From the Department of Physiology and Pharmacology

Karolinska Institutet, Stockholm, Sweden

CIRCULATING FACTORS AFFECTING SKELETAL MUSCLE METABOLISM

Stockholm 2024

Circulating factors affecting skeletal muscle metabolism

Thesis for Licentiate Degree

By

Elena Caria

The thesis will be defended in public at Rockefeller lecture hall, Karolinska Institutet, Stockholm at 13.00 on June 10th 2024.

Principal Supervisor:

Professor Anna Krook
Karolinska Institutet
Department of Physiology and Pharmacology
Section of Integrative Physiology

Co-supervisor(s):

Professor Juleen R. Zierath
Karolinska Institutet
Department of Molecular Medicine
and Surgery
Section of Integrative Physiology

Dr Mutsumi Katayama
Karolinska Institutet
Department of Physiology and Pharmacology
Section of Integrative Physiology

Examination Board:

Docent Rongrong Fan
Karolinska Institutet
Department of Medicine, Huddinge

Docent Sergiu-Bogdan Catrina
Karolinska Institutet
Department of Molecular Medicine
and Surgery
Section of Growth and Metabolism

Dr Susanne Keipert
Stockholm University
Department of Molecular Biosciences
The Wenner-Gren Institute

Popular science summary of the thesis

The muscles in our body serve a purpose far beyond movement. They are integral to our overall health, playing a critical role in managing blood sugar levels through their response to the insulin hormone. However, in type 2 diabetes, this system becomes dysfunctional. Muscles become less responsive to insulin, a condition known as insulin resistance, leading to elevated blood sugar levels and possibly failure of vital organs such as the heart, the kidney and the blood vessels.

Exercise, a powerful tool in the prevention and management of type 2 diabetes, prompts an adaptive response in our muscles, improving their sensitivity to insulin. This enhancement is vital for controlling blood sugar levels and mitigating the risks associated with type 2 diabetes. Understanding how exercise induces these beneficial changes can unlock new strategies to combat insulin resistance.

In this thesis, we want to zoom in on how the muscle responds to circulating factors that are either altered in the context of type 2 diabetes or in response to exercise training. **In study I,** we focus on deciphering the role of glutamine in the modulation of insulin sensitivity. Our findings suggest that higher levels of circulating glutamine might make the muscles more insulin sensitive, likely through different mechanisms. So far, we have identified two key pathways: one involves modulating the muscle's inflammatory response to the disease, and the other involves reducing levels of a protein called GRB10, which is known to inhibit the muscle response to insulin. Other studies have pointed to higher levels of circulating glutamine as protective against the development of type 2 diabetes, so we cannot exclude a future where glutamine is supplemented to help in the prevention or treatment of type 2 diabetes. **In study II**, we focus on a different class of circulating molecules that have been proposed to act as molecular messengers between the cells and the tissues in our body: microRNAs. microRNAs are tiny molecules that act as 'molecular switches' in our cells, turning on and off specific pathways to elicit specific cellular responses. Specifically, we investigated a class of microRNAs that are packed in vesicles. These vesicles protect microRNAs from degradation and are thought to act as vehicles, carrying around microRNAs in the circulation and allowing them to reach their tissue or cell of destination and deliver their message. We found that after three weeks of regular exercise, the levels of certain microRNAs, particularly miR-136-3p, increase in the bloodstream. This specific microRNA has shown a remarkable ability to enhance glucose uptake and improve mitochondrial function in muscle cells, independent of insulin. By studying the pathways regulated by this microRNA, we hope to uncover new mechanisms through which exercise promotes muscle health.

Abstract

The overall aim of this doctoral thesis was to study the role of circulating factors in modulating skeletal muscle metabolism. To this end, we investigated two different scenarios: Type 2 diabetes, which is characterised by impaired skeletal muscle metabolism and reduced insulin sensitivity, and exercise training, which induces various adaptations in skeletal muscle that ultimately improve insulin sensitivity and substrate handling.

Specifically, the aims of the studies presented in the thesis were to determine whether an increase in the circulating levels of the amino acid glutamine improves whole-body and skeletal muscle metabolism and insulin sensitivity **(Study I)**, and whether three weeks of endurance exercise training alters the microRNA cargo of circulating extracellular vesicles **(Study II).**

Study I revealed that increasing circulating levels of the amino acid glutamine improves whole-body glucose homeostasis and skeletal muscle insulin action. This effect was attributed to the modulation of inflammatory gene expression and the downregulation of Growth factor receptor-bound protein (GRB10), an inhibitor of insulin signalling. **Study II** found that three weeks of supervised endurance exercise training changes the microRNA content of serum-derived extracellular vesicles, specifically increasing the content of microRNA miR-136-3p. microRNA miR-136-3p increases glucose uptake, oxygen consumption rate and extracellular acidification rate in human skeletal muscle cells. In skeletal muscle, miR-136-3p directly targets NRDC, an exercise- and inactivity-responsive gene, although the metabolic effects induced by mir-136-3p are not solely mediated through the silencing of NRDC.

List of scientific papers

I. Dollet L, Kuefner M, **Caria E**, Rizo-Roca D, Pendergrast L, Abdelmoez AM, Karlsson HKR, Björnholm M, Dalbram E, Treebak JT, Harada J, Naslund E, Rydén M, Zierath JR, Pillon NJ, Krook A. Glutamine regulates skeletal muscle immunometabolism in Type 2 Diabetes. Diabetes. 2022;71:624-636. doi: 10.2337/db20-0814

II. Katayama M, **Caria E**, Yagüe Sanz A, Barrès R, Caidahl K, Wiklander OPB, El-Andaloussi S, Zierath JR, Krook A. Exercise-training-induced exosomal miR-136-3p modulates mitochondrial function by targeting NRDC in human skeletal muscle. Unpublished, in manuscript.

Contents

List of abbreviations

BCA	Bicinchoninic Acid
BMI	Body Mass Index
CCL2	Chemokine (C-C Motif) Ligand 2
CO_2	Carbon Dioxide
ECL	Enhanced Chemiluminescence
EDL	Extensor Digitorum Longus
EVs	Extracellular Vesicles
FCCP	Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
GRB10	Growth Factor Receptor Bound Protein 10
HCl	Hydrochloric Acid
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
IL6	Interleukin 6
IRS	Insulin Receptor Substrate
miRNA	microRNA
NaOH	Sodium Hydroxide
NRDC	Nardilysin (N-arginine dibasic convertase)
NTA	Nanoparticle Tracking Analysis
PCR	Polymerase Chain Reaction
PI3K	Phosphatidylinositol 3-kinase
PIP3	phosphatidylinositol 4,5-bisphosphate 3
PVDF	Polyvinylidene Fluoride
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
TBS	Tris-buffered Saline

1   Literature review

1.1   Skeletal muscle plays a central role in glucose homeostasis

In healthy individuals, blood glucose concentrations are rigorously maintained within a narrow range of 4-6 mmol/L. This tight regulation is mainly mediated by the counter-regulatory actions of the hormones glucagon, which elevates plasma glucose levels during periods of fasting, and insulin, which facilitates glucose uptake and storage postprandially (1,2).

Skeletal muscle is a primary site of insulin-stimulated glucose disposal. Under hyperinsulinaemic euglycaemic clamp conditions, skeletal muscle accounts for approximately 70-80% of insulin-stimulated glucose uptake in healthy individuals (3). In response to an increase in plasma glucose concentrations, pancreatic β-cells release insulin (4). Insulin action is dependent on the insulin receptor, and although all cells express insulin receptors, in response to increased glycaemia, insulin specifically targets liver to reduce hepatic glucose output, and adipose and skeletal muscle tissue to increase glucose uptake and storage (5–7). This process involves specific signalling events within the muscle fibres (7–11). Specifically, insulin binds to its receptor, a heterotetrameric tyrosine kinase, thereby triggering auto-phosphorylation of its intracellular domain and subsequent phosphorylation of insulin receptor substrates (IRS) proteins, of which the most studied in the context of skeletal muscle metabolism are IRS1 and IRS2 (12,13). Growth factor receptor-binding protein 10 (GRB10) interacts with the phosphorylated insulin receptor, inhibiting the association of downstream effectors, including IRSs, in what appears to serve as a negative feedback regulatory mechanism within the insulin signalling pathway (14,15).

Phosphorylated IRSs act as docking proteins for phosphatidylinositol 3-kinase (PI3K). PI3K is a heterodimeric enzyme composed of a regulatory subunit (p85) and a catalytic subunit (p110). The regulatory subunit of PI3K has Src homology 2 domains that recognise and bind to the phosphorylated tyrosine residues on IRS proteins. When associated with IRS, PI3K undergoes a conformational change that allows the catalytic subunit to convert phosphatidylinositol 4,5-bisphosphate into phosphatidylinositol 3,4,5-trisphosphate (PIP3) in the plasma membrane. PIP3 serves as a second messenger that recruits and activates proteins having PH domains, including phosphoinositide-dependent kinase-1 (PDK1), the mechanistic target of rapamycin complex 2 (mTORC2), and the serine/threonine kinase Akt. Akt, also known as protein kinase B (PKB), is then activated through phosphorylation by PDK1 and mTORC2, on Thr308 and Ser473, respectively. Once activated, Akt - particularly Akt2 - can phosphorylate a wide array of substrates, including TBC1 domain family member 4 (TBC1D4), also known as the Akt substrate of 160 kDa (AS160) (12,16,17). TBC1D4/AS160 is a Rab GTPase-activating protein that, when unphosphorylated, constitutively deactivates a select group of Rab GTPases

involved in GLUT4 vesicle trafficking, by converting them from their active GTP-bound state to their inactive GDP-bound state (18,19). The inhibitory phosphorylation (Thr642) of TBCD14 by Akt removes this inhibitory effect on its target Rab GTPases, allowing their action in facilitating GLUT4 vesicle trafficking to the plasma membrane (20–22). Thus, a key metabolic outcome of insulin signalling within the muscle fibre is the translocation and fusion of intracellular GLUT4 vesicles into the plasma membrane and transverse tubules. Furthermore, AKT-dependent inactivation of glycogen synthase kinase 3 (GSK3) removes the inhibitory phosphorylation of glycogen synthase, increasing the rate at which glucose is stored as glycogen (23).

1.2   Decreased skeletal muscle insulin sensitivity is a hallmark of type 2 diabetes

Diabetes is a chronic disease that leads to disabling and life-threatening complications, ultimately reducing life expectancy and quality of life (24–26). According to the last edition of the IDF diabetes atlas, 536.6 million adults worldwide were affected by diabetes in 2021. This was calculated as 10.5% of the global adult population, with numbers expected to rise to 783.2 million (global prevalence of 12.2%) in 2045 (27). Notably, around 90% of these cases are type 2 diabetes (27).

Diabetes is diagnosed when fasting plasma glucose concentrations are higher than 125 mg/dL (7.0 mmol/L) on two distinct occasions or if glucose levels exceed 200 mg/dL (11.1 mmol/L) following a two-hour oral glucose tolerance test. The pathogenesis of the disease is highly heterogeneous, encompassing various subtypes (28), but typically involves defects in both insulin secretion and insulin action for clinical manifestation to occur (29–32).

Impaired insulin sensitivity, termed insulin resistance, is a hallmark of type 2 diabetes, and is often present before the onset of overt hyperglycaemia (29–32). The molecular basis of skeletal muscle insulin resistance appears to be a post receptor defect and involves alterations in the insulin signalling pathway leading to GLUT4 translocation, although where the primary defect occurs within the insulin signalling pathway remains an open question (32–34). Both defects in proximal insulin signalling, namely at the level of IRS, PI3K and AKT, and in more distal components of the insulin signalling pathway, including AS160 and GSK3, have been described and proposed as the underlying cause of skeletal muscle insulin resistance (7,33).

Obesity and overweight (35), and particularly an accumulation of visceral body fat (36), are well-established risk factors for the development of type 2 diabetes, and often coexist with type 2 diabetes, exacerbating the disease's progression towards its complications (37–39). Defined broadly as a body mass index of 30 Kg/m² or greater (40), obesity is characterised by adipose tissue remodelling and expansion, leading to increases in both fat cell size (hypertrophy) and number (hyperplasia) (41). As obesity progresses, adipose depots expand

beyond tissue's capacity and undergo several pathologic programs that include chronic hypoxia, fibrosis, and chronic low-grade inflammation (42). A paradigm shift in the understanding of metabolic diseases occurred in the 1990s when it was observed that conditioned medium from endotoxin-stimulated macrophages induced insulin resistance in adipocytes (43), suggesting the involvement of immune-derived mediators in modulating insulin action. Tumor necrosis factor alpha (TNFα) was subsequently identified and found to be highly expressed in the adipose tissue of several mouse models of obesity, as well as in people with obesity (44,45). These observations laid the foundations for a field called immunometabolism, which investigates the intersection between immune responses and metabolic processes (46,47). Insights into the signalling events linking obesity, inflammation, and impaired insulin action were provided by the identification of two key molecules downstream of the TNF receptors: the inhibitor of kappa B kinase (IKK) and c-Jun NH2-terminal kinase (JNK) (48,49). These kinases directly phosphorylate key inhibitory residues within insulin receptor substrates (IRS), inhibiting insulin signalling (48,49). Further research demonstrated that macrophage infiltration of adipose tissue in obesity is crucial for inducing whole-body insulin resistance (50), and that inflammation occurs at all metabolically relevant sites (51), including skeletal muscle, contributing to the development and progression of insulin resistance (51).

1.3   Increased skeletal muscle insulin sensitivity is a hallmark of exercise training

Current public health guidelines adopt exercise as a fundamental tool in the prevention, management, and treatment of type 2 diabetes. In part, this is because a single exercise session (acute exercise) increases the utilisation of internal skeletal muscle energy depots and triggers a coordinated response among various tissues to accommodate the increased energy demands of working muscle (52–54).

Skeletal muscle glucose uptake is ≈50 times greater than baseline upon cessation of a single exhaustive bout of endurance exercise at ≈100% of $\dot{V}O_{2max}$ (55). This upregulation of glucose transport is primarily attributed to the insulin-independent translocation of GLUT4-containing vesicles to the sarcolemmal membrane and transverse tubules (56), governed by a complex interplay of various factors, including Ca^{2+} transients, metabolic stimuli, and mechano-transduction (57), with a degree of redundancy between pathways (58).

Exercise also increases skeletal muscle insulin sensitivity through a combination of greater insulin-stimulated perfusion of muscle capillaries (59) and the redistribution of intramuscular GLUT4 into insulin-responsive storage vesicles (60). Such refined vesicular trafficking and subsequent sarcolemmal enrichment of GLUT4 in the post exercise period increases skeletal muscle membrane permeability to glucose upon insulin stimulation (61). These mechanisms combine to sensitise skeletal muscle to insulin for up to 48 hours after exercise cessation,

enhancing glucose delivery while augmenting the capacity for glucose uptake and disposal within the muscle fibre (59).

When the cellular and molecular events initiated by acute exercise are repeated over time, through regular sessions of exercise (exercise training), this lays the foundation for long-term adaptations in skeletal muscle (53). Such adaptations establish a new functional threshold for muscle, enhancing metabolism, exercise capacity and performance, while conferring health benefits such as increased peripheral insulin sensitivity and overall metabolic flexibility (52–54,62).

Training-induced improvements in muscle oxidative capacity often corresponds to the expansion and modification of the mitochondrial proteome (63–65) in type I and type II fibres (66). Resistance training can increase mitochondrial respiratory capacity (67), but the overall impact of this training modality on mitochondrial phenotype is frequently less potent than observed after high-intensity interval (63,65,67) and endurance (66) training. The greater effect of cardiorespiratory-type exercise on mitochondrial biogenesis could be related to the additive effects of these exercise bouts on the nuclear localisation of regulators of mitochondrial transcription (such as, PGC1α1 and transcription factor EB (TFEB)) (68) and the upregulation of mitochondrial ribosomes (63,65,66) and mitochondrial protein synthesis (65).

Consistent exercise training further augments the protein content of sarcoplasmic and mitochondrial enzymes involved in fatty acid, malate/aspartate shuttle, and glucose metabolic pathways in both type I and type II fibres (66). For example, lactate dehydrogenase B (LDHB) is increased after periods of either endurance (69) or resistance (70) training. LDHB has greater affinity for the lactate to pyruvate reaction and, alongside improved mitochondrial density, LDH tetramers with a higher abundance of the LDHB isoform could enhance lactate clearance in trained muscle via more efficient conversion to pyruvate for subsequent oxidation (69) . Further suggestion of metabolic refinement in skeletal muscle with regular exercise can be seen through the increased expression of key proteins involved in glycogen synthesis (71), NAD+ biosynthesis and branched-chain amino acid degradation, as well as ubiquinone biosynthesis (5-demethoxyubiquinone hydroxylase) (64).

Acute exercise typically results in the enrichment of various metabolites, proteins, and nucleic acids within the circulation. These molecules, collectively termed ´exercise factors´ or ´exerkines´, are at the forefront of current scientific inquiry due to their presumed roles in coordinating the adaptive responses of individual tissues with whole-body physiology (72). Beyond the immediate changes induced by exercise, the baseline profile of these circulating exercise factors may be modified by regular exercise training.

1.4 Skeletal muscle metabolic adaptations to circulating factors

Skeletal muscle displays remarkable plasticity, defined as the ability to adapt and remodel in response to a variety of internal and external stimuli. This characteristic is essential for muscle function and overall health, enabling the muscle to respond to environmental cues such as changes in nutrient availability, physical activity levels, and energy demands. The adaptive response of skeletal muscle is often coordinated by a diverse array of circulating factors. Skeletal muscle responds to circulating hormones such as insulin and insulin-like growth factors, as well as to cytokines, metabolites, and nucleic acids. Notably, the levels of these circulating factors can vary in response to physiological stimuli like exercise or due to pathological states, such as type 2 diabetes, triggering adaptive and maladaptive processes within the muscle fibre.

1.4.1 Circulating amino acids and metabolism

Recent advances in metabolomics have significantly contributed to understanding type 2 diabetes by identifying biomarkers and revealing the metabolic pathways involved in disease development and progression (73,74). Particularly, obesity and type 2 diabetes are associated with altered levels of certain amino acids in the blood (73,75–79). The circulating levels of branched-chain amino acids, namely leucin, isoleucine and valine, have been shown elevated ahead of clinical manifestation (78,80,81), as well as in overt type 2 diabetes, suggesting a role of these amino acids in the pathogenesis of the disease. Later research has shown a dysregulation in the catabolism of branched-chain amino acids in various metabolic tissues, including skeletal muscle (77,82), linking it to the pathogenesis of insulin resistance (77,83,84). Conversely, modifying the circulating levels of these amino acid appears to improve metabolic health. For example, lowering circulating branched-chain amino acids levels by inhibiting the branched-chain ketoacid dehydrogenase kinase (BDK) or by overexpression of the protein phosphatase, Mg^{2+}/Mn^{2+} dependent 1K (PPM1K) in the liver has been shown to improve glucose tolerance in rats (85). Similarly, an increase in the uptake and oxidation of mitochondrial branched-chain amino acids in brown adipose tissue enhances their clearance from the circulation in mice. Conversely, when branched-chain amino acids catabolism in brown adipose tissue is defective, it results in impaired clearance of these amino acids, which leads to the development of diet-induced obesity and glucose intolerance (86). Elevated circulating levels of the aromatic amino acids phenylalanine and tyrosine, and lower levels of glycine, have also been linked to type 2 diabetes (78,87,88); whether they have a causal role in the pathogenesis of the disease has yet to be fully elucidated, but changes in their circulating concentrations are thought to be indirect markers of insulin sensitivity (89). Glutamine and glutamate, important in various cellular processes like the TCA cycle and epigenetic regulation, show changed levels in type 2 diabetes. High plasma glutamate levels were associated with increased risk of type 2 diabetes, while higher glutamine levels and a

better glutamine-to-glutamate ratio were protective against the disease (87,88,90,91). White adipose tissue plays a crucial role in maintaining this ratio by releasing glutamine and absorbing glutamate, suggesting that disruptions in this process in obesity might lead to metabolic diseases. In an untargeted analysis of polar metabolites released from white adipose tissue of obese female individuals, glutamine was one of the most significantly altered metabolites in obesity (92). In the same study, glutamine was found to modulate the expression of proinflammatory pathways in white adipocytes (92). In macrophages, alpha-ketoglutarate was shown to promote the anti-inflammatory M2 activation via epigenetic reprogramming of genes, while also suppressing pro-inflammatory responses in M1 macrophages by inhibiting the NF-KB pathway (93).

1.4.2 Circulating miRNAs and metabolism

1.4.2.1 *miRNAs are post-transcriptional regulators of gene expression*

MicroRNAs (miRNAs) are small, non-coding RNA molecules that mainly act intracellularly as post-transcriptional regulators of gene expression (94–96). miRNAs are initially transcribed from DNA by RNA polymerase II as part of a longer primary transcript known as pri-miRNA, which is then processed into a shorter precursor miRNA (pre-miRNA) by the RNase III-type endonuclease Drosha (97,98). This pre-miRNA is exported to the cytoplasm by exportin 5 (99), and it is further processed by RNase III-type endonuclease Dicer into a short double-stranded RNA molecule known as the miRNA duplex, typically around 22 nucleotides long (94–96). This duplex consists of two strands: the mature miRNA strand (guide strand) and the passenger strand (miRNA* or star strand). The miRNA duplex is then unwound by RNA helicases, and the individual miRNA strands are separated and incorporated into the RNA-induced silencing complex (RISC) (94,96). The miRNA within RISC pairs with complementary sequences on target messenger RNA (mRNA) molecules. This pairing typically occurs at the 3' untranslated region (3' UTR) of the mRNA, although miRNA binding can also occur at other regions (100). The binding of miRNA to mRNA does not usually result in perfect base pairing. Imperfect pairing allows a single miRNA to bind to multiple different mRNA targets, not limited to perfectly complementary sequences. This versatility means that miRNAs can regulate a broad network of genes and participate in multiple cellular processes or pathways simultaneously. At the same time, this provides redundancy in gene regulation. Several miRNAs can target the same mRNA, and a single miRNA can target multiple mRNAs involved in similar pathways. This redundancy helps ensure robust gene expression, even if one pathway component is altered. Lastly, the degree of complementarity between a miRNA and its target can affect the mechanism of gene silencing; whether it leads to mRNA degradation or translational repression (101). While both mechanisms ultimately lead to lower protein levels of miRNA targets, this versatility permits a single miRNA to exert variable effects across different cellular contexts. Such adaptability enhances the precision of gene expression regulation, allowing

cells to dynamically adjust to fluctuating environmental and physiological conditions in key tissues such as muscle.

Muscle cells are enriched in the so-called myomiRs miR-1, -133a, -133b, -206, -208a, -208b, -486, and -499, which have a well-established role in skeletal muscle development (102,103). Indeed, skeletal muscle-specific Dicer knockout mice die perinatally and are characterised by skeletal muscle hypoplasia (104,105). Several studies have also highlighted the roles of a number of miRNAs in the regulation of metabolic pathways in skeletal muscle (106,107). For example, miRNA let-7 inhibits multiple components of the insulin signal transduction pathway, including the insulin receptor and IRS2 in mouse muscle (108,109). Members of the miR-29 members, specifically miR-29a and miR-29c, are increased in skeletal muscle of people with type 2 diabetes, and negatively regulate insulin-stimulated glucose uptake, glycogen synthesis and hexokinase activity, as well as lipid oxidation (110). miR-126 regulates angiogenesis, at least in part, by repressing the PI3K regulatory subunit (111). miR-486, -21, -26a, -216a, and -217 appear to positively influence PI3K/Akt signalling by targeting PTEN for inhibition (112–114). miR-20b regulates insulin-stimulated glycogen synthesis via AKT signalling (115).

The emerging roles of miRNAs in the regulation of skeletal muscle metabolism and remodelling have prompted several research groups to study these molecules in the context of exercise metabolism, the main hypothesis being that exercise alters the expression of miRNAs as a means to regulate pathways involved in exercise adaptations. An acute bout of endurance exercise increases the expression of Drosha, Dicer and Exportin-5 in vastus lateralis biopsies of healthy male individuals (116), suggesting that miRNA-mediated post-transcriptional regulation is needed for post-exercise adaptations in skeletal muscle. Similarly, the expression of various miRNAs in skeletal muscle is altered by acute exercise or exercise training (116–121). Some of the exercise-responsive miRNAs have been validated to regulate substrate metabolism in skeletal muscle and mediate the adaptive response to acute exercise or training. For example, miR-19b-3p was increased after two weeks of endurance training in healthy males and regulates glucose metabolism in mouse and human skeletal muscle (122). miR-29c expression is decreased in the skeletal muscle of endurance-trained rats and also in human skeletal muscle after 14 days of endurance exercise training. This miRNA negatively regulates insulin-stimulated glucose metabolism and lipid oxidation in healthy male individuals (110). Acute endurance exercise has been reported to influence the expression of miRNA species, such as miR-23 and miR-696, which are involved in regulating the expression of PGC-1α (123). Targeting of the PGC-1 family of transcriptional co-activators has wide implications in the context of exercise metabolism considering their roles in mitochondrial biogenesis, insulin sensitivity and glucose homeostasis. In general, these examples highlight

the function of miRNAs in the regulation of skeletal muscle metabolism and exercise adaptations.

1.4.2.3 miRNAs as bioactive cargoes of extracellular vesicles

Besides acting intracellularly, miRNAs can be secreted into the circulation as the cargo of small bilipid membrane vesicles named extracellular vesicles (EVs) (124). The release of EVs from cells is being increasingly appreciated as an important mechanism of intercellular communication (125–128). The term "extracellular vesicle" is used to address diverse subpopulations that can differ in size, morphology, composition and biogenesis. Classically, these subpopulations consist of exosomes and ectosomes, also known as microvesicles. Exosomes are typically 50–150 nm in size and originate from the endosomal pathway within the cell. They are formed as intraluminal vesicles within multivesicular bodies and are released into the extracellular space when the multivesicular bodies fuse with the plasma membrane. Ectosomes are typically 50–1000 nm in size and are generated through external budding of the plasma membrane (125,126,128).

EVs carry proteins, lipids, and nucleic acids, and have been found in different body fluids, including blood/serum/plasma, as well as in the interstitial space of different tissues, suggesting a role in both endocrine and autocrine/paracrine signalling. For miRNAs especially, these vesicles protect the EV-miRNA in the circulation when en-route to the target cells. There is a growing amount of evidence supporting the ability of EVs to transfer their miRNA cargoes to recipient cells in a way that significantly affects their functionality (124). For example, exosomes derived from adipose tissue macrophages of obese mice induce glucose intolerance and insulin resistance when transplanted into lean mice (129). Conversely, exosomes derived from adipose tissue macrophages of lean mice improve glucose tolerance and insulin sensitivity when administered to obese recipients. This appears to be mediated by their miRNA cargo, specifically miR-155, which is overexpressed in the exosomes derived from the adipose tissue macrophages of obese mice (129). Similarly, adipose tissue secretes exosomes containing microRNAs capable of regulating gene expression in the liver (130). EV trafficking, therefore, represents an important biological process, which has been shown to mediate metabolic effects.

Some studies found that EVs become enriched in number in the circulation in response to acute exercise or exercise training (131–136), although other studies could not recapitulate these findings (137–139). EV profiling studies also found changes in the EV cargoes in response to acute exercise and training. Specifically, EV-associated miRNA profiling revealed a transient increase in several miRNAs after acute exercise (131,134,135,138,140). Collectively, these observations in the field have led to speculation about potential roles of EV-associated miRNAs as 'exerkines' in mediating exercise-induced metabolic adaptations.

In conclusion, skeletal muscle responds to a variety of circulating factors, including amino acids and EV-associated miRNAs, which can elicit profound metabolic effects. The levels of these circulating factors can be altered under conditions of disease such type 2 diabetes, possibly contributing to the progression of the disease, as well as in response to exercise, possibly contributing to exercise-induced metabolic adaptations.

2 Research aims

The overall aim of this thesis was to study the role of circulating factors in the context of skeletal muscle metabolism. To this end, we investigated two different scenarios; Type 2 diabetes, which is characterised by impaired skeletal muscle metabolism and reduced insulin sensitivity, and exercise training, which is associated with various metabolic adaptations that ultimately improve skeletal muscle insulin sensitivity and substrate handling.

Specifically, the aims of the two studies presented in the thesis were to determine:

1. Will an increase in the circulating levels of the amino acid glutamine improve whole body and skeletal muscle metabolism/insulin action?

2. Will three weeks of exercise training alter the miRNA cargo of circulating extracellular vesicles?

3. Does exercise-induced circulating miRNA miR-136-3p play a role in the metabolic adaptations of skeletal muscle to exercise?

3   Materials and methods

3.1   Ethical considerations

Study I was conducted with the approval of the Regional Ethics Committee of Stockholm, Sweden. This study involved obtaining blood samples and muscle biopsies from human participants. All participants were fully informed of the risks associated with the sample collection, and written consent forms were obtained in accordance with the Declaration of Helsinki. The cohort included participants with type 2 diabetes, who stood to benefit first-hand from the research conducted.

Study I also involved animal experiments, which were approved by the Stockholm North Animal Ethical Committee (Stockholm, Sweden). The animals were housed in groups of up to four, had access to food and water *ad libitum*, and were provided with nesting and basic enrichment materials.

Some of the animals were placed on a high-fat diet, which resulted in significant weight gain and the development of insulin resistance. This is an established model commonly used in diabetes research. The increased body weight typically leads to reduced locomotive activity and could lower the quality of life for the animals. The mice were monitored regularly to ensure that they showed no signs of severe pain and that their suffering remained below the humane endpoints, beyond which we would need to euthanise them. The potential to uncover new knowledge that could benefit individuals with type 2 diabetes was considered sufficient justification for the potential suffering of the mice.

Study II was conducted with the approval of the Regional Ethics Committee of Stockholm, Sweden. Informed consent was obtained from all participants in accordance with the Declaration of Helsinki. We recruited both male and female participants, an inclusive approach that ensures the research outcomes are applicable to a broader segment of the population. However, the limited sample size restricted our ability to discern sex-specific differences, which is a limitation of our research.

Studies I and II also included work on satellite cells obtained from muscle biopsies from human volunteers. These cells were obtained with the approval of the regional ethics committee of Stockholm, Sweden. All the participants were informed of the risks involved with the procedure of obtaining skeletal muscle biopsies and written informed consents were obtained from all the participants according to the declaration of Helsinki. The use of skeletal muscle satellite cells allows us to study the molecular mechanisms of skeletal muscle metabolism and reduces our dependence on animal models, which is consistent with the 3Rs principles.

3.2   Human cohorts

Study I is a part of a larger cross-sectional observational study designed for assessing the influence of type 2 diabetes on the plasma and skeletal muscle metabolome of study participants (82). Specifically, the subset used in study I consists of 14 males with normal glucose tolerance and 19 males with type 2 diabetes. Clinical characteristics of the study participants are presented in **Table 1.** Plasma and *vastus lateralis* skeletal muscle biopsies were collected after an overnight fast. Participants with type 2 diabetes, who were treated daily with metformin and/or sulfonylurea, were instructed to delay taking their medications until all the sampling procedures were concluded. This delay, along with the requirement for an overnight fast, aimed to avoid the immediate effects of both medications and recent dietary intake on metabolite levels. This approach ensures that the study measures the chronic, rather than acute, metabolic state associated with T2D.

Clinical parameters	Lean NGT	Lean T2D	Overweight NGT	Overweight T2D	OWE effect	T2D effect
n size	7	6	7	13	-	-
Age (years)	57.3 ± 9.5	65.3 ± 2.7	61.4 ± 10.2	63.6 ± 6.1	p=0.721	p=0.097
BMI [kg/m^2]	24.1 ± 0.58	24.6 ± 0.36	27.2 ± 1.65	27.6 ± 1.2	p<0.001***	p=0.321
W/H ratio	0.91 ± 0.04	0.98 ± 0.06	0.94 ± 0.05	0.99 ± 0.06	p=0.296	p=0.007**
fP-glucose [mmol/L]	5.27 ± 0.4	8.42 ± 2.28	5.36 ± 0.32	8.77 ± 1.76	P=0.679	p<0.001***
S-insulin [pmol/L]	40.1 ± 15.4	44.2 ± 17	64.8 ± 17.8	85.9 ± 37.5	p=0.002**	p=0.193
HbA1c [mmol/mol]	34 ± 5.32	51 ± 6.13	38.1 ± 2.54	51.9 ± 6.42	0.241	p<0.001***
HOMA-IR	1.61 ± 0.5	3.03 ± 1.48	2.27 ± 0.73	5.51 ± 2.92	p=0.033*	p=0.002**
S-C-peptide [nmol/L]	0.59 ± 0.12	0.69 ± 0.15	0.72 ± 0.131	0.99 ± 0.40	p=0.035*	p=0.057
P-TG [mmol/L]	0.89 ± 0.33	1.19 ± 0.44	1.21 ± 0.71	1.47 ± 0.78	p=0.212	p=0.240
P-Chol [mmol/L]	4.94 ± 0.53	4.35 ± 0.85	5.56 ± 0.67	4.65 ± 0.76	p=0.099	p=0.005**
P-HDL [mmol/L]	1.24 ± 0.13	1.42 ± 0.21	1.36 ± 0.26	1.21 ± 0.312	p=0.523	p=0.869
P-LDL [mmol/L]	3.3 ± 0.54	2.43 ± 0.76	3.67 ± 0.44	2.77 ± 0.83	p=0.174	p=0.001**

Table 1. Clinical characteristics of participants in Study I: Data are presented as mean ± SEM. Effects of BMI and type 2 diabetes (T2D) were measured by two-way ANOVA. *P < 0.05. **P < 0.01. ***P < 0.001. FPG, fasting plasma glucose; TG, triglyceride; W/H, waist to hip.

Study II used a pre-post experimental design to identify training-responsive, EV-associated miRNAs. 12 healthy volunteers who had not been involved in any formal exercise training for more than 2 hours per week for at least two years. This is a sub-cohort of a previous study (141). Participants underwent three weeks of supervised endurance training consisting of two sessions per day, six days a week. All participants were assessed with the same battery of assessments at baseline and 24 hours after the completion of the intervention (**Table 2**). To extract extracellular vesicles, serum samples collected at baseline and 24 hours post-intervention were used.

	Baseline	After training
n	12	12
Sex (M, F)	(4,8)	(4,8)
Age (y)	26 ± 0.7	26 ± 0.7
Body weight (kg)	66.4 ± 3.3	66.1 ± 3.2
BMI (kg/m^2)	22.6 ± 0.6	22.5 ± 0.6
Fat mass (kg)	16.2 ± 1.8	15.7 ± 1.8
Fat mass (%)	24.3 ± 2.2	23.7 ± 2.2
Lean mass (kg)	50.2 ± 2.9	50.4 ± 2.9
HbA1c (%)	4.3 ± 0.1	4.2 ± 0.1
VO$_2$max (l/min)	2.96 ± 0.28	3.33 ± 0.26**
VO$_2$max (ml/kg/min)	44.32 ± 3.08	50.39 ± 2.91**
VO$_2$@RER$_{1.0}$ (l/min)	2.16 ± 0.16	2.47 ± 0.25*
RER$_{1.0}$ (% VO$_2$max)	73.99 ± 2.16	73.36 ± 2.08
CK-MB (ng/ml)	3.1 ± 0.4	3.7 ± 0.3**
Insulin	270.8 ± 50.48	285.9 ± 0.23
Leptin	16587.9 ± 3717.77	29.4 ± 0.23**

Table 2. Anthropometric and clinical characteristics of subjects in study II: Data were analysed using paired t-test and are represented as mean ± SEM. *P < 0.05 and ** P < 0.01. BMI, Body Mass Index; HbA1c, glycated haemoglobin; VO2max, oxygen uptake at peak exercise; RER1.0, respiratory exchange ratio; VO2max@RER1.0, oxygen uptake at RER=1.0; CK-MB, creatine kinase

3.3 Isolation of extracellular vesicles

EVs were isolated from serum using a precipitation-based commercial kit (miRCURY Exosome Isolation Kit – Serum and Plasma, Exiqon, Vedbaek, Denmark). The process involves mixing the samples with a polymer solution. The polymer, being hydrophilic, interacts with the water molecules in the solution, reducing the solubility of EVs. As a result, EVs aggregate and precipitate out of the solution, and can be collected by a simple low-speed centrifugation. This results in a sample that contains a mixture of different types of EVs, including exosomes, ectosomes and possibly other particles like protein aggregates. Because this method does not selectively isolate exosomes based on their size, density, or specific surface markers, the

resulting preparation is referred to as "EVs" or "exosome-enriched EVs" rather than purely exosomal.

3.4 Nanoparticle tracking analysis (NTA)

EV sample preparations were then analysed using Nanoparticle Tracking Analysis (NTA). NTA provides information about the size distribution of particles in the sample. As exosomes and other small EVs typically range from 30 to 150 nm in diameter, NTA confirmed that a significant portion of the particles in the preparation fell within this size range. Specifically, samples were loaded into the sample chamber of an NS500 unit (NanoSight, Amesbury, U.K.), and five 1-min videos of each sample were recorded. The size and concentration of particles was calculated using NTA 2.3 software (NanoSight).

3.5 RNA extraction

Different RNA methods were used depending on sample and scope.

For extraction of RNA from EV sample preparations, miRCURY RNA Isolation Kit (Exiqon) was used. This kit is specifically designed for the extraction of total RNA, including microRNA and other small RNAs. An RNA Spike-in Template Kit (Exiqon) was used to monitor the efficiency of the RNA extraction process and to provide a reference for quantitative assays. The use of MS2 RNA (Roche, Basel, Switzerland) as carrier RNA helped by providing more material for the extraction reagents to bind to, thereby reducing the loss of the small RNA molecules that are the actual targets of the study.

For extraction of total RNA from skeletal muscle tissue samples, TRIzol extraction method was used. Lastly, the E.Z.N.A. Total RNA Kit (Omega Bio-tek) was used to extract total RNA from human skeletal muscle cells in study I, while miRNeasy mini kit (Qiagen) was used in study II to guarantee extraction of total RNA including miRNAs. RNA concentration was determined by spectrophotometry. cDNA was synthesised from 0.5-1 µg RNA using the High-Capacity cDNA Reverse Transcription kit according to manufacturer's instructions.

3.6 Animal experiments

Study I employed a total of three mouse experiments, all using mice fed a high-fat diet as a model of diet-induced obesity and insulin resistance. Beginning at 6 (Experiment I and II) or 8 (Experiment III) weeks of age, mice were placed on either a standard chow diet (CD), comprising 4% of calories from fat, or a high-fat diet (HFD) consisting of 60% calories from fat. The diet was maintained until termination, for a total period of 15 weeks (Experiment I and II) or 17 weeks (Experiment III). Starting 14 days before termination, the HFD-fed mice were randomized to receive daily intraperitoneal injections of either glutamine (1 g/kg body weight) or PBS (20 mL/kg body weight). CD-fed mice were also injected with PBS (20 mL/kg

body weight) to serve as a control for the HFD effects. Body composition was determined in conscious mice using the EchoMRI-100 system (Echo Medical Systems). On day 15, glycaemia was measured after 4 hours of fasting using a glucometer (One Touch Ultra 2 Glucose Meter; LifeScan), and animals were then euthanized under general anesthaesia by avertin injection. Plasma insulin levels were quantified using the Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem). To assess insulin tolerance (experiment II), mice were fasted for 4 hours and the rate of glucose disappearance was measured between 0 and 15 minutes after intravenous injection of insulin (0.75 UI/Kg body weight). To assess glucose tolerance (experiment III), mice were fasted for 4 hours and glycaemia was monitored for 150 minutes after intraperitoneal injection of glucose (1.5 g/kg body weight). *Extensor digitorum longus*, *soleus* and *quadriceps* muscle were dissected and snap-frozen in liquid nitrogen for *ex vivo* insulin signalling, RT-qPCR and microarray analyses.

3.7 Cell experiments

Primary cells were isolated from *vastus lateralis* skeletal muscle biopsies derived from healthy volunteers. After biopsies were obtained using the open muscle biopsy method, satellite cells were isolated and cultures were established based on protocols described elsewhere (142). In brief, biopsies were cleaned from residual connective and fat tissues and digested in a solution containing collagenase IV, trypsin, bovine serum albumin (BSA), and 1% penicillin/streptomycin in Ham's F-10 medium. Undigested tissue was allowed to settle, and the supernatant containing liberated cells, including satellite cells, was collected and centrifuged for 10 min at 350 g. The cell pellet was resuspended in media and incubated in a non-coated (bacteriological) petri dish for 1 hour to selectively promote adherence of non-myogenic cells. The supernatant was then transferred into culture flasks, and cells were grown to confluence (80%) before the first trypsinisation and subculture step. This first flask (after the first trypsinisation) was marked 'passage 1'. For cell experiments reported in Study I and II, vials at passage 3-5 were thawed and expanded in DMEM containing 20% FBS and 1% anti-anti. Once the number of cells required for experiments was reached, myoblasts were grown to 100% confluence before inducing differentiation by switching to media containing 2% FBS. Insulin and apotransferrin were supplemented in the differentiation media to aid the fusion process. Myotube formation was monitored under the microscope and cells were harvested or used for terminal experiments ten days after the initiation of differentiation. In the high/low glutamine experiments in Study I, myotubes were exposed to media containing either 0.5 or 10 mmol/L final concentration glutamine (Life Technologies) beginning at day 8 of differentiation for a total of 48 hours. In the glutaminase 1 inhibitor experiment in study I, cells were treated with BPTES at 10 μmol/L final concentration (Sigma-Aldrich, SML0601) for 24 hours prior to harvest. In the LPS experiments in Study I, LPS (Sigma-Aldrich, L2630) was added to the cell medium at a concentration of 100 ng/mL for 24 h prior to harvest. For the silencing experiments in Study I, myotubes were transfected on day 6 and 8 of differentiation

with either a negative control siRNA (Ambion Silencer Select Negative Control #2, 4390847) or an siRNA against GRB10 (Ambion Silencer Select siRNA, s6125), at 10 mmol/L final concentration. For the miR-136-3p transfection and NRDC silencing experiments in Study II, the same double transfection protocol on day 6 and day 8 of differentiation was chosen when the readout was metabolism.

3.8 Metabolic profiling of cells

3.8.1 Glucose Uptake

Myotubes were starved for 4 hours in no-serum, low-glucose DMEM (5.5 mM glucose as opposed to 25 mM; no. 21885; Gibco) prior to 60-minute incubation in the absence (basal) or presence of insulin at a maximal concentration (120 nmol/L). Cells were washed with PBS and incubated for 15 minutes in glucose- and serum-free DMEM (no. 11966; Gibco) containing radiolabeled 2-[1,2-3H]deoxy-d-glucose (1 mCi/mL and 80.7 Ci/mmol; MT911; Moravek) and unlabeled 2-deoxy-d-glucose (10 µmol/L). Cell monolayers were washed three times with ice-cold PBS and lysed in 0.03% SDS. The cell lysate was counted in a liquid scintillation counter, and results were normalised to total protein content/well, as measured using BCA Protein Assay Kit (no. 23225; Thermo Fisher Scientific, Rockford, IL).

3.8.2 Glycogen synthesis

After a 4 hour serum starvation in low-glucose DMEM, myotubes were pre-incubated in the absence (basal) or presence of a maximal concentration of insulin (120 nM) for 30 minutes before adding 1 µL/well of radiolabelled d-[U-14C] glucose (1 mCi/mL and 250 mCi/mmol; no. NEC042B005MC; PerkinElmer, Waltham, MA) for 90 minutes. Cell monolayers were washed three times with ice-cold PBS and lysed in 0.03% SDS. Carrier glycogen (no. G0885; Merck) was added to the cell lysates (2 mg/sample) and samples were heated to 99°C for 1 hour. Addition of 95% ethanol and subsequent overnight incubation at -20C allowed the precipitation of glycogen, which was then collected in the form of pellet through centrifugation for 15 minutes at 10,000 g and resuspended in distilled water. Radioactivity was counted in a liquid scintillation counter and normalized to total protein content per well.

3.8.3 Glucose oxidation

Myotubes were first serum-starved for 4 hours in low-glucose DMEM, followed by a 4-hour incubation in fresh serum-free DMEM containing 5.5 mM unlabeled glucose and 2 mCi/mL d-[U-14C] glucose. After incubation, a small cup containing 0.5 M NaOH was carefully placed in each well. The plates were then sealed with a plastic seal, and 2 M HCl was injected into the medium using a needle syringe to capture the CO_2 released by the cells into the NaOH. The [14C]-CO_2 trapped in the NaOH was measured using a liquid scintillation counter. Cells were washed with PBS and lysed in 0.5 M NaOH. The pH was neutralized by adding 2 M HCl, which

facilitated protein quantification via the BCA assay. The results were later normalized based on the protein content.

3.8.4 Fatty acid oxidation

Palmitate oxidation was determined by incubating cells with a mix of 25 µM non-labeled palmitic acid and [9,10-3H]palmitic acid for 6 hours. The amount of $3H_2O$ released into culture media was determined by scintillation counting, and results were reported relative to total cellular protein content.

3.8.5 Protein synthesis

After a 4-hour serum starvation in low-glucose DMEM (1 g/L, cat.no #21885), myotubes were incubated with puromycin (1 µM final concentration) for 30 minutes before collection. Cells were then washed once in ice-cold PBS and harvested in protein lysis buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1% Triton X-100, 10% glycerol, 20 mM Tris pH 7.8, 10 mM NaF, 1 mM EDTA, 1 mM PMSF, 0.5 mM Na_3VO_4 and 1x PIC). Lysates were gently rotated for 30 min at 4°C before being subjected to centrifugation (12,000 g for 15 min at 4°C). Supernatants were then stored at -80°C until protein quantification via BCA assay and subsequent sample preparation for immunoblot analysis.

3.8.6 Lactate assay

Lactate released by cells into culture medium was measured using a colourimetric assay. Cell medium was filtered through 3 kDa using centrifugation (14000 g; 15 min at 4°C), and 50 µL of sample was mixed with 150 µL of assay buffer (Tris (50 mM pH 8), NAD (7.5 mM), N-methylphenazonium methyl sulfate (250 µM), p-iodonitrotetrazolium violet (500 µM), and lactate dehydrogenase (4 U.mL-1)). Samples were incubated for 20 minutes, after which absorbance was read at 490 nm.

3.9 Microarray analysis

Microarray analysis in Study I was performed using the Affymetrix GeneChip Human Transcriptome Array 2.0 and the mouse Clariom D array, both from Thermo Fisher Scientific. In Study II, the GeneChip Human Gene 2.0 ST arrays from Thermo Fisher Scientific were used.

3.10 Immunoblotting

Extensor digitorum longus (EDL) and soleus muscles were incubated for 20 minutes in Krebs-Henseleit buffer solution under continuous gassing (95% oxygen/5% carbon dioxide) at 30°C in the absence (basal) or presence of insulin (0.36 nmol/L, Actrapid; Novo Nordisk). Muscles were then snap-frozen in liquid nitrogen and kept at -80C until further processing. To extract protein, tissues and cells were lysed in ice-cold protein lysis buffer (137 mM NaCl, 2.7 mM KCl,

1 mM MgCl2, 1% Triton X-100, 10% glycerol, 20 mM Tris pH 7.8, 10 mM NaF, 1 mM EDTA, 1 mM PMSF, 0.5 mM Na3VO4 and 1x PIC) using a tissue lyser and a manual scraper, respectively. The protein content of lysates was determined using BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein were diluted in Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl) and separated on SDS-PAGE (#3450124, Criterion XT Precast gels, BioRad, Hercules, CA, US). Protein was transferred to a polyvinyl fluoride (PVDF) membrane (IPVH00010, Immobilon-P, Merck Millipore), and Ponceau S staining (#P7170, Sigma-Aldrich, St. Louis, MO, USA) was utilised to verify the uniform loading of samples and to serve as a quality control measure for the transfer process. Results were normalised to total protein per lane. Membranes were blocked using 5% (w/v) nonfat dry milk in Tris-buffered saline (TBS) with Tween 20 prior to incubation in primary antibody dilutions overnight (~16 h) at 4°C under gentle agitation. Species-appropriate horseradish peroxidase-conjugated secondary antibodies, diluted 1:25,000 (v/v) in 5% nonfat dried milk in TBS-Tween were used, and protein amounts were visualized using chemiluminescence (#RPN2106 ECL and #RPN2235 ECL select, GE Healthcare, Chicago, IL, US).

3.11 Statistical analysis

Normality of the data was assessed using the Shapiro–Wilk test. When the data were confirmed to be normally distributed, appropriate parametric tests were selected based on the experimental design. For experiments involving two groups and one parameter, a t-test was employed; a dependent t-test was used for paired data, and an independent t-test was used for unpaired data. For designs that included more than two groups, a one-way ANOVA was utilised to compare group means. When the experimental design included two variables, a two-way ANOVA was used to explore both individual and interactive effects of the factors. When the data did not follow a normal distribution, non-parametric equivalents of these tests were used instead. The Spearman correlation test was applied to explore relationships between metabolite levels and clinical parameters in Study I.

4 Results

4.1 STUDY I: Glutamine regulates skeletal muscle immunometabolism

4.1.1 Plasma glutamine levels are associated with BMI and HOMA-IR

We analysed the plasma and skeletal muscle metabolome of 14 males with normal glucose tolerance and 19 males with T2D. Plasma and skeletal muscle metabolites were correlated with clinical parameters associated with adiposity or dysregulated glucose and lipid metabolism. This analysis found that plasma glutamine levels were inversely associated with BMI (Figure 1A and Supplemental figure 2A), HOMA-IR (Figure 1B and Supplemental figure 2A), serum insulin (Supplemental figure 2A), and serum C-peptide levels (Supplemental figure 2A). HOMA-IR and serum C-peptide also correlated negatively with skeletal muscle glutamine levels (Supplemental figure 2A). Plasma glutamine level showed a trend to decrease in people with T2D (p=0.06, Supplemental figure 1H), and stratification of study participants based on BMI revealed that plasma glutamine levels were decreased specifically in individuals who are overweight (BMI, 25.1 – 29.9 kg.m2) and have T2D, but not in individuals with a normal BMI (18.5 – 25 kg.m2) and T2D diagnosis (Figure 1E). Subsequent stratification of participants based on quartiles of plasma glutamine levels revealed that the individuals having the highest glutamine levels had the lowest BMI and HOMA-IR, with a progressively higher BMI and HOMA-IR observed in descending quartiles (Figure 1J-K). Based on these associations, we hypothesised a role of glutamine in protecting against overweight-induced insulin resistance.

4.1.2 Glutamine treatment reverses the adverse effects of a high fat diet on glucose metabolism in mice

To assess whether the modulation of circulating glutamine levels would affect whole-body glucose homeostasis, we injected glutamine in mice fed a high-fat diet. The administration of a high-fat diet for 15-17 weeks resulted in an increase of body weight (Figure 2B), adiposity (Figure 2C), glycaemia (Figure 2D), plasma insulin levels (Figure 2E), and HOMA-IR (Figure 2F), along with an impaired glucose tolerance (Figure 2G-H) and impaired insulin sensitivity (Figure 2I-J). After 13-15 weeks, the high fat diet group was randomised into receiving PBS or glutamine injections for two weeks prior to termination. Daily administration of glutamine was able to reverse or prevent the high fat diet effects on glycaemia (Figure 2D), while also ameliorating those on plasma insulin (Figure 2E), HOMA-IR (Figure 2F), glucose tolerance (Figure 2G-H) and peripheral insulin sensitivity (Figure 2I-J). This was independent of any effect on adiposity as body weight and fat mass were not altered by glutamine treatment (Figure 2B-C). Possibly accounting for the improved glucose homeostasis, the levels of phosphorylated, inactive AS160 upon insulin-stimulation were higher in the skeletal muscle of glutamine-treated, high fat diet-fed mice (Figure 2M). Of note, this effect on skeletal

muscle insulin signalling was seen in EDL muscle, but not in soleus muscle (Supplemental figure 3C), possibly suggesting a fibre-type specificity in the response to glutamine.

4.1.3 Glutamine attenuates inflammatory gene expression in the skeletal muscle of mice fed a high fat diet

To investigate the molecular changes that occur in skeletal muscle in response to glutamine treatment, microarray analysis was conducted on quadriceps muscle from mice. 1272 genes were differentially expressed in high fat diet-fed, PBS-treated mice as compared to chow-fed, PBS-treated mice (Figure 3D). 1269 genes were differentially expressed in high fat diet-fed, glutamine-treated mice as compared to chow-fed, PBS-treated mice (Figure 3D). Of note, only 625 of these genes were overlapping, suggesting that a 2-week glutamine treatment altered the skeletal muscle transcriptome of mice fed a high fat diet (Figure 3D). Furthermore, the fold change of the genes altered between the high fat diet-fed, glutamine-treated and high fat diet-fed, PBS-treated conditions correlated inversely with the fold change of the genes altered between high fat diet-fed PBS-treated and chow-fed PBS treated mice (r=0.7, p=1.2e-08), suggesting the glutamine treatment could reverse some of the high fat diet-induced changes on the skeletal muscle transcriptome (Figure 3F). Among the 51 differentially expressed genes between the high fat diet-fed, glutamine-treated mice and the high fat diet-fed, PBS-treated controls, the majority were downregulated (Figure 3C). Pathway enrichment analysis identified a significant enrichment of these downregulated genes in inflammatory pathways (Figure 3E). Subsequent qPCR for cytokines and markers of immune cell infiltration showed that inflammation was attenuated in glutamine-treated, high fat diet-fed mice (Figure 3G).

4.1.4 GRB-10 expression is regulated by glutamine

We next correlated the fold changes of genes that were altered between the glutamine-treated mice and their high fat diet-fed controls, with the fold changes of the genes that were differentially expressed between the high and low quartile of glutamine in our human cohort (Figure 4A). This analysis facilitated the identification of a subset of genes that were responding similarly, in humans and mice, to higher circulating glutamine levels. Of these genes that responded similarly, GRB10 transcript level was lower in the skeletal muscle of high fat diet-fed, glutamine-treated mice (Figure 4D), and in the skeletal muscle of participants belonging to the highest quartile of plasma glutamine concentration (Figure 4C). GRB10 is an adaptor protein that interacts with the insulin tyrosine kinase receptor (143). Silencing of GRB10 gene was shown to increase insulin-stimulated glucose uptake, as well as insulin-stimulated AKT phosphorylation in cultured human skeletal muscle cells (144). To understand whether these were just associations or whether glutamine levels could indeed regulate GRB10 expression, we exposed human myotubes to culture media supplemented with high or low glutamine levels, in the absence or presence of a glutaminase inhibitor

(BPTES). GRB10 expression was decreased in cells exposed to high extracellular levels of glutamine compared to low, and this was reversed when BPTES was added to the medium (Figure 5G).

4.1.5 CCL2 and IL6 expression is regulated by extracellular glutamine levels in primary human myotubes

In light of the microarray results pointing to inflammatory pathways being downregulated in response to glutamine administration (Figure 3), and in light of the existing literature linking glutamine to cytokine production in immune cells and adipocytes (92,93), we exposed human primary skeletal muscle cells to high or low extracellular glutamine levels during 8 days differentiation and assessed gene expression of CCL2 and IL6. Both CCL2 and IL6 transcripts were lower when the cells were exposed to higher glutamine concentrations (Figure 5B-C). CCL2 and IL6 release into cell culture media followed a similar then (p=0.06 for both) as measured by ELISA (Supplemental figure 3F-G). Exposure to BPTES reversed the effect on IL6 but not CCL2 (Figure 5E-F).

4.1.6 The effects of glutamine on GRB10 and inflammatory gene expression are not interrelated

Skeletal muscle inflammation and insulin resistance are interconnected in the context of obesity and type 2 diabetes (51), with chronic low-grade inflammation contributing at least partly to the development of insulin resistance in skeletal muscle. We hypothesised that the effect of glutamine on GRB10 expression might be secondary to its impact on inflammation. To test our hypothesis, we exposed human skeletal muscle cells to a well-established inflammatory stimulus, namely LPS. As expected, LPS-treatment markedly increased the expression of CCL2 and IL6 (Figure 4G-H), but did not affect GRB10 expression (Figure 4I), suggesting that changes in inflammatory pathways likely do not play a role in GRB10 transcriptional regulation. Furthermore, silencing of GRB10 in human skeletal muscle cells did not affect the LPS-induced upregulation of IL6 and CCL2 (Figure 4J-K). These results suggest that effects of glutamine on GRB10 expression and on the expression of inflammatory genes CCL2 and IL6 are likely regulated independently.

4.2 STUDY II:

4.2.1 miR-136-3p is increased in serum extracellular vesicles after training

Figure 1 provides an outline of the study design used for Study II
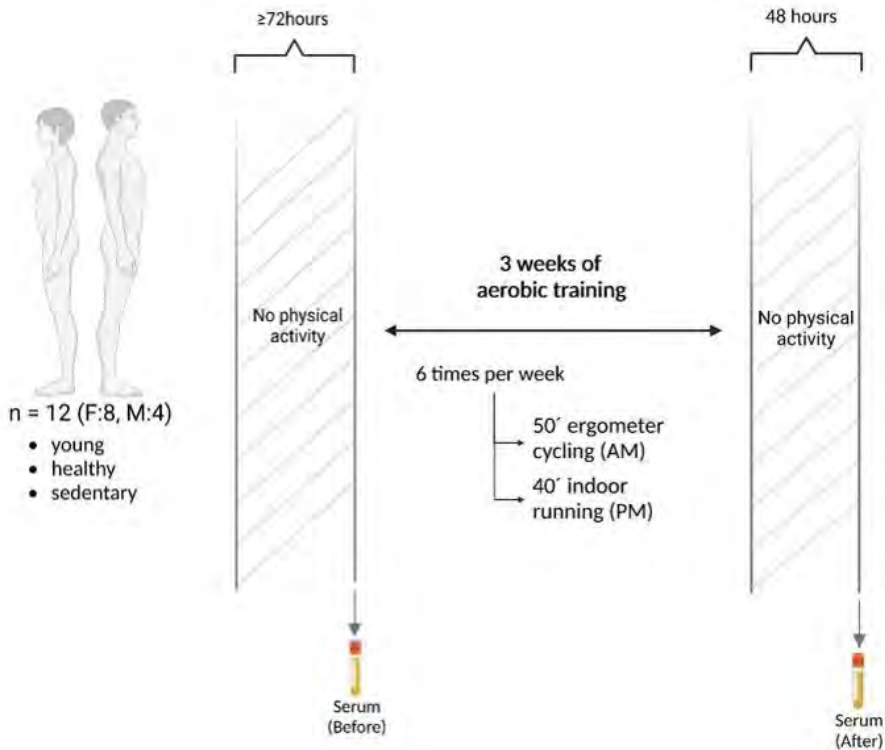

Figure 1: Outline of intervention and sample collection in study II

To assess whether our training intervention resulted in changes in the circulating levels of EV-associated miRNAs, we first used a pre-designed miRNA-focused qPCR panel on a select group of four participants. This allowed us to screen for 179 miRNAs whose expression has previously been reported in human serum or plasma. This initial screening identified miR-136-3p, miR-28-5p, miR-208a-3p, miR-139-5p, miR-92b-3p, miR-375, miR-1, and miR-421 as miRNAs that were differentially expressed following training (Table 3). We then proceeded to determine the expression of these miRNAs in our whole cohort, using targeted miRNA assays. This analysis confirmed that the expression of miR-136-3p, miR-139-5p, and miR-1 was increased in serum EVs after training (Table 3). miR-136-3p was prioritised for more in-depth downstream analyses.

4.2.2   miR-136-3p in extracellular vesicles can enter cultured myotubes

We next incubated human myotubes with serum EVs loaded with Cy3-labelled miR-136-3p, and fluorescence imaging confirmed the presence of Cy3-miR-136-3p throughout the cytoplasm (Figure 2A). Notably, fluorescence was absent in cells exposed to unencapsulated Cy3-miR-136-3p (data not shown), indicating that EVs are essential for the delivery of miR-136-3p into human myotubes. However, similar effectiveness in delivery was observed for EVs derived HEK293 culture medium (Figure 2B). To test whether the miRNA sequence itself directed this targeting, we introduced EVs containing a TexasRed-labeled control RNA with a sequence different from miR-136-3p (TexasRed-control RNA, Figure 2C), and observed uptake comparable to that of miR-136-3p. This suggests that the delivery of miRNAs into myotubes via EVs is efficient but does not depend on the origin of the EVs or the miRNA sequence.

4.2.3   miR-136-3p targets NRDC in human skeletal muscle cells

To identify potential targets of miR-136-3p in human skeletal muscle, human myotubes were transfected with either a miR-136-3p mimic or a negative control miRNA mimic, followed by microarray analysis. Since miRNAs primarily function by repressing gene expression, we focused on the downregulated genes as potential direct targets of miR-136-3p. Ten candidate genes were selected based on fold change (Table 4). Out of these, five had a predicted binding site for miR-136-3p. Among these genes, NRDC was enriched in human skeletal muscle (Figure 3A), and was markedly downregulated at both transcript and protein levels upon transfection with miR-136-3p in primary human myotubes (Figure 3D-E). To determine whether NRDC is a direct target of miR-136-3p, we employed a luciferase reporter assay. We constructed a luciferase reporter vector where the sequence of interest of NRDC mRNA, which contains the predicted miR-136-3p binding sites, was cloned downstream of the luciferase gene. This plasmid was then transfected into HEK293 cells, alongside either the miR-136-3p mimic or a control miRNA mimic. The activity of the luciferase enzyme was measured as luminescence. The decrease in luminescence observed upon transfection with miR-136-3p, coupled with a dose-dependent restoration of luminescence when a miR-136-3p inhibitor is added, confirms the direct targeting of NRDC by miR-136-3p (Figure 3C).

4.2.4   NRDC is altered in response to training and inactivity in human skeletal muscle

We then explored whether the interaction we observed *in vitro,* where miR-136-3p targets NRDC, would be practically relevant in the context of the skeletal muscle response to exercise training. We did not have access to skeletal muscle biopsies to directly test this hypothesis in our cohort. However, we interrogated the MetaMEx database, a comprehensive resource for meta-analysis of skeletal muscle transcriptomic responses to diverse modes of acute exercise and exercise training, as well as inactivity (145). We selected datasets with study designs and cohort characteristics that matched our study. We found that both six weeks of endurance

training (Figure 4A) and two months of endurance training led to a reduction in NRDC expression in human skeletal muscle (Figure 4B). Additionally, NRDC expression was increased in human skeletal muscle following 14 days of limb immobilisation. These results suggest that the regulation of NRDC by miR-136-3p observed in-vitro may indeed have physiological implications for how skeletal muscle adapts to training.

4.2.5 miR-136-3p transfection, but not NRDC silencing, increases glucose uptake in human myotubes

Next, we investigated the potential roles of miR-136-3p and NRDC in modulating the metabolic response of skeletal muscle to exercise. Primary human myotubes were transfected with either a miR-136-3p mimic or a siRNA against NRDC, along with their respective controls. We then performed a battery of metabolic assays to evaluate substrate metabolism. Specifically, we used radiolabelled substrate assays to assess glucose uptake, glucose oxidation, glycogen synthesis, fatty acid oxidation. Notably, miR-136-3p transfection resulted in increased glucose uptake in the absence of insulin stimulation (Figure 5A), whereas no changes were observed in glucose oxidation (Figure 5B), glycogen synthesis (Figure 5C), and fatty acid oxidation (Figure 5D). Lactate release was also not altered by miR-136-3p transfection (Figure 5F), as measured using a colorimetric assay. To assess protein synthesis, we utilised the SUnSET assay, which involves the measurement of puromycin incorporation into newly synthesised proteins. The results indicated that miR-136-3p transfection did not alter protein synthesis (Figure 5E). Additionally, we included insulin-stimulated conditions to determine the effect of miR-136-3p on the insulin responsiveness of these cells (data not shown), and found that miR-136-3p did not alter insulin-stimulated substrate metabolism.

4.2.6 Both miR-136-3p transfection and NRDC silencing increase extracellular acidification rate

Endurance training increases mitochondrial content and capacity in human skeletal muscle. To assess whether miR-136-3p and NRDC could play a role in mediating such adaptations, we transfected human primary myotubes with either a miR-136-3p mimic or a siRNA against NRDC, along with their respective controls. Transfection with miR-136-3p led to an increase in basal and maximal oxygen consumption rate, whereas NRDC silencing had no effect (Figure 6A-C). Notably, both miR-136-3p transfection and NRDC increased extracellular acidification rate (Figure B).

5 DISCUSSION

In Study I, we explored the role of glutamine in the context of skeletal muscle insulin resistance. We found that plasma glutamine levels correlated with BMI and HOMA-IR in a cohort of male individuals with normal glucose tolerance or type 2 diabetes. Using an in-vivo model of diet-induced obesity and insulin resistance, we show that a systemic increase in circulating glutamine levels improves insulin sensitivity and restores glucose homeostasis. At the skeletal muscle level, we found that glutamine regulates the expression of genes involved in inflammatory pathways and the insulin response. Mechanistically, we determined that the metabolism of glutamine by glutaminase is necessary for regulating IL6 and GRB10 gene expression, but not for CCL2.

Our comparative analysis of plasma and skeletal muscle metabolic profiles aimed to uncover potential contributors to the progression of skeletal muscle insulin resistance in type 2 diabetes and to identify novel metabolic pathways that may be disrupted in this context. We analysed metabolomic datasets from males with either normal glucose tolerance or type 2 diabetes to identify metabolites that exhibit significant associations with clinical indicators of metabolic dysregulation. Plasma glutamine and glutamate showed inverse associations with BMI and HOMA-IR, in line with existing literature showing that a high glutamine-to-glutamate ratio is associated with reduced risk of incident type 2 diabetes (146) and insulin sensitivity in obese patients (92). This association, along with the observation that circulating glutamine levels were selectively altered in participants with both overweight and T2D diagnosis, prompted us to investigate glutamine in the context of obesity-induced insulin resistance. Skeletal muscle is a primary site of insulin action and glutamine is primarily synthesised in skeletal muscle (147). We observed a mild positive correlation between intramuscular and plasma glutamine (r = 0.37, p < 0.01). However, the plasma differences between normal and overweight individuals with T2D did not translate in the muscle, with intramuscular glutamine levels correlated with HOMA-IR but not with BMI and showing no changes across groups after stratification of participants based on BMI and only a trend to a decrease in the participants with type 2 diabetes before stratification. This may be due, at least in part, to the narrow range of BMI in our cohort, with a mean BMI of 24.6 and 27.6 kg/m^2 for the normal and overweight individuals, respectively, with several participants within one point of the normal/overweight cut-off. Muscle tissue might have more robust mechanisms to maintain its glutamine levels, possibly due to its role in glutamine synthesis and storage (147), making the detection of differences in muscle glutamine levels more challenging with the low n-size after stratification. It is also possible that other tissues contribute to the more pronounced differences in glutamine concentration in plasma. For example, glutamine metabolism has been shown to be dysregulated in adipose tissue in obese female individuals (92,148).

In this study, we observed that two weeks of glutamine treatment are sufficient to reverse the deleterious effects of a high-fat diet on whole-body glucose homeostasis and insulin sensitivity in mice. We did not directly measure insulin-stimulated glucose transport in skeletal muscle, but the enhanced insulin signalling leading to GLUT4 translocation in EDL muscle suggests that skeletal muscle contributes, at least partly, to the improvements we measured in systemic glucose metabolism following glutamine treatment. Of note, insulin signalling was not affected by glutamine treatment in *soleus* muscle. This may be due to a fibretype specificity in glutamine uptake and accumulation (149). We further identified GRB10, an inhibitor of insulin signalling (143), as a glutamine-responsive gene in skeletal muscle. GRB10 expression is lower both in *vastus lateralis* muscle of participants with higher circulating glutamine and in *gastrocnemius* muscle of glutamine-treated mice. In parallel, exposure of primary human skeletal muscle cells to high glutamine levels led to a decrease in GRB10 gene expression. This was reversed by the addition of a glutaminase inhibitor, BPTES, indicating that the effect on GRB10 expression is indeed mediated by glutamine through its metabolism. GRB10 is an adaptor protein that interacts with the insulin receptor through its SH2 and PH domains (143). Global deletion of Grb10 in mice leads to a phenotype characterised by improved glucose homeostasis and peripheral insulin sensitivity (15,150–152). Muscle-specific Grb10-deficient mice exhibit enhanced insulin signalling at the level of phospho-Akt and increased skeletal muscle glucose uptake (15). In human skeletal muscle cells, knocking down Grb10 enhances insulin-induced PI3K/Akt signalling and glucose uptake (144). Based on this literature, we concluded that the glutamine-induced downregulation of GRB10 gene expression may have contributed, at least partly, to the enhanced insulin signalling in the skeletal muscle of our glutamine-treated mice. Altogether, our findings suggest a mechanistic link between glutamine metabolism and the modulation of insulin signalling pathways, mediated by changes in GRB10 expression.

In study II, we explored the molecular adaptations induced by endurance training, focusing on the role of extracellular vesicles and their miRNA cargo. While the diameter and serum concentration of these vesicles remained unchanged, we found that their miRNA composition was altered following aerobic exercise training. Specifically, we found increased levels of miR-1-3p, miR-139-5p, and miR-136-3p. We hypothesized that skeletal muscle might be one of the target tissues of EV-associated miR-136-3p, and determined that cultured primary human myotubes can incorporate miR-136-3p packed in extracellular vesicles. We further identified NRDC as a direct target of miR-136-3p in human skeletal muscle cells, which is likely of practical relevance in the context of training adaptations as NRDC transcript is downregulated in human skeletal muscle in response to aerobic training. We further investigated the metabolic roles of miR-136-3p in primary human myotubes and found that miR-136-3p increases glucose uptake independent of insulin, and increases mitochondrial oxygen consumption rate. Overexpression of miR-136-3p and silencing of NRDC increase ECAR, which

can be indicative of a change in cellular metabolism potentially through impacting mitochondrial function.

Our training intervention resulted in no change in the size or concentration of circulating EVs. The question of whether exercise leads to an increase in the release of EVs into the circulation remains a subject of debate in the current literature (153). The initial observation that exercise would lead to an increased number of circulating EVs led to speculations about their role in facilitating cell-to-cell communication to orchestrate the body's integrated response to exercise. Subsequent studies have provided mixed results; this variability could stem from differences in exercise intensity, duration, and the fitness levels of participants. Additionally, the methods used to isolate and characterise EVs vary widely, potentially affecting the consistency of results across studies. The temporal sampling points post-exercise could influence the observed levels of circulating EVs (137), as the release and clearance dynamics of EVs may change rapidly and lead to substantial discrepancies in results. This is of particular relevance in training studies, where the time between the last exercise bout and sampling can complicate distinguishing between the acute responses to exercise and the new baseline established by chronic training. Further, individual biological variability, such as age, sex, and metabolic health status, might modulate the EV response to exercise (154), leading to different results observed depending on the cohort examined. Of note, more research on extracellular vesicles has shown that the number of extracellular vesicles, albeit indicative, is not the main determinant for functional effects (128). A difference in their cargo alone has proven sufficient to suggest that they may be released with specific functional roles within a certain physiological or pathological context. Indeed, we found enrichment of extracellular vesicle-associated miRNAs, namely miR-1-3p, miR-139-5p, and miR-136-3p. miR-139p-5p and miR-136-3p have not previously been shown to be exercise-responsive, although have been highlighted as a biomarker for major depressive disorder and cancer (155–157). This highlights a potential functional subpopulation of extracellular vesicles in serum.

We hypothesised skeletal muscle could be a target tissue of miR-136-3p, and we showed that myotubes can incorporate labelled miR-136-3p packed in extracellular vesicles. This is in line with similar uptake studies that show that extracellular vesicles are readily uptaken by target cells. However, this method simplifies the uptake in-vivo, because other cells might be more prone to uptake the extracellular vesicle-associated miR-136-3p. Similar studies have used an in-vitro approach by using a co-culture method (158,159).In general, in-vivo experiments have demonstrated that the liver is the main target of extracellular vesicles (136,160–162), however this may stem from the role of liver in the clearance of extracellular vesicles, rather than from functional targeting of liver, and it is unknown if this is the case for the miR-136-3p extracellular vesicles. Future experiments with in-vivo administered extracellular vesicles could further elaborate.

We also showed that miR-136-3p directly targets NRDC in skeletal muscle cells. NRDC is a zinc peptidase that is involved in different cellular processes. In metabolic tissues, it has been shown to be involved in the maintenance of body thermogenesis in brown adipose tissue (163) and to regulate glucose-stimulated insulin secretion in the pancreas (164). In skeletal muscle NRDC is enriched, however the function of NRDC is unknown. Following exercise, in the MetaMex database, NRDC expression in skeletal muscle was decreased in response to aerobic exercise training and increased in response to inactivity. Potentially, an explanation of this phenotype could be due to miR-136-3p following exercise. However, previous research has shown that miR-136-3p is not increased following exercise in skeletal muscle (122). Therefore, it could be that external miR-136-3p is responsible for the change in NRDC, however further studies are required.

Lastly, this study found that miR-136-3p may play a role in the regulation of energy metabolism in human primary myotubes. Specifically, miR-136-3p transfection results in increased glucose uptake independently of insulin, similar to what happens in-vivo in skeletal muscle with exercise and training. miR-136-3p transfection also results in an increase in both basal and maximal oxygen consumption rates, indicating enhanced mitochondrial respiration. Neither the increase in glucose uptake nor the increase in oxygen consumption rate could be recapitulated by NRDC silencing in the same model of primary human myotubes, suggesting that the miR-136-3p mediates these processes independent from NRDC. Conversely, both miR-136-3p transfection and NRDC silencing result in increased extracellular acidification rates, possibly indicating increased glycolytic flux. However, lactate release into the cell culture medium is not affected by either condition. Overall, our findings indicate that the metabolic changes triggered by miR-136-3p are not solely due to its silencing effect on NRDC. This suggests that additional pathways are involved in the metabolic adaptations driven by miR-136-3p. In line with the biology of miRNAs, which typically target multiple mRNAs to regulate various pathways, these results prompt further research into the metabolic roles of miR-136-3p.

In conclusion, this thesis examines the molecular mechanisms regulating insulin sensitivity in muscle and how these are influenced by circulating factors, integrating muscle tissue physiology. In Study I, we highlight a role for the amino acid glutamine in improving whole body insulin sensitivity. In Study II, we explored the molecular adaptations induced by endurance training, focusing on the role of exosome-enriched extracellular vesicles and their miRNA cargo. In this context of increased insulin sensitivity, we found that miR-136-3p enhances glucose uptake without altering the insulin response of skeletal muscle cells.

6   Conclusions

Based on the data presented in this thesis we conclude:

1. Increasing circulating levels of the amino acid glutamine improves whole body glucose homeostasis and skeletal muscle insulin action.

2. Three weeks of supervised endurance exercise training changes the abundance of extracellular vesicle-associated miRNAs, specifically increasing the content of miRNA miR-136-3p.

3. microRNA miR-136-3p increases skeletal muscle glucose uptake. miRNA miR-136-3p directly targets expression of NRDC in human skeletal muscle, although this does not mediate miR-136-3p effects on glucose uptake.

7 Points of perspective

This thesis has explored the roles of circulating factors, particularly glutamine and miR-136-3p, in modulating skeletal muscle metabolism in the contexts of type 2 diabetes and exercise training. The findings provide evidence that these factors influence muscle physiology, suggesting potential pathways that may be of clinical relevance. However, further research is needed to fully understand and utilise these pathways for clinical benefit.

Further research could aim to understand whether glutamine levels are decreased in the skeletal muscle of people with type 2 diabetes, which would enhance the physiological relevance of our in-vitro and in-vivo findings. Given that both our results and others suggest a role for BMI, measuring glutamine in muscle biopsies from a larger cohort of patients— including individuals with a larger gap in BMI and with or without type 2 diabetes— could definitively answer whether skeletal muscle fibres in-vivo are exposed to lower levels of extracellular glutamine. Moreover, exploring the causes of lower circulating glutamine levels might reveal whether skeletal muscle is a driver of these plasma differences. If glutamine metabolism is dysregulated in skeletal muscle, the clinical implications of this study could extend beyond glutamine supplementation to uncover pathways that can be targeted pharmacologically to improve skeletal muscle insulin sensitivity. Our study was limited to measuring mRNA levels of GLUL and GLS in muscle biopsies at baseline. Although the transcriptome of skeletal muscle does not differ significantly between people with type 2 diabetes and those with normal glucose tolerance (82,165), the physiology of muscle and how it handles metabolic challenges, such as a meal or exercise, does undergo pathologic changes that are a hallmark of the disease (32). Including analyses that provide information on glutamine metabolism in skeletal muscle in-vivo in patients with type 2 diabetes both at baseline and after a metabolic challenge could unmask such dysregulations. For example, integrating studies of metabolic flux could be highly informative. While our study provides insights into the basal levels of glutamine in individuals with type 2 diabetes, which are informative for understanding the metabolic milieu to which tissues are chronically exposed, it is limited to static measurements of metabolites at a single point in time. Metabolic flux analysis would allow us to observe the rates at which glutamine is produced and utilised within skeletal muscle. This dynamic approach can uncover how glutamine metabolism responds to physiological stresses such as exercise or a nutritional challenge. If human data conclude that dysregulation of glutamine metabolism is indeed a hallmark of type 2 diabetes, integrating these data with in-vitro and in-vivo studies—where key enzymes in glutamine metabolism like GLUL or GLS are knocked out, silenced, or overexpressed specifically in skeletal muscle cells or in mouse skeletal muscle—would help understand the consequences of this metabolic dysregulation on insulin sensitivity and whole-body energy metabolism.

In study II, our findings indicate that while miR-136-3p facilitates an increase in insulin-independent glucose uptake and increases oxygen consumption rate, these effects are largely independent of NRDC. These effects are likely mediated through additional targets that have not been explored. Furthermore, despite increased glucose uptake and oxygen consumption rate, glucose oxidation remained unchanged, prompting questions about the metabolic fate of the glucose. This disconnect suggests that miR-136-3p likely activates additional pathways that we have not been fully explored yet. Further investigation into the pathways regulated by miR-136-3p is needed to fully understand its role in the regulation of energy metabolism. Moreover, studying miR-136-3p in other contexts, such as type 2 diabetes, could reveal whether the pathways it regulates have implications in metabolic health.

The observed increase in extracellular acidification rate upon silencing NRDC in human skeletal muscle cells highlights a potentially significant metabolic role for NRDC that merits further exploration. Notably, NRDC is enriched in skeletal muscle and responds dynamically to physiological stimuli such as exercise training and periods of inactivity. This responsiveness suggests that NRDC could play a critical role in skeletal muscle homeostasis. The development of animal models, specifically mice with a skeletal muscle-specific deletion of NRDC, could be help understanding the functional roles on NRDC is skeletal muscle.

Studying the origin and the biodistribution of EV-associated miR-136-3p following training could increase the relevance of our in-vitro findings and bridge them with in-vivo scenarios. We show that skeletal muscle cells can incorporate EV-associated miR-136-3p, which in turn induces phenotypic changes in these cells. Additionally, NRDC is downregulated in training studies which suggests both that the targeting of NRDC by miR-136-3p and the targeting of muscle by miR-136-3p could indeed be a physiologically relevant response to training. However, investigating the mechanisms regulating the release of miR-136-3p in-vivo in response to training, identifying the specific tissues involved, and understanding the factors that govern miR-136-3p loading into EVs could deepen our understanding of training adaptations, as well as their effects on skeletal muscle metabolism and insulin sensitivity.

8 Acknowledgements

To my Supervisors, Anna, Juleen, and Mutsumi, thank you for the opportunity to be a PhD student in your lab and for your guidance throughout this journey.

To past and present members of the Integrative Physiology Group, working with you has been both inspiring and fun. Thank you for what you have taught me, thank you for your support and for every word of encouragement, thank you for every laugh!

To Christian, to my family, to my friends, I hope you don´t need this page to know how much you mean to me. Your presence makes all the difference. Thank you for your endless support in everything I do.

9 References

1. Bansal P, Wang Q. Insulin as a physiological modulator of glucagon secretion. Am J Physiol Endocrinol Metab. 2008 Oct;295(4):E751-761.

2. Norton L, Shannon C, Gastaldelli A, DeFronzo RA. Insulin: The master regulator of glucose metabolism. Metabolism. 2022 Apr;129:155142.

3. DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP. The Effect of Insulin on the Disposal of Intravenous Glucose: Results from Indirect Calorimetry and Hepatic and Femoral Venous Catheterization. Diabetes. 1981;30(12):1000–7.

4. Campbell JE, Newgard CB. Mechanisms controlling pancreatic islet cell function in insulin secretion. Nat Rev Mol Cell Biol. 2021 Feb;22(2):142–58.

5. DeFronzo RA, Ferrannini E, Hendler R, Felig P, Wahren J. Regulation of splanchnic and peripheral glucose uptake by insulin and hyperglycemia in man. Diabetes. 1983 Jan;32(1):35–45.

6. Katz LD, Glickman MG, Rapoport S, Ferrannini E, Defronzo RA. Splanchnic and Peripheral Disposal of Oral Glucose in Man.

7. Petersen MC, Shulman GI. Mechanisms of Insulin Action and Insulin Resistance. Physiol Rev. 2018;98(4):2133–223.

8. Boucher J, Kleinridders A, Kahn CR. Insulin receptor signaling in normal and insulin-resistant states. Cold Spring Harb Perspect Biol. 2014 Jan 1;6(1):a009191.

9. Leto D, Saltiel AR. Regulation of glucose transport by insulin: traffic control of GLUT4. Nat Rev Mol Cell Biol. 2012 May 23;13(6):383–96.

10. Sylow L, Tokarz VL, Richter EA, Klip A. The many actions of insulin in skeletal muscle, the paramount tissue determining glycemia. Cell Metab. 2021 Apr;33(4):758–80.

11. Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol. 2006;7(2):85–96.

12. Bouzakri K, Zachrisson A, Al-Khalili L, Zhang BB, Koistinen HA, Krook A, et al. siRNA-based gene silencing reveals specialized roles of IRS-1/Akt2 and IRS-2/Akt1 in glucose and lipid metabolism in human skeletal muscle. Cell Metab. 2006;4(1):89–96.

13. Long YC, Cheng Z, Copps KD, White MF. Insulin Receptor Substrates Irs1 and Irs2 Coordinate Skeletal Muscle Growth and Metabolism via the Akt and AMPK Pathways. Mol Cell Biol. 2011;31(3):430–41.

14. Hansen H, Svensson U, Zhu J, Laviola L, Giorgino F, Wolf G, et al. Interaction between the Grb10 SH2 Domain and the Insulin Receptor Carboxyl Terminus (∗). J Biol Chem. 1996 Apr 12;271(15):8882–6.

15. Holt LJ, Brandon AE, Small L, Suryana E, Preston E, Wilks D, et al. Ablation of Grb10 Specifically in Muscle Impacts Muscle Size and Glucose Metabolism in Mice. Endocrinology. 2018 Mar 1;159(3):1339–51.

16. Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB, et al. Insulin Resistance and a Diabetes Mellitus-Like Syndrome in Mice Lacking the Protein Kinase Akt2 (PKBβ). Science. 2001;292(5522):1728–31.

17. Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ. Akt1/PKBα Is Required for Normal Growth but Dispensable for Maintenance of Glucose Homeostasis in Mice. J Biol Chem. 2001;276(42):38349–52.

18. Klip A, McGraw TE, James DE. Thirty sweet years of GLUT4. J Biol Chem. 2019 Jul 26;294(30):11369–81.

19. Sun Y, Bilan PJ, Liu Z, Klip A. Rab8A and Rab13 are activated by insulin and regulate GLUT4 translocation in muscle cells. Proc Natl Acad Sci U S A. 2010;107(46):19909–14.

20. Klip A, Sun Y, Chiu TT, Foley KP. Signal transduction meets vesicle traffic: the software and hardware of GLUT4 translocation. Am J Physiol-Cell Physiol. 2014;306(10):C879–86.

21. Mîinea CP, Sano H, Kane S, Sano E, Fukuda M, Peränen J, et al. AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. Biochem J. 2005;391(Pt 1):87–93.

22. Sano H, Kane S, Sano E, Mîinea CP, Asara JM, Lane WS, et al. Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. J Biol Chem. 2003;278(17):14599–602.

23. Katz A. A century of exercise physiology: key concepts in regulation of glycogen metabolism in skeletal muscle. Eur J Appl Physiol. 2022;122(8):1751–72.

24. Collaboration TERF. Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. The Lancet. 2010 Jun 26;375(9733):2215–22.

25. Fowler MJ. Microvascular and Macrovascular Complications of Diabetes. Clin Diabetes. 2008 Apr 1;26(2):77–82.

26. Heald AH, Stedman M, Davies M, Livingston M, Alshames R, Lunt M, et al. Estimating life years lost to diabetes: outcomes from analysis of National Diabetes Audit and Office of National Statistics data. Cardiovasc Endocrinol Metab. 2020;9(4):183–5.

27. Sun H, Saeedi P, Karuranga S, Pinkepank M, Ogurtsova K, Duncan BB, et al. IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. Diabetes Res Clin Pract. 2022;183:109119.

28. Ahlqvist E, Storm P, Käräjämäki A, Martinell M, Dorkhan M, Carlsson A, et al. Novel subgroups of adult-onset diabetes and their association with outcomes: a data-driven cluster analysis of six variables. Lancet Diabetes Endocrinol. 2018 May;6(5):361–9.

29. Chatterjee S, Khunti K, Davies MJ. Type 2 diabetes. The Lancet. 2017 Jun 3;389(10085):2239–51.

30. DeFronzo RA, Tripathy D. Skeletal Muscle Insulin Resistance Is the Primary Defect in Type 2 Diabetes. Diabetes Care. 2009 Nov;32(Suppl 2):S157–63.

31. Færch K, Johansen NB, Witte DR, Lauritzen T, Jørgensen ME, Vistisen D. Relationship between insulin resistance and β-cell dysfunction in subphenotypes of prediabetes and type 2 diabetes. J Clin Endocrinol Metab. 2015 Feb;100(2):707–16.

32. Roden M, Shulman GI. The integrative biology of type 2 diabetes. Nature. 2019;576(7785):51–60.

33. James DE, Stöckli J, Birnbaum MJ. The aetiology and molecular landscape of insulin resistance. Nat Rev Mol Cell Biol. 2021 Nov;22(11):751–71.

34. White MF, Kahn CR. Insulin action at a molecular level - 100 years of progress. Mol Metab. 2021;52:101304.

35. Jayedi A, Soltani S, Motlagh SZT, Emadi A, Shahinfar H, Moosavi H, et al. Anthropometric and adiposity indicators and risk of type 2 diabetes: systematic review and dose-response meta-analysis of cohort studies. BMJ. 2022 Jan 18;376:e067516.

36. Shen F, Guo C, Zhang D, Liu Y, Zhang P. Visceral adiposity index as a predictor of type 2 diabetes mellitus risk: A systematic review and dose-response meta-analysis. Nutr Metab Cardiovasc Dis NMCD. 2024 Apr;34(4):811–22.

37. Chan JM, Rimm EB, Colditz GA, Stampfer MJ, Willett WC. Obesity, fat distribution, and weight gain as risk factors for clinical diabetes in men. Diabetes Care. 1994 Sep;17(9):961–9.

38. Ruze R, Liu T, Zou X, Song J, Chen Y, Xu R, et al. Obesity and type 2 diabetes mellitus: connections in epidemiology, pathogenesis, and treatments. Front Endocrinol. 2023;14:1161521.

39. Zheng Y, Ley SH, Hu FB. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. Nat Rev Endocrinol. 2018 Feb;14(2):88–98.

40. González-Muniesa P, Mártinez-González MA, Hu FB, Després JP, Matsuzawa Y, Loos RJF, et al. Obesity. Nat Rev Dis Primer. 2017 Jun 15;3:17034.

41. Choe SS, Huh JY, Hwang IJ, Kim JI, Kim JB. Adipose Tissue Remodeling: Its Role in Energy Metabolism and Metabolic Disorders. Front Endocrinol. 2016;7:30.

42. Crewe C, An YA, Scherer PE. The ominous triad of adipose tissue dysfunction: inflammation, fibrosis, and impaired angiogenesis. J Clin Invest. 2017 Jan 3;127(1):74–82.

43. Pekala P, Kawakami M, Vine W, Lane MD, Cerami A. Studies of insulin resistance in adipocytes induced by macrophage mediator. J Exp Med. 1983;157(4):1360–5.

44. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. 1993 Jan 1;259(5091):87–91.

45. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J Clin Invest. 1995 May;95(5):2409–15.

46. Hotamisligil GS. Foundations of Immunometabolism and Implications for Metabolic Health and Disease. Immunity. 2017 Sep 19;47(3):406–20.

47. Hotamisligil GS. Inflammation, metaflammation and immunometabolic disorders. Nature. 2017 Feb 8;542(7640):177–85.

48. Arkan MC, Hevener AL, Greten FR, Maeda S, Li ZW, Long JM, et al. IKK-beta links inflammation to obesity-induced insulin resistance. Nat Med. 2005 Feb;11(2):191–8.

49. Gao Z, Hwang D, Bataille F, Lefevre M, York D, Quon MJ, et al. Serine Phosphorylation of Insulin Receptor Substrate 1 by Inhibitor κB Kinase Complex *. J Biol Chem. 2002;277(50):48115–21.

50. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest. 2003;112(12):1821–30.

51. Wu H, Ballantyne CM. Skeletal muscle inflammation and insulin resistance in obesity. J Clin Invest. 2017 Jan 3;127(1):43–54.

52. Ashcroft SP, Stocks B, Egan B, Zierath JR. Exercise induces tissue-specific adaptations to enhance cardiometabolic health. Cell Metab. 2024;36(2):278–300.

53. Egan B, Zierath JR. Exercise metabolism and the molecular regulation of skeletal muscle adaptation. Cell Metab. 2013 Feb 5;17(2):162–84.

54. Smith JAB, Murach KA, Dyar KA, Zierath JR. Exercise metabolism and adaptation in skeletal muscle. Nat Rev Mol Cell Biol. 2023 Sep;24(9):607–32.

55. Katz A, Broberg S, Sahlin K, Wahren J. Leg glucose uptake during maximal dynamic exercise in humans. Am J Physiol. 1986 Jul;251(1 Pt 1):E65-70.

56. Goodyear LJ, Hirshman MF, King PA, Horton ED, Thompson CM, Horton ES. Skeletal muscle plasma membrane glucose transport and glucose transporters after exercise. J Appl Physiol Bethesda Md 1985. 1990 Jan;68(1):193–8.

57. Sylow L, Kleinert M, Richter EA, Jensen TE. Exercise-stimulated glucose uptake - regulation and implications for glycaemic control. Nat Rev Endocrinol. 2017 Mar;13(3):133–48.

58. Sylow L, Møller LLV, Kleinert M, D'Hulst G, De Groote E, Schjerling P, et al. Rac1 and AMPK Account for the Majority of Muscle Glucose Uptake Stimulated by Ex Vivo Contraction but Not In-vivo Exercise. Diabetes. 2017 Jun;66(6):1548–59.

59. Sjøberg KA, Frøsig C, Kjøbsted R, Sylow L, Kleinert M, Betik AC, et al. Exercise Increases Human Skeletal Muscle Insulin Sensitivity via Coordinated Increases in Microvascular Perfusion and Molecular Signaling. Diabetes. 2017;66(6):1501–10.

60. Knudsen JR, Steenberg DE, Hingst JR, Hodgson LR, Henriquez-Olguin C, Li Z, et al. Prior exercise in humans redistributes intramuscular GLUT4 and enhances insulin-stimulated sarcolemmal and endosomal GLUT4 translocation. Mol Metab. 2020 Sep;39:100998.

61. McConell GK, Sjøberg KA, Ceutz F, Gliemann L, Nyberg M, Hellsten Y, et al. Insulin-induced membrane permeability to glucose in human muscles at rest and following exercise. J Physiol. 2020 Jan;598(2):303–15.

62. Bergouignan A, Antoun E, Momken I, Schoeller DA, Gauquelin-Koch G, Simon C, et al. Effect of contrasted levels of habitual physical activity on metabolic flexibility. J Appl Physiol. 2013 Feb;114(3):371–9.

63. Granata C, Caruana NJ, Botella J, Jamnick NA, Huynh K, Kuang J, et al. High-intensity training induces non-stoichiometric changes in the mitochondrial proteome of human skeletal muscle without reorganisation of respiratory chain content. Nat Commun. 2021 Dec 3;12(1):7056.

64. Hostrup M, Lemminger AK, Stocks B, Gonzalez-Franquesa A, Larsen JK, Quesada JP, et al. High-intensity interval training remodels the proteome and acetylome of human skeletal muscle. eLife. 2022 May 31;11:e69802.

65. Robinson MM, Dasari S, Konopka AR, Johnson ML, Manjunatha S, Esponda RR, et al. Enhanced Protein Translation Underlies Improved Metabolic and Physical Adaptations to Different Exercise Training Modes in Young and Old Humans. Cell Metab. 2017 Mar 7;25(3):581–92.

66. Deshmukh AS, Steenberg DE, Hostrup M, Birk JB, Larsen JK, Santos A, et al. Deep muscle-proteomic analysis of freeze-dried human muscle biopsies reveals fiber type-specific adaptations to exercise training. Nat Commun. 2021 Jan 12;12(1):304.

67. Porter C, Reidy PT, Bhattarai N, Sidossis LS, Rasmussen BB. Resistance Exercise Training Alters Mitochondrial Function in Human Skeletal Muscle. Med Sci Sports Exerc. 2015 Sep;47(9):1922–31.

68. Andrade-Souza VA, Ghiarone T, Sansonio A, Santos Silva KA, Tomazini F, Arcoverde L, et al. Exercise twice-a-day potentiates markers of mitochondrial biogenesis in men. FASEB J Off Publ Fed Am Soc Exp Biol. 2020 Jan;34(1):1602–19.

69. Liang X, Liu L, Fu T, Zhou Q, Zhou D, Xiao L, et al. Exercise Inducible Lactate Dehydrogenase B Regulates Mitochondrial Function in Skeletal Muscle. J Biol Chem. 2016 Dec 2;291(49):25306–18.

70. Koh JH, Pataky MW, Dasari S, Klaus KA, Vuckovic I, Ruegsegger GN, et al. Enhancement of anaerobic glycolysis - a role of PGC-1α4 in resistance exercise. Nat Commun. 2022 Apr 28;13(1):2324.

71. Hostrup M, Onslev J, Jacobson GA, Wilson R, Bangsbo J. Chronic β2 -adrenoceptor agonist treatment alters muscle proteome and functional adaptations induced by high intensity training in young men. J Physiol. 2018 Jan 15;596(2):231–52.

72. Chow LS, Gerszten RE, Taylor JM, Pedersen BK, van Praag H, Trappe S, et al. Exerkines in health, resilience and disease. Nat Rev Endocrinol. 2022 May;18(5):273–89.

73. Gonzalez-Franquesa A, Burkart AM, Isganaitis E, Patti ME. What Have Metabolomics Approaches Taught Us About Type 2 Diabetes? Curr Diab Rep. 2016 Aug;16(8):74.

74. Johnson CH, Ivanisevic J, Siuzdak G. Metabolomics: beyond biomarkers and towards mechanisms. Nat Rev Mol Cell Biol. 2016 Jul;17(7):451–9.

75. Felig Philip, Marliss Errol, Cahill George F. Plasma Amino Acid Levels and Insulin Secretion in Obesity. N Engl J Med. 1969 Oct 9;281(15):811–6.

76. Morze J, Wittenbecher C, Schwingshackl L, Danielewicz A, Rynkiewicz A, Hu FB, et al. Metabolomics and Type 2 Diabetes Risk: An Updated Systematic Review and Meta-analysis of Prospective Cohort Studies. Diabetes Care. 2022 Apr 1;45(4):1013–24.

77. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab. 2009 Apr;9(4):311–26.

78. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, et al. Metabolite profiles and the risk of developing diabetes. Nat Med. 2011;17(4):448–53.

79. Würtz P, Soininen P, Kangas AJ, Rönnemaa T, Lehtimäki T, Kähönen M, et al. Branched-chain and aromatic amino acids are predictors of insulin resistance in young adults. Diabetes Care. 2013 Mar;36(3):648–55.

80. Flores-Guerrero JL, Osté MCJ, Kieneker LM, Gruppen EG, Wolak-Dinsmore J, Otvos JD, et al. Plasma Branched-Chain Amino Acids and Risk of Incident Type 2 Diabetes: Results from the PREVEND Prospective Cohort Study. J Clin Med. 2018 Dec 4;7(12):513.

81. Ramzan I, Ardavani A, Vanweert F, Mellett A, Atherton PJ, Idris I. The Association between Circulating Branched Chain Amino Acids and the Temporal Risk of Developing Type 2 Diabetes Mellitus: A Systematic Review & Meta-Analysis. Nutrients. 2022 Oct 20;14(20):4411.

82. Sjögren RJO, Rizo-Roca D, Chibalin AV, Chorell E, Furrer R, Katayama S, et al. Branched-chain amino acid metabolism is regulated by ERRα in primary human myotubes and is further impaired by glucose loading in type 2 diabetes. Diabetologia. 2021;64(9):2077–91.

83. Lynch CJ, Adams SH. Branched-chain amino acids in metabolic signalling and insulin resistance. Nat Rev Endocrinol. 2014;10(12):723–36.

84. Newgard CB. Interplay between lipids and branched-chain amino acids in development of insulin resistance. Cell Metab. 2012;15(5):606–14.

85. White PJ, McGarrah RW, Grimsrud PA, Tso SC, Yang WH, Haldeman JM, et al. The BCKDH Kinase and Phosphatase Integrate BCAA and Lipid Metabolism via Regulation of ATP-Citrate Lyase. Cell Metab. 2018;27(6):1281-1293.e7.

86. Yoneshiro T, Wang Q, Tajima K, Matsushita M, Maki H, Igarashi K, et al. BCAA catabolism in brown fat controls energy homeostasis through SLC25A44. Nature. 201AD;572(7771):614–9.

87. Sun Y, Gao HY, Fan ZY, He Y, Yan YX. Metabolomics Signatures in Type 2 Diabetes: A Systematic Review and Integrative Analysis. J Clin Endocrinol Metab. 2020 Apr 1;105(4):dgz240.

88. Würtz P, Mäkinen VP, Soininen P, Kangas AJ, Tukiainen T, Kettunen J, et al. Metabolic signatures of insulin resistance in 7,098 young adults. Diabetes. 2012;61(6):1372–80.

89. Klein MS, Shearer J. Metabolomics and Type 2 Diabetes: Translating Basic Research into Clinical Application. J Diabetes Res. 2016;2016:3898502.

90. Liu X, Zheng Y, Guasch-Ferré M, Ruiz-Canela M, Toledo E, Clish C, et al. High plasma glutamate and low glutamine-to-glutamate ratio are associated with type 2 diabetes: Case-cohort study within the PREDIMED trial. Nutr Metab Cardiovasc Dis NMCD. 2019;29(10):1040–9.

91. Walford GA, Ma Y, Clish C, Florez JC, Wang TJ, Gerszten RE, et al. Metabolite Profiles of Diabetes Incidence and Intervention Response in the Diabetes Prevention Program. Diabetes. 2016;65(5):1424–33.

92. Petrus P, Lecoutre S, Dollet L, Wiel C, Sulen A, Gao H, et al. Glutamine Links Obesity to Inflammation in Human White Adipose Tissue. Cell Metab. 2020;31(2):375-390.e11.

93. Liu PS, Wang H, Li X, Chao T, Teav T, Christen S, et al. α-ketoglutarate orchestrates macrophage activation through metabolic and epigenetic reprogramming. Nat Immunol. 2017 Sep;18(9):985–94.

94. Bartel DP. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. Cell. 2004;116(2):281–97.

95. Gebert LFR, MacRae IJ. Regulation of microRNA function in animals. Nat Rev Mol Cell Biol. 2019 Jan;20(1):21–37.

96. O'Brien J, Hayder H, Zayed Y, Peng C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. Front Endocrinol [Internet]. 2018 [cited 2024 May 12];9. Available from: https://www.frontiersin.org/journals/endocrinology/articles/10.3389/fendo.2018.00402/full

97. Denli AM, Tops BBJ, Plasterk RHA, Ketting RF, Hannon GJ. Processing of primary microRNAs by the Microprocessor complex. Nature. 2004;432(7014):231–5.

98. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, et al. The nuclear RNase III Drosha initiates microRNA processing. Nature. 2003;425(6956):415–9.

99. Okada C, Yamashita E, Lee SJ, Shibata S, Katahira J, Nakagawa A, et al. A High-Resolution Structure of the Pre-microRNA Nuclear Export Machinery. Science. 2009;326(5957):1275–9.

100. Chipman LB, Pasquinelli AE. MiRNA Targeting – Growing Beyond the Seed. Trends Genet TIG. 2019 Mar;35(3):215–22.

101. Saxena S, Jónsson ZO, Dutta A. Small RNAs with Imperfect Match to Endogenous mRNA Repress Translation: IMPLICATIONS FOR OFF-TARGET ACTIVITY OF SMALL INHIBITORY RNA IN MAMMALIAN CELLS *. J Biol Chem. 2003;278(45):44312–9.

102. Horak M, Novak J, Bienertova-Vasku J. Muscle-specific microRNAs in skeletal muscle development. Dev Biol. 2016;410(1):1–13.

103. Siracusa J, Koulmann N, Banzet S. Circulating myomiRs: a new class of biomarkers to monitor skeletal muscle in physiology and medicine. J Cachexia Sarcopenia Muscle. 2018;9(1):20–7.

104. Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, et al. Dicer is essential for mouse development. Nat Genet. 2003;35(3):215–7.

105. O'Rourke JR, Georges SA, Seay HR, Tapscott SJ, McManus MT, Goldhamer DJ, et al. Essential role for Dicer during skeletal muscle development. Dev Biol. 2007 Nov 15;311(2):359–68.

106. Agbu P, Carthew RW. MicroRNA-mediated regulation of glucose and lipid metabolism. Nat Rev Mol Cell Biol. 2021 Jun;22(6):425–38.

107. Massart J, Katayama M, Krook A. microManaging glucose and lipid metabolism in skeletal muscle: Role of microRNAs. Biochim Biophys Acta. 2016 Dec;1861(12 Pt B):2130–8.

108. Frost RJA, Olson EN. Control of glucose homeostasis and insulin sensitivity by the Let-7 family of microRNAs. Proc Natl Acad Sci U S A. 2011;108(52):21075–80.

109. Zhu H, Shyh-Chang N, Segrè AV, Shinoda G, Shah SP, Einhorn WS, et al. The Lin28/let-7 axis regulates glucose metabolism. Cell. 2011 Sep 30;147(1):81–94.

110. Massart J, Sjögren RJO, Lundell LS, Mudry JM, Franck N, O'Gorman DJ, et al. Altered miR-29 Expression in Type 2 Diabetes Influences Glucose and Lipid Metabolism in Skeletal Muscle. Diabetes. 2017 Apr 12;66(7):1807–18.

111. Fish JE, Santoro MM, Morton SU, Yu S, Yeh RF, Wythe JD, et al. miR-126 regulates angiogenic signaling and vascular integrity. Dev Cell. 2008;15(2):272–84.

112. Huse JT, Brennan C, Hambardzumyan D, Wee B, Pena J, Rouhanifard SH, et al. The PTEN-regulating microRNA miR-26a is amplified in high-grade glioma and facilitates gliomagenesis in-vivo. Genes Dev. 2009;23(11):1327–37.

113. Kato M, Putta S, Wang M, Yuan H, Lanting L, Nair I, et al. TGF-β activates Akt kinase via a microRNA-dependent amplifying circuit targeting PTEN. Nat Cell Biol. 2009;11(7):881–9.

114. Small EM, O'Rourke JR, Moresi V, Sutherland LB, McAnally J, Gerard RD, et al. Regulation of PI3-kinase/Akt signaling by muscle-enriched microRNA-486. Proc Natl Acad Sci U S A. 2010;107(9):4218–23.

115. Katayama M, Wiklander OPB, Fritz T, Caidahl K, El-Andaloussi S, Zierath JR, et al. Circulating Exosomal miR-20b-5p Is Elevated in Type 2 Diabetes and Could Impair Insulin Action in Human Skeletal Muscle. Diabetes. 2019 Mar 1;68(3):515–26.

116. Russell AP, Lamon S, Boon H, Wada S, Güller I, Brown EL, et al. Regulation of miRNAs in human skeletal muscle following acute endurance exercise and short-term endurance training. J Physiol. 2013;591(18):4637–53.

117. Camera DM, Smiles WJ, Hawley JA. Exercise-induced skeletal muscle signaling pathways and human athletic performance. Free Radic Biol Med. 2016;98:131–43.

118. Margolis LM, Lessard SJ, Ezzyat Y, Fielding RA, Rivas DA. Circulating MicroRNA Are Predictive of Aging and Acute Adaptive Response to Resistance Exercise in Men. J Gerontol A Biol Sci Med Sci. 2017;72(10):1319–26.

119. Margolis LM, Hatch-McChesney A, Allen JT, DiBella MN, Carrigan CT, Murphy NE, et al. Circulating and skeletal muscle microRNA profiles are more sensitive to sustained aerobic exercise than energy balance in males. J Physiol. 2022;600(17):3951–63.

120. Ogasawara R, Akimoto T, Umeno T, Sawada S, Hamaoka T, Fujita S. MicroRNA expression profiling in skeletal muscle reveals different regulatory patterns in high and low responders to resistance training. Physiol Genomics. 2016;48(4):320–4.

121. Telles GD, Libardi CA, Conceição MS, Vechin FC, Lixandrão ME, DE Andrade ALL, et al. Time Course of Skeletal Muscle miRNA Expression after Resistance, High-Intensity Interval, and Concurrent Exercise. Med Sci Sports Exerc. 2021 Aug 1;53(8):1708–18.

122. Massart J, Sjögren RJO, Egan B, Garde C, Lindgren M, Gu W, et al. Endurance exercise training-responsive miR-19b-3p improves skeletal muscle glucose metabolism. Nat Commun. 2021 Dec;12(1):5948.

123. Safdar A, Abadi A, Akhtar M, Hettinga BP, Tarnopolsky MA. miRNA in the regulation of skeletal muscle adaptation to acute endurance exercise in C57Bl/6J male mice. PloS One. 2009;4(5):e5610.

124. Mori MA, Ludwig RG, Garcia-Martin R, Brandão BB, Kahn CR. Extracellular miRNAs: From Biomarkers to Mediators of Physiology and Disease. Cell Metab. 2019 Oct 1;30(4):656–73.

125. Colombo M, Raposo G, Théry C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu Rev Cell Dev Biol. 2014;30:255–89.

126. Doyle LM, Wang MZ. Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. Cells. 2019;8(7):727.

127. EL Andaloussi S, Mäger I, Breakefield XO, Wood MJA. Extracellular vesicles: biology and emerging therapeutic opportunities. Nat Rev Drug Discov. 2013 May;12(5):347–57.

128. van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. Nat Rev Mol Cell Biol. 2018 Apr;19(4):213–28.

129. Ying W, Riopel M, Bandyopadhyay G, Dong Y, Birmingham A, Seo JB, et al. Adipose Tissue Macrophage-Derived Exosomal miRNAs Can Modulate In-vivo and In-vitroInsulin Sensitivity. Cell. 2017;171(2):372-384.e12.

130. Thomou T, Mori MA, Dreyfuss JM, Konishi M, Sakaguchi M, Wolfrum C, et al. Adipose-derived circulating miRNAs regulate gene expression in other tissues. Nature. 2017;542(7642):450–5.

131. Annibalini G, Contarelli S, Lucertini F, Guescini M, Maggio S, Ceccaroli P, et al. Muscle and Systemic Molecular Responses to a Single Flywheel Based Iso-Inertial Training Session in Resistance-Trained Men. Front Physiol. 2019 May 9;10:554.

132. Bei Y, Xu T, Lv D, Yu P, Xu J, Che L, et al. Exercise-induced circulating extracellular vesicles protect against cardiac ischemia-reperfusion injury. Basic Res Cardiol. 2017 Jul;112(4):38.

133. Frühbeis C, Helmig S, Tug S, Simon P, Krämer-Albers EM. Physical exercise induces rapid release of small extracellular vesicles into the circulation. J Extracell Vesicles. 2015;4:28239.

134. Ma C, Wang J, Liu H, Chen Y, Ma X, Chen S, et al. Moderate Exercise Enhances Endothelial Progenitor Cell Exosomes Release and Function. Med Sci Sports Exerc. 2018 Oct;50(10):2024–32.

135. Oliveira GP, Porto WF, Palu CC, Pereira LM, Petriz B, Almeida JA, et al. Effects of Acute Aerobic Exercise on Rats Serum Extracellular Vesicles Diameter, Concentration and Small RNAs Content. Front Physiol [Internet]. 2018 May 24 [cited 2024 May 15];9. Available from:
https://www.frontiersin.org/journals/physiology/articles/10.3389/fphys.2018.00532/full

136. Whitham M, Parker BL, Friedrichsen M, Hingst JR, Hjorth M, Hughes WE, et al. Extracellular Vesicles Provide a Means for Tissue Crosstalk during Exercise. Cell Metab. 2018;27(1):237-251.e4.

137. Darragh IAJ, McNamee N, Daly R, Pacheco SM, O'Driscoll L, Egan B. The separation and identification of circulating small extracellular vesicles from endurance-trained, strength-trained and recreationally active men. J Physiol. 2023 Nov;601(22):5075–91.

138. Hou Z, Qin X, Hu Y, Zhang X, Li G, Wu J, et al. Longterm Exercise-Derived Exosomal miR-342-5p: A Novel Exerkine for Cardioprotection. Circ Res. 2019 Apr 26;124(9):1386–400.

139. Lovett JAC, Durcan PJ, Myburgh KH. Investigation of Circulating Extracellular Vesicle MicroRNA Following Two Consecutive Bouts of Muscle-Damaging Exercise. Front Physiol. 2018;9:1149.

140. Doncheva AI, Romero S, Ramirez-Garrastacho M, Lee S, Kolnes KJ, Tangen DS, et al. Extracellular vesicles and microRNAs are altered in response to exercise, insulin sensitivity and overweight. Acta Physiol Oxf Engl. 2022 Dec;236(4):e13862.

141. Czepluch F, Barrès R, Caidahl K, Olieslagers S, Krook A, Rickenlund A, et al. Strenuous physical exercise adversely affects monocyte chemotaxis. Thromb Haemost. 2011;105(01):122–30.

142. Al-Khalili L, Chibalin AV, Kannisto K, Zhang BB, Permert J, Holman GD, et al. Insulin action in cultured human skeletal muscle cells during differentiation: assessment of cell surface GLUT4 and GLUT1 content. Cell Mol Life Sci CMLS. 2003 May;60(5):991–8.

143. Holt LJ, Siddle K. Grb10 and Grb14: enigmatic regulators of insulin action--and more? Biochem J. 2005 Jun 1;388(Pt 2):393–406.

144. Edick AM, Auclair O, Burgos SA. Role of Grb10 in mTORC1-dependent regulation of insulin signaling and action in human skeletal muscle cells. Am J Physiol Endocrinol Metab. 2020;318(2):E173–83.

145. Pillon NJ, Gabriel BM, Dollet L, Smith JAB, Sardón Puig L, Botella J, et al. Transcriptomic profiling of skeletal muscle adaptations to exercise and inactivity. Nat Commun. 2020 Dec;11(1):470.

146. Cheng S, Rhee EP, Larson MG, Lewis GD, McCabe EL, Shen D, et al. Metabolite profiling identifies pathways associated with metabolic risk in humans. Circulation. 2012 May 8;125(18):2222–31.

147. Cruzat V, Macedo Rogero M, Noel Keane K, Curi R, Newsholme P. Glutamine: Metabolism and Immune Function, Supplementation and Clinical Translation. Nutrients. 2018 Oct 23;10(11):1564.

148. Lecoutre S, Maqdasy S, Petrus P, Ludzki A, Couchet M, Mejhert N, et al. Glutamine metabolism in adipocytes: a bona fide epigenetic modulator of inflammation. Adipocyte. 2020 Dec;9(1):620–5.

149. Onan MC, Fisher JS, Ju JS, Fuchs BC, Bode BP. Type I diabetes affects skeletal muscle glutamine uptake in a fiber-specific manner. Exp Biol Med Maywood NJ. 2005 Oct;230(9):606–11.

150. Holt LJ, Lyons RJ, Ryan AS, Beale SM, Ward A, Cooney GJ, et al. Dual ablation of Grb10 and Grb14 in mice reveals their combined role in regulation of insulin signaling and glucose homeostasis. Mol Endocrinol Baltim Md. 2009 Sep;23(9):1406–14.

151. Smith FM, Holt LJ, Garfield AS, Charalambous M, Koumanov F, Perry M, et al. Mice with a Disruption of the Imprinted Grb10 Gene Exhibit Altered Body Composition, Glucose Homeostasis, and Insulin Signaling during Postnatal Life. Mol Cell Biol. 2007 Aug;27(16):5871–86.

152. Wang L, Balas B, Christ-Roberts CY, Kim RY, Ramos FJ, Kikani CK, et al. Peripheral disruption of the Grb10 gene enhances insulin signaling and sensitivity in-vivo. Mol Cell Biol. 2007 Sep;27(18):6497–505.

153. Estébanez B, Jiménez-Pavón D, Huang CJ, Cuevas MJ, González-Gallego J. Effects of exercise on exosome release and cargo in in-vivo and ex vivo models: A systematic review. J Cell Physiol. 2021 May;236(5):3336–53.

154. Rigamonti AE, Bollati V, Pergoli L, Iodice S, De Col A, Tamini S, et al. Effects of an acute bout of exercise on circulating extracellular vesicles: tissue-, sex-, and BMI-related differences. Int J Obes 2005. 2020 May;44(5):1108–18.

155. Liang JQ, Liao HR, Xu CX, Li XL, Wei ZX, Xie GJ, et al. Serum Exosome-Derived miR-139-5p as a Potential Biomarker for Major Depressive Disorder. Neuropsychiatr Dis Treat. 2020;16:2689–93.

156. Sun H, Dai J, Chen M, Chen Q, Xie Q, Zhang W, et al. miR-139-5p Was Identified as Biomarker of Different Molecular Subtypes of Breast Carcinoma. Front Oncol. 2022;12:857714.

157. Zhang H da, Jiang L hong, Sun D wei, Li J, Tang J hai. MiR-139-5p: promising biomarker for cancer. Tumour Biol J Int Soc Oncodevelopmental Biol Med. 2015 Mar;36(3):1355–65.

158. Czernek L, Chworos A, Duechler M. The Uptake of Extracellular Vesicles is Affected by the Differentiation Status of Myeloid Cells. Scand J Immunol. 2015;82(6):506–14.

159. Németh K, Varga Z, Lenzinger D, Visnovitz T, Koncz A, Hegedűs N, et al. Extracellular vesicle release and uptake by the liver under normo- and hyperlipidemia. Cell Mol Life Sci. 2021;78(23):7589–604.

160. Driedonks T, Jiang L, Carlson B, Han Z, Liu G, Queen SE, et al. Pharmacokinetics and biodistribution of extracellular vesicles administered intravenously and intranasally to Macaca nemestrina. J Extracell Biol. 2022;1(10):e59.

161. Gangadaran P, Li XJ, Lee HW, Oh JM, Kalimuthu S, Rajendran RL, et al. A new bioluminescent reporter system to study the biodistribution of systematically injected tumor-derived bioluminescent extracellular vesicles in mice. Oncotarget. 2017 Dec 15;8(66):109894–914.

162. Wiklander OPB, Nordin JZ, O'Loughlin A, Gustafsson Y, Corso G, Mäger I, et al. Extracellular vesicle in-vivo biodistribution is determined by cell source, route of administration and targeting. J Extracell Vesicles. 2015;4(1):26316.

163. Hiraoka Y, Matsuoka T, Ohno M, Nakamura K, Saijo S, Matsumura S, et al. Critical roles of nardilysin in the maintenance of body temperature homoeostasis. Nat Commun. 2014 May;5(1):3224.

164. Nishi K, Sato Y, Ohno M, Hiraoka Y, Saijo S, Sakamoto J, et al. Nardilysin Is Required for Maintaining Pancreatic β-Cell Function. Diabetes. 2016 Oct 1;65(10):3015–27.

165. Pillon NJ, Smith JAB, Alm PS, Chibalin AV, Alhusen J, Arner E, et al. Distinctive exercise-induced inflammatory response and exerkine induction in skeletal muscle of people with type 2 diabetes. Sci Adv. 2022;8(36):eabo3192.