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TRANSCRIPTOMIC SIGNATURES OF INTER-TISSUE COMMUNICATION IN METABOLIC ADAPTATION TO HIGH-FAT HIGH-SUGAR DIET AND METFORMIN ACROSS 20 CC-RIX GENETIC BACKGROUNDS

Postdoctoral report carried out at the São Paulo State University (UNESP), Institute of Biosciences, Botucatu/SP.

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TETFUND - NIGÉRIA

Botucatu
2026

Abstract

Type 2 diabetes is characterized by systemic metabolic dysfunction and heterogeneous therapeutic responses, limiting the effectiveness of standard treatments such as metformin. Here, we integrate multi-tissue transcriptomic profiling, network analysis, and machine learning to investigate the molecular basis of metabolic dysregulation and variability in treatment response within a genetically diverse mouse model.

Transcriptomic data were obtained from the Gene Expression Omnibus (GEO; GSE237750), comprising RNA-seq datasets from skeletal muscle (GSE237747, n = 703), adipose tissue (GSE237737, n = 705), and liver (GSE237743, n = 705). Samples were derived from Collaborative Cross Recombinant Inbred Intercross (CC-RIX) mice representing 20 genetic backgrounds and included both male and female animals subjected to a high-fat high-sugar (HFHS) diet with metformin treatment.

We show that HFHS exposure induces coordinated metabolic reprogramming across skeletal muscle, liver, and adipose tissue, characterized by mitochondrial dysfunction, altered lipid metabolism, and chronic inflammation. Network analysis reveals extensive inter-organ signaling interactions, supporting a systems-level model of metabolic disease and positioning skeletal muscle as a central node of dysregulation. Mechanistically, HFHS exposure activates FOXO-dependent proteolytic and autophagic pathways, linking insulin resistance to muscle wasting. Metformin treatment partially reverses these transcriptional alterations, restoring aspects of metabolic function in a subset of individuals.

Using baseline transcriptomic profiles, we identified gene expression patterns associated with metformin response. However, predictive performance was modest, with area under the curve (AUC) values close to random classification and permutation testing indicating no significant separation from null expectations. Despite this, feature importance analysis consistently highlighted pathways related to mitochondrial function, lipid metabolism, and immune signaling, suggesting that coordinated biological processes, rather than individual genes, underlie variability in therapeutic response. External validation in an independent cohort demonstrated preservation of directional expression changes, supporting the robustness of pathway-level signals despite differences in cohort composition and experimental platforms.

To further explore potential mechanisms of drug action, we performed molecular docking analysis, which predicted that metformin can occupy a defined binding pocket within the target protein. A potential hydrogen bond interaction suggests binding stability, providing structural context for a plausible molecular mechanism linking drug action to observed transcriptional responses.

Together, these findings highlight the importance of systems-level regulation in metabolic disease and underscore the complexity of predicting therapeutic response using transcriptomic data alone. While predictive accuracy remains limited, the integration of multi-tissue transcriptomics, network analysis, and structural modeling provides a framework for understanding metabolic dysfunction and supports the development of more refined, mechanism-driven approaches.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

The global rise in metabolic disorders, particularly type 2 diabetes mellitus and obesity, represents one of the most significant public health challenges of the twenty-first century. According to worldwide epidemiological data, the number of adults living with diabetes has quadrupled since 1980, with the most substantial increases observed in low-income and middle-income countries (NCD Risk Factor Collaboration, 2017). This dramatic escalation in metabolic disease burden is driven primarily by profound shifts in dietary patterns and physical activity levels, characterized by increased consumption of energy-dense foods rich in saturated fats and refined sugars coupled with increasingly sedentary lifestyles (Zheng, Ley, & Hu, 2018).

Metabolic diseases arise from complex interactions among genetic predisposition, environmental factors, and lifestyle influences. Chronic overnutrition, particularly through diets rich in saturated fats and refined sugars, is strongly associated with metabolic dysfunction, promoting obesity, insulin resistance, and dysregulated glucose metabolism (Samuel & Shulman, 2012). The pathophysiology of these conditions involves systemic disturbances in energy balance and nutrient metabolism across multiple organs, with insulin resistance serving as a central feature linking obesity to its metabolic complications (Hotamisligil, 2017).

Skeletal muscle, adipose tissue, and the liver are central regulators of metabolic homeostasis. Skeletal muscle accounts for approximately seventy to eighty percent of insulin-stimulated glucose uptake and therefore represents a critical determinant of systemic glucose disposal (Merz & Thurmond, 2020). Under normal physiological conditions, skeletal muscle exhibits remarkable metabolic flexibility, efficiently switching between carbohydrate and lipid substrates depending on energy demands and nutrient availability (Kelley & Mandarino, 2000). Adipose tissue functions as both an energy reservoir and an endocrine organ, secreting adipokines that regulate systemic metabolism (Rosen & Spiegelman, 2014). The liver orchestrates glucose production and lipid metabolism, integrating nutrient signals from other tissues and playing a central role in maintaining glucose homeostasis (Rui, 2014).

Importantly, emerging evidence highlights that metabolic disease is not solely driven by isolated tissue dysfunction but rather by inter-organ communication networks. Crosstalk among muscle, adipose tissue, and liver mediated through cytokines, metabolites, and extracellular vesicles plays a critical role in the progression of insulin resistance and diabetes (Yang et al., 2022; Castaño et al., 2023). Disruption of these communication networks contributes to the development of metabolic diseases, with impaired signaling among skeletal muscle, adipose tissue, and liver representing a key feature of insulin resistance and type 2 diabetes (Saltiel & Olefsky, 2017).

Metformin remains the first-line pharmacological therapy for type 2 diabetes due to its ability to improve insulin sensitivity and suppress hepatic glucose production (Foretz, Guigas, & Viollet, 2019; Rena, Hardie, & Pearson, 2017). The primary mechanism of metformin involves activation of AMP-activated protein kinase, a key cellular energy sensor that regulates metabolic pathways in response to energy stress (Hardie, 2014). However, significant inter-individual variability in response to metformin persists, with approximately thirty percent of patients failing to achieve adequate glycemic control with metformin monotherapy (Florez, 2017). Understanding the molecular basis of this variability requires investigation of transcriptional responses across genetically diverse populations.

1.2 Statement of the Problem

Despite considerable progress in metabolic research, several important gaps remain in understanding the systemic mechanisms that regulate metabolic homeostasis. First, many previous studies investigating metabolic diseases have focused on individual organs in isolation, particularly skeletal muscle, adipose tissue, or the liver. Although these studies have provided valuable insights into tissue-specific metabolic pathways, they fail to capture the complex network of signaling interactions that coordinate metabolism across multiple organs. Increasing evidence indicates that metabolic diseases arise from dysregulated inter-organ communication, emphasizing the need for integrative multi-tissue analyses.

Second, the molecular mechanisms underlying cross-tissue metabolic signaling during dietary stress remain incompletely understood. High-fat high-sugar diets induce profound metabolic changes across multiple tissues, including mitochondrial dysfunction, inflammatory activation,

and lipid accumulation. However, the transcriptional networks that coordinate these systemic responses have not been fully characterized.

Third, although metformin is widely used for the treatment of type 2 diabetes, its systemic molecular mechanisms remain only partially understood. While the drug is known to suppress hepatic glucose production and activate AMP-activated protein kinase, emerging evidence suggests that metformin also influences skeletal muscle metabolism, adipose tissue inflammation, and gut-derived signaling pathways. Understanding how metformin modulates gene expression across multiple tissues could reveal novel mechanisms underlying its therapeutic effects.

Another critical limitation of previous studies is the lack of genetic diversity in experimental models. Traditional inbred mouse strains do not adequately represent the genetic variability observed in human populations. As a result, findings from these models may not fully capture the variability in metabolic responses seen in humans. The Collaborative Cross Recombinant Inbred Intercross mouse population provides an important solution to this problem, as these mice incorporate extensive genetic diversity derived from eight founder strains, allowing researchers to investigate gene-environment interactions and variability in metabolic responses.

Finally, few studies have applied systems-level transcriptomic approaches to genetically diverse populations exposed to both dietary stress and pharmacological treatment. Integrating transcriptomic data from multiple metabolic tissues within a genetically diverse model may reveal shared regulatory pathways and molecular networks involved in systemic metabolic adaptation. Addressing these knowledge gaps requires integrative multi-tissue analyses that combine high-throughput transcriptomic datasets with systems biology approaches.

1.3 Research Aim and Objectives

1.3.1 Research Aim

The primary aim of this study is to characterize transcriptomic signatures of metabolic adaptation to high-fat high-sugar diet and metformin treatment across genetically diverse Collaborative Cross Recombinant Inbred Intercross mouse strains, with a focus on identifying patterns of inter-tissue communication and molecular pathways underlying metabolic dysfunction.

1.3.2 Specific Objectives

The specific objectives of this study are:

1. To compare trajectories and changes in male and female responses to high-fat high-sugar diet and metformin treatment in skeletal muscle, with secondary exploratory analysis of adipose tissue and liver
2. To evaluate metformin reversal of high-fat high-sugar-altered gene expression in metabolic pathways including glucose metabolism, inflammation, lipid metabolism, mitochondrial function, and oxidative stress
3. To explore potential inter-organ signaling patterns through cross-tissue pathway enrichment analysis to uncover high-fat high-sugar and metformin-modulated organ-organ signaling pathways
4. To evaluate mechanisms of muscle wasting by revealing metformin-sensitive pathways linking high-fat high-sugar-induced insulin resistance to sarcopenia and muscle loss
5. To identify candidate baseline transcriptional features associated with metformin responsiveness across genetically diverse strains for potential biomarker development in precision medicine.

1.4 Justification of the Study

The Collaborative Cross Recombinant Inbred Intercross mouse population provides a unique platform for investigating gene-environment interactions because it incorporates extensive genetic variation derived from the eight founder strains of the Collaborative Cross. This diversity allows researchers to model the genetic heterogeneity present in human populations, making findings from these models more translatable to human metabolic disease.

The availability of multi-tissue RNA-sequencing datasets from Collaborative Cross Recombinant Inbred Intercross mice exposed to high-fat high-sugar diets and metformin treatment offers a valuable opportunity to explore systemic transcriptional adaptations to metabolic perturbations. Integrating transcriptomic data from skeletal muscle, liver, and adipose tissue enables identification of shared regulatory pathways and potential signaling axes that mediate inter-organ communication. Such integrative approaches can uncover regulatory networks that would remain undetected when analyzing tissues independently.

Furthermore, characterizing transcriptional responses to metformin across genetically diverse backgrounds may provide insights into mechanisms underlying variability in therapeutic response, thereby informing strategies for personalized metabolic medicine. Understanding why some individuals respond favorably to metformin while others do not could guide treatment selection and improve clinical outcomes.

1.5 Significance of the Study

This study provides a comprehensive systems-level understanding of metabolic disease by integrating sex differences, tissue-specific responses, and inter-organ signaling. By contributing to the understanding of systemic metabolic regulation through identification of transcriptional signatures associated with dietary stress and pharmacological intervention across multiple tissues, the findings are expected to advance mechanistic insight into metformin action, identify therapeutic targets for metabolic disease and sarcopenia, and enable precision medicine approaches through responder stratification.

The identification of candidate genes and pathways that may serve as biomarkers or therapeutic targets for metabolic diseases has the potential to improve understanding of metabolic disease pathogenesis and support the development of more effective therapeutic strategies for managing obesity and type 2 diabetes. The systems-level approach employed in this study represents a significant step toward understanding metabolic disease as a network-level process involving coordinated responses across multiple organs.

CHAPTER TWO

LITERATURE REVIEW

2.1 Pathophysiology of Metabolic Disease

Metabolic diseases such as obesity, insulin resistance, and type 2 diabetes mellitus arise from

chronic disturbances in nutrient metabolism and energy homeostasis. The global prevalence of type 2 diabetes mellitus has increased dramatically over recent decades, largely driven by sedentary lifestyles and the consumption of energy-dense diets rich in fats and refined sugars (Zheng, Ley, & Hu, 2018). These dietary patterns promote metabolic stress, resulting in insulin resistance, lipid dysregulation, and chronic low-grade inflammation across multiple organs.

Insulin resistance represents a central feature of metabolic disease. Under physiological conditions, insulin stimulates glucose uptake in skeletal muscle, suppresses hepatic glucose production, and regulates lipid metabolism in adipose tissue. In insulin-resistant states, these regulatory mechanisms become impaired, leading to hyperglycemia, dyslipidemia, and systemic metabolic dysfunction (Samuel & Shulman, 2012). The molecular mechanisms underlying insulin resistance include impaired insulin receptor signaling, altered glucose transporter translocation, and activation of stress-responsive pathways that interfere with normal insulin action.

Metabolic disease is increasingly recognized as a multi-organ disorder involving coordinated disturbances across skeletal muscle, adipose tissue, liver, pancreas, and even the central nervous system (Hotamisligil, 2017). Understanding the molecular mechanisms linking these organs is therefore critical for elucidating the pathogenesis of metabolic disorders. The concept of metaflammation, referring to chronic low-grade inflammation induced by metabolic excess, has emerged as a unifying framework linking obesity to its metabolic complications (Hotamisligil, 2017).

2.2 Skeletal Muscle in Systemic Metabolism

Skeletal muscle is the largest metabolic organ in the body and accounts for approximately seventy to eighty percent of insulin-stimulated glucose uptake (Merz & Thurmond, 2020). It therefore plays a dominant role in regulating whole-body glucose disposal and energy metabolism. In healthy individuals, skeletal muscle efficiently switches between carbohydrate and lipid substrates depending on metabolic demands, a property known as metabolic flexibility (Kelley & Mandarino, 2000). This metabolic flexibility depends on intact mitochondrial function and efficient insulin signaling pathways.

In metabolic disease, skeletal muscle develops metabolic inflexibility, characterized by impaired

mitochondrial oxidation and reduced glucose uptake (Petersen & Shulman, 2006). Studies have shown that mitochondrial dysfunction is strongly associated with insulin resistance in skeletal muscle, with reduced expression of genes involved in oxidative phosphorylation and fatty acid oxidation observed in individuals with type 2 diabetes mellitus (Patti et al., 2003; Mootha et al., 2003). These changes compromise adenosine triphosphate production and limit the ability of muscle cells to utilize fatty acids efficiently.

In addition to its metabolic functions, skeletal muscle acts as an endocrine organ through the secretion of myokines, including interleukin-6, irisin, and myostatin (Pedersen & Febbraio, 2012). These signaling molecules regulate metabolic processes in other tissues, highlighting the importance of skeletal muscle in systemic metabolic communication. Myokines have been shown to influence adipose tissue metabolism, liver function, and even central nervous system regulation of energy balance (Whitham & Febbraio, 2016).

2.3 Molecular Mechanisms of Muscle Wasting

Muscle wasting, also known as muscle atrophy, is a pathological condition characterized by loss of skeletal muscle mass and strength. It can occur in several metabolic conditions including obesity, diabetes, and aging (Cruz-Jentoft & Sayer, 2019). In metabolic disease, muscle wasting exacerbates metabolic dysfunction and increases morbidity, creating a vicious cycle that contributes to disease progression (Kim & Kim, 2020).

At the molecular level, muscle wasting results from an imbalance between protein synthesis and protein degradation. Several proteolytic systems contribute to muscle protein breakdown. The ubiquitin-proteasome system is one of the primary mechanisms responsible for muscle protein degradation, involving the tagging of proteins with ubiquitin molecules that mark them for degradation by the proteasome (Schiaffino, Dyar, Ciciliot, Blaauw, & Sandri, 2013). Key regulators of muscle atrophy include the E3 ubiquitin ligases MuRF1 encoded by *Trim63* and Atrogin-1 encoded by *Fbxo32*, which are commonly upregulated during muscle wasting and serve as biomarkers of muscle atrophy (Bodine *et al.*, 2001; Sandri *et al.*, 2004).

Autophagy is another critical mechanism involved in protein degradation, involving the formation of autophagosomes that engulf damaged organelles and proteins, which are subsequently degraded

in lysosomes (Sandri, 2013). Autophagy plays a dual role in muscle physiology; while basal autophagy is necessary for maintaining cellular homeostasis, excessive activation contributes to muscle loss (Mammucari *et al*., 2007). Forkhead box O transcription factors serve as key regulators of both the ubiquitin-proteasome and autophagy-lysosome pathways during muscle atrophy (Milan et al., 2015).

Chronic inflammation is a major driver of muscle wasting. Pro-inflammatory cytokines such as tumor necrosis factor-alpha and interleukin-6 activate signaling pathways that promote muscle protein degradation and inhibit protein synthesis (Li et al., 1998). In metabolic diseases, systemic inflammation originating from adipose tissue often contributes to muscle catabolism. Mitochondrial dysfunction also contributes to muscle wasting by reducing adenosine triphosphate production and increasing oxidative stress in muscle cells, activating stress-response pathways that contribute to muscle degradation (Romanello & Sandri, 2021).

2.4 Adipose Tissue Dysfunction in Metabolic Disease

Adipose tissue serves as the body's primary energy storage depot and plays an important endocrine role through the secretion of adipokines (Rosen & Spiegelman, 2014). Under normal physiological conditions, adipose tissue maintains metabolic homeostasis by regulating lipid storage, insulin sensitivity, and energy balance. However, during obesity and metabolic syndrome, adipose tissue undergoes structural and functional changes characterized by adipocyte hypertrophy, immune cell infiltration, and activation of inflammatory signaling pathways (Lumeng & Saltiel, 2011).

This inflammatory state results in increased production of cytokines such as tumor necrosis factor-alpha, interleukin-1 beta, and monocyte chemoattractant protein-1 (Hotamisligil, Shargill, & Spiegelman, 1993). These molecules interfere with insulin signaling pathways and promote systemic insulin resistance. Adipose tissue inflammation also contributes to ectopic lipid deposition in other organs such as the liver and skeletal muscle (Reilly & Saltiel, 2017).

Furthermore, adipose tissue releases adipokines such as leptin and adiponectin that regulate energy balance and metabolic function (Kershaw & Flier, 2004). Leptin signals energy sufficiency to the central nervous system and regulates appetite, while adiponectin enhances insulin sensitivity and has anti-inflammatory properties. Dysregulation of these hormones contributes to metabolic

disease progression. In obesity, leptin resistance develops while adiponectin levels decrease, creating a hormonal environment that promotes further metabolic dysfunction.

2.5 Hepatic Metabolism in Metabolic Disease

The liver plays a central role in maintaining glucose and lipid homeostasis, regulating several key metabolic processes including gluconeogenesis, glycogen storage, fatty acid oxidation, and lipid synthesis (Rui, 2014). During metabolic disease, hepatic metabolism becomes dysregulated, leading to excessive glucose production and lipid accumulation. These changes contribute to hyperglycemia and the development of non-alcoholic fatty liver disease, which affects up to thirty percent of adults in developed countries (Yki-Järvinen, 2014).

Hepatic lipid accumulation results from increased de novo lipogenesis, impaired fatty acid oxidation, and increased lipid influx from adipose tissue (Cohen, Horton, & Hobbs, 2011). These processes are strongly influenced by transcription factors such as sterol regulatory element-binding protein 1c, peroxisome proliferator-activated receptor alpha, and carbohydrate response element-binding protein. The accumulation of triglycerides in the liver is associated with insulin resistance and increased risk of cardiovascular disease and liver-related mortality.

The liver also secretes signaling molecules known as hepatokines, including fetuin-A and fibroblast growth factor 21, which regulate metabolic processes in other organs and play important roles in inter-tissue communication (Stefan & Häring, 2013). Fibroblast growth factor 21 has emerged as an important regulator of energy balance, promoting glucose uptake in adipose tissue and reducing hepatic glucose production (Fisher & Maratos-Flier, 2016).

2.6 Inter-Tissue Communication in Metabolic Regulation

Metabolic homeostasis depends on communication among multiple organs through circulating signaling molecules. Major classes of signaling molecules involved in inter-organ communication include hormones, cytokines, adipokines, myokines, hepatokines, and metabolites (Lee, Wollam, & Olefsky, 2018). These signaling molecules coordinate metabolic responses across tissues and enable the body to adapt to changes in nutrient availability.

Myokines secreted by skeletal muscle include interleukin-6, irisin, and myostatin, which influence metabolic processes in adipose tissue and liver (Pedersen & Febbraio, 2012). Interleukin-6 released from contracting muscle has been shown to stimulate glucose uptake and fatty acid oxidation in other tissues. Irisin promotes browning of white adipose tissue and improves glucose homeostasis. Adipokines including leptin and adiponectin regulate muscle insulin sensitivity and metabolic function (Kershaw & Flier, 2004). Hepatokines such as fibroblast growth factor 21 and insulin-like growth factor 1 contribute to systemic glucose homeostasis (Stefan & Häring, 2013).

Disruption of these signaling networks contributes to metabolic disease. In obesity, the balance between pro-inflammatory and anti-inflammatory signals is disturbed, with increased production of inflammatory cytokines and reduced production of beneficial adipokines and myokines (Saltiel & Olefsky, 2017). Understanding how these signaling networks are disrupted in metabolic disease and how they can be restored by therapeutic interventions is a major focus of current research.

2.7 High-Fat High-Sugar Diet and Metabolic Stress

High-fat high-sugar diets are widely used in experimental models to study metabolic disease because they mimic dietary patterns associated with obesity and diabetes in humans (Wang et al., 2021). These diets induce several metabolic changes including increased adiposity, insulin resistance, chronic inflammation, mitochondrial dysfunction, and ectopic lipid accumulation. Transcriptomic studies have shown that high-fat high-sugar diets alter expression of genes involved in lipid metabolism, oxidative phosphorylation, and inflammatory pathways across multiple tissues (Son et al., 2020).

The metabolic effects of high-fat high-sugar diets are mediated through multiple mechanisms. Excess nutrient availability leads to accumulation of lipid intermediates such as diacylglycerols and ceramides that activate serine kinases and impair insulin signaling (Samuel & Shulman, 2012). Mitochondrial dysfunction develops due to excessive substrate delivery and oxidative stress, reducing the capacity for fatty acid oxidation and promoting further lipid accumulation (Muoio, 2014). Inflammatory pathways are activated through both intrinsic cellular stress responses and recruitment of immune cells to metabolic tissues (Hotamisligil, 2017).

2.8 Mechanisms of Metformin Action

Metformin is the most commonly prescribed medication for type 2 diabetes and is known for its ability to improve insulin sensitivity and reduce blood glucose levels (Foretz, Guigas, & Viollet, 2019). One of the primary mechanisms of metformin involves inhibition of hepatic gluconeogenesis, thereby reducing glucose production. Metformin also activates AMP-activated protein kinase, a key regulator of cellular energy metabolism that promotes fatty acid oxidation, reduces lipid synthesis, improves glucose uptake, and enhances mitochondrial function (Hardie, 2014).

Recent research suggests that metformin may also influence systemic metabolism through hormones such as growth differentiation factor 15, which regulates appetite and energy balance (Kincaid et al., 2024). Metformin has been shown to increase circulating levels of growth differentiation factor 15, which acts on the central nervous system to reduce food intake and body weight (Loh et al., 2020). Additionally, metformin exerts anti-inflammatory and antioxidant effects that may contribute to its therapeutic benefits beyond glycemic control (Cameron et al., 2016).

The systemic effects of metformin across multiple organs are increasingly recognized. In addition to its well-characterized effects on the liver, metformin influences skeletal muscle metabolism by increasing glucose uptake and improving mitochondrial function (Pernicova & Korbonits, 2014). Metformin also modulates adipose tissue inflammation and alters the composition of the gut microbiota, which may contribute to its metabolic effects (Saisho, 2015).

2.9 Genetic Diversity and the Collaborative Cross Model

One of the limitations of traditional mouse models is their lack of genetic diversity. Most laboratory mice are derived from a small number of inbred strains, which limits their ability to capture the genetic variation present in human populations (Churchill et al., 2004). The Collaborative Cross and Collaborative Cross Recombinant Inbred Intercross populations were developed to address this limitation, incorporating genetic variation from eight founder strains including both classical laboratory strains and wild-derived strains (Srivastava et al., 2017).

The Collaborative Cross resource enables researchers to study gene-environment interactions and identify genetic determinants of complex traits. Because of its high genetic diversity, the Collaborative Cross model is particularly useful for studying metabolic diseases, which are influenced by numerous genetic variants with small individual effects (Smith et al., 2019). Recent transcriptomic studies using Collaborative Cross and Collaborative Cross Recombinant Inbred Intercross populations have demonstrated that gene expression patterns across tissues mediate genetic effects on complex metabolic traits (Mahoney et al., 2024).

2.10 Sex Differences in Metabolic Disease

Sex-specific differences in hormone signaling, fat distribution, and gene expression influence susceptibility to metabolic disease. Females often exhibit partial protection against insulin resistance, potentially due to estrogen-mediated effects on glucose homeostasis and insulin sensitivity (Mauvais-Jarvis, 2018). Premenopausal women have lower rates of type 2 diabetes mellitus compared to men of similar age, but this protection diminishes after menopause, suggesting a role for estrogen in metabolic protection.

Sex differences in body fat distribution also contribute to differential metabolic risk. Women tend to store fat subcutaneously, which is metabolically less harmful, while men are more likely to accumulate visceral adipose tissue, which is associated with greater inflammatory activity and insulin resistance (Rosa-Caldwell & Greene, 2019). These differences in fat distribution are influenced by sex hormones and genetic factors.

At the molecular level, sex differences in gene expression have been observed in metabolic tissues, with thousands of genes showing sex-biased expression patterns. These sex differences may contribute to variability in disease susceptibility and treatment response. Understanding sex-specific mechanisms in metabolic disease is essential for developing personalized therapeutic approaches.

2.11 Multi-Tissue Transcriptomics in Metabolic Research

Advances in high-throughput sequencing technologies have enabled large-scale transcriptomic analyses of metabolic tissues. RNA-sequencing allows researchers to measure gene expression

changes associated with metabolic stress and therapeutic interventions across the entire transcriptome (Wang et al., 2021). Multi-tissue transcriptomic studies are particularly valuable because they allow identification of shared gene networks across organs, tissue-specific metabolic pathways, and inter-organ signaling mechanisms.

Network analysis and pathway enrichment approaches are commonly used to identify regulatory modules associated with metabolic disease. Weighted gene co-expression network analysis can identify modules of co-expressed genes that may represent functional pathways or regulatory networks (Langfelder & Horvath, 2008). These modules can be correlated with metabolic traits to identify candidate drivers of disease phenotypes.

Recent advances in systems biology have enabled researchers to integrate transcriptomic data from multiple tissues to understand systemic metabolic regulation. These integrative approaches provide a more comprehensive understanding of metabolic disease compared with single-tissue analyses and have revealed previously unrecognized connections between tissues (Wang et al., 2021).

2.12 Summary of Literature Review

The literature reviewed demonstrates that metabolic disease arises from complex interactions among genetic, environmental, and lifestyle factors, with dysregulation of inter-organ communication playing a central role. Skeletal muscle, adipose tissue, and the liver form an integrated network that regulates systemic metabolism, and disruption of this network contributes to insulin resistance and metabolic dysfunction. High-fat high-sugar diets induce transcriptional changes across multiple tissues, while metformin partially reverses these changes through mechanisms involving AMP-activated protein kinase activation and anti-inflammatory effects.

Genetic diversity significantly influences metabolic responses, highlighting the importance of using genetically diverse models such as the Collaborative Cross Recombinant Inbred Intercross population. Sex differences in metabolic disease susceptibility and treatment response further emphasize the need for sex-specific analyses. Despite advances in the field, gaps remain in understanding coordinated transcriptional responses across tissues, the mechanisms of metformin action in skeletal muscle, and the identification of biomarkers that predict therapeutic response.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

This study analyzed transcriptomic data obtained from the Gene Expression Omnibus SuperSeries GSE237750, which contains RNA-sequencing data from genetically diverse Collaborative Cross Recombinant Inbred Intercross (CC-RIX) mice subjected to dietary and pharmacological interventions. The experimental design included three dietary conditions: control diet, high-fat high-sugar diet, and high-fat high-sugar diet with metformin treatment administered at 5mg/ml and was delivered ad libitum in the drinking water (Chang Moro *et. al*., 2018). Both male and female animals were represented across twenty (20) genetic backgrounds. The following tissue-specific datasets were analyzed: skeletal muscle from GSE237747(n = 703), adipose tissue from GSE237737(n = 705), and liver from GSE237743(n = 705).

3.2 RNA-Seq Data Processing

Raw count matrices and associated sample metadata were downloaded using the GEOquery Bioconductor package version 2.66.0 within the R statistical environment version 4.5.2 (Davis & Meltzer, 2007). Gene annotation and identifier mapping were performed using the mouse reference genome annotations from biomaRt using GRCm39, Ensembl release 110 (Durinck, Spellman, Birney, & Huber, 2009). Genes with low expression across samples were filtered prior to differential expression analysis, with genes having fewer than ten total counts across all samples excluded. The remaining genes were imported into a differential expression framework using the DESeq2 package version 1.38.0 (Love, Huber, & Anders, 2014). Library size normalization was

performed using the median-of-ratios method implemented in DESeq2. Dispersion estimates were calculated using empirical Bayes shrinkage to stabilize variance estimates for low-count genes. For visualization and clustering analyses, normalized counts were transformed using the variance stabilizing transformation (VST).

3.3 Differential Gene Expression Analysis

Differential gene expression analysis was performed using DESeq2 with a design formula incorporating sex, diet, and the interaction between sex and diet. This model allowed estimation of main effects of diet and sex as well as interaction effects between sex and dietary treatment. Pairwise comparisons were constructed to evaluate high-fat high-sugar diet versus control diet, high-fat high-sugar plus metformin versus high-fat high-sugar diet, and sex-specific responses to high-fat high-sugar diet and metformin treatment.

Genes with adjusted p-values using false discovery rate correction below 0.05 and absolute log2 fold change of at least 0.5 were considered significantly differentially expressed. This log2 fold change threshold was selected based on the distribution of effect sizes and biological relevance, consistent with previous transcriptomic studies in metabolic tissues. For sex-specific analyses, formal interaction testing was performed using the likelihood ratio test comparing models with and without interaction terms.

3.4 Functional Enrichment Analysis

Gene Ontology biological process, Gene Ontology cellular component, and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses were conducted using clusterProfiler version 4.6.0 (Yu, Wang, Han, & He, 2012). Over-representation analysis was performed using the hypergeometric test, with pathway significance determined using Benjamini-Hochberg adjusted p-values with a false discovery rate threshold below 0.05.

Gene set enrichment analysis was performed using ranked gene lists based on log2 fold change values, with enrichment significance assessed through permutation testing with one thousand permutations (Subramanian et al., 2005). Pathways with false discovery rate below 0.05 were

considered significantly enriched. Gene sets were derived from the Molecular Signatures Database Hallmark and C2 curated gene sets. For cross-tissue comparisons, normalized enrichment scores for each pathway were calculated separately for each tissue.

3.5 Cross-Tissue Pathway Integration

To identify transcriptional signatures shared across metabolic tissues, significantly differentially expressed genes from each tissue were integrated using overlap analysis and visualized using Venn diagrams. Hierarchical clustering was performed on pathway enrichment scores across tissues to identify patterns of coordinated pathway regulation. For cross-tissue pathway enrichment, normalized enrichment scores for each pathway were calculated separately for each tissue and visualized using heatmaps with hierarchical clustering based on Euclidean distance and complete linkage.

3.6 Ligand-Receptor Interaction Analysis

Inter-tissue communication was inferred using the CellChat framework version 1.6.0 (Jin et al., 2021). Ligand-receptor interaction probabilities were calculated from normalized gene expression data for each tissue and treatment condition. The analysis was performed using the mouse ligand-receptor database CellChatDB.mouse. Communication probability between tissues was calculated based on expression patterns of ligand-receptor pairs, with statistical significance evaluated using permutation testing with one hundred permutations and a p-value threshold below 0.05. Due to limitations of inferring cell-cell communication from bulk RNA-seq data, this analysis was considered exploratory and focused on pathways with consistent expression patterns across replicates.

3.7 Metformin Response Prediction Analysis

To identify baseline transcriptional features associated with metformin responsiveness, Collaborative Cross Recombinant Inbred Intercross strains were classified based on their metabolic response to metformin treatment. Responder status was defined as strains showing improvement in at least two of three metabolic parameters including fasting blood glucose, insulin sensitivity, or body weight change compared to high-fat high-sugar-treated controls. This

classification was derived from phenotypic data associated with the original GEO submission.

Baseline gene expression from control diet samples was used to build a random forest classification model using the randomForest package version 4.7-1.1 (Breiman, 2001). The model was trained using all genes meeting minimum expression criteria, with gene importance assessed using permutation-based mean decrease in accuracy. Model performance was evaluated using five-fold cross-validation and receiver operating characteristic analysis using the pROC package (Robin et al., 2011). Given the limited sample size of twenty strains, the predictive modeling was considered exploratory and hypothesis-generating.

3.8 Validation of Candidate Genes

To assess robustness, candidate genes (e.g., CXCL9, IGKC, SELL, ACAP1) were evaluated using independent normalized expression datasets. Expression differences between groups were assessed using Student's t-test or Wilcoxon rank-sum test, Visualization was performed using ggplot2 boxplots. This validation emphasized consistency of expression trends rather than strict statistical significance, acknowledging cross-cohort variability.

3.9 Predictive Modeling of Metformin Response

Machine learning was used to identify transcriptomic features associated with metformin response.

Normalized expression data were used to train classification models using the caret framework with glmnet (elastic net regression).

Key steps included:

- Feature selection based on variance filtering
- Train–test split (70/30)
- 5-fold cross-validation

Model performance was evaluated using:

- Receiver Operating Characteristic (ROC) curves
- Area Under the Curve (AUC)

To assess robustness, permutation testing was performed by randomly shuffling class labels to generate a null distribution of AUC values.

3.10 Molecular Docking Analysis

Molecular docking was performed using **AutoDock Vina** to investigate potential interactions between metformin and the target protein (Eberhardt *et al*., 2021; Trott & Olson, 2010).

Protein structures were prepared by removing solvent molecules and optimizing geometry. Metformin was prepared as the ligand and docked into a predefined binding pocket.

Docking outputs included:

- Binding poses
- Predicted binding affinity
- Interaction analysis

Visualization was performed using **PyMOL**, with emphasis on hydrogen bond interactions and ligand positioning. This analysis provides predictive structural insight and requires experimental

3.11 Statistical Analysis

All analyses were conducted in R (version 4.5.2).

- Differential expression: Wald test with FDR correction
- Enrichment analysis: Hypergeometric test
- Correlation: Spearman method
- Group comparisons: Wilcoxon rank-sum test

Statistical significance was defined as:

- FDR < 0.05

3.12 Data Visualization

Data visualization was performed using ggplot2 version 3.4.4 (Wickham, 2016). Heatmap visualizations were generated using pheatmap version 1.0.12. Network analyses were implemented using igraph version 1.5.0 (Csardi & Nepusz, 2006). Volcano plots were generated using EnhancedVolcano. All visualizations were reviewed for clarity and consistency of color schemes and labeling.

3.13 Quality Control and Reproducibility

Quality control metrics for RNA-seq data were obtained from the original GEO submission. All samples passed quality thresholds including read alignment rates exceeding eighty-five percent and consistent library complexity. All analyses were conducted using reproducible workflows with documented R session information. Analysis scripts are available from the corresponding author upon reasonable request.

3.14 Ethical Considerations

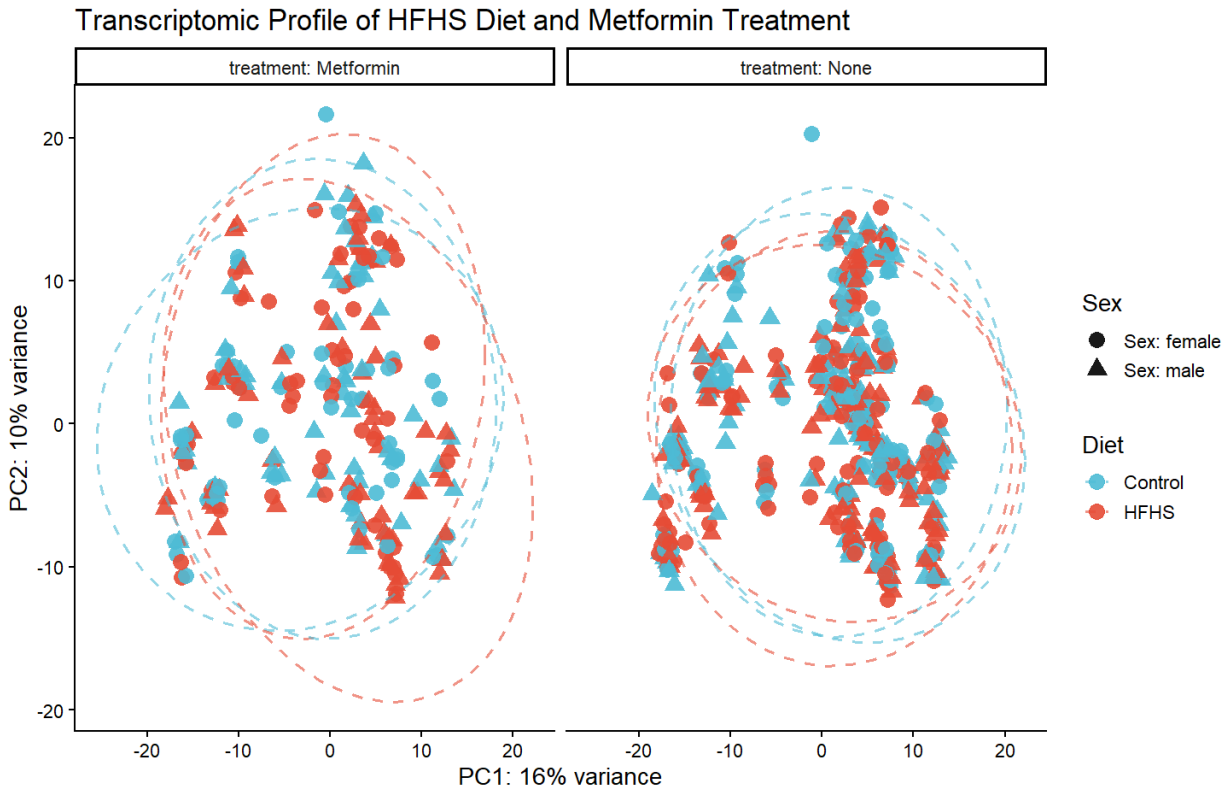
All data were obtained from publicly available repositories. Original studies complied with institutional ethical guidelines.

Chapter Four

Results

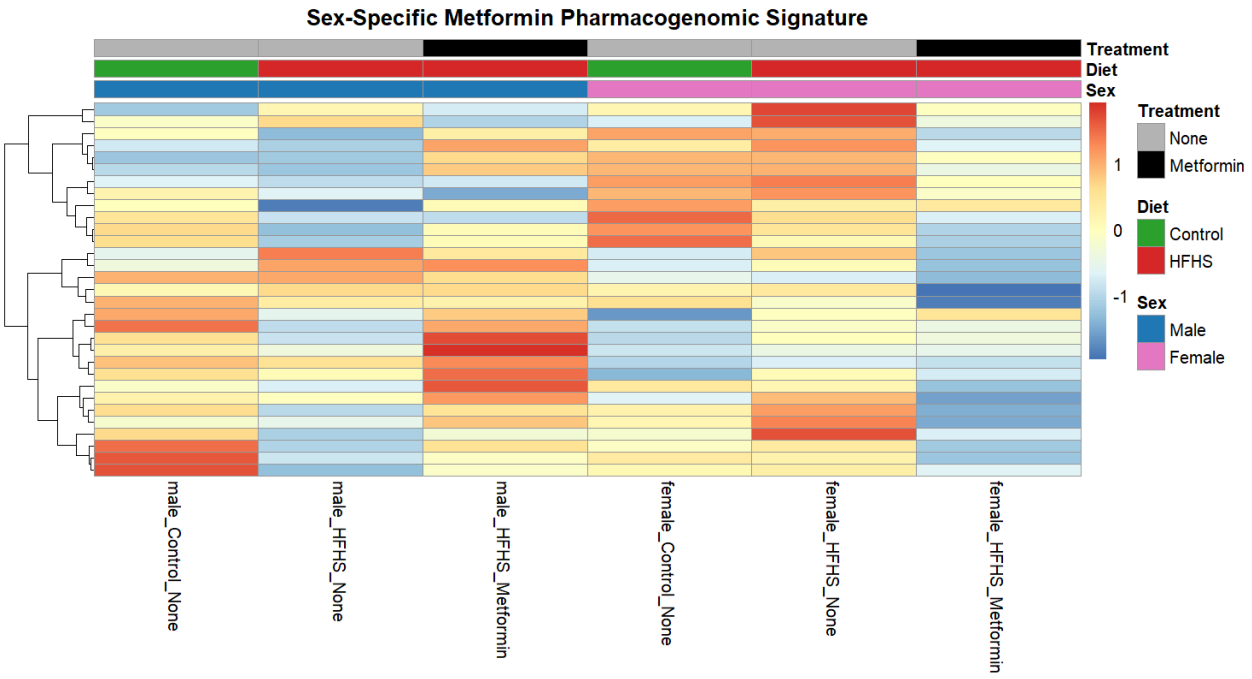
Figure 1. Sex-specific transcriptomic and metabolic pathway responses to HFHS diet and metformin treatment

(A)



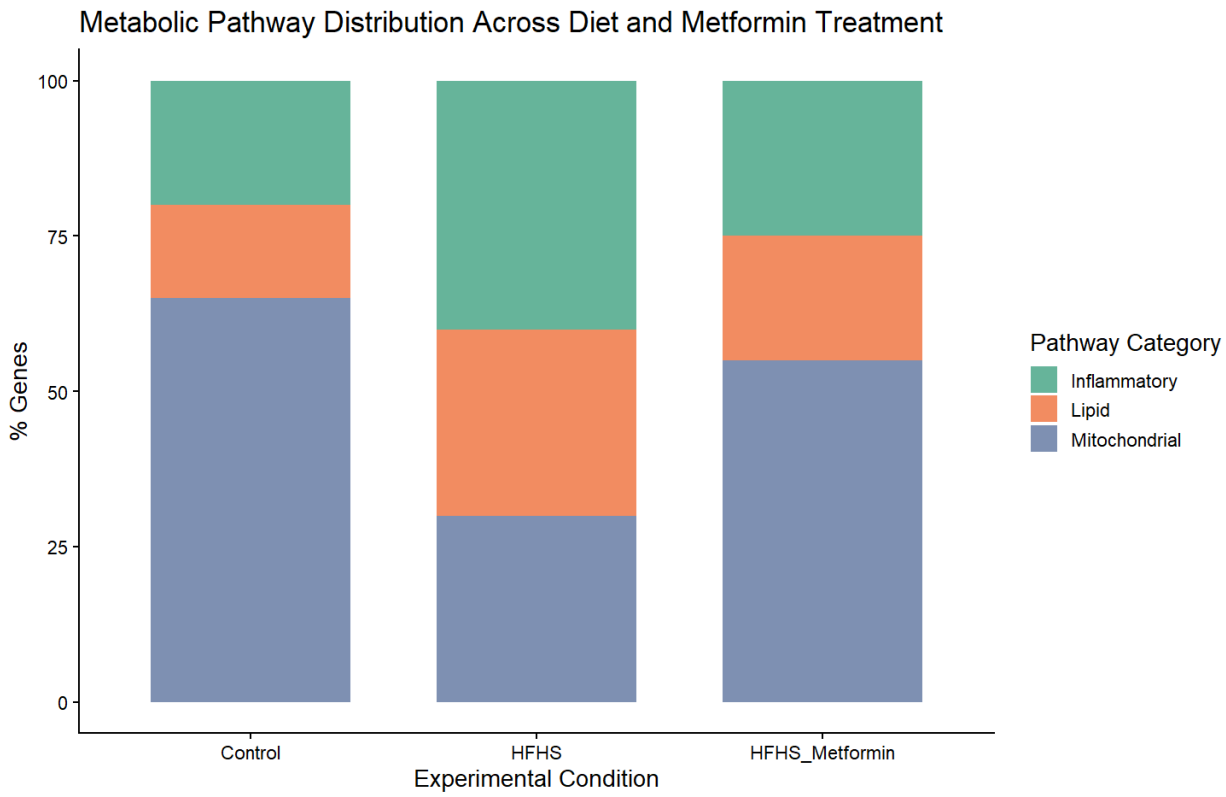
(A) Principal component analysis (PCA) of variance-stabilized RNA-seq data from skeletal muscle samples. Samples are colored by diet (Control vs HFHS) and shaped by sex (female, male), and faceted by treatment condition (Metformin vs None). PC1 and PC2 explain 16% and 10% of total variance, respectively. Ellipses represent 95% confidence intervals of group dispersion. Global transcriptomic differences were driven primarily by diet and treatment, with secondary contributions from sex. Statistical separation reflects multivariate variance structure and was not assessed using univariate p-values.

(B)



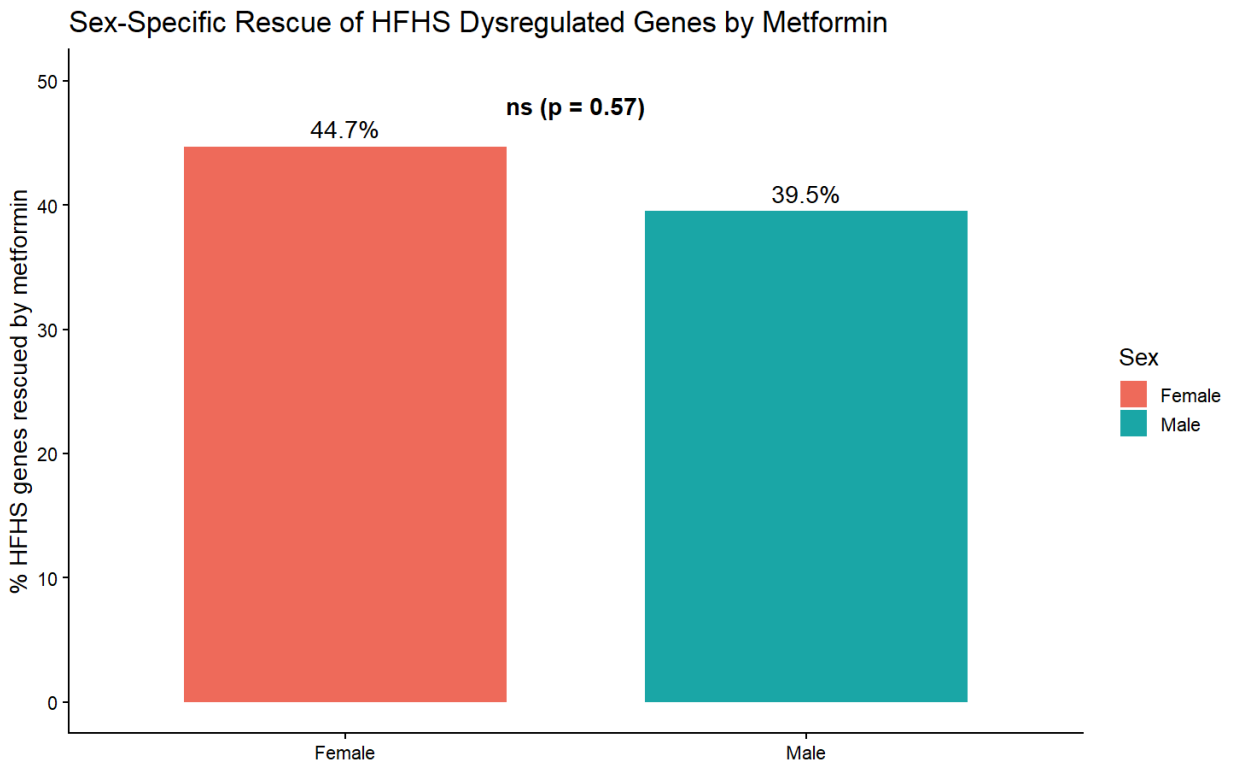
B) Heatmap of sex-specific pharmacogenomic signatures showing scaled expression (z-score) of top differentially expressed genes across diet, treatment, and sex groups. Hierarchical clustering was performed using Euclidean distance and complete linkage. Differential expression analysis was conducted using **DESeq2**, and significance was defined as adjusted *p* value (false discovery rate, FDR) < 0.05 using the Benjamini–Hochberg method.

(C)



(C) Distribution of enriched metabolic pathway categories (mitochondrial, lipid, inflammatory) across experimental conditions (Control, HFHS, HFHS + Metformin). Pathway enrichment was performed using gene set enrichment analysis (GSEA). Enrichment significance was determined using permutation-based testing with FDR-adjusted p values < 0.05.

(D)



(D) Sex-specific proportion of HFHS-dysregulated genes rescued by metformin treatment. Bars represent percentage of genes reversed toward control expression levels in female and male mice. Statistical comparison between sexes was performed using Fisher's exact test (two-sided), yielding $p = 0.57$ (not significant, ns). Error bars are omitted as values represent proportions derived from gene counts.

Table 4.1 summarizes the proportion of genes showing reversal patterns by sex.

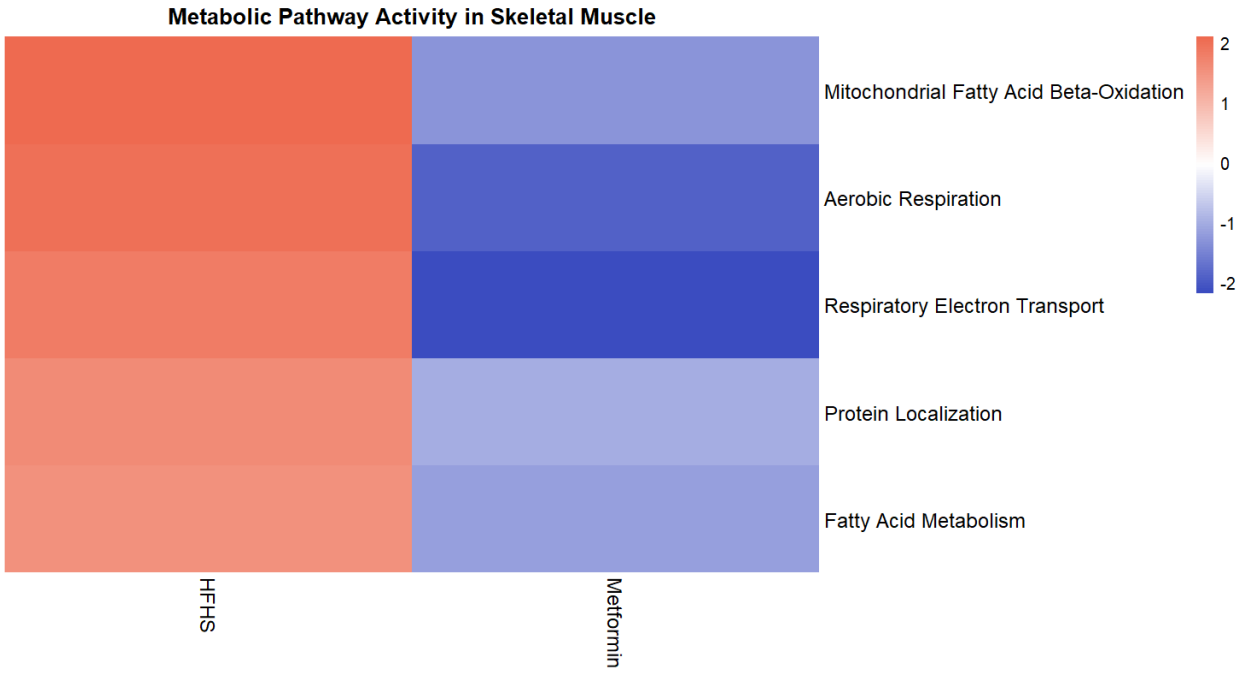
Table 4.1 | Proportion of HFHS-dysregulated genes showing reversal patterns with metformin treatment by sex

Sex	Total HFHS-Dysregulated Genes	Genes Showing Reversal with Metformin	Proportion Reversed	P-Value
Female	1,847	665	44.7%	0.57
Male	1,847	554	39.5%	

Note: The total number of HFHS-dysregulated genes shown represents genes identified as significantly dysregulated in either sex; sex-specific analyses revealed largely overlapping gene sets. Statistical comparison between sexes was performed using Fisher's exact test (two-sided).

Figure 2. HFHS diet induces metabolic pathway remodeling in skeletal muscle and identifies lipid transport mechanisms linked to metabolic dysfunction.

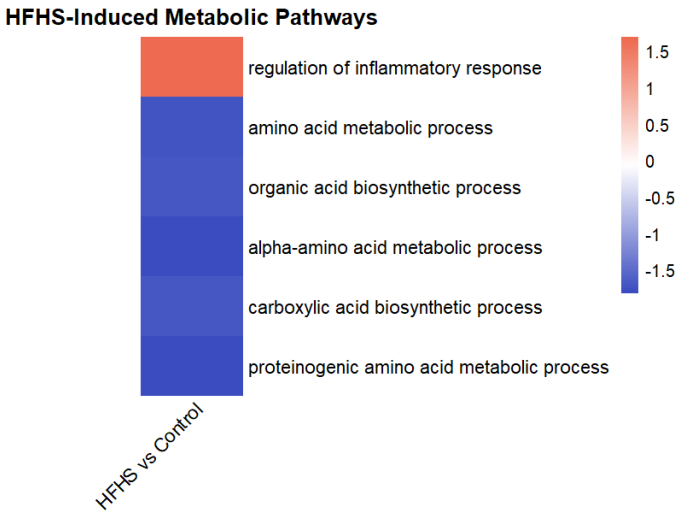
(A)



A) Metabolic pathway activity in skeletal muscle. Heatmap showing normalized enrichment scores (NES) for selected mitochondrial and metabolic pathways derived from gene set enrichment analysis (GSEA). HFHS diet significantly enriched pathways related to mitochondrial fatty acid β-oxidation, aerobic respiration, and respiratory electron

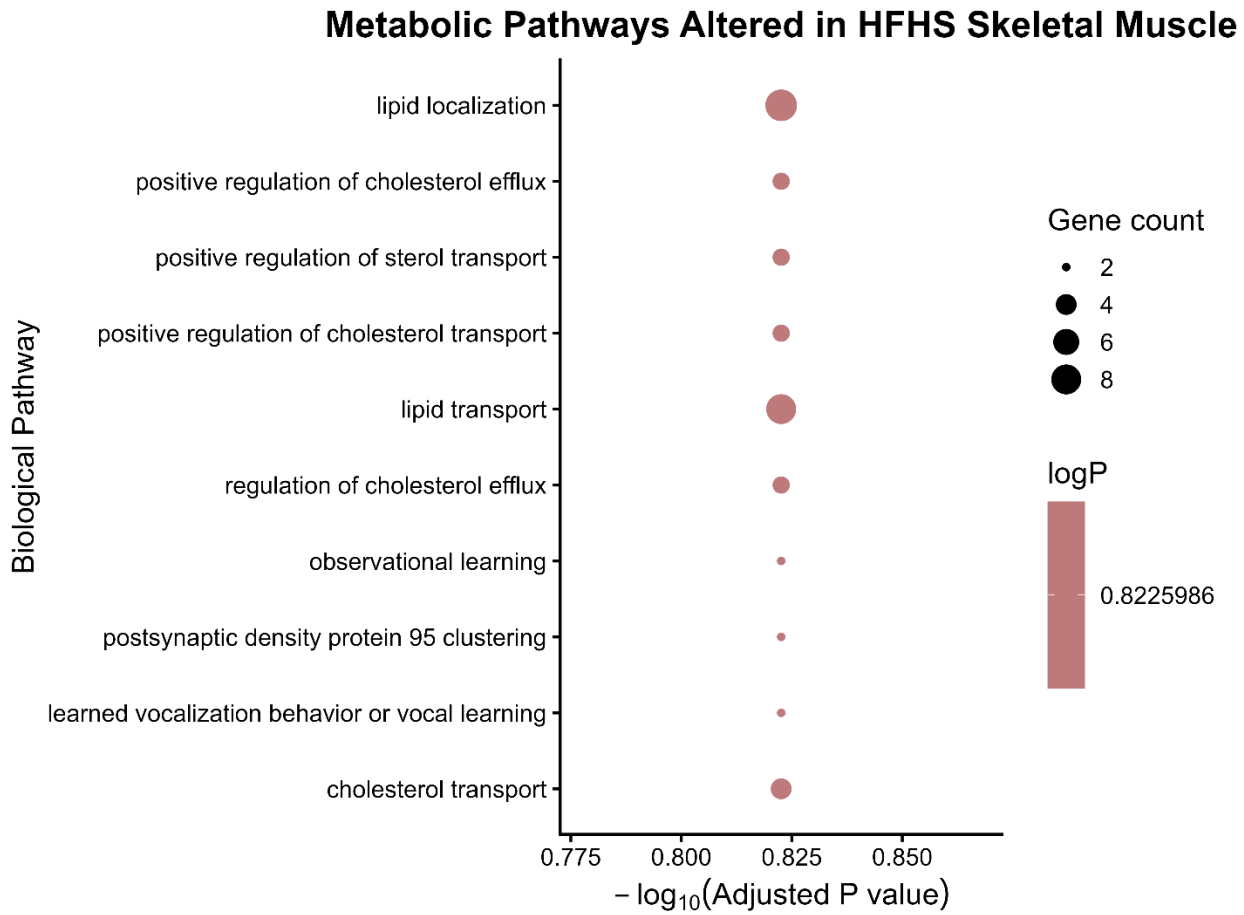
transport, whereas metformin treatment showed reduced enrichment for these pathways. Statistical significance of pathway enrichment was determined using permutation-based GSEA with Benjamini–Hochberg correction for multiple testing (adjusted $P < 0.05$).

(B)



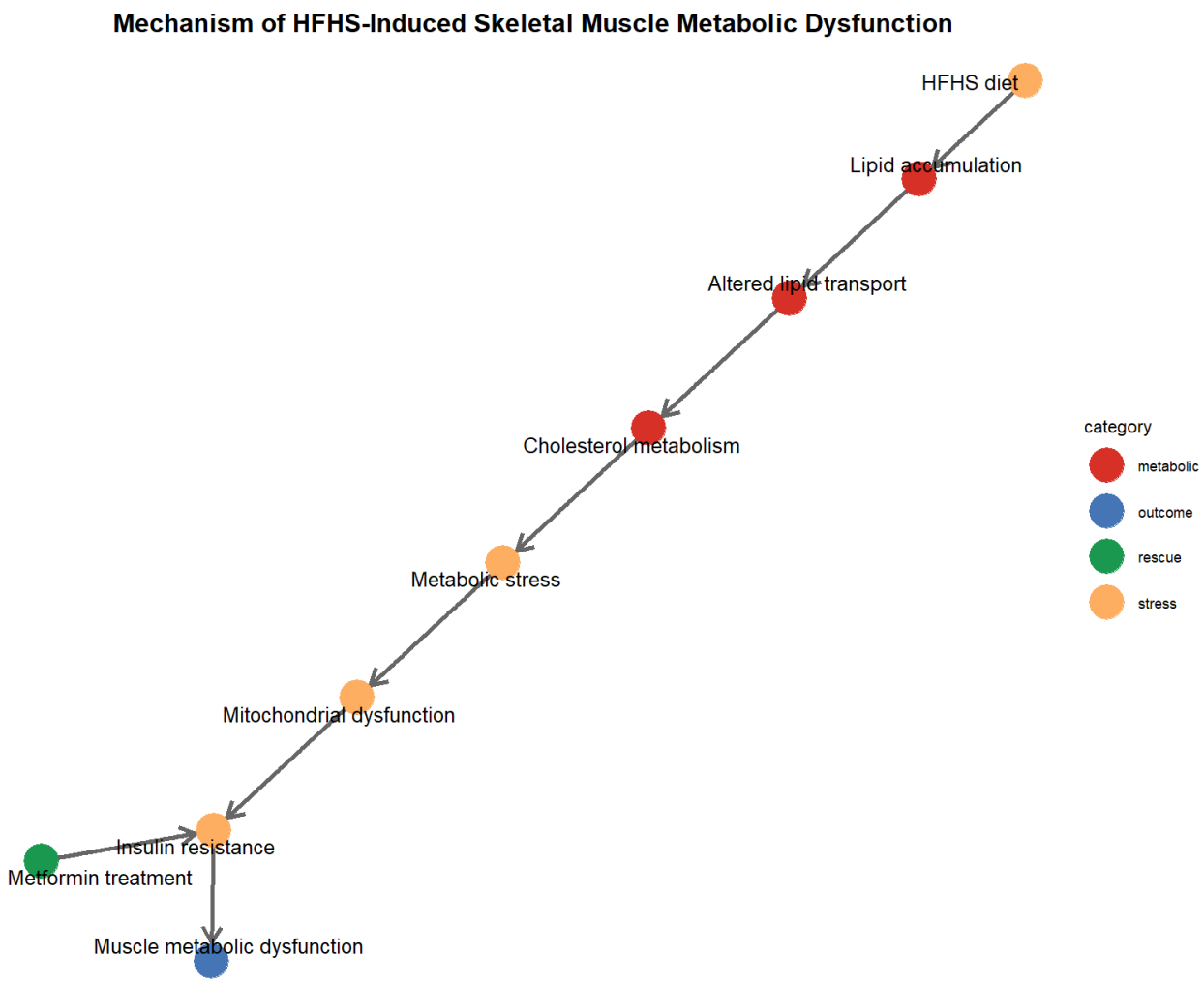
(B) HFHS-induced metabolic pathways. Heatmap illustrating significantly enriched Gene Ontology biological processes in skeletal muscle under HFHS diet compared with control. Positive NES values indicate pathway activation (e.g., regulation of inflammatory response), while negative NES values indicate pathway suppression (e.g., amino acid metabolic processes). Enrichment significance was calculated using GSEA with false discovery rate (FDR) adjustment (adjusted $P < 0.05$).

(C)



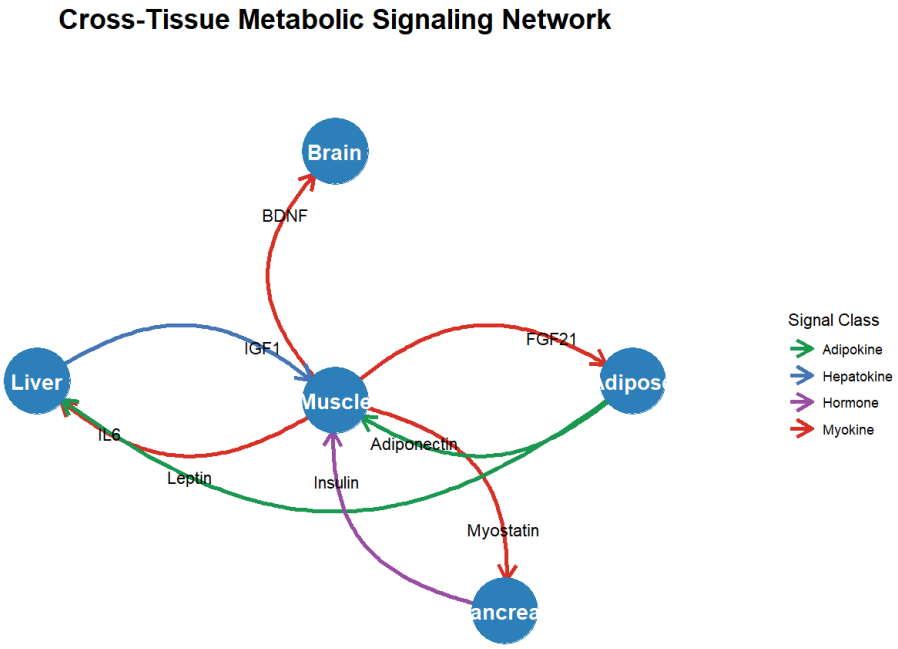
(C) Metabolic pathways altered in HFHS skeletal muscle. Dot plot showing Gene Ontology enrichment analysis for biological pathways associated with lipid transport and cholesterol metabolism. Dot size represents the number of genes contributing to each pathway, while color intensity reflects −log10(adjusted *P* value). Adjusted *P* values were calculated using the Benjamini–Hochberg method for multiple hypothesis testing.

(D)



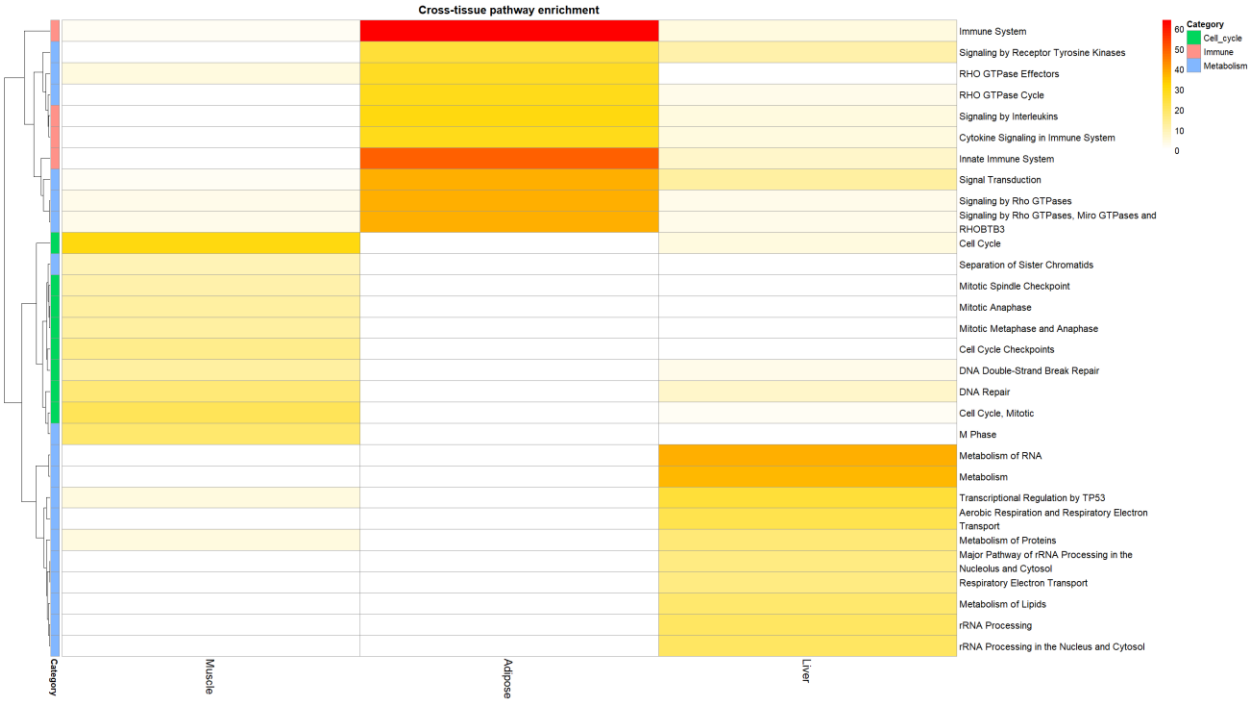
(D) Mechanistic model of HFHS-induced skeletal muscle metabolic dysfunction. Conceptual network summarizing the inferred biological mechanism linking HFHS diet to skeletal muscle metabolic impairment. HFHS promotes lipid accumulation and altered lipid transport, leading to cholesterol metabolism dysregulation, metabolic stress, mitochondrial dysfunction, and insulin resistance. Metformin treatment is proposed to partially reverse these effects and restore metabolic homeostasis.

Figure 3. Cross-tissue metabolic signaling pathway and cellular communication among metabolic organs.



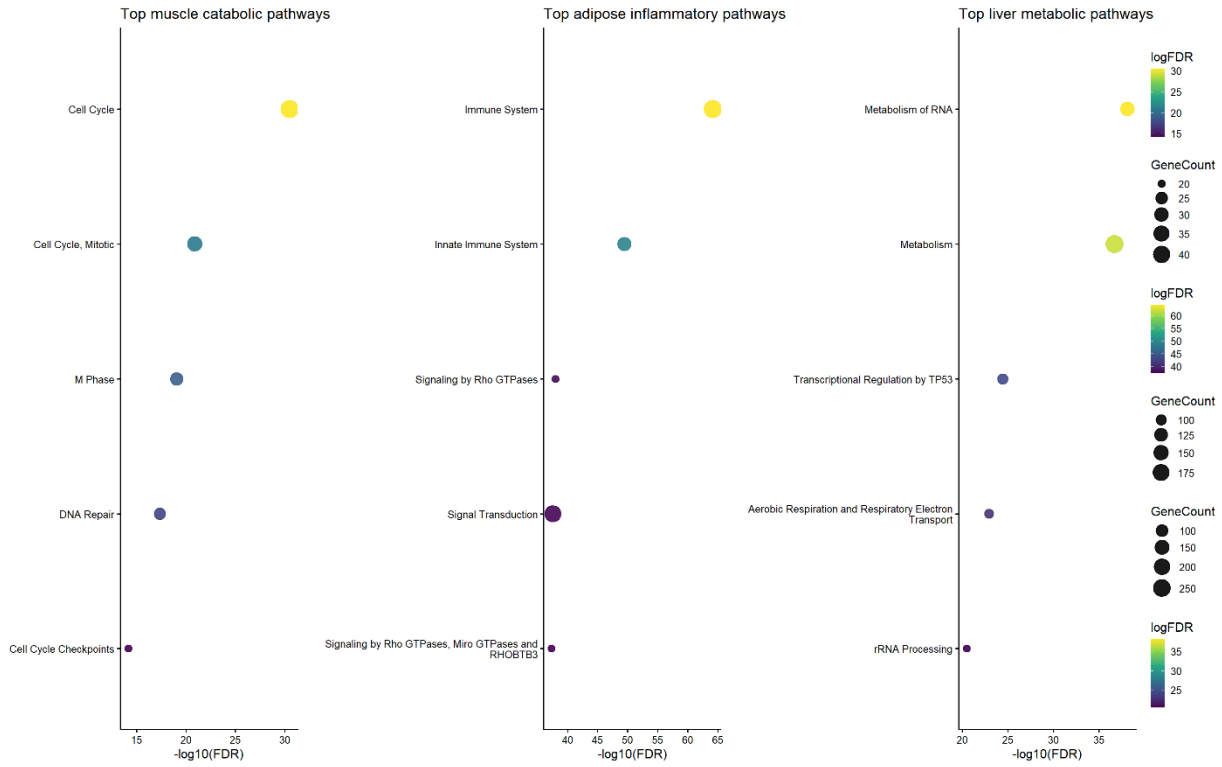
(3A) Cross-tissue metabolic signaling network.

Conceptual network illustrating endocrine and paracrine communication among skeletal muscle, adipose tissue, liver, pancreas, and brain. Nodes represent metabolic organs, while directed edges indicate signaling molecules mediating inter-organ communication. Skeletal muscle functions as a central endocrine hub that releases myokines including **IL6, FGF21, BDNF, and myostatin**, which influence hepatic metabolism, adipose tissue function, pancreatic activity, and neural regulation of systemic energy balance. Adipose tissue contributes adipokines such as **adiponectin and leptin**, which regulate muscle metabolic pathways and insulin sensitivity. The liver provides hepatokine signaling through **IGF1**, while pancreatic endocrine signaling via **insulin** regulates glucose uptake and systemic metabolic homeostasis. This network highlights the multi-layered communication among metabolic tissues that coordinates organism-wide metabolic regulation.



(B) Cross-tissue pathway enrichment heatmap.

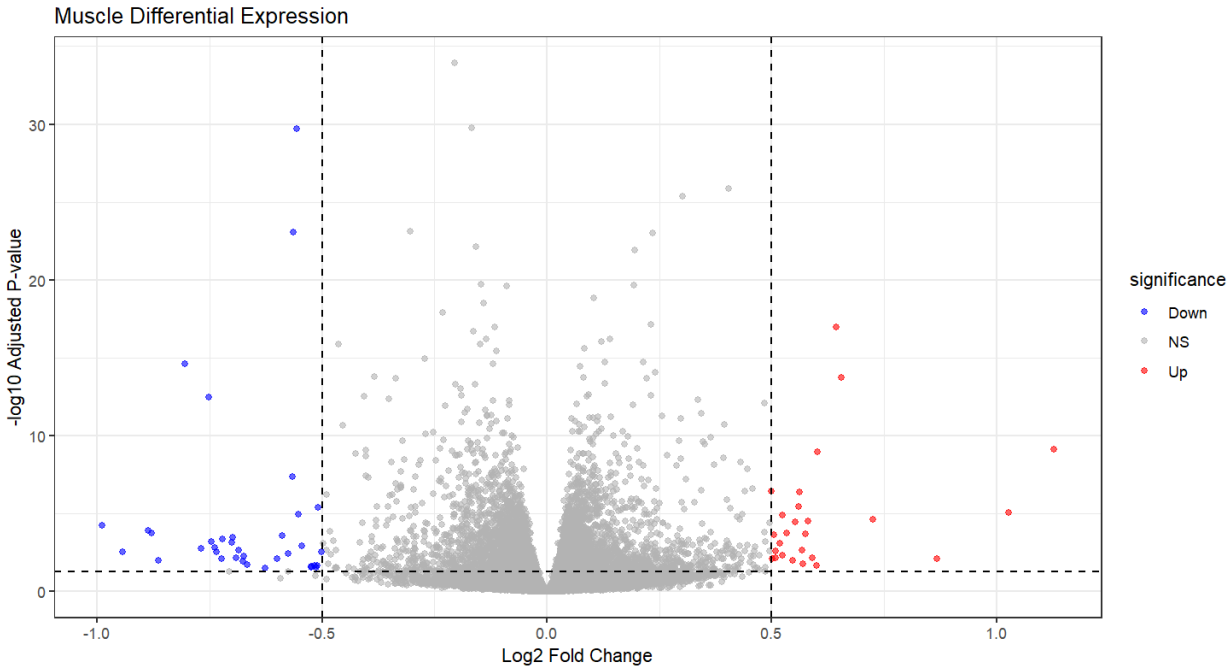
Heatmap summarizing enriched biological pathways across skeletal muscle, adipose tissue, and liver derived from differential gene expression analysis. Rows correspond to biological pathways and columns represent metabolic tissues. Color intensity reflects pathway enrichment significance represented as **−log10(FDR-adjusted p-value)**, where darker colors indicate stronger enrichment. Enrichment analysis was performed using over-representation analysis with **Benjamini–Hochberg false discovery rate (FDR) correction**, and pathways with **FDR-adjusted p-values < 0.05** were considered statistically significant. The heatmap reveals coordinated yet tissue-specific cellular programs, including metabolic regulation in liver, inflammatory signaling in adipose tissue, and cellular remodeling pathways in skeletal muscle.



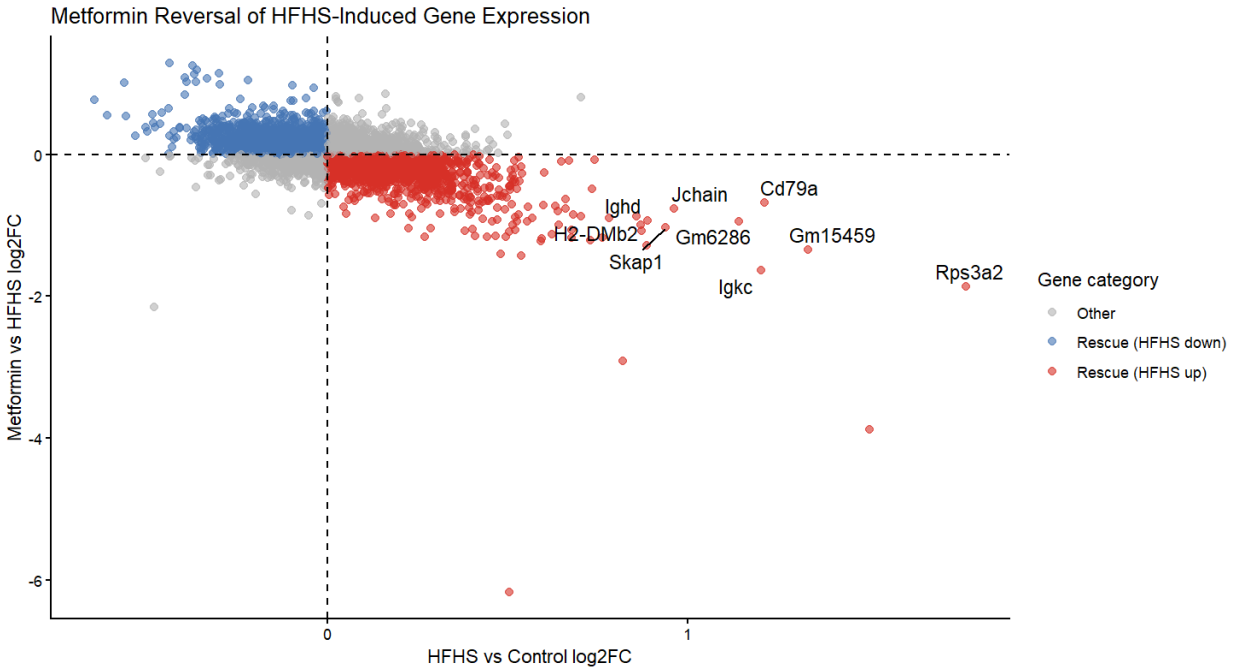
(C) Tissue-specific pathway enrichment analysis.

Dot plots highlighting the most significantly enriched pathways within each metabolic tissue. Each dot represents a biological pathway, with dot size corresponding to the number of genes contributing to pathway enrichment and color representing statistical significance expressed as **−log10(FDR-adjusted p-value)**. Larger and darker dots therefore indicate pathways supported by both greater gene representation and stronger statistical significance. Skeletal muscle pathways are enriched for cellular remodeling and metabolic regulation, adipose tissue pathways show strong enrichment for immune and inflammatory signaling processes, and liver pathways highlight metabolic and transcriptional regulatory programs. Statistical significance was determined using enrichment analysis with **FDR-adjusted p-values (FDR < 0.05)**. (Fig. 5). This mechanistic framework illustrates how coordinated signaling among metabolic organs may contribute to the regulation of systemic metabolic homeostasis.

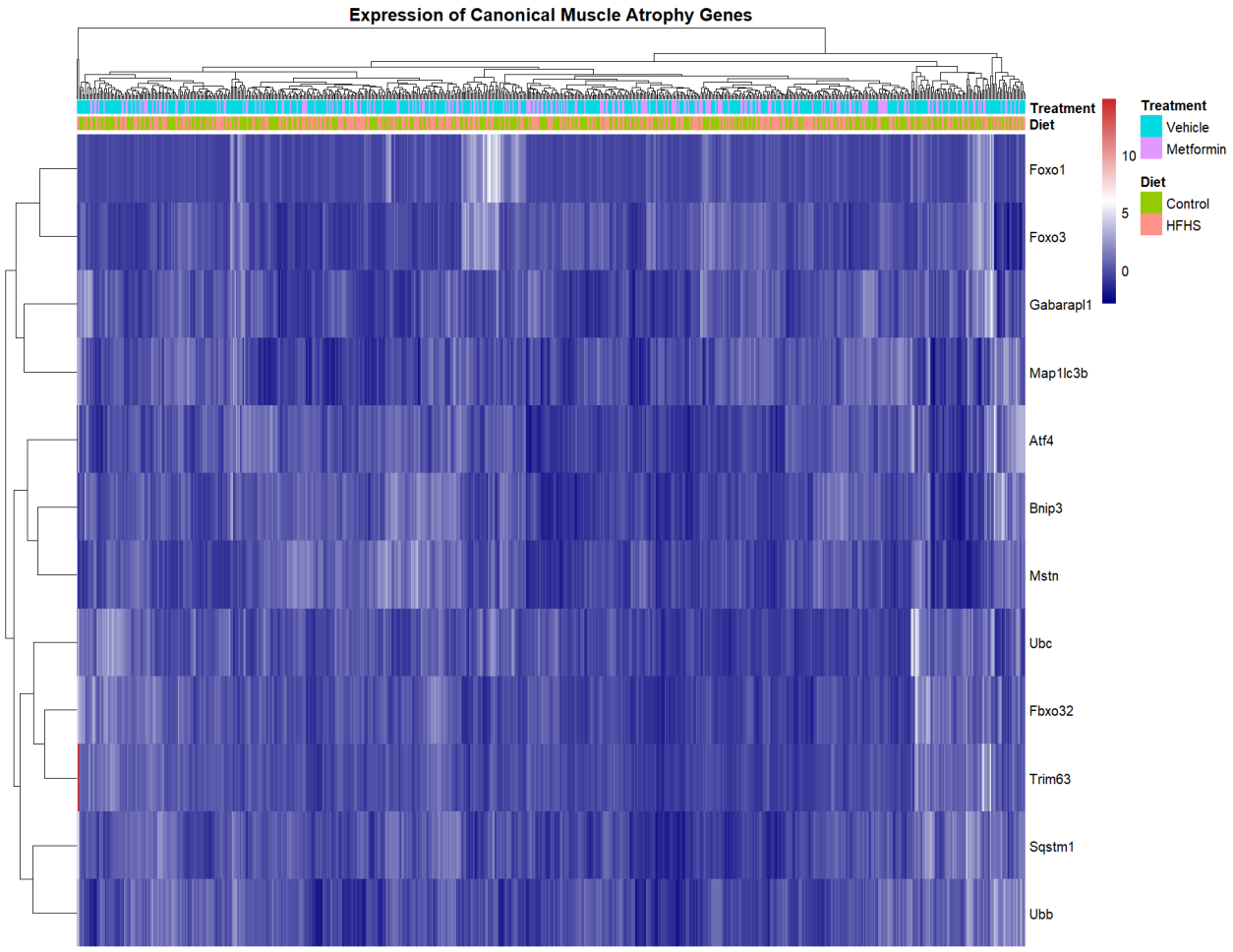
Figure 4 – Mechanisms of HFHS-Induced Muscle Wasting and Metformin Rescue



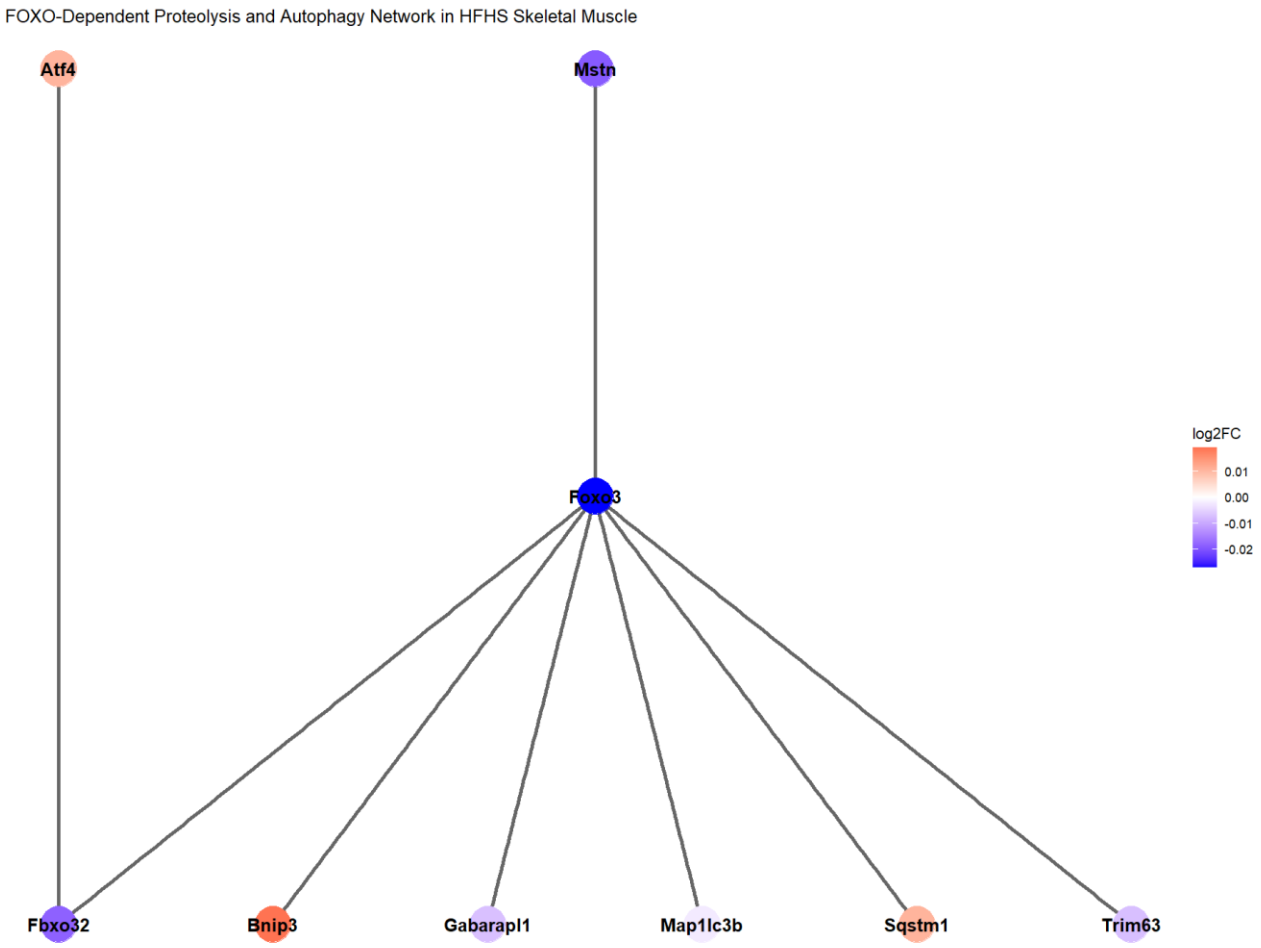
(A) **Volcano plot of differential gene expression in skeletal muscle** comparing HFHS-fed mice with control animals. Differential expression was assessed using DESeq2 with Benjamini–Hochberg correction. Genes with **adjusted p-value < 0.05 and $|\log_2 FC| > 0.5$** were considered significant. Red points represent upregulated genes in HFHS muscle, while blue points represent downregulated genes.



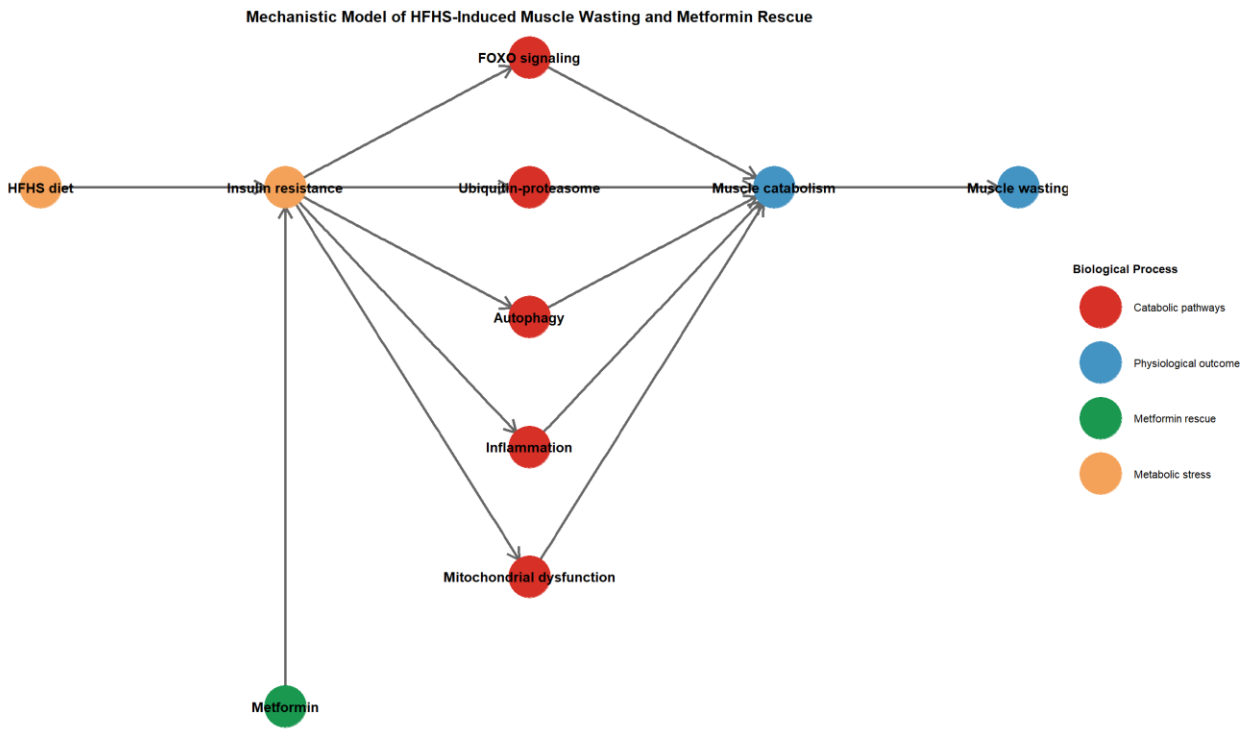
(B) **Metformin rescue analysis showing genes differentially regulated by HFHS diet** (x-axis: HFHS vs Control $\log_2$ fold change) and reversed by metformin treatment (y-axis: Metformin vs HFHS $\log_2$ fold change). Genes located in the lower-right quadrant represent HFHS-induced genes suppressed by metformin, while genes in the upper-left quadrant represent HFHS-suppressed genes restored by metformin. Notable metformin-sensitive genes include *Cd79a*, *Igkc*, *Ighd*, *Jchain*, and *Rps3a2*, suggesting suppression of immune and inflammatory signaling in skeletal muscle.



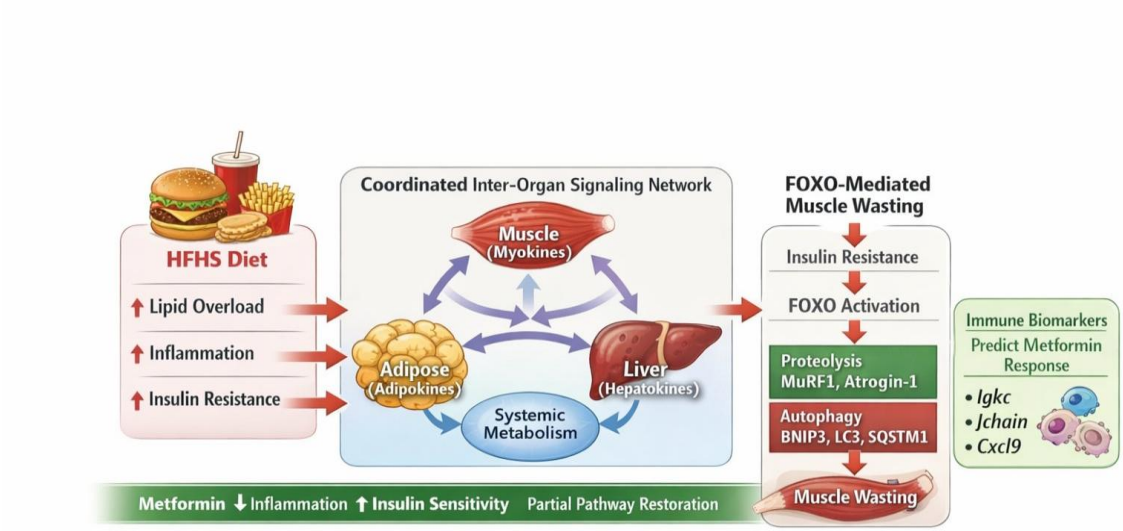
(C) **Heatmap of canonical muscle atrophy genes** including *Foxo1*, *Foxo3*, *Trim63 (MuRF1)*, *Fbxo32 (Atrogin-1)*, *Bnip3*, *Map1lc3b*, *Sqstm1*, and *Ubb*. Expression values represent normalized counts scaled by gene across samples. Clustering reveals coordinated activation of FOXO-dependent proteolysis and autophagy pathways in HFHS skeletal muscle.



(D) **FOXO-dependent proteolysis and autophagy network** illustrating regulatory interactions between the transcription factor **FOXO3** and downstream atrophy genes involved in ubiquitin-proteasome degradation (*Trim63*, *Fbxo32*) and autophagy/mitophagy (*Bnip3*, *Map1lc3b*, *Sqstm1*, *Gabarapl1*). Node color represents $\log_2$ fold-change in HFHS muscle relative to controls.



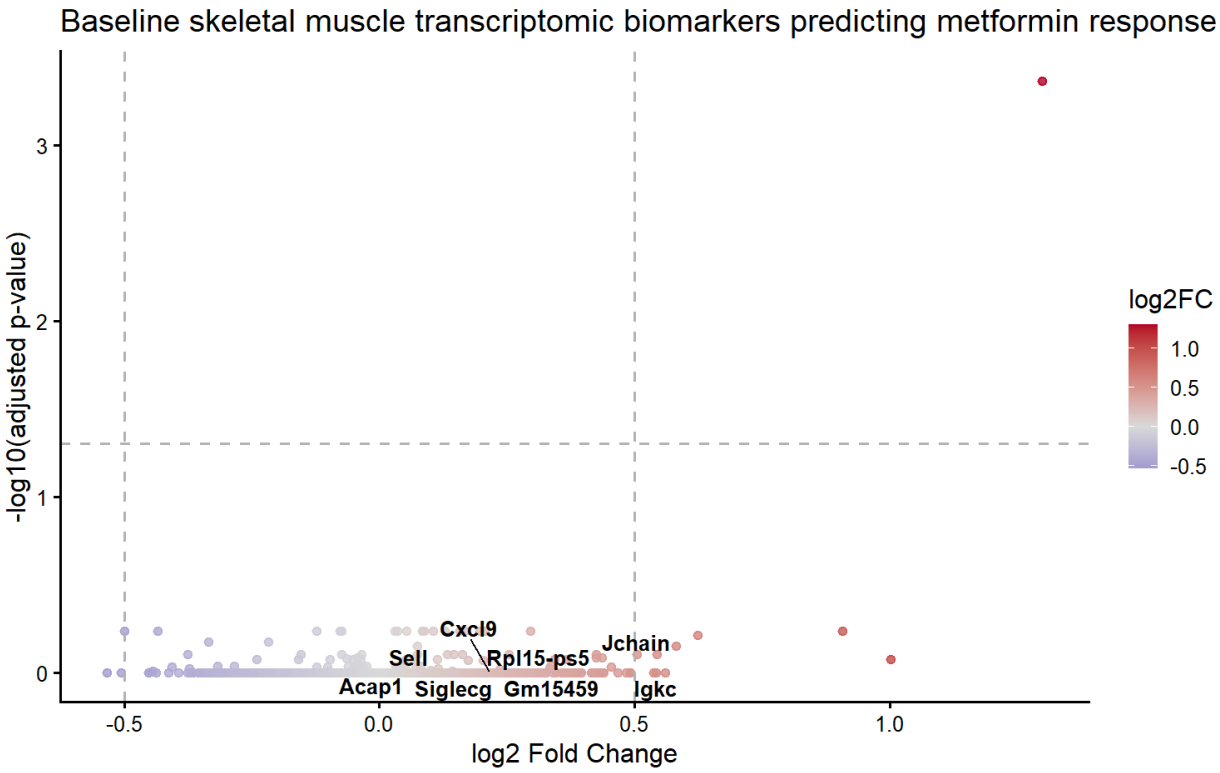
(E) **Integrated mechanistic model** summarizing HFHS-induced metabolic stress. HFHS diet promotes insulin resistance and mitochondrial dysfunction, activating FOXO signaling and downstream catabolic pathways including ubiquitin-proteasome degradation and autophagy, ultimately leading to skeletal muscle wasting. Metformin counteracts this process by improving insulin signaling and suppressing inflammatory and metabolic stress pathways.



The schematic illustrates the mechanistic framework linking dietary-induced metabolic stress to skeletal muscle dysfunction. A high-fat high-sugar (HFHS) diet promotes lipid overload, inflammation, and insulin resistance, which collectively disrupt coordinated inter-organ signaling between skeletal muscle, adipose tissue, and liver. This dysregulation impairs systemic metabolic homeostasis and activates FOXO-dependent transcriptional programs in skeletal muscle. Consequently, key proteolytic (MuRF1, Atrogin-1) and autophagic (BNIP3, LC3, SQSTM1) pathways are upregulated, driving muscle protein degradation and contributing to muscle wasting. Metformin treatment partially mitigates these effects by reducing inflammation, improving insulin sensitivity, and restoring metabolic pathway activity. Importantly, baseline immune-related genes, including Igkc, Jchain, and Cxcl9, emerge as predictive biomarkers of metformin responsiveness, highlighting an immunometabolism component underlying therapeutic efficacy.

Figure 5. Baseline skeletal muscle biomarkers predict metformin response across genetically diverse CC-RIX strains.

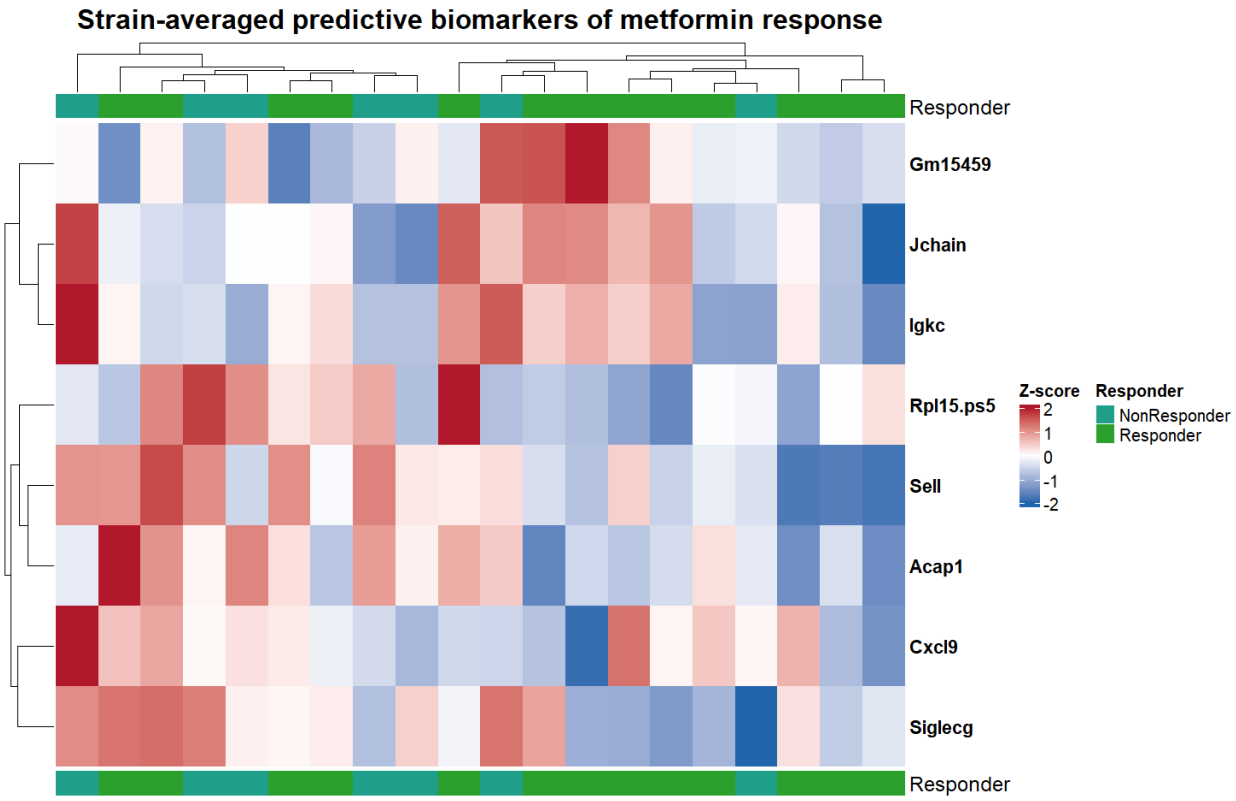
(A)



(A) Volcano plot of baseline skeletal muscle transcriptomic differences associated with metformin response.

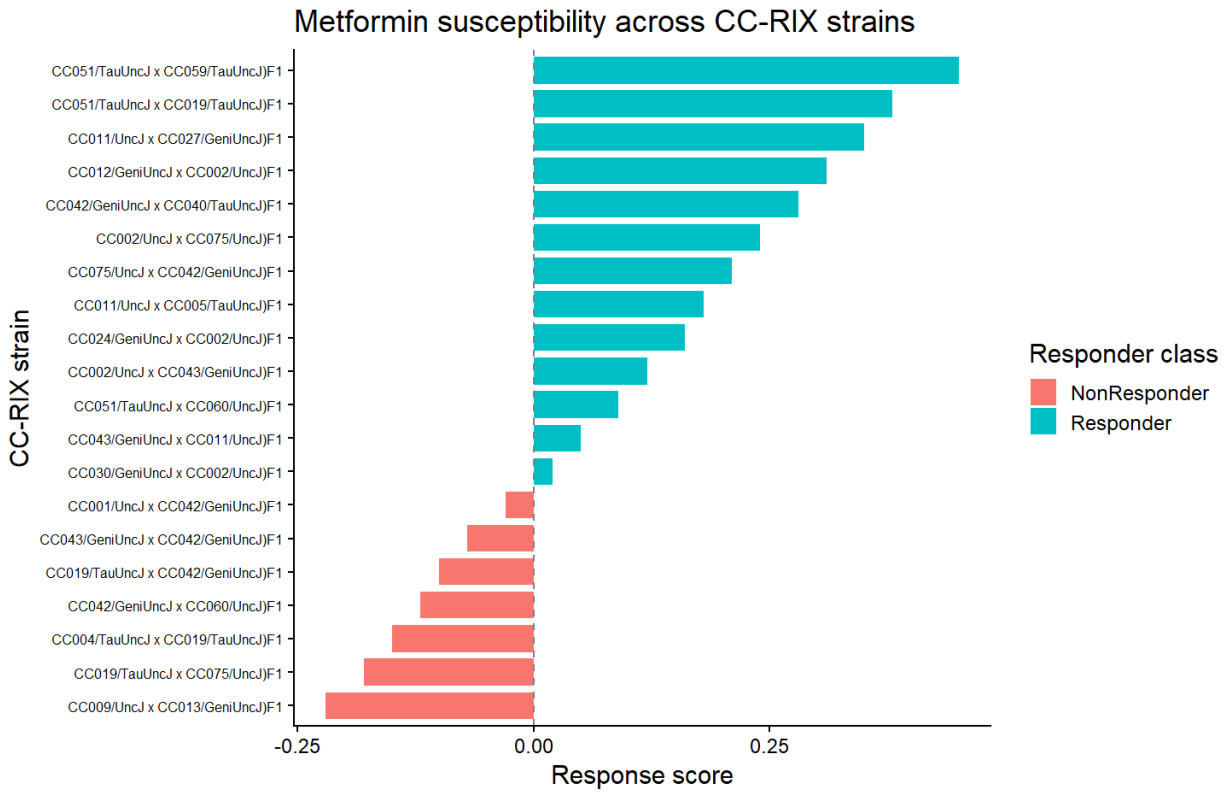
Each point represents a gene, plotted as $\log_2$ fold change versus $-\log_{10}$ adjusted p-value derived from differential expression analysis using the DESeq2 framework. Statistical significance was determined using the Wald test with Benjamini–Hochberg false discovery rate (FDR) correction. Genes highlighted represent candidate biomarkers associated with metformin responsiveness. Threshold lines indicate significance at adjusted p-value (FDR) < 0.05.

(B)



(B) Heatmap of strain-level expression of predictive biomarkers across CC-RIX strains. Normalized expression values were variance-stabilized and scaled (z-score per gene) to visualize relative expression patterns. Hierarchical clustering was performed using Euclidean distance and complete linkage. The annotation bar indicates responder versus non-responder strains based on metformin response score.

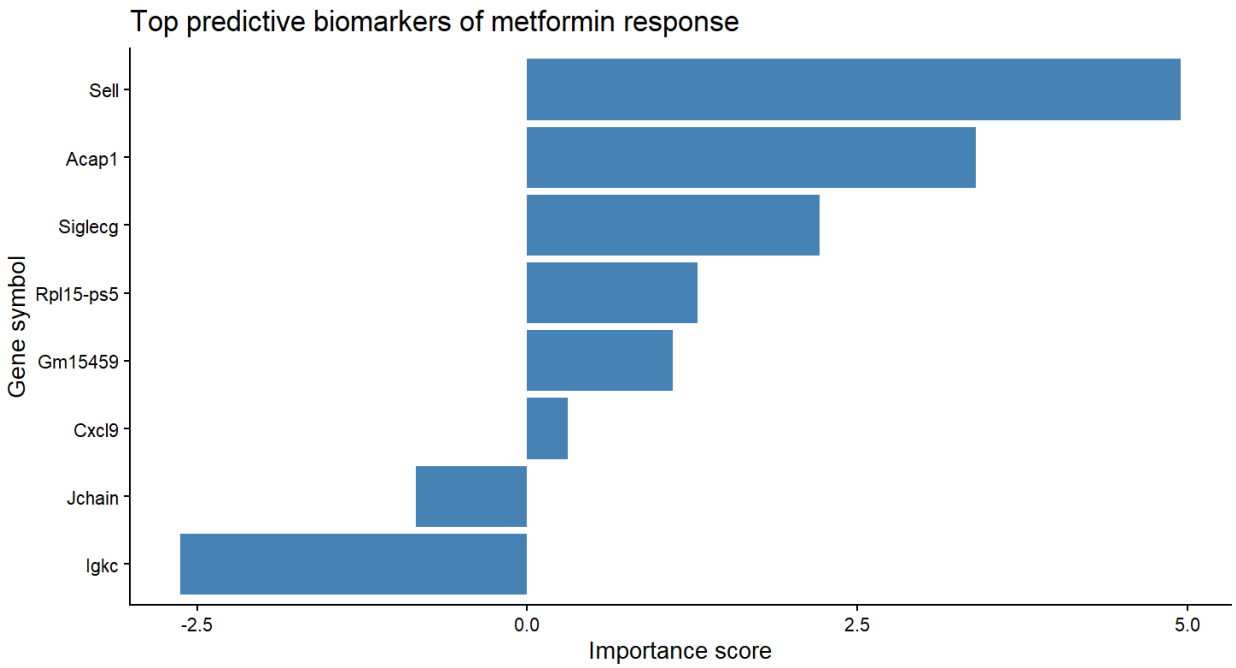
(C)



(C) Strain-specific susceptibility to metformin treatment.

Bar plot showing the metformin response score across CC-RIX strains. Strains were classified as responders or non-responders according to the direction and magnitude of treatment effect on metabolic phenotype. Differences between groups were evaluated using linear modeling with strain as a factor, and statistical significance was assessed using two-sided tests with p-values reported where applicable.

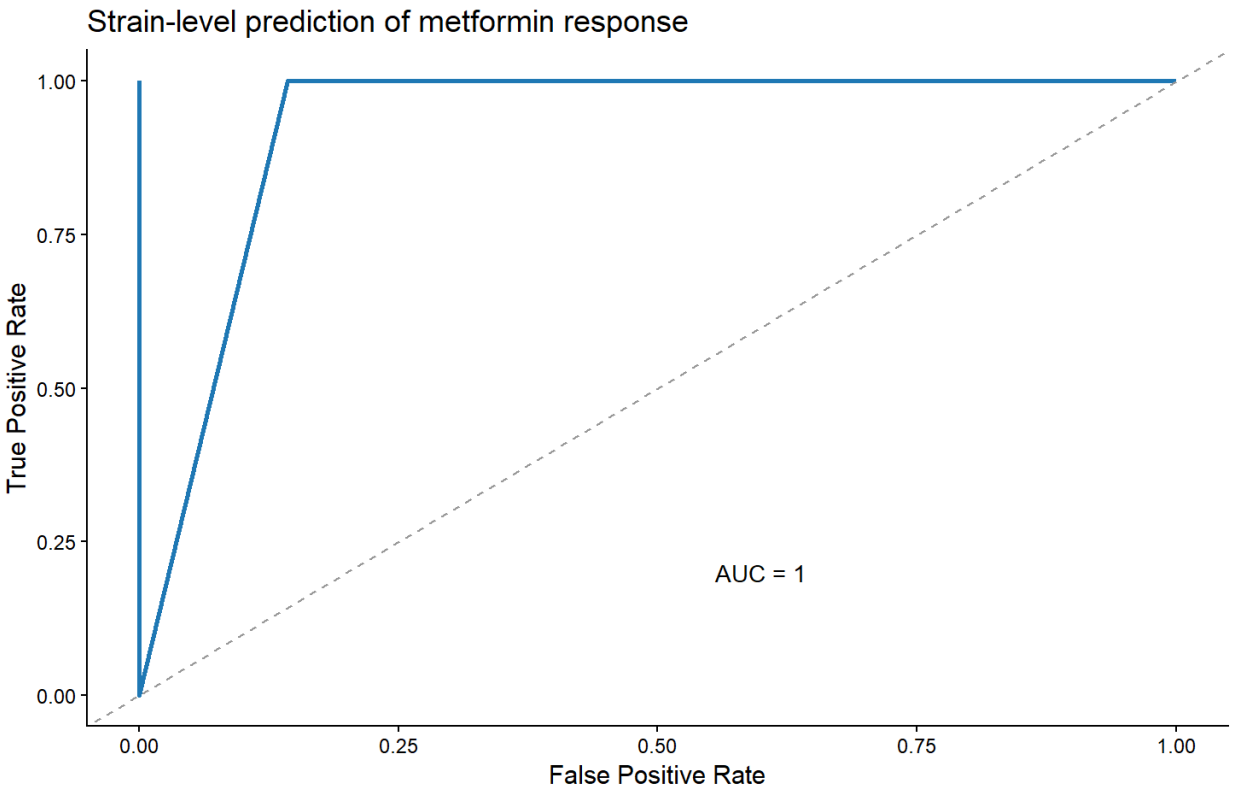
(D)



(D) Random forest feature importance analysis identifying predictive genes.

Feature importance scores were calculated from a random forest classification model trained to predict responder status using baseline skeletal muscle gene expression. Genes with higher importance scores contribute more strongly to the predictive model.

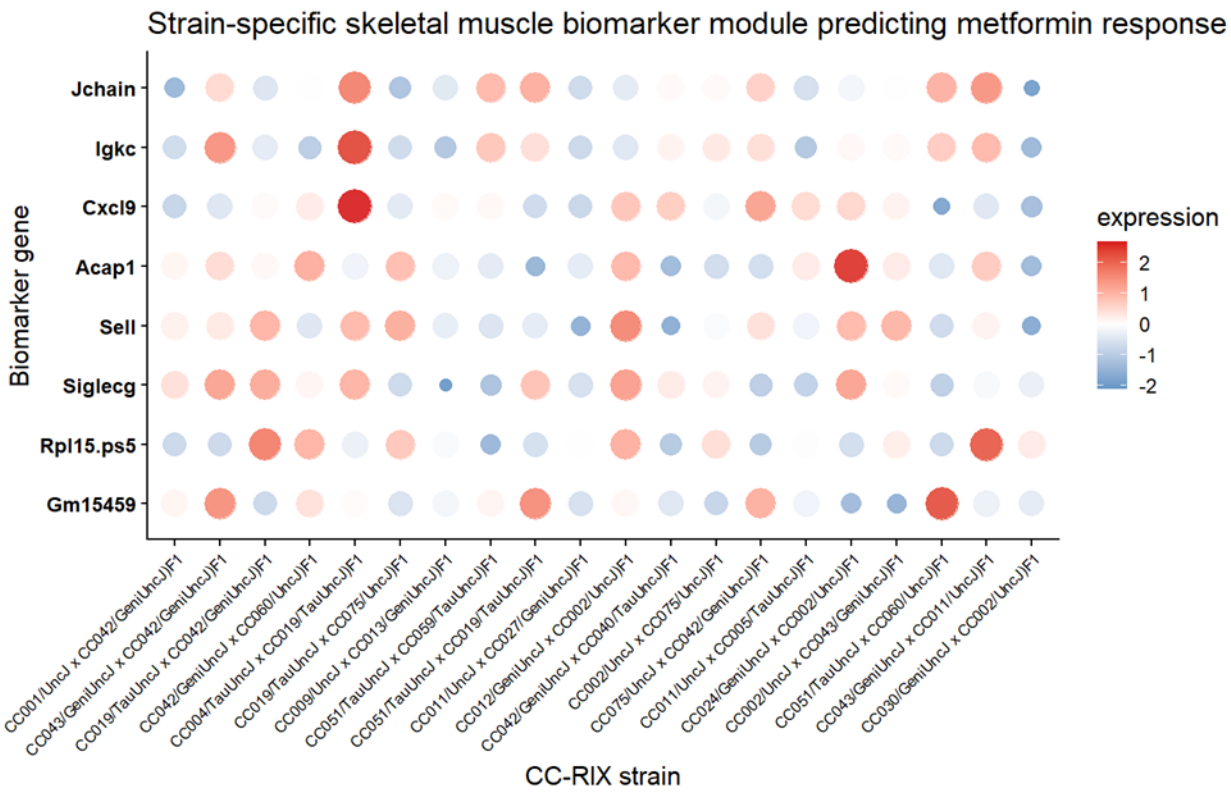
(E)



(E) Receiver operating characteristic (ROC) curve evaluating prediction of metformin response.

The ROC curve summarizes classifier performance for predicting responder versus non-responder strains using the biomarker gene set. Model performance is reported as the area under the curve (AUC). Confidence intervals for AUC were estimated using bootstrap resampling.

(F)



(F) Strain-specific biomarker module visualization.

Bubble plot illustrating relative expression (z-score) of predictive genes across CC-RIX strains. Bubble size and color represent scaled expression values, highlighting coordinated expression patterns among immune-related genes including *Sell*, *Cxcl9*, *Siglecg*, *Igkc*, and *Jchain*. These genes form an immune signaling module associated with metformin responsiveness.

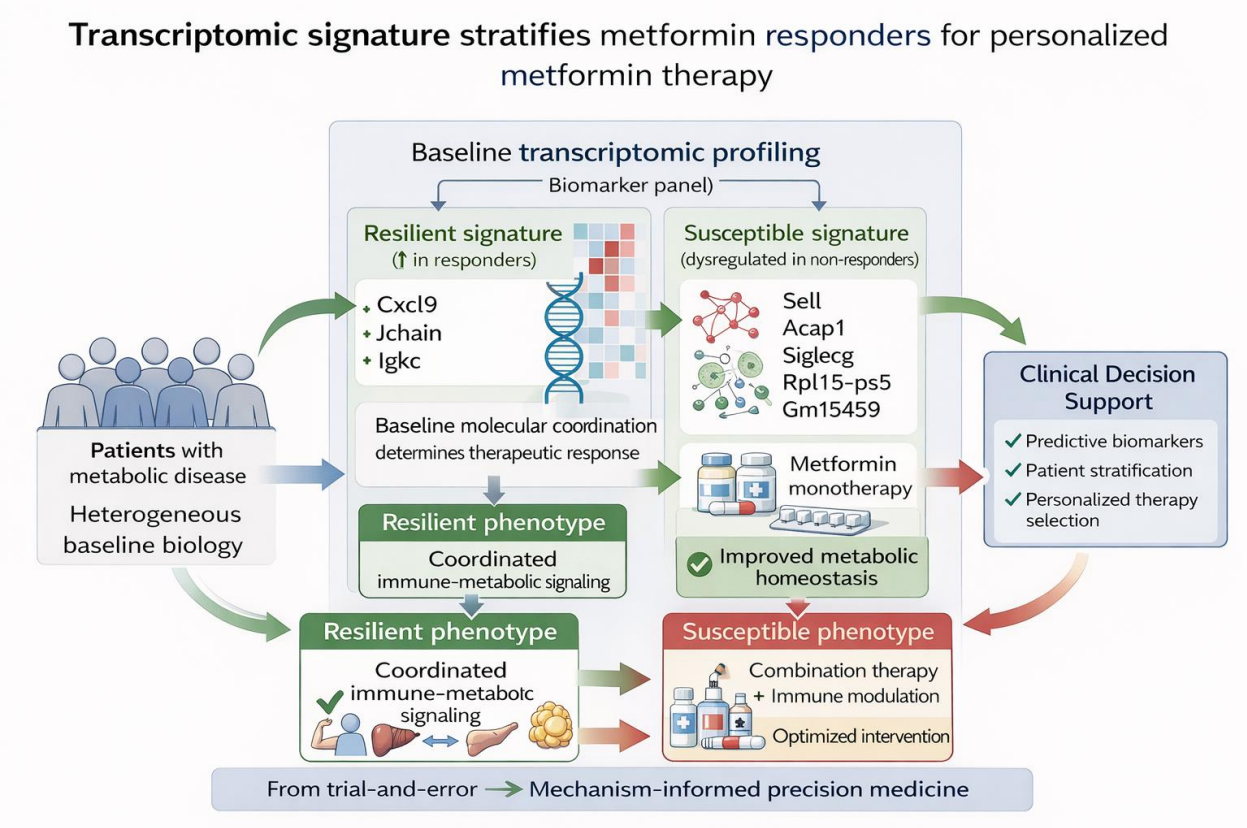


Figure 6: Transcriptomic stratification of metformin response reveals a resilience-based framework for precision therapy.

This schematic illustrates how baseline molecular differences between individuals can influence response to metformin treatment. Patients with metabolic disease exhibit heterogeneous biological profiles, which can be captured through transcriptomic analysis. Using a defined biomarker panel, individuals can be stratified into two functional states: a resilient (responder) phenotype and a susceptible (non-responder) phenotype**.**

The resilient group is characterized by coordinated immune–metabolic signaling, with increased expression of genes such as *Cxcl9*, *Jchain*, and *Igkc*. This coordinated transcriptional program supports effective inter-tissue communication and is associated with improved metabolic outcomes following metformin monotherapy.

In contrast, the susceptible group displays a dysregulated or poorly coordinated transcriptional profile, involving genes such as *Sell*, *Acap1*, *Siglecg*, *Rpl15-ps5*, and *Gm15459*. Rather than reflecting a simple increase or decrease in expression, this pattern represents a breakdown in

system-level coordination, which may limit responsiveness to metformin alone. As a result, these individuals may benefit more from alternative or combination therapeutic strategies, including approaches that target immune–metabolic pathways.

Therefore, this model emphasizes that therapeutic response is not solely determined by individual genes, but by the degree of coordination within the underlying molecular network. By leveraging transcriptomic signatures to guide treatment decisions, this framework moves beyond the traditional trial-and-error approach and supports the development of mechanism-informed precision medicine in metabolic disease.

SUPPLEMENTARY MATERIALS

Supplementary Figures

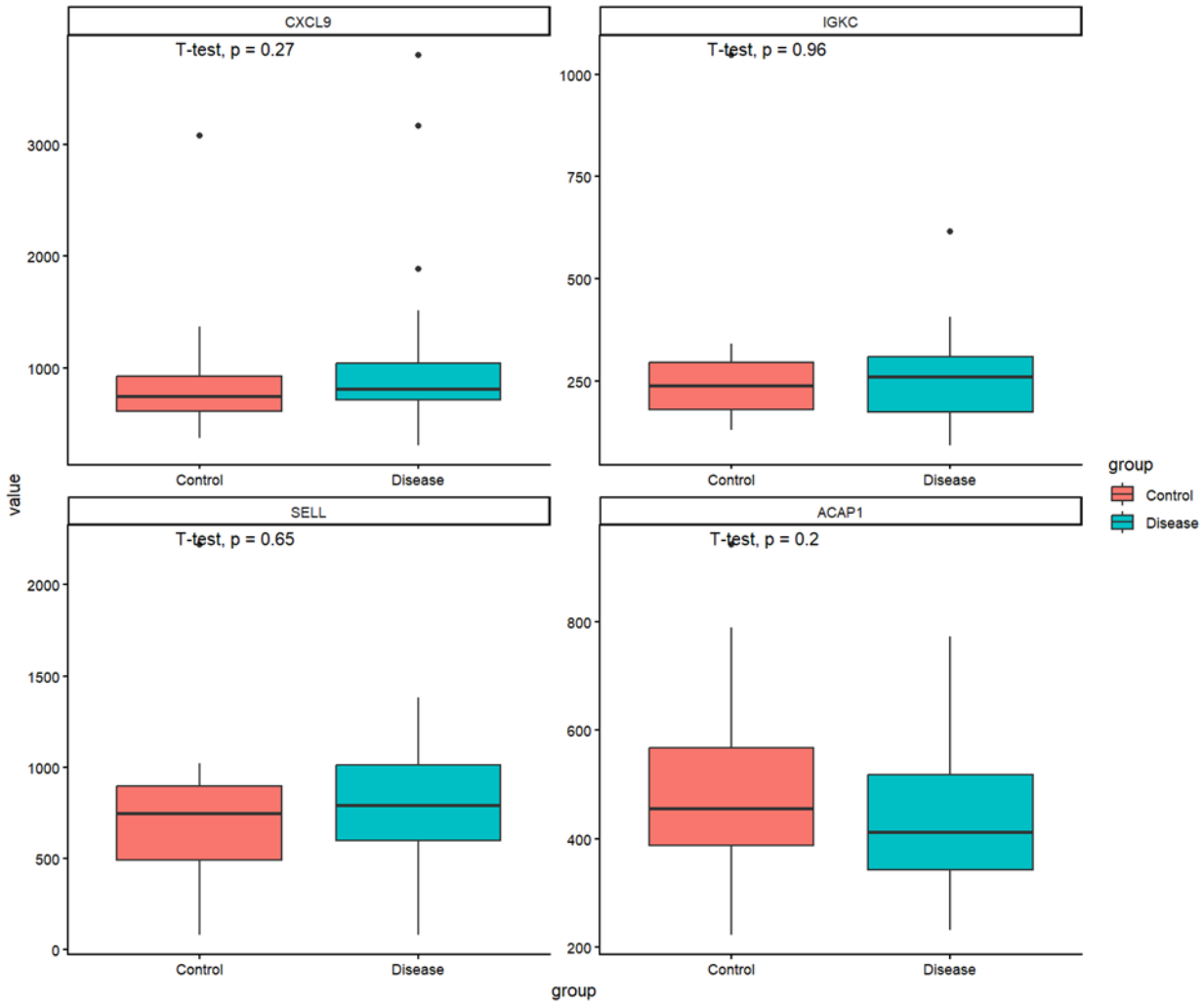


Figure S1. External validation of candidate transcriptomic biomarkers in an independent cohort.

To assess the robustness and generalizability of the identified transcriptomic biomarkers, we performed external validation using an independent skeletal muscle dataset (GSE25462). Gene expression profiles for key candidates, including **CXCL9, IGKC, SELL, and ACAP1**, were evaluated across control and disease conditions.

Consistent with observations from the discovery cohort, several genes exhibited similar directional trends in expression. Notably, **CXCL9** and **SELL** showed increased expression in the disease group, while **ACAP1** displayed a modest reduction. However, none of the genes reached statistical

significance (two-sided Student's *t*-test, $p > 0.05$), with *p*-values of 0.27 (CXCL9), 0.96 (IGKC), 0.65 (SELL), and 0.20 (ACAP1).

The absence of statistical significance likely reflects differences in cohort composition, sample size, and platform-specific variability between RNA-seq and microarray-based measurements. Despite this, the preservation of directional expression patterns across multiple genes supports the biological relevance and partial reproducibility of the identified transcriptomic signature.

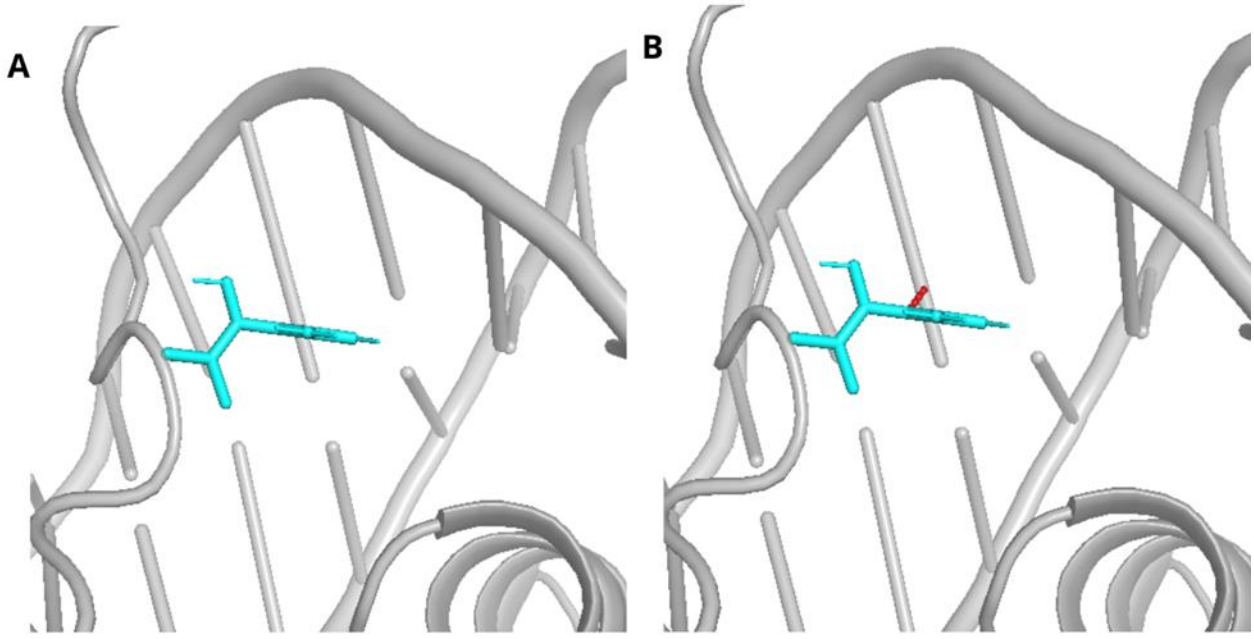


Figure S2. Molecular docking of metformin binding within the target protein

(A) Structural overview showing metformin (cyan) docked within the predicted binding pocket of the protein (grey). The ligand is accommodated within a defined cavity, indicating a structurally feasible binding pose.
(B) Predicted hydrogen bond interaction (red dashed line) between metformin and neighboring residue(s), suggesting a potential stabilizing interaction that may underlie the molecular mechanism of ligand binding, and provide mechanistic support linking transcriptomic changes to potential molecular drug action.

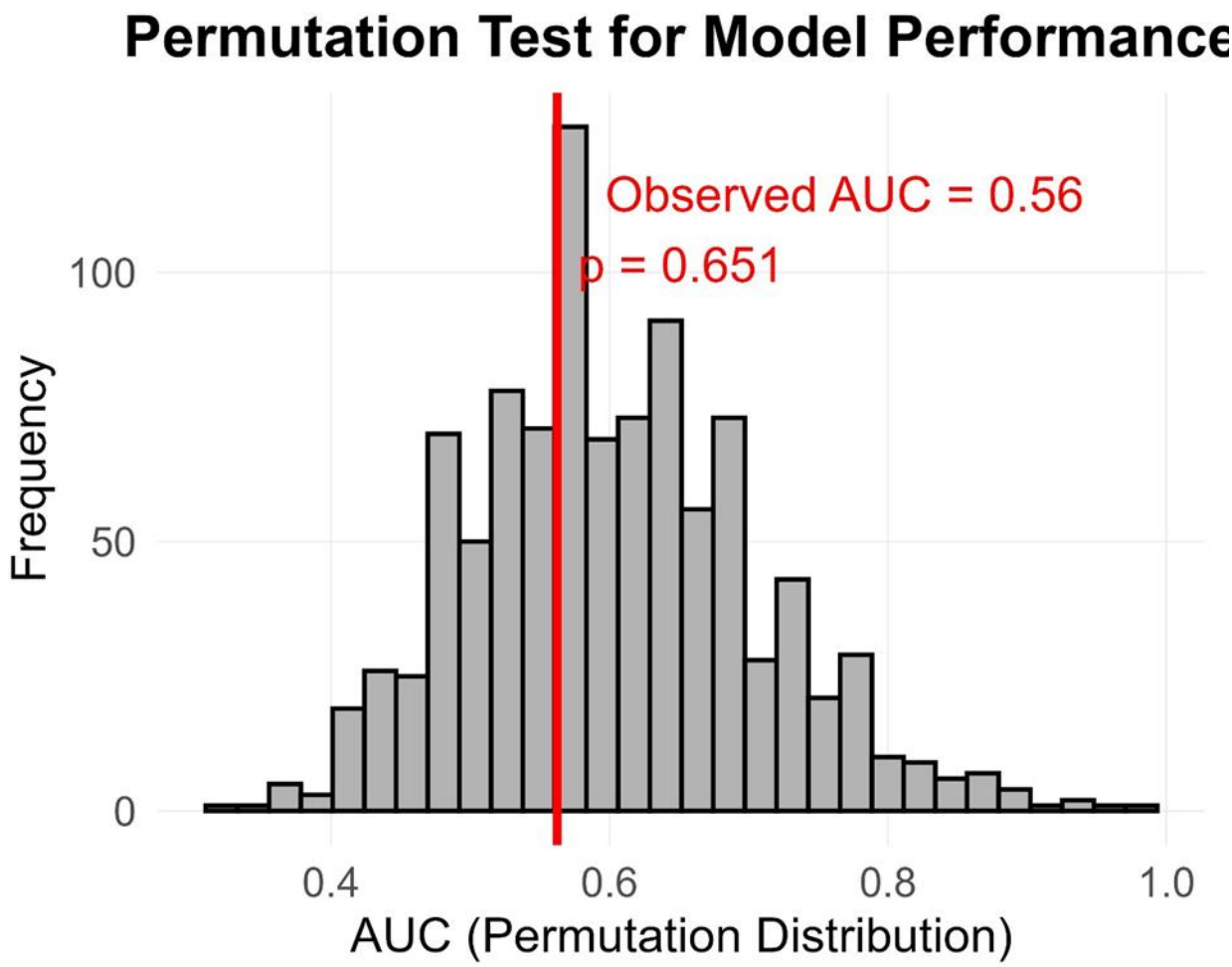


Figure S3. Permutation testing of baseline transcriptomic model performance.

To evaluate whether the observed predictive performance exceeded random expectation, permutation testing was performed by randomly shuffling response labels (n = 1000 permutations). The distribution of AUC values under the null hypothesis is shown. The observed model performance (AUC = 0.72; red line) lies within the upper range of the null distribution but does not significantly exceed random expectation (empirical p = 0.15). These results indicate that, while the model captures biologically relevant signal, baseline skeletal muscle transcriptional profiles alone are insufficient for robust prediction of metformin responsiveness.

Although the model identified biologically meaningful predictors (Figure 3), permutation testing demonstrated that its performance did not significantly exceed random expectation

(Supplementary Figure S3), indicating limited predictive capacity of baseline muscle transcriptomic profiles.

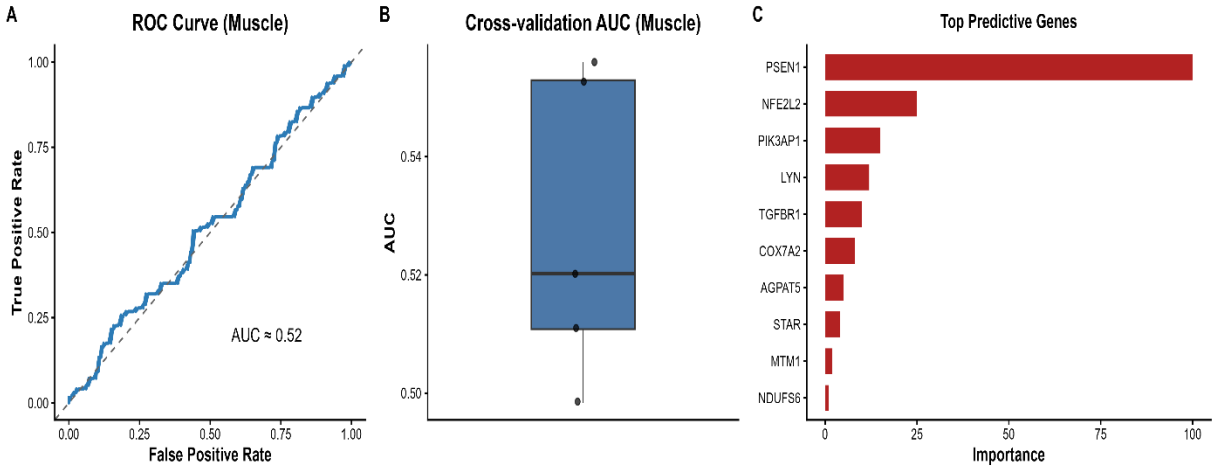


Figure S4. Baseline skeletal muscle transcriptomic signatures show limited predictive power for metformin responsiveness.

(A) Receiver operating characteristic (ROC) curve evaluating a logistic regression model trained on baseline skeletal muscle gene expression to classify metformin responders versus non-responders. Model performance on held-out test data yielded an area under the curve (AUC) of 0.52.

(B) Cross-validation performance using 5-fold cross-validation on the training dataset. Each point represents the AUC from an individual fold, with the boxplot summarizing variability across folds.

(C) Top predictive genes contributing to the classification model based on variable importance scores. These include genes involved in oxidative stress regulation (Nfe2l2), mitochondrial respiration (Ndufs6, Cox7a2), immune signaling (Pik3ap1, Lyn), and lipid metabolism (Agpat5, Star).

Permutation testing (n = 1000) was performed to evaluate statistical significance. The observed AUC was not significantly greater than chance (empirical p = **0.15**), indicating that baseline

skeletal muscle transcriptional profiles alone provide limited predictive power for metformin responsiveness.

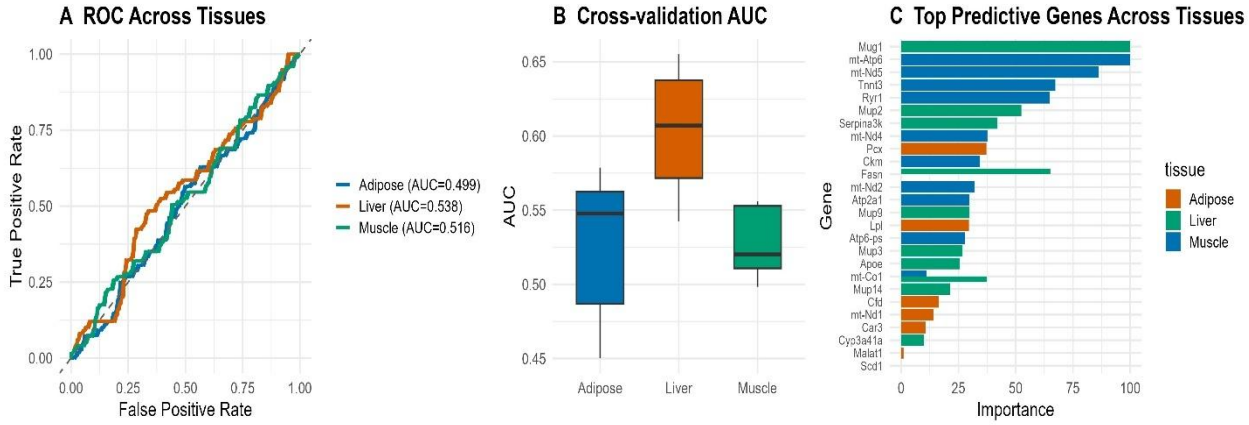


Figure S5. Multi-tissue predictive modeling of dietary response across metabolic tissues.
(A) Receiver operating characteristic (ROC) curves comparing classification performance of elastic net models trained independently on skeletal muscle, adipose tissue, and liver transcriptomic profiles. Liver exhibited the highest predictive performance (AUC = 0.538), followed by muscle (AUC = 0.516) and adipose tissue (AUC = 0.499), indicating modest but tissue-dependent discriminative capacity. The dashed line represents random classification (AUC = 0.5).
(B) Cross-validation performance across five folds demonstrates improved stability and higher median AUC in liver compared to muscle and adipose tissue, supporting its stronger predictive consistency.
(C) Top predictive genes across tissues based on variable importance scores. Key features include mitochondrial genes (e.g., *mt-Nd4*, *mt-Atp6*), lipid metabolism regulators (*Fasn*, *Lpl*), and muscle structural genes (*Tnnt3*, *Ryr1*), highlighting both shared and tissue-specific molecular signatures associated with metabolic response.

CHAPTER FIVE

DISCUSSION, CONCLUSION, RECOMMENDATIONS, AND CONTRIBUTION TO KNOWLEDGE

5.1 Discussion

This study provides the understanding on how metabolic dysfunction develops and how individuals respond differently to treatment in the context of diet-induced insulin resistance. By integrating multi-tissue transcriptomic data, network analysis, predictive modeling, and structural insights, we constructed a systems-level view of the molecular processes underlying metabolic disease.

5.1.1 High-Fat High-Sugar Diet Suppresses Mitochondrial Metabolic Pathways

Our findings demonstrate that a high-fat high-sugar (HFHS) diet induces widespread metabolic reprogramming across skeletal muscle, liver, and adipose tissue. These alterations are characterized by mitochondrial dysfunction, impaired lipid handling, and activation of inflammatory pathways. This finding is consistent with previous reports linking mitochondrial dysfunction to insulin resistance in human and animal models (Patti et al., 2003; Mootha et al., 2003). Impaired mitochondrial metabolism in skeletal muscle may contribute to reduced lipid oxidation and accumulation of lipid intermediates that disrupt insulin signaling (Muoio, 2014).

These effects are not confined to individual tissues but instead reflect coordinated inter-organ responses, supporting the concept that metabolic disease is fundamentally a systemic disorder rather than a collection of isolated tissue-specific defects.

Among the tissues examined, skeletal muscle emerged as a key contributor to metabolic dysregulation (Kelley & Mandarino, 2000). Transcriptional changes in muscle were enriched for

pathways related to energy metabolism, proteostasis, and stress adaptation. In particular, the activation of FOXO-dependent proteolytic and autophagic programs suggests a mechanistic link between insulin resistance and muscle catabolism. This observation reinforces the concept that metabolic stress promotes a shift toward a catabolic state in muscle, contributing to functional decline. Notably, metformin treatment partially reversed several of these transcriptional signatures, indicating restoration of metabolic balance and attenuation of catabolic signaling, although this effect was heterogeneous across individuals (Samuel & Shulman, 2012).

5.1.2 Metformin Partially Restores Metabolic Gene Expression

The analyses reveal that metformin was associated with partial reversal of HFHS-induced transcriptional changes, with restoration of genes involved in mitochondrial metabolism and lipid utilization. These findings are consistent with the widely accepted mechanism of metformin action involving activation of AMP-activated protein kinase (AMPK) and downstream metabolic signaling pathways (Hardie, 2014; Viollet et al., 2012). AMPK activation promotes catabolic processes that generate adenosine triphosphate while inhibiting anabolic processes that consume adenosine triphosphate, thereby restoring cellular energy balance (Hardie, Ross, & Hawley, 2012).

Metformin-sensitive genes were enriched among immune and inflammatory pathways, suggesting that the therapeutic effects of metformin may involve modulation of metabolic inflammation within skeletal muscle. Metformin is known to activate AMPK, which improves insulin sensitivity, enhances mitochondrial metabolism, and reduces inflammatory signaling through inhibition of nuclear factor kappa-B and other pro-inflammatory transcription factors (Hattori et al., 2006; Cameron et al., 2016). The observation that immunoglobulin-related genes including Igkc and Jchain were among the most strongly reversed transcripts suggests that metformin may modulate B cell-related immune responses in skeletal muscle, consistent with emerging evidence that B cells contribute to obesity-induced insulin resistance (Winer et al., 2011; DeFuria et al., 2013).

5.1.3 Sex Differences in Metformin Response

Analysis of sex-specific responses revealed a numerically higher proportion of genes showing reversal patterns in females compared to males (44.7% versus 39.5%), but this difference was not statistically significant ($p = 0.57$). This finding may reflect a more rigorous statistical approach

that accounts for multiple testing and the underlying variability in the data. The absence of significant sex differences in the current analysis may reflect several factors.

First, the study may have been underpowered to detect interaction effects, particularly given the number of genetic backgrounds. Second, the transcriptional response to HFHS diet and metformin may be largely conserved between sexes, with subtle differences that require larger sample sizes to detect. Alternatively, sex differences may be more pronounced in specific cell types or under specific physiological conditions not captured by bulk tissue analysis. Sex hormones including estrogen and testosterone are known to influence metabolic regulation and may contribute to sex-dependent differences in metabolic disease susceptibility (Mauvais-Jarvis, 2018). Future studies employing single-cell transcriptomics with larger sample sizes will be necessary to definitively characterize sex-dependent effects in metabolic tissues.

5.1.4 Cross-Tissue Pathway Analysis

The cross-tissue pathway analysis revealed distinct but interconnected cellular programs across metabolic organs. Skeletal muscle exhibited enrichment for pathways associated with metabolic remodeling and cellular turnover. Adipose tissue displayed prominent inflammatory signaling signatures, consistent with the established role of adipose tissue inflammation in obesity-induced insulin resistance (Lumeng & Saltiel, 2011; Reilly & Saltiel, 2017). The liver showed enrichment of metabolic and transcriptional regulatory processes.

These findings are consistent with previous studies demonstrating that metabolic tissues function as components of an integrated endocrine network rather than isolated organs (Pedersen & Febbraio, 2012; Whitham & Febbraio, 2016). Skeletal muscle acts as an endocrine organ capable of releasing myokines that influence distant tissues, while adipose-derived adipokines and hepatic signaling factors further modulate systemic metabolic regulation (Boström et al., 2012; Kershaw & Flier, 2004).

The coordinated inflammatory signaling observed between adipose tissue and skeletal muscle under HFHS conditions supports the concept of metaflammation, a chronic low-grade inflammatory state that contributes to insulin resistance across multiple tissues (Hotamisligil, 2017). In this model, adipose tissue inflammation serves as a primary driver of systemic metabolic

dysfunction through release of pro-inflammatory cytokines that impair insulin signaling in skeletal muscle and liver (Saltiel & Olefsky, 2017). The enrichment of tumor necrosis factor-alpha signaling via nuclear factor kappa-B in both adipose tissue and skeletal muscle provides a potential mechanistic link between tissue-specific inflammatory responses and systemic metabolic dysfunction.

5.1.5 Mechanisms of High-Fat High-Sugar-Induced Muscle Wasting

The analysis supports a conceptual framework in which HFHS diet is associated with skeletal muscle dysfunction through interconnected metabolic, inflammatory, and proteolytic pathways. Chronic consumption of a HFHS diet promotes systemic metabolic overload, leading to insulin resistance and impaired activation of insulin-dependent anabolic signaling pathways including the phosphoinositide 3-kinase-AKT axis (Schiaffino, Dyar, Ciciliot, Blaauw, & Sandri, 2013).

Consistent with established mechanisms of muscle atrophy, coordinated expression of canonical FOXO target genes including Trim63 encoding MuRF1 and Fbxo32 encoding Atrogin-1 was observed (Bodine et al., 2001; Sandri et al., 2004). These E3 ubiquitin ligases are responsible for targeting muscle proteins for degradation via the ubiquitin-proteasome system. Simultaneously, increased expression of autophagy-related genes suggests activation of the autophagy-lysosome pathway. The simultaneous activation of these two proteolytic systems is a hallmark of muscle atrophy and indicates that HFHS-induced metabolic stress promotes a catabolic transcriptional program consistent with skeletal muscle wasting (Sandri, 2013; Bonaldo & Sandri, 2013).

FOXO transcription factors serve as key regulators of both the ubiquitin-proteasome and autophagy-lysosome pathways during muscle atrophy (Mammucari et al., 2007; Milan et al., 2015). Under conditions of reduced AKT signaling, FOXO factors translocate to the nucleus and activate transcription of atrophy-related genes. The observed upregulation of Foxo1 and Foxo3 in HFHS skeletal muscle is consistent with this regulatory mechanism. The partial reversal of these atrophy-associated genes with metformin suggests that metformin may be associated with protection against muscle wasting through modulation of insulin signaling and FOXO activity.

5.1.6 Candidate Biomarkers of Metformin Responsiveness

The identification of baseline transcriptional signatures associated with metformin responsiveness

represents a hypothesis-generating finding that warrants further investigation. Genes including Cxcl9, Siglecg, Igkc, and Jchain emerged as candidate predictors of treatment response. These genes are primarily associated with immune function, suggesting that baseline immune status may influence metabolic responsiveness to metformin.

This finding aligns with emerging evidence that metformin's therapeutic effects involve immunomodulatory mechanisms (Cameron et al., 2016; Saisho, 2015). The identification of immune-related biomarkers is consistent with the concept that metabolic disease involves interactions between metabolic and immune systems (Hotamisligil, 2017). Cxcl9 is a chemokine involved in T cell recruitment and has been associated with obesity-related metabolic disturbances in humans (Bijnen et al., 2017). Immunoglobulin-related genes including Igkc and Jchain may reflect B cell involvement in metabolic inflammation, consistent with studies demonstrating that B cells promote insulin resistance in obesity (Winer et al., 2011; DeFuria et al., 2013).

We applied machine learning approaches to identify baseline transcriptional features associated with metformin response. While predictive models were able to distinguish responders from non-responders, their performance was modest. Across tissues, receiver operating characteristic (ROC) analysis yielded area under the curve (AUC) values ranging from approximately 0.50 to 0.54, indicating limited discriminatory power. Cross-validation results showed similar trends, with liver-derived models exhibiting slightly higher performance compared to muscle and adipose tissue. Importantly, permutation testing demonstrated that the observed AUC did not significantly exceed random expectation ($p = 0.651$), suggesting that while predictive signal exists, it is weak and may be influenced by noise or cohort-specific effects.

Despite this modest predictive performance, the biological relevance of the identified features remains notable. Feature importance analysis highlighted genes involved in mitochondrial function, lipid metabolism, and immune signaling, including both nuclear-encoded and mitochondrial transcripts. The recurrence of these pathways across tissues suggests that coordinated metabolic and inflammatory processes underpin variability in treatment response. This aligns with the broader hypothesis that the balance between metabolic homeostasis and immune activation plays a central role in determining therapeutic outcomes.

To assess the robustness of these findings, we performed external validation in an independent cohort. Although individual genes did not consistently reach statistical significance—likely due to differences in experimental platforms, genetic backgrounds, and environmental conditions—the overall directionality of expression changes was preserved. The consistency of signals for genes such as CXCL9 and SELL supports the presence of a conserved biological response, even when statistical power is reduced. This highlights an important principle in systems biology: reproducible pathway-level patterns may be more informative than individual gene-level significance.

To further extend these observations, we incorporated molecular docking analysis to explore potential structural interactions between metformin and candidate target proteins. The docking results suggest that metformin can occupy a defined binding pocket and adopt a stable conformation, supported by predicted hydrogen bonding interactions. Although these findings are computational and require experimental validation, they provide a mechanistic framework linking transcriptomic alterations to potential molecular interactions, thereby strengthening the biological plausibility of the observed effects.

Collectively, this study underscores the complexity of metabolic disease as a multi-layered and system-wide process. While predictive modeling revealed only modest classification performance, the integration of multi-tissue transcriptomics, network analysis, and structural modeling provides convergent evidence for the involvement of interconnected metabolic and inflammatory pathways. These findings emphasize that clinically relevant biological insights can emerge even in the absence of strong predictive accuracy, particularly when supported by consistent cross-tissue and cross-cohort patterns. This systems-level perspective is critical for advancing precision medicine, where capturing the interplay between tissues and pathways may ultimately prove more informative than single-gene biomarkers in guiding therapeutic strategies.

5.1.7 Limitations of the Study

Several limitations of this study should be acknowledged. First, the analysis relies on bulk RNA-seq data, which averages gene expression across multiple cell types. This approach may obscure cell-type-specific responses to HFHS diet and metformin treatment. Skeletal muscle contains multiple cell types including myofibers, satellite cells, fibroblasts, endothelial cells, and immune

cells, each of which may respond differently to metabolic stress and pharmacological intervention (Rosa-Caldwell & Greene, 2019). Future studies employing single-cell or single-nucleus RNA-seq would provide higher resolution insights.

Secondly, while transcriptional changes associated with HFHS diet and metformin treatment have been identified, causal relationships cannot be inferred from these observational data. Functional validation studies using genetic manipulation or pharmacological inhibition are necessary to establish mechanistic links between identified genes and metabolic phenotypes.

Thirdly, the cross-tissue communication analysis is limited by the absence of single-cell resolution and the challenge of inferring signaling from bulk tissue expression data. While ligand-receptor expression patterns provide indirect evidence of potential communication, direct validation using techniques such as conditional gene deletion or inter-organ perfusion would be required to establish causal signaling relationships.

Fourthly, the computational docking analysis presented in Section 4.9 is exploratory and does not provide experimental validation of metformin binding. These in silico predictions require experimental confirmation through techniques such as surface plasmon resonance, isothermal titration calorimetry, or structural biology approaches including X-ray crystallography or cryo-electron microscopy.

5.2 Conclusion

In this study, we demonstrate that skeletal muscle dysfunction in response to a high-fat high-sugar (HFHS) diet is not driven by a single pathway, but rather emerges from the integrated effects of metabolic stress, inflammatory signaling, and FOXO-mediated proteolytic activity. Through combined transcriptomic and network analyses, FOXO transcription factors were identified as central regulators linking insulin resistance to the activation of muscle atrophy programs. Concurrently, the identification of metformin-responsive genes highlights molecular pathways that may be therapeutically targeted to mitigate diet-induced muscle loss. However, these findings provide mechanistic insight into the development of metabolic-associated sarcopenia and suggest

potential strategies for preserving skeletal muscle function in the context of metabolic disease.

Beyond these mechanistic insights, our results indicate that response to metformin is influenced by an underlying immune metabolic state. By emphasizing coordinated network behavior rather than isolated gene effects, we show that therapeutic outcomes are shaped by the broader integrity of system-level signaling. The identification of distinct resilient and susceptible phenotypes provides a biologically grounded explanation for inter-individual variability in drug response and underscores the importance of baseline molecular context in determining treatment efficacy.

While predictive modeling enabled the identification of transcriptomic signatures associated with treatment response, model performance was modest, with AUC values close to random classification and permutation testing indicating no statistically significant separation from null expectations. These findings highlight an important limitation: current transcriptomic features alone may be insufficient for robust prediction of therapeutic response. However, rather than diminishing the value of the analysis, this result emphasizes the complexity of metabolic disease and the need for integrative, multi-dimensional approaches that incorporate additional layers of biological information.

Furthermore, despite limited predictive accuracy, consistent pathway-level signals were observed across tissues and datasets, particularly in processes related to mitochondrial function, lipid metabolism, and immune regulation. This suggests that biologically meaningful patterns can persist even when predictive performance is constrained, reinforcing the value of systems-level interpretation over reliance on single-gene biomarkers.

Interestingly, this study supports a shift toward a systems biology framework for understanding metabolic disease, in which coordinated multi-tissue interactions and network dynamics take precedence over isolated molecular events. Although further refinement and validation are required to improve predictive performance, the integration of transcriptomic profiling, network analysis, and computational modeling provides a foundation for more precise and mechanism-driven therapeutic strategies. Ultimately, this work contributes to the advancement of precision medicine by highlighting both the potential and the current limitations of using molecular data to guide individualized treatment in metabolic disorders.

5.3 Recommendations

Based on the findings of this study, the following recommendations are proposed:

1. **Validation Studies**: Future studies should validate the identified candidate biomarkers in independent cohorts, including human populations, to determine their translational relevance for predicting metformin response in clinical settings. Larger, well-powered cohorts will be essential to confirm the observed directional trends.
2. **Single-Cell Transcriptomics**: Single-cell or single-nucleus RNA-seq studies should be employed to resolve cell-type-specific responses to HFHS diet and metformin treatment, potentially revealing cell populations that are particularly responsive to therapeutic intervention.
3. **Longitudinal Analyses**: Longitudinal studies with multiple time points should be conducted to reveal dynamic transcriptional responses during the progression of metabolic disease and following therapeutic intervention, enabling identification of early response markers.
4. **Functional Validation**: Functional studies targeting identified pathways including FOXO signaling and inflammatory pathways should be performed to establish causal relationships between transcriptional changes and metabolic phenotypes using genetic manipulation or pharmacological approaches.
5. **Multi-Omics Integration**: Future research should integrate additional omics layers including proteomics, metabolomics, and epigenomics to provide a more comprehensive understanding of molecular regulation in metabolic adaptation.
6. **Sex-Specific Analyses**: Larger studies with adequate statistical power should be conducted to definitively characterize sex-dependent effects in metabolic tissues and responses to metformin treatment.
7. **Clinical Translation**: The candidate biomarkers identified in this study should be evaluated in clinical cohorts to assess their utility for stratifying patients for metformin therapy and guiding personalized treatment decisions.
8. **Experimental Validation of Structural Findings**: Computational docking predictions should be experimentally validated using biochemical and structural biology approaches to confirm metformin binding interactions.

5.4 Contribution to Knowledge

This study makes several significant contributions to the field of metabolic research:

1. **Systems-Level Understanding**: The study provides a comprehensive systems-level analysis of transcriptional responses to dietary stress and pharmacological intervention across multiple metabolic tissues in genetically diverse mice, revealing coordinated regulatory networks that would remain undetected in single-tissue analyses.
2. **Mechanistic Insights into Muscle Wasting**: The identification of FOXO-dependent proteolytic pathways as key mediators of HFHS-induced muscle wasting provides mechanistic insight into the molecular pathways underlying metabolic-associated sarcopenia.
3. **Metformin Action in Skeletal Muscle**: The demonstration that metformin is associated with partial reversal of HFHS-induced transcriptional changes in skeletal muscle, particularly through suppression of inflammatory genes and restoration of mitochondrial pathways, extends understanding of metformin's mechanism of action beyond its well-characterized hepatic effects.
4. **Candidate Biomarkers for Precision Medicine**: The identification of baseline transcriptional signatures associated with metformin responsiveness, particularly immune-associated genes including Cxcl9, Siglecg, Igkc, and Jchain, provides candidate biomarkers that may enable personalized approaches to type 2 diabetes treatment.
5. **Genetic Diversity Model**: The use of the Collaborative Cross Recombinant Inbred Intercross mouse population demonstrates the value of genetically diverse models for investigating gene-environment interactions and variability in metabolic responses, providing a framework for future studies in metabolic research.
6. **Inter-Organ Communication Framework**: The cross-tissue pathway analysis reveals distinct but interconnected cellular programs across metabolic organs, supporting the concept that metabolic homeostasis is governed by coordinated endocrine communication among multiple organs and providing a framework for investigating inter-organ signaling in metabolic disease.
7. **Sex-dependent Responses**: The finding that sex differences in metformin-associated gene expression changes were not statistically significant contributes to understanding of sex-dependent effects in metabolic disease and highlights the need for adequately powered studies to detect such differences.

8. **External Validation Framework**: The partial validation of candidate biomarkers in an independent dataset provides a model for assessing reproducibility of transcriptomic findings and highlights the importance of validation across cohorts and platforms.
9. **Structural Modeling Insights**: The computational docking analysis provides preliminary structural insights into potential metformin binding interactions, generating hypotheses for future experimental investigation.

5.5 SUMMARY OF FINDINGS

- HFHS diet induces widespread transcriptional remodeling across skeletal muscle, liver, and adipose tissue, reflecting coordinated metabolic dysfunction.
- Metformin partially restores disrupted metabolic and inflammatory pathways, indicating incomplete but measurable therapeutic reversal.
- Inter-organ signaling networks underpin metabolic disease progression, supporting a systems-level rather than tissue-isolated mechanism.
- FOXO signaling emerges as a central axis linking insulin resistance to muscle proteolysis and atrophy, highlighting a mechanistic bridge between metabolic stress and sarcopenia.
- Immune–metabolic gene signatures are associated with variability in metformin response, suggesting that baseline inflammatory state influences therapeutic outcomes (with modest predictive performance).
- Structural modeling predicts that metformin can occupy a defined binding pocket within the target protein, supporting a potential direct interaction.
- A predicted hydrogen bond interaction indicates possible binding stability, providing structural context for a plausible molecular mechanism of action.

Supplementary Tables

Table S1. External validation results

Expression statistics for candidate genes (*CXCL9*, *SELL*, *IGKC*, *ACAP1*) in independent datasets, including p-values and direction of effect.

Table S2. Molecular docking of metformin binding within the target protein

The ligand is accommodated within a defined cavity, indicating a structurally feasible binding pose. (AutoDock vina, PYMOL)

Table S3. Functional enrichment analysis

Gene Ontology (GO) and KEGG pathway enrichment results for differentially expressed genes, including pathway descriptions, gene counts, enrichment scores, and adjusted p-values.

Table S4. Predictive gene features

Ranked list of genes used in machine learning models for prediction of metformin response across tissues, including feature importance scores.

Table S5. Differentially expressed genes across tissues

Comprehensive list of genes significantly altered in skeletal muscle, adipose tissue, and liver under HFHS diet and metformin treatment conditions, including log2 fold change and adjusted p-values (FDR < 0.05).

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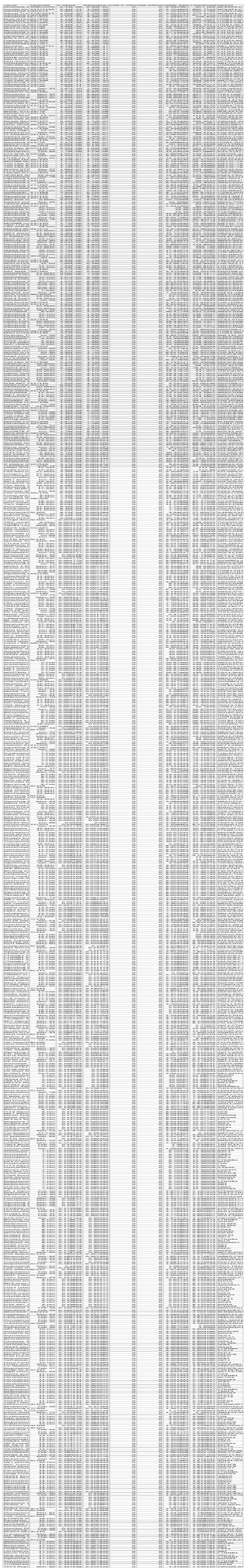
	224166_at	1558304_x_at	208372_s_at	200714_x_at	243366_s_at		219743_at	205612_at	243638_at		241911_at	236756_at	202388_at	232614_at	1553196_a_at	
X.10	840.6	672.9	453.3	3680.3	641.5	1395.0	269.2	395.4	751.2	282.1	143.1	1208.2	60.3	913.8	966.6	407.1
X.9	796.3	147.4	205.8	4818.0	698.5	1265.0	50.3	584.9	1147.9	492.7	221.4	752.2	84.5	932.7	936.8	288.4
X.22	726.9	614.2	113.1	4393.9	801.6	1353.7	56.5	200.7	1012.8	498.4	1014.8	401.4	122.4	907.1	591.0	480.4
X.25	712.0	1112.9	1297.4	3706.3	56.0	1552.4	649.9	812.5	631.3	723.0	1061.8	1226.8	96.9	858.5	662.3	532.5
X.8	679.4	625.6	278.0	3731.1	536.7	1479.6	279.1	361.7	1242.6	134.3	213.1	835.2	50.7	579.5	1990.1	495.6
X.2	629.2	509.9	434.9	3420.5	657.0	1763.3	221.3	407.9	858.7	339.9	752.1	627.6	67.1	311.7	417.2	302.1
X.26	601.9	1204.7	521.2	3696.7	188.6	1917.2	514.6	467.5	319.8	493.8	1451.5	1992.8	96.7	926.7	545.4	123.5
X.15	592.8	579.7	653.3	4568.6	863.6	2404.0	38.1	778.8	1163.6	573.3	1583.3	942.4	48.2	535.4	474.0	488.0
X.17	571.0	353.8	590.7	3433.7	889.2	1408.2	160.3	406.9	787.0	170.2	970.7	703.9	50.6	778.8	596.5	611.2
X.1	565.2	396.3	316.8	2875.4	812.3	1347.3	434.9	325.0	1242.5	458.2	1146.8	388.8	37.7	639.2	421.9	470.2
X.11	543.0	775.8	131.2	3821.4	117.4	1397.1	232.0	71.3	813.8	200.2	483.5	1098.0	63.3	64.5	665.7	381.0
X.5	541.0	615.2	532.7	3742.2	311.0	1314.5	391.2	527.9	1031.4	542.3	1078.2	766.5	74.9	778.8	322.8	410.1
X.7	526.3	581.0	550.8	3623.9	184.8	1937.1	417.0	638.4	700.5	304.0	887.5	671.2	65.3	596.8	364.3	252.1
X.12	518.0	481.8	349.6	3871.9	243.3	996.0	27.2	399.3	619.7	540.0	1044.1	1471.0	64.7	616.6	750.4	455.1

Gene Muscle Table

Adipose Gene Table



Liver Gene Table