of EV biogenesis and uptake pathways will be required to define the spatial and temporal dynamics of EV-mediated lipid signaling. Integration of EV profiling with organ-resolved metabolic flux analyses may further clarify how EVs contribute to systemic metabolic coordination.

Therapeutic relevance.

Elucidating EV trafficking rules and target specificity could enable therapeutic strategies aimed at disrupting pathogenic EV signaling, enhancing beneficial EV communication, or engineering EVs as targeted delivery platforms for lipid-modifying or antioxidant interventions.

Limitation 4. Incomplete bidirectional integration between iron stress and adiponectin-EV signaling

Integrative limitation.

Although I established parallel roles for iron-driven ferroptotic vulnerability in cardiomyocytes and adiponectin-associated EV lipid remodeling, direct bidirectional interactions between these axes were not experimentally interrogated. Specifically, the impact of iron status on adiponectin secretion, EV lipid biogenesis, and EV cargo remodeling was not examined. Conversely, whether adiponectin signaling directly modulates cellular iron handling, ferroptotic sensitivity, or iron-dependent lipid remodeling under CMD conditions remains unresolved. As a result, the current framework integrates iron stress and adipokine signaling conceptually rather than through demonstrated causal feedback mechanisms.

Future directions.

Future studies combining controlled manipulation of iron availability with adiponectin gain- and loss-of-function models will be required to establish causal feedback relationships between these pathways. Integrating iron tracer approaches, EV lipid flux analyses, and cell type-specific perturbation of adiponectin receptors or iron transport machinery may further resolve hierarchical regulation across this axis.

Therapeutic relevance.

Clarifying bidirectional coupling between iron metabolism and adipokine-EV signaling could enable rational combinatorial interventions targeting both iron handling and adipokine pathways, potentially enhancing therapeutic efficacy while minimizing off-target metabolic disruption.

Limitation 5. Opportunities for system-level integration and therapeutic convergence

Integrative limitation.

The current framework primarily addresses lipid remodeling within metabolic and inflammatory axes but does not yet incorporate higher-order regulatory systems, including neuroendocrine control, circadian regulation, immune–metabolic feedback loops, and tissue regeneration dynamics.

Future directions.

Expanding the framework to include multi-organ, multi-omics, and systems-level modeling may reveal emergent regulatory principles governing lipid-centered stress adaptation. Incorporation of sex-specific analyses, spatial lipidomics, and single-cell resolution approaches will further refine mechanistic granularity.

Therapeutic relevance.

System-level integration may uncover convergent intervention nodes capable of simultaneously modulating metabolic resilience, inflammatory tone, and tissue repair capacity, thereby enabling multi-target therapeutic strategies for CMD.

4.3 Conclusions and Significance

I established lipid remodeling as an integrative axis linking iron-dependent cellular stress responses with adipokine-associated metabolic regulation across cellular and intercellular contexts in CMD. By demonstrating that PUFA-enriched membrane lipid composition sensitized cells to ferroptotic injury and that adiponectin deficiency was associated with conserved EV lipid remodeling, this dissertation provided mechanistic coherence to processes that are often studied in isolation. Iron availability, lipid composition, and adipokine signaling were shown to converge on a shared lipid-centered stress landscape that shapes cellular vulnerability, inflammatory tone, and metabolic responsiveness.

This framework reframed CMD not solely as a disorder of nutrient excess or hormonal imbalance, but as a condition of impaired lipid-centered stress adaptation. Membrane lipid composition emerged as a biologically meaningful regulatory layer that integrates intracellular redox sensitivity with intercellular metabolic communication. Within this paradigm, ferroptotic vulnerability represented a measurable manifestation of lipid–iron imbalance, whereas EV lipid remodeling reflected the systemic propagation of metabolic stress signals.

From a translational perspective, the identification of lipid-centered vulnerabilities and EV-associated lipid signatures provided a conceptual foundation for biomarker development and therapeutic stratification. Targeting lipid remodeling pathways, iron handling mechanisms, or EV-mediated signal propagation may offer complementary strategies to restore metabolic resilience and limit tissue injury in CMD. Together, the findings of this dissertation advanced a mechanistic framework that bridges molecular lipid biology with systemic metabolic pathophysiology and establishes a platform for future therapeutic innovation.

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6. Appendices

Appendix A: List of Publications

1. **Cho S***, Sung HK*, Nguyen K, et al. Lipidomic Analysis of Plasma Extracellular Vesicles from Adiponectin Deficient Mice or Metabolic Syndrome Patients Reveals Pro-Oxidative and Pro-Inflammatory Lipid Signatures Correlating with Metabolic Dysfunction. *Journal of Extracellular Vesicles*. 2026 Feb;15(2):e70229.
2. **Cho S**, Tam E, Nguyen K, Lei Y, et al. ω -6 PUFA-enriched membrane phospholipid composition increases the susceptibility of Cardiomyocytes to iron-induced ferroptosis and inflammation. *Apoptosis*. 2025 Jun;30(5-6):1614-1627.
3. **Cho, S.**, Dadson, K., Sung, H. K., et al. Cardioprotection by the adiponectin receptor agonist ALY688 in a preclinical mouse model of heart failure with reduced ejection fraction (HFrEF). *Biomedicine & Pharmacotherapy*. 2024 Feb;171:116119.
4. **Cho S**, Ying F, & Sweeney G. Sterile inflammation and the NLRP3 inflammasome in cardiometabolic disease. *Biomedical Journal*. 2023 Oct;46(5):100624.
5. **Cho S**, Sung HK, Tang J, et al. Lipid and Iron Dysregulation as Key Determinants of Ferroptosis in Cardiometabolic Disease. *Under review in Progress in Lipid Research*
6. Sung HK*, **Cho S***, Lei Y, et al. Extracellular Vesicles from Adiponectin-Deficient Mice Mediate Skeletal Muscle Metabolic Dysfunction and Insulin Resistance. *In Preparation*
7. Lei Y, Li B, Gosse T, **Cho S**, et al. Systemic Metabolomic Remodeling in Pressure Overload-induced Heart Failure Indicates Modulation of a Gut–Liver–Heart Axis by the Adiponectin Receptor Agonist ALY688. *Metabolites*. 2026, 16(1), 38.
8. Nguyen K, Tang J, **Cho S**, et al. Salubrinal promotes phospho-eIF2 α -dependent activation of UPR leading to autophagy-mediated attenuation of iron-induced insulin resistance. *Molecular Metabolism*. 2024 83:101921.
9. Oh E, Lee J, **Cho S**, Kim, SW, et al. Gossypetin Prevents the Progression of Nonalcoholic Steatohepatitis by Regulating Oxidative Stress and AMP-Activated Protein Kinase. *Molecular Pharmacology*. 2023 Nov;104(5):214-229.
10. Tam E, Sung HK, Lam NH, You S, **Cho S**, et al. Role of Mitochondrial Iron Overload in Mediating Cell Death in H9c2 Cells. *Cells*. 2022 Dec 28;12(1):118.
11. Tam E, Reno C, Nguyen K, **Cho S**, & Sweeney, G. Importance of Autophagy in Mediating Cellular Responses to Iron Overload in Cardiomyocytes. *Reviews in Cardiovascular Medicine*. 2022 May 7;23(5):167.

Appendix B: Study 3

Adiponectin Deficiency in Mice Remodels Circulating Extracellular Vesicle Proteomes and Alters Skeletal Muscle Metabolic Signaling.

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Key words: extracellular vesicles, adiponectin, proteomics, insulin resistance, metabolism

Abstract

Extracellular vesicles (EVs) are nanoscale biological entities that operate at the biointerface between tissues by transporting molecular cargo and modulating recipient-cell function. While adiponectin is a master regulator of systemic metabolism, whether adiponectin status governs the nanoscale composition and bioactivity of circulating EVs under dietary stress remains unresolved. This study examines how adiponectin deficiency reprograms EV proteomic cargo and alters EV–cellular interactions in skeletal muscle independently of systemic glycaemic phenotype.

Plasma EVs are isolated from wild-type and adiponectin-deficient mice fed standard chow or a high-fat diet and analyzed by quantitative proteomics. EV bioactivity is evaluated in L6 skeletal muscle cells using assays of mitochondrial respiration, insulin signaling, and glucose uptake, coupled with proteomic profiling of EV-exposed recipient cells.

Despite comparable glucose intolerance between high-fat diet–fed groups, EV proteomic landscapes diverge markedly by adiponectin status. EVs from adiponectin-deficient mice are selectively depleted of mitochondrial and glycolytic enzymes and impair mitochondrial respiration and insulin responsiveness in recipient muscle cells. Correlation analyses reveal genotype-dependent reorganization of EV protein–phenotype coupling, with metabolic programs enriched in wild-type EVs and immune–stress pathways predominating in adiponectin-deficient EVs.

Together, these findings establish adiponectin as a regulator of EV-mediated nano-biointerfaces and identify circulating EVs as hormone-sensitive nanoscale systems that encode and transmit metabolic dysfunction across tissues.

Introduction

Maintenance of metabolic homeostasis requires coordinated communication among peripheral organs to integrate nutrient availability and cellular function across tissues¹. This systemic coordination relies not only on soluble factors but also on nanoscale carriers that operate at biological interfaces to transmit molecular information between cells and organs²⁻⁴. Disruption of such inter-organ communication networks contributes to impaired substrate utilization and metabolic inflexibility under conditions of nutrient excess and stress²⁻⁴.

Extracellular vesicles (EVs) have emerged as nanoscale biological systems that mediate intercellular communication by transporting proteins, lipids, nucleic acids, and metabolites across tissue boundaries⁵⁻⁷. Circulating EV abundance and cargo composition are altered in obesity and insulin resistance and correlate with metabolic dysfunction⁸⁻¹¹. However, despite growing descriptive evidence, how EV molecular signatures encode systemic metabolic states and translate into functional responses in recipient tissues remains incompletely understood. In particular, the principles governing hormonal regulation of EV cargo composition and bioactivity are poorly defined.

Adiponectin is a pleiotropic adipokine with insulin-sensitizing and anti-inflammatory properties that plays a central role in systemic energy homeostasis^{12,13}. Circulating adiponectin levels decline in human obesity and type 2 diabetes and are associated with impaired metabolic control and increased cardiometabolic risk¹⁴⁻¹⁷. Genetic loss of adiponectin or its receptors (AdipoR1/2) exacerbates stress-induced insulin resistance and tissue inflammation¹⁸⁻²¹, whereas pharmacological activation of adiponectin pathways improves metabolic outcomes across multiple disease contexts^{16,22-25}. Notably, adiponectin has been identified as an EV-associated protein and has been shown to enhance EV biogenesis²⁶⁻²⁸, suggesting that adiponectin signaling may directly influence EV production and molecular composition, and downstream bioactivity. Whether adiponectin deficiency reprograms circulating EV proteomes and EV-mediated insulin signaling remains unknown.

Skeletal muscle represents a major biointerface for systemic metabolic regulation, accounting for the majority of insulin-stimulated glucose disposal and serving as a key determinant of whole-body metabolic flexibility²⁻⁴. Under nutrient excess, skeletal muscle develops mitochondrial dysfunction, impaired insulin signaling and altered substrate utilization, contributing directly to

systemic insulin resistance²⁹⁻³³. EVs derived from adipose tissue and other metabolic organs can modulate skeletal muscle metabolism and inflammatory signaling^{6,7,26,34-37}, positioning muscle as a biologically relevant recipient tissue for interrogating EV bioactivity at the nano-cellular interface.

Here, this study tests the hypothesis that adiponectin status governs the proteomic composition and functional properties of circulating EVs during high-fat diet (HFD)-induced metabolic stress. Using an adiponectin knockout (KO) mouse model, quantitative proteomic profiling of plasma EVs is integrated with functional assays and proteomic analysis of EV-exposed skeletal muscle cells¹⁹. This approach defines how adipokine signaling reshapes EV nano-biointerfaces and alters EV-mediated metabolic communication across tissues.

Results

Metabolic phenotyping indicates glucose intolerance in response to HFD.

Wild-type (WT) and adiponectin-deficient (KO) mice were subjected to a 4-week high-fat diet (HFD) regimen, followed by glucose tolerance testing (GTT) and plasma-derived extracellular vesicle (EV) characterization (Fig.1A). Both genotypes exhibited comparable body weight gain following HFD feeding relative to baseline ($p < 0.01$ within each genotype; Fig.S1A). Circulating adiponectin levels in WT mice were not significantly altered by HFD, whereas adiponectin was undetectable in KO plasma (Fig.1B). Blood glucose levels after overnight (18 h) fasting was modestly higher in KO mice than in WT controls ($p < 0.05$; Fig.S1B). GTT demonstrated impaired glucose tolerance in HFD-fed groups of both genotypes, reflected by delayed glucose clearance and increased area under the curve (AUC; Fig.S1C-D).

EV number, size and composition are altered by adiponectin deficiency and diet.

Plasma EVs were isolated by size-exclusion chromatography (SEC) and validated by marker profiling. Canonical EV markers (CD63, Alix and Flotillin-1) were detected across all groups, whereas non-EV markers (Calnexin and β -actin) were not detected (Fig.1C). EV-associated adiponectin was present exclusively in WT-derived EVs and was not altered by HFD (Fig.1C-D). Cryo-electron microscopy confirmed vesicular morphology (Fig.1E, inset).

Nanoparticle tracking analysis (NTA) showed increased EV particle concentration in KO and HFD groups (Fig.1E-F). Median particle size increased in WT EVs following HFD, whereas no size shift was observed in KO EVs (Fig. 1G). These measurements demonstrate genotype- and diet-dependent differences in EV abundance and size distribution. EV proteomic profiling was therefore performed to assess associated changes in cargo composition.

Adiponectin deficiency and HFD alter the EV proteome.

LC-MS/MS proteomic analysis was performed on plasma EVs ($n = 6$ per group). A total of 714 proteins were identified, of which 250 overlapped with Vesiclepedia and ExoCarta databases, while 464 were not previously annotated as EV-associated proteins (Fig.1H). Partial least squares-discriminant analysis (PLS-DA) revealed clear separation of EV proteomic profiles according to both genotype and dietary condition (Fig.1I). Volcano plot analysis identified numerous differentially expressed proteins (DEPs; $p < 0.05$, $|\log_2FC| > 1.2$; Fig.1J-K, Fig.S2A-B).

Gene Ontology enrichment analysis (GO) of DEPs highlighted changes in catabolic and energy-related pathways under HFD conditions, including carbohydrate metabolism, monocarboxylic acid metabolism, tricarboxylic acid (TCA) cycle activity, phosphagen metabolism and glycogen phosphorylase activity (Fig.1L-M, Fig.S2C-D). Kyoto Encyclopedia of Genes and Genomes pathway analysis in the WT-HFD versus KO-HFD comparison identified enrichment of carbon metabolism, the TCA cycle, amino acid biosynthesis and cysteine/methionine metabolism (Fig.S2E). Heatmap visualization showed relative enrichment of proteins within these metabolic pathways in WT-HFD EVs and lower abundance in KO-HFD EVs (Fig.1N). Mitochondrial and glycolytic enzymes, including citrate synthase (CS), malate dehydrogenase 2 (MDH2), triose phosphate isomerase 1 (TPI1) and aldolase A (ALDOA), were prominent in WT-derived EVs together with adiponectin (ADIPOQ) (Fig.1N).

Diet-driven reorganization of EV proteome–glucose tolerance coupling is constrained by adiponectin status.

To examine the relationship between EV protein abundance and glucose tolerance, EV proteomic data were analyzed relative to GTT AUC. Kendall's tau correlations were calculated between EV abundance and GTT AUC in WT and KO mice (Fig.2A). EV proteins were assigned to four quadrants based on the direction and statistical significance of correlations in each genotype. Comparison of WT (x-axis) and KO (y-axis) backgrounds demonstrated divergence in proteins associated with glucose intolerance, with many proteins showing significant coupling in only one genotype (Q2 and Q4) rather than being conserved genotypes (Q1 and Q3) (Fig.2A).

Proteins within each quadrant were subjected to GO biological process enrichment analysis (Fig.2B). Proteins positively correlated with glucose intolerance in WT mice but negatively correlated in KO mice (Q4) were enriched for metabolic and catabolic processes, including small-molecule and organic acid metabolism and oxidative stress-related pathways. Proteins positively correlated with glucose intolerance in KO mice but negatively correlated in WT mice (Q2) were enriched for immune- and host-response processes, including adaptive immune responses, plasminogen activation, coagulation and inflammatory pathways.

Correlation structures were further examined under normal chow and HFD conditions (Fig.S2F-G). Under normal chow, EV-GTT correlations were clustered near zero. HFD broadened the

distribution of correlation coefficients, with a greater fraction of proteins exhibiting stronger positive or negative associations with GTT AUC.

EVs from adiponectin-deficient mice impair mitochondrial respiration and insulin signaling in skeletal muscle cells.

Functional assays were subsequently performed to evaluate the effects of EVs on L6 skeletal muscle recipient cells. Seahorse XF analysis showed no statistically significant differences in basal or maximal oxygen consumption rates between cells treated with EVs from KO mice and those treated with WT EVs ($p > 0.05$), although the lowest mean values were observed in the KO-HFD EV-treated group (Fig. 3A–B). Proton leak and non-mitochondrial respiration showed minimal variation across groups (Fig.S3A), whereas the OCR/ECAR ratio was lower in KO-derived EV-treated cells under high-glucose conditions (Fig.S3C).

Insulin responsiveness was assessed using L6 cells expressing a GFP-FoxO1 reporter (Fig.3C-E). Insulin dose-response analysis showed EC50 values of 2.63 nM for WT EV-treated cells and 1.69 nM for WT-HFD EV-treated cells, whereas EC50 values increased to 11.71 nM and 9.64 nM following KO EV and KO-HFD EV treatment, respectively (Fig.3E; Fig.S3D-E). Immunoblot analysis showed reduced insulin-stimulated Akt (Ser473) phosphorylation in KO EV-treated cells (Fig.3F). In L6-GLUT4myc cells, EVs from KO-HFD mice reduced insulin-stimulated glucose uptake relative to WT EVs (Fig.3G).

Proteomic remodeling in skeletal muscle cells following EV exposure.

LC-MS/MS profiling was performed on L6 cells treated with EVs from each experimental group (Fig.4A). PLS-DA separated PBS-treated cells from EV-treated cells and differentiated KO- and KO-HFD EV-treated groups from WT and WT-HFD EV-treated groups (Fig.4B).

Volcano plot analysis identified multiple DEPs ($p < 0.05$, $|\log_2FC| > 1.2$), including more than 180 downregulated proteins in KO-HFD versus WT-HFD EV-treated cells (Fig.4C-D; Fig.S4A-B). GO enrichment analysis showed over-representation of catabolic and energy-linked processes, including pathways related to the TCA cycle, ATPase activity and protein metabolic turnover (Fig.4E-F; Fig.S4C-D). KEGG pathway analysis identified enrichment of carbon metabolism, the TCA cycle, amino acid biosynthesis and pyruvate metabolism in the comparison between WT and KO EV-treated cells (Fig.4G).

Heatmap visualization showed differential abundance of multiple metabolic proteins across EV treatment groups (Fig. 4H). Mitochondrial and glycolytic proteins were relatively more abundant in KO EV-treated cells than in WT EV-treated cells, whereas WT-HFD EV-treated cells retained enrichment of proteins associated with oxidative metabolism (Fig.4H).

Together, these findings indicate that EV exposure is associated with substantial remodeling of recipient-cell proteomic profiles, with adiponectin status influencing the balance between metabolic and stress-associated protein networks.

Discussion

This study supports the concept that adiponectin status governs how metabolic stress is encoded and transmitted through circulating EV nano-biointerfaces, independently of overt glycaemic phenotype. Although WT and KO mice developed comparable glucose intolerance under short-term HFD feeding, EV-phenotype coupling patterns diverged markedly by genotype, revealing distinct molecular programs across metabolic and immune-related pathways. Integration of EV profiling with functional assays in skeletal muscle cells further demonstrated that EVs derived from KO mice were associated with reduced mitochondrial respiratory capacity and impaired insulin responsiveness, whereas EVs from WT mice preserved metabolic function. Together, these observations indicate that EV proteomic composition and bioactivity reflect adipokine-dependent regulation of systemic stress signaling rather than glucose tolerance alone.

A key strength of this study lies in the systems-level analytical framework used to interrogate EV-mediated nanoscale communication across physiological and cellular scales. Rather than focusing on individual EV markers, rank-based correlation mapping enabled identification of coordinated protein networks whose associations with glucose tolerance differed by genotype and dietary context. This approach captures emergent properties of EV signaling that are not readily detected by conventional differential expression analyses. Complementary functional validation in recipient cells and proteomic profiling of EV-treated cells further strengthened biological interpretation while maintaining appropriate caution regarding causality.

Previous studies have reported altered EV abundance and cargo composition in obesity and insulin resistance⁸⁻¹¹, and have demonstrated that adipose-derived EVs can modulate metabolic and inflammatory responses in target tissues^{26,37,38}. However, much of this work has emphasized descriptive profiling or isolated EV components, with limited integration of systemic phenotype

coupling or functional consequence^{26,37,38}. The present study extends this literature by demonstrating that adipokine status reshapes not only EV cargo composition but also the structure of EV-phenotype associations and downstream cellular responses. In contrast to reports emphasizing immune-dominant EV signatures in metabolic disease³⁹, the genotype-dependent divergence observed here highlights the coexistence of metabolic and immune programs within circulating EV pools, consistent with EVs functioning as heterogeneous nanoscale assemblies rather than uniform disease markers. These findings further suggest that adiponectin signaling influences the balance between these programs under dietary stress.

Several limitations warrant consideration. The cellular origin and tissue specificity of circulating EV populations cannot be resolved in the present study and circulating EVs likely represent heterogeneous contributions from multiple metabolic and immune compartments. Although group sizes were sufficient to detect robust EV–phenotype associations, larger cohorts will be required to validate weaker or context-dependent coupling patterns. The analyses focused on EV proteomic cargo, whereas other EV components, including lipids, metabolites and nucleic acids, may also contribute to EV bioactivity and metabolic regulation. Consistent with this, recent work demonstrated that adiponectin deficiency also remodels EV lipidomic profiles⁴⁰, underscoring the multimodal nature of EV cargo. In addition, proteomic profiling cannot fully distinguish vesicle-intrinsic proteins from proteins adsorbed onto the EV surface, which may influence quantitative interpretation of EV composition⁴¹. Finally, correlation-based analyses preclude direct causal inference between specific EV cargo components and metabolic outcomes.

These findings raise several important questions for future research. Defining the tissue sources and biogenesis pathways of functionally distinct EV subpopulations will be critical for mechanistic resolution. Longitudinal studies will be required to determine how EV-phenotype coupling evolves during disease progression and therapeutic intervention. Integrating multi-omic EV profiling with cell-type–resolved functional models may further clarify how adipokine signalling shapes inter-organ communication at the nanoscale.

In summary, this work positions adiponectin as a regulator of EV-mediated nano-biointerfaces and demonstrates that circulating EV proteomic organization captures biologically meaningful information beyond conventional glycaemic measures. Beyond biomarker potential, these findings highlight circulating EVs as hormone-sensitive nanoscale signaling systems that encode and

transmit metabolic stress across tissues, thereby contributing to adaptive or maladaptive inter-organ metabolic communication under dietary stress.

Materials and methods

Animal models

All animal experimental protocols were approved by York University Animal Care Committee where the facility conformed to Canadian Council on Animal Care guidelines. All mice used in this study were male wild-type (WT) and adiponectin deficient (KO) littermate-matched mice (C57BL/6 background⁴². Experimental animals were housed in a temperature-controlled environment under 12h light and 12h dark conditions, with free access to food and water. Mice were fed chow diet (LabDiet 5001) or 60% fat diet (Research Diets, D12492) for 4 weeks from 6 weeks old. Animals were allocated to dietary groups based on genotype. Given the genotype-defined nature of the experimental groups and the use of objective outcome measures, formal randomization and investigator blinding were not applied. All the tissues were frozen in liquid nitrogen and stored at -80°C after collection. Blood was collected in EDTA-coated tube by cardiac puncture, and the plasma was separated by centrifugation. No adverse events or unexpected animal losses were observed during the study.

Intraperitoneal glucose tolerance test (IP-GTT)

GTT was performed after 3 weeks of HFD feeding. Mice were fasted for 16 hours, followed by *i.p.* injection of glucose (Sigma, 68270) at 2 g/kg in sterile PBS. Blood glucose levels were measured using a glucometer at 0-, 15-, 30-, 45-, 60-, 90-, and 120-minute time points via tail vein blood collection.

Size-exclusion chromatography isolation of Plasma-EV

Plasma EVs were isolated by size-exclusion chromatography (SEC) under International Society of Extracellular Vesicles (ISEV)-concordant conditions⁴³. SEC using qEV columns (IZON, Cambridge, MA) was the primary method to isolate EV from plasma samples. In all experiments, fractions 3 to 6 were collected and used for subsequent EV characterization and downstream applications as per manufacturer's recommendations. The isolated EV were either used immediately or stored at -80°C until usage.

and validated by marker profiling.

Size and particle quantification by Nanoparticle Tracking Analysis (NTA)

To determine the number and size of particles in the samples, plasma EV were pooled by groups, diluted in PBS, and analyzed using the NanoSight Pro system (Malvern Instruments, Saint-Laurent, Canada). Thirty-second videos of EV were recorded at room temperature across 3–5 independent experiments. The videos were averaged and analyzed using NS Xplorer software. For size calibration, 100 nm polystyrene beads (Malvern Instruments, Saint-Laurent, Canada) were used as described previously⁴⁴.

Cryogenic electron microscopy

For cryogenic-electron microscopy, EV were loaded onto glow-discharged Quantifoil R 1.2/1.3 holey carbon grids (Electron Microscopy Sciences). A 3 µL aliquot of EV suspension was applied to each grid, blotted for 2–3 seconds under conditions of approximately 95% humidity and 4°C, using a Vitrobot Mark IV (Thermo Fisher Scientific), followed by rapid vitrification in liquid ethane. Cryogenic-electron microscopy imaging was conducted on a Talos L120C transmission electron microscope (Thermo Fisher Scientific).

Proteomic analysis of EV cargo

Isolated EV were lysed in 5% SDS (Fisher Chemical), 200 mM TRIS (Fisher BioReagents™) pH 8.5 with 10 mM TCEP (60C x 25 minutes). Free disulfides were alkylated with 40 mM IAA (RT, dark, 30 minutes) prior to proteolytic digestion by S-TRAP micro (see attached for the SOP from the vendor that we use). Peptide containing eluates were lyophilized and rehydrated at 10 ng/uL prior to loading on EvoTips and analysis by library free DIA using an Evosep One system coupled to a Bruker timsTOF HT MS operated in DIA-PASEF mode. Samples were separated on an EV1137 column @ 30SPD. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE⁴⁵ partner repository with the dataset identifier PXD070917.

Cell culture (Wild-type L6, Akt biosensor & L6-GLUT4myc cells)

L6 rat skeletal muscle cells were grown in alpha-minimum essential medium (AMEM) (Wisent; #210-010) containing 10% fetal bovine serum (FBS) (Wisent; #080-150) and 1% antibiotic/antimycotic (Gibco, #15240062) at 37°C and 5% CO₂.

Proteomic analysis of recipient cell lysates

For recipient cell proteomics, wild-type L6 cells were seeded on 6-well plate until reaching 80-90% confluency. Confluent cells were treated with EV isoalted from mice plasma, at the concentration of 10 µg/ mL . After 24 h of EV treatment, cells were trypsinized and centrifuged for 5 minutes at 2000 rpm. Cell pellets were collected and lysed in NP40 lysis buffer for 30 min on ice. Cell lysate was then subjected to in-solution digestion following protocol by Promega (Cat# V5280). Gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using gProfiler, and further analysis and visualization were used metaboanalyst and R-studio.

Western blotting analysis

For EV characterization, the EV containing in PBS were lysed in SDS lysis buffer. For assessing insulin sensitivity *in vitro*, L6 myotubes were seeded on a 6-well plate. After reaching 80-90% confluency, cells were treated with plasma-EV (10 µg/mL) for 24 h. After treatment, protein extraction was collected using lysis buffer. Lysed EV and cell lysates were denatured at 95°C for 5 min. Lysates were run on 8% or 15% SDS-PAGE gels conducted at 90 V for 2 h. The gels were transfered to a polyvinylidene difluoride (PVDF) membrane. Membranes were submerged in 3% bovine serum albumin (BSA) blocking buffer for 1 h, followed by overnight incubation in 1:1000 dilution of primary antibodies: CD63 (ThermoFisher; #PA5-92370), Alix (Cell Signaling; #92880), Flotillin-1 (abcam; #133497), calnexin (abcam; #ab22595), β-Actin (Cell Signaling; #4970), phospho-Akt s473 (Cell Signaling; #9271), adiponectin (ImmunoDiagnostics; #12010) at 4°C. Membranes were washed with TBS-T three times, followed by 1 h of secondary antibody incubation. The secondary antibody used was anti-rabbit immunoglobulin G horseradish peroxidase-conjugated antibody (Cell Signaling; #7074) at 1:5000 dilution. Membranes were washed with Clarity Western ECL Substrate and visualized using the BioRad ChemiDoc imager. Western blot band intensity was quantified using ImageJ software, and phosphor-Akt normalized

to total Akt (Cell Signaling; #9272). Uncropped immunoblot image is provided in Supporting information.

Metabolic flux assays

L6 cells were seeded in a Seahorse XF96 Cell Culture Microplate (Agilent) and cultured overnight at 37 °C in standard growth medium to allow cell attachment. Once cells reached 70–80% confluence, they were treated with EVs derived from experimental mice for 24 hours. After EV treatment, the medium was replaced with Seahorse XF Base Medium (supplemented with 25 mM glucose, 2 mM L-glutamine, and 1 mM pyruvate), and cells were equilibrated for 1 h in a non-CO₂ incubator. Mitochondrial respiration and real-time metabolic flux were measured using the Agilent Seahorse XF Cell Mito Stress Test Kit (Agilent, #103015-100) according to the manufacturer's instructions. The following compounds were sequentially injected at the indicated time points: oligomycin (2 µM, 18 min) to inhibit ATP synthase, FCCP (3 µM, 36 min) to uncouple oxidative phosphorylation, and rotenone/antimycin A (0.5 µM each, 54 min) to inhibit complex I and III. Hoechst 33342 (1 µg/mL) was included for cell normalization. Basal, ATP-linked, maximal, and non-mitochondrial respiration rates were calculated from oxygen consumption rate (OCR) traces using Wave Desktop Software (v2.6, Agilent). Data were normalized to cell number per well.

Kinetics and dose response of insulin sensitivity measurement using Akt biosensor cells

FoxO1-transfected L6 cells were used to monitor insulin sensitivity in real-time⁴⁶. Cells were grown on a µ-slide 4-well chambered polymer cover slip till 80% confluency. Cells were treated with plasma-EVs diluted in 0% AMEM media at the concentration of 10 µg/ mL for 24 h. After treatment, cells were starved for 90 min, followed by 10 nM insulin stimulation. Cells were imaged at 20x objective (Nikon A1 confocal microscope) in a 5% CO₂ live-cell chamber, over a span of 20 min to observe fluorescence translocation. For the dose-response study, cells were stimulated with different serial dilutions of insulin, starting from 100 nM. Nuclear fluorescence signal was measured after 20 min of insulin treatment for dose response study and after every 2 min for kinetics study. Data quantitation was performed using ImageJ software.

Insulin sensitivity measured by glucose uptake assay

L6-GLUT4myc cells were seeded on 96-well plate at a confluency of 10,000 cells/ well. After reaching desired confluency, cells were treated with EV at the concentration of 10 μ g/ mL for 24 h. After treatment, cells were stimulated with insulin 100 nM for 15 min. Glucose uptake assay was performed following the protocol previously described⁴⁶.

Bioenergetics analysis of EV-treated cells Seahorse assay

L6 skeletal muscle cells were seeded onto Seahorse XF96 Cell Culture Microplates and allowed to adhere overnight. After 24 hours of treatment with EV, mitochondrial respiration was assessed using the Seahorse XF Mito Stress Test Kit (Agilent, Cat# 103015-100) according to the manufacturer's instructions. Measurements were performed using the Seahorse XF Analyzer, and key parameters of mitochondrial function were calculated using Seahorse Wave software.

Statistical analysis

Statistical analyses were performed in GraphPad Prism 10.4 using one-way ANOVA with Tukey's multiple comparisons test, with $p < 0.05$ considered statistically significant. Sample sizes were determined based on prior studies using comparable experimental designs and were sufficient to detect biologically meaningful differences in metabolic and proteomic outcomes. For *in vivo* experiments, the experimental unit was a single mouse. For EV functional assays and recipient-cell proteomics, the experimental unit was an independent EV preparation derived from plasma samples of individual mice; where indicated, EVs were pooled within experimental groups prior to application to independent cell culture experiments to minimize inter-individual variability. Proteomics datasets were processed and visualized using MetaboAnalyst and RStudio, including pathway enrichment analysis and generation of Venn diagrams and volcano plots based on significantly altered features ($p < 0.05$ and $|\log_2FC| > 1.0$ or 1.2, as indicated). Data distribution and variance were assessed visually prior to statistical testing.

Data Availability

EV proteomics data are available via ProteomeXchange with identifier PXD070917. Other datasets for this study are available on Supplementary Table 1.

Author contributions

H.K. Sung and S. Cho contributed equally to this work.

Hye Kyoung Sung: Conceptualization, Writing-Review & Editing, Project administration

Sungji Cho: Conceptualization, Methodology, Formal analysis, Investigation, Writing-Original Draft, Writing-Review & Editing, Visualization, Project administration

Yubin Lei: Investigation, Methodology, Visualization

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Christoph H. Borchers: Resources

Dylan Burger: Writing-Review & Editing

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Gary Sweeney: Conceptualization, Resources, Writing-Review & Editing, Supervision, Funding acquisition

Disclosure statement

All authors declare no conflict of interest with the contents of this manuscript.

Declaration of Generative AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work the author used ChatGPT-5.2 in order to improve the readability and language of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

Abbreviations

AMP-activated protein kinase (AMPK); cryogenic-electron microscopy (Cryo-EM); citrate synthase (CS); extracellular acidification rate (ECAR); extracellular vesicle (EV); gene ontology (GO); high-fat diet (HFD); Kyoto Encyclopedia of Genes and Genomes (KEGG); adiponectin knockout (KO);; nanoparticle tracking analysis (NTA); oxygen consumption rate (OCR); principal

component analysis (PCA); partial least squares-discriminant analysis (PLS-DA); size-exclusion chromatography (SEC); tricarboxylic acid (TCA); wild-type (WT)

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Figures

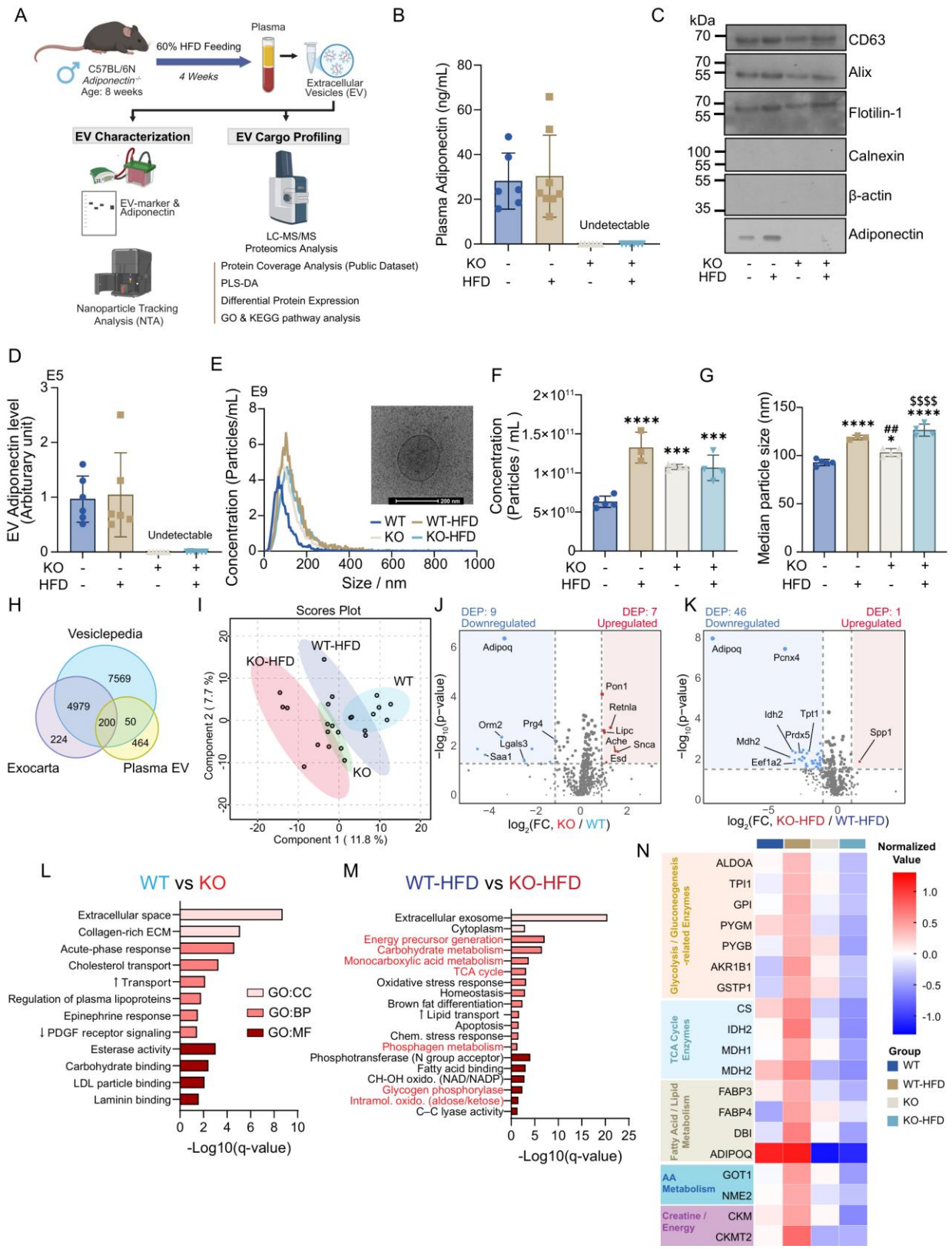


Figure 1. Adiponectin deficiency and HFD remodel circulating EV abundance and cargo composition.

(A) Experimental schematic illustrating the study design.

(B) Plasma adiponectin levels measured by ELISA (ng/mL; n = 6–9 per group).

(C) Immunoblot validation of EV markers (CD63, Alix, Flotillin-1), absence of non-EV markers (calnexin, β -actin), and detection of adiponectin in isolated EVs.

(D) Quantification of EV-associated adiponectin levels by LC–MS/MS (arbitrary units; n = 6 per group).

(E) Representative plasma EV size distribution profiles determined by nanoparticle tracking analysis (NTA; particles/mL) and a representative cryo-electron microscopy (cryo-EM) image.

(F) EV particle concentration (particles/mL) and (G) Median EV particle size (nm) measured by NTA.

(H) Venn diagram comparing identified EV proteins with Vesiclepedia, ExoCarta, and published plasma EV proteomic datasets.

(I) Partial least squares–discriminant analysis (PLS-DA) of plasma EV proteomes.

(J–K) Volcano plots showing differentially expressed EV proteins (DEPs) in WT vs KO (J) and WT-HFD vs KO-HFD (K) comparisons.

(L–M) Gene Ontology (GO) enrichment analysis of EV proteins altered by genotype (L) or by genotype under HFD (M).

(N) Heatmap showing the averaged expression levels of proteins identified within the KEGG-enriched pathways shown in Fig.S2E (n = 6 per group).

Data are presented as mean ± SD. Statistical significance: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 (one-way ANOVA); Symbol key: * vs WT; # vs WT-HFD; \$ vs KO.

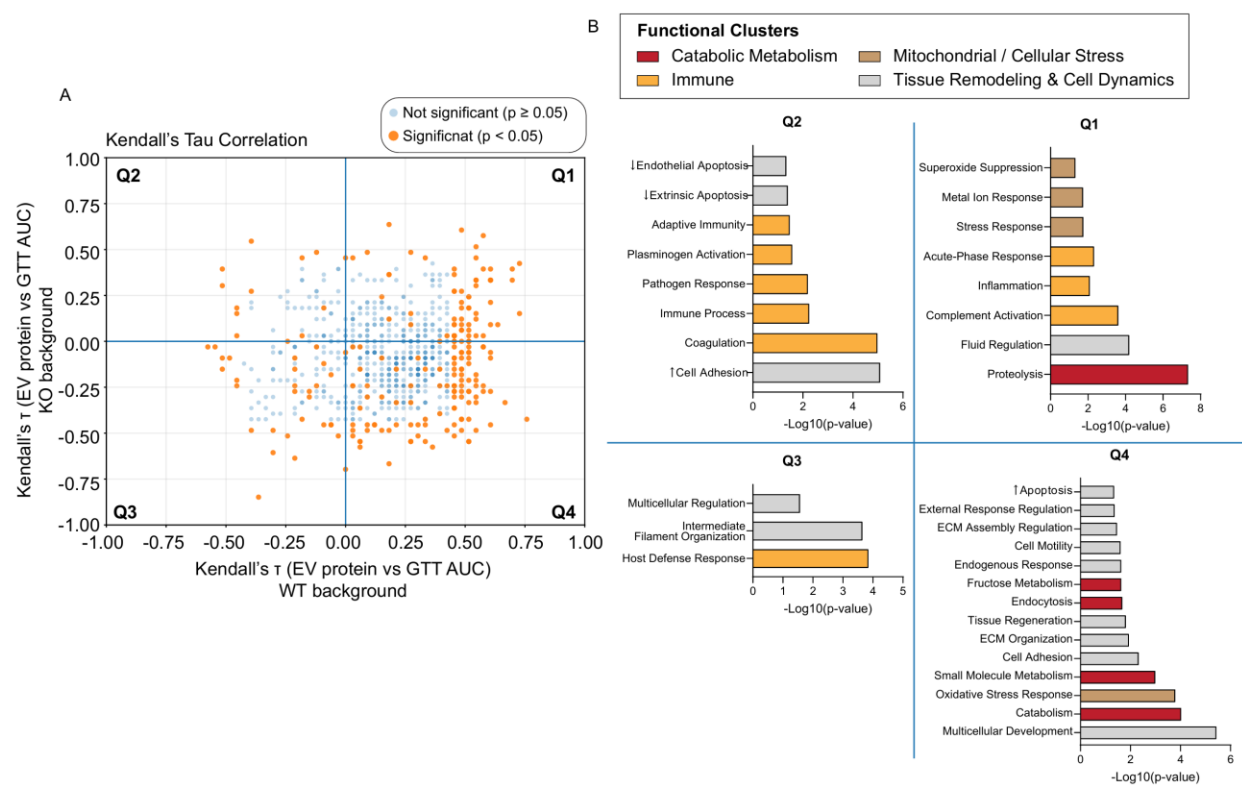


Figure 2. Associations between extracellular vesicle proteomic profiles and glucose tolerance differ by genotype.

(A) Quadrant-based Kendall's tau correlation analysis between EV protein abundance and glucose tolerance test (GTT) area under the curve (AUC), comparing WT (x-axis) and KO (y-axis) backgrounds. Each point represents an individual EV protein. Proteins are assigned to four quadrants (Q1-Q4) based on the direction and statistical significance ($p < 0.05$) of their correlations in each genotype.

(B) Gene Ontology (GO) Biological process enrichment analysis of significantly correlated EV proteins within each quadrant, grouped into four major functional clusters ($p < 0.05$): catabolic metabolism, immune processes, mitochondrial/cellular stress responses, and tissue remodeling/cell dynamics.

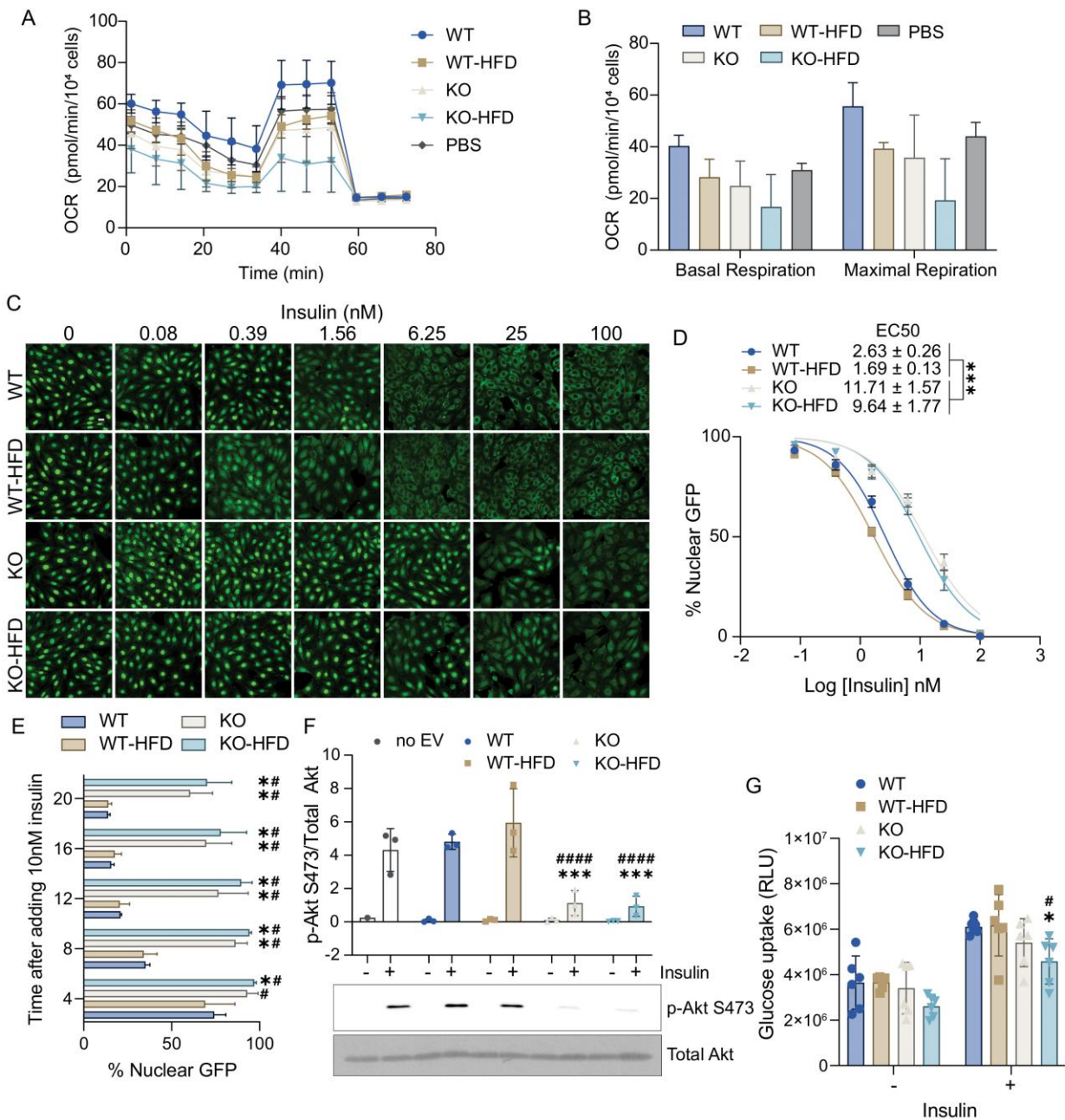


Figure 3. Adiponectin-deficient EVs impair insulin signaling and metabolic function in skeletal muscle cells.

(A) Oxygen consumption rate (OCR) profiles in L6 skeletal muscle cells treated with EVs and (B) quantification of basal and maximal respiration following EV treatment.

(C) Representative images of insulin-induced nuclear translocation of a GFP-tagged FOXO reporter across increasing insulin concentrations.

(D) Dose–response curves and EC₅₀ values for insulin-stimulated FOXO nuclear exclusion.

(E) Time-course quantification of FOXO nuclear localization following insulin stimulation.

(F) Immunoblot analysis and quantification of insulin-stimulated Akt phosphorylation (Ser473), normalized to total Akt.

(G) Glucose uptake measured in L6 skeletal muscle cells following EV treatment in the presence or absence of insulin.

Data are presented as mean $\pm$ SD. Statistical significance was determined by one-way ANOVA, except for panel G, which was analyzed by two-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ##### $P < 0.0001$. Symbol key: * vs WT; # vs WT-HFD.

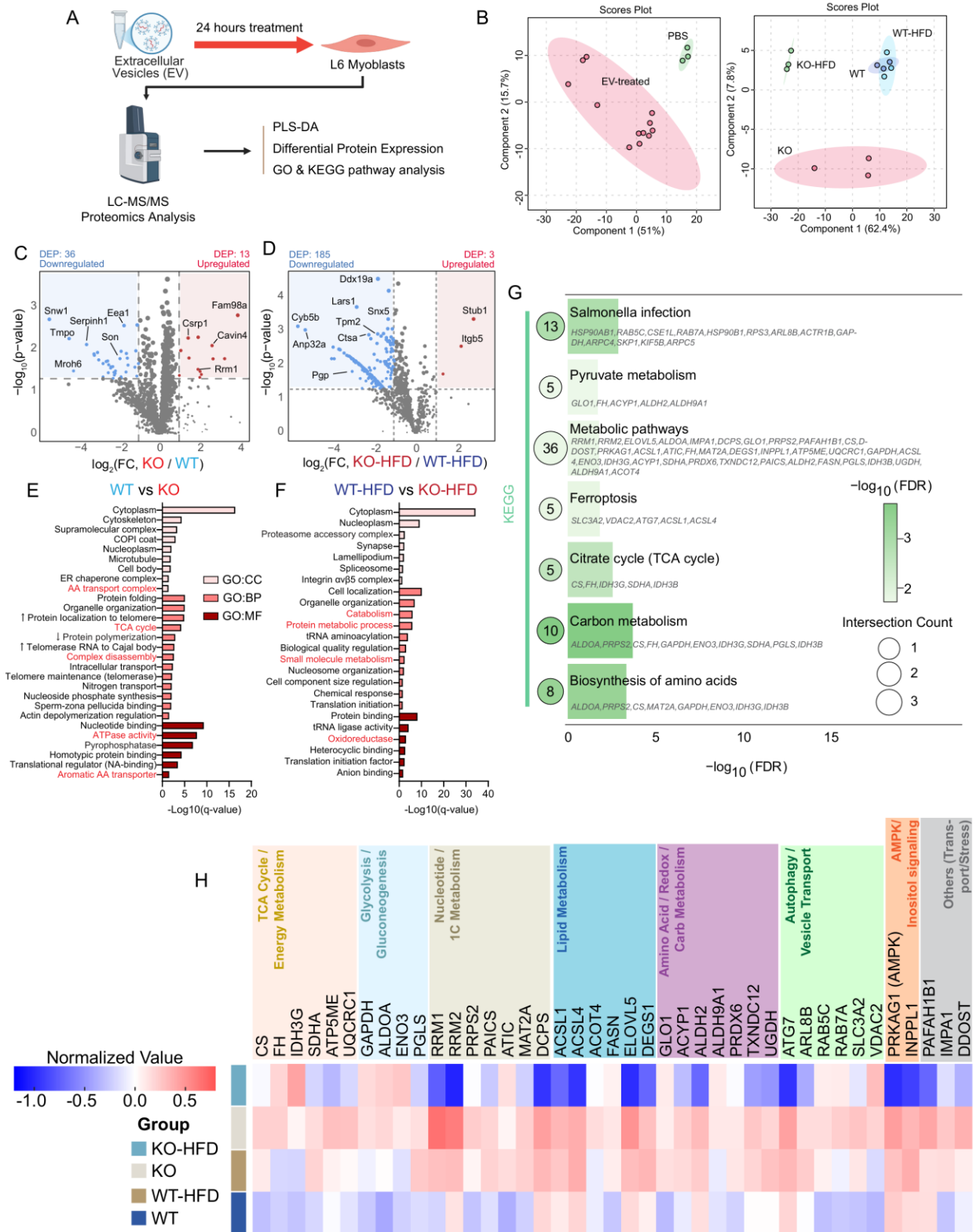


Figure 4. EV-mediated remodeling of recipient-cell proteome highlights metabolic reprogramming

(A) Experimental workflow illustrating EV treatment of L6 skeletal muscle cells followed by LC–MS/MS–based proteomic analysis.

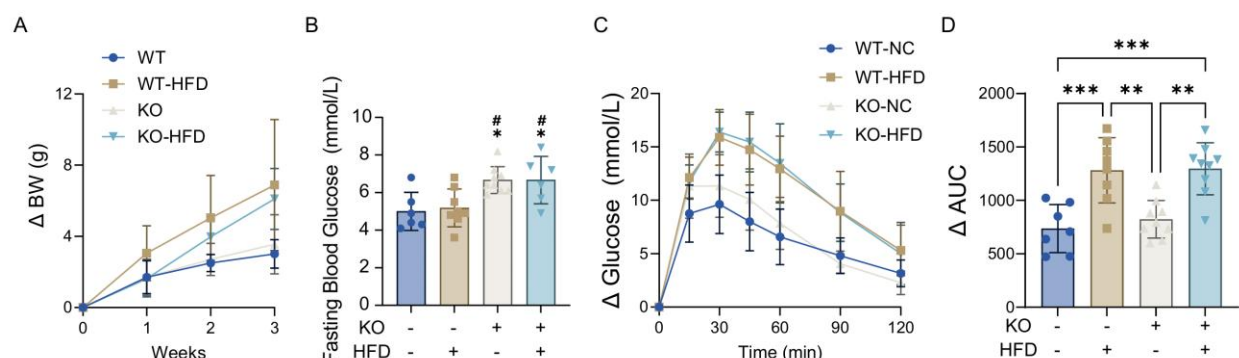
(B) Partial least squares–discriminant analysis (PLS-DA) demonstrating separation between PBS- and EV-treated cells, as well as among EV treatment groups (n = 3 per group).

(C–D) Volcano plots showing differentially expressed cellular proteins following treatment with KO versus WT EVs under normal chow (NC; C) or HFD (D) conditions.

(E–F) GO enrichment analysis of cellular proteomic changes induced by WT versus KO EVs (E) and WT-HFD versus KO-HFD EVs (F).

(G) KEGG pathway enrichment analysis highlighting alterations in carbon metabolism, TCA cycle, amino acid biosynthesis, and ferroptosis-related pathways in comparison between WT and KO EV-treated cells.

(H) Heatmap of differentially expressed metabolic and signaling proteins in EV-treated cells, grouped by functional pathways (n = 3 per group).



Supplementary Figure 1. Metabolic phenotyping confirms systemic insulin resistance in

adiponectin-deficient mice.

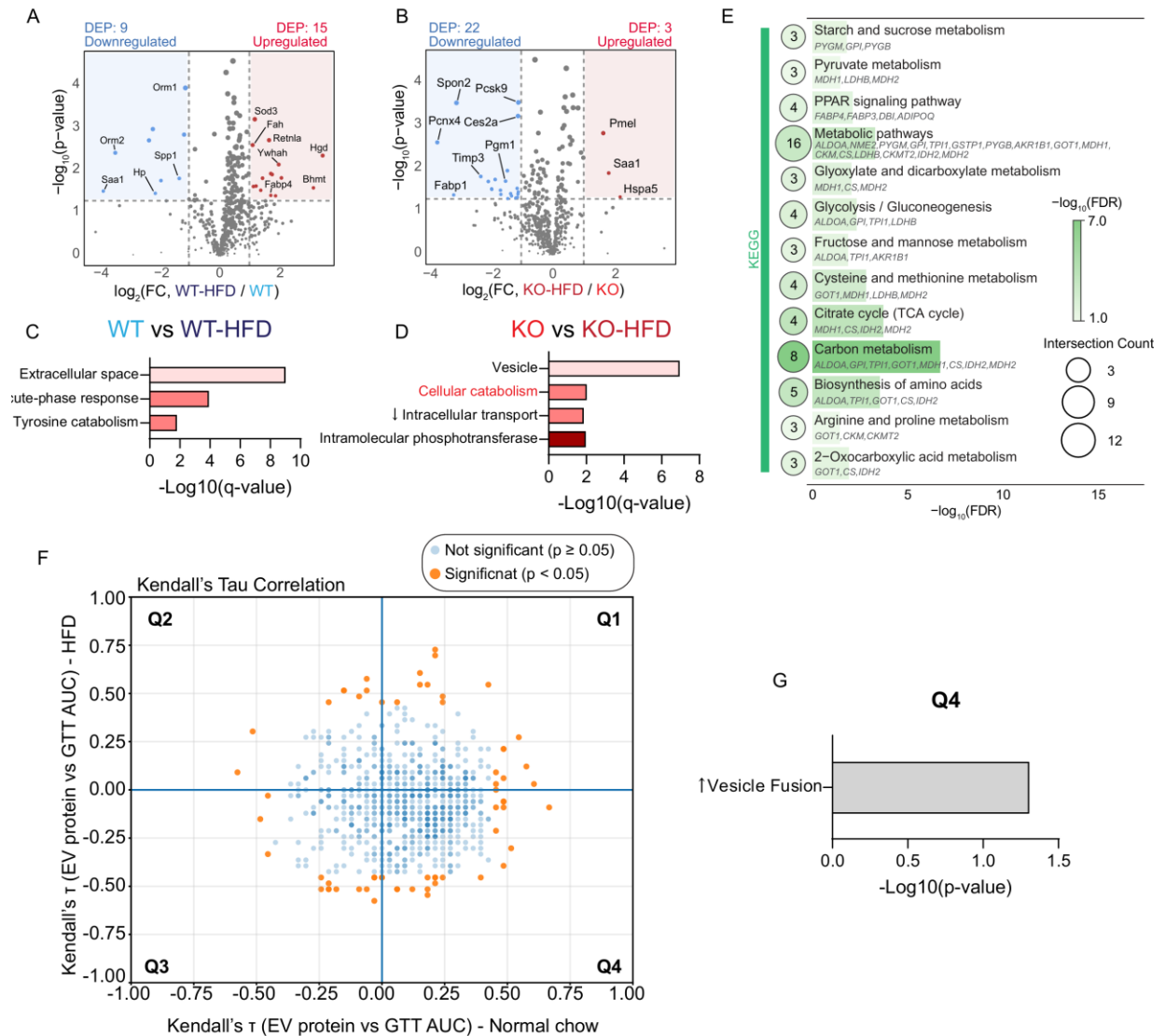
(A) Body weight (BW) gain over the dietary intervention period (n = 6–9 per group).

(B) Fasting blood glucose levels (mmol/L; n = 6–9 per group).

(C) Glucose tolerance test (GTT) curves.

(D) Normalized GTT area under the curve (AUC), calculated after subtraction of fasting glucose levels.

Data are presented as mean $\pm$ SD. Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, (one-way ANOVA); Symbol key: * vs WT; # vs WT-HFD



Supplementary Figure 2. Diet-dependent remodeling of EV proteomes and their association with glucose intolerance.

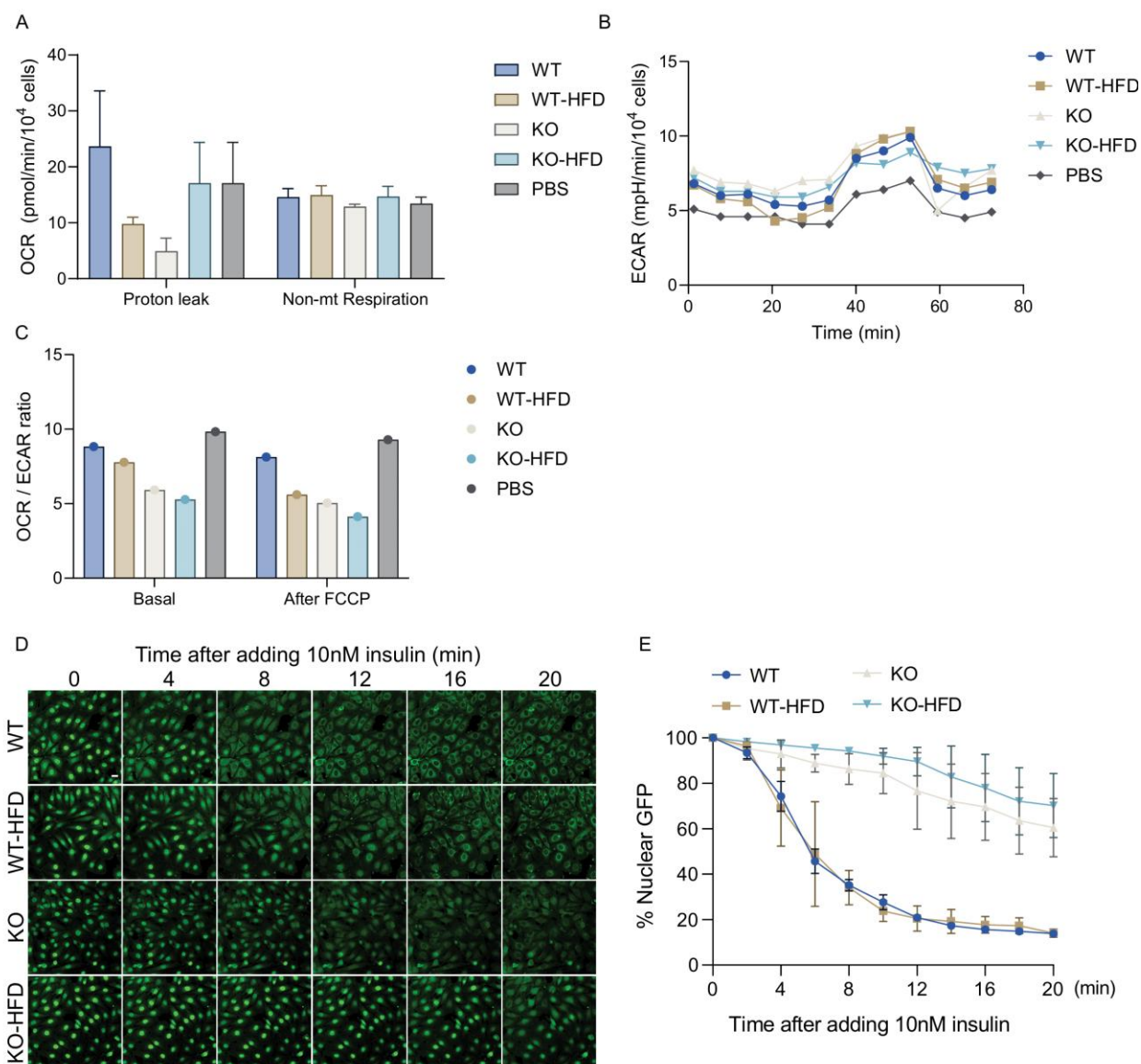
(A–B) Volcano plots showing differentially expressed EV proteins (DEPs) in WT vs WT-HFD (A) and KO vs KO-HFD (B) comparisons.

(C–D) Gene Ontology (GO) enrichment analysis of EV proteins altered by diet in WT (C) or KO (D) mice.

(E) KEGG pathway analysis comparing differentially expressed EV proteins between WT-HFD and KO-HFD groups.

(F) Quadrant-based Kendall's tau correlation analysis between EV protein abundance and GTT AUC, comparing normal chow and HFD conditions. Proteins are categorized into four quadrants (Q1–Q4) based on correlation direction and significance.

(G) GO Biological Process enrichment analysis of significantly correlated proteins within quadrant Q4 ($p < 0.05$).



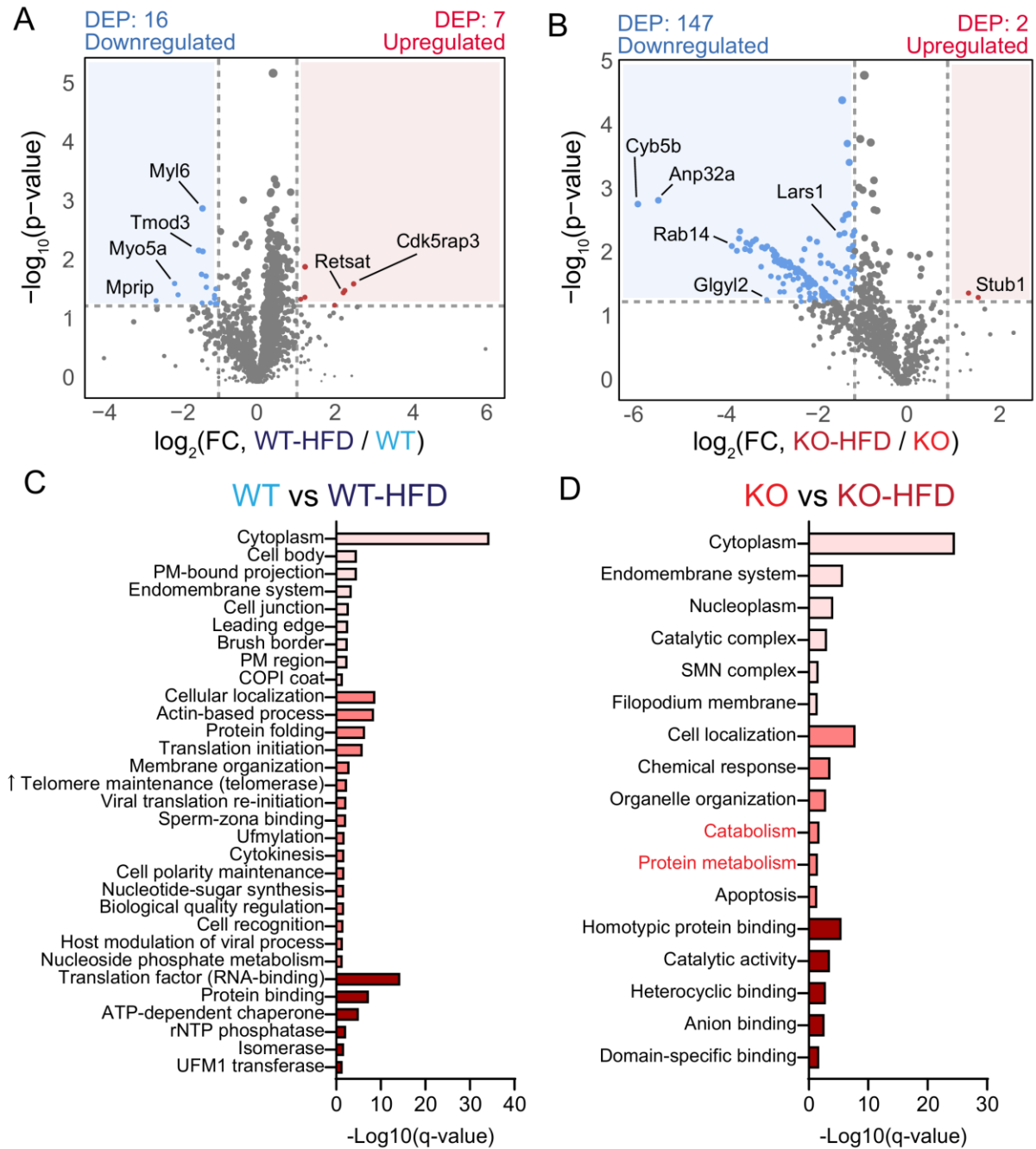
Supplementary Figure 3. Additional metabolic responses of L6 skeletal muscle cells to EV treatment.

(A) Proton leak and non-mitochondrial respiration (pmol/min/10⁴ cells; Seahorse assay).

(B) Extracellular acidification rate (ECAR; mpH/min/10⁴ cells) and (C) OCR/ECAR ratio in EV-treated cells.

(D–E) Representative images and quantification of nuclear GFP intensity over time in L6 Akt-biosensor cells (n = 3; scale bar = 20 μ m).

Data are presented as mean $\pm$ SEM for panels A and C, mean $\pm$ SD for E, and mean values only for B.



Supplementary Figure 4. EV-induced remodeling of recipient-cell proteomes under dietary stress.

(A–B) Volcano plots showing differentially expressed cellular proteins following treatment with WT vs WT-HFD EVs (A) and KO vs KO-HFD EVs (B).

(C–D) GO enrichment analysis of cellular proteomic changes induced by WT vs WT-HFD EVs (C) and KO vs KO-HFD EVs (D).

Appendix C: Copyright Permission

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Harnessing the therapeutic potential of exercise in extracellular vesicle-based therapy in metabolic disease associated cardiovascular complications

Author: Nimna Perera, Miles J. De Blasio, Mark A. Febbraio

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