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SKELETAL MUSCLE RESPONSES TO PHYSICAL ACTIVITY IN HEALTH AND METABOLIC DISEASE

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Skeletal Muscle Responses to Physical Activity in Health and Metabolic Disease

Thesis for Doctoral Degree (Ph.D.)

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"Before you judge a person, walk a mile in their shoes. After that who cares? They're a mile away and you've got their shoes!" – Billy Connolly

Popular science summary of the thesis

Physical inactivity is detrimental to overall metabolic wellbeing. Engaging in sedentary behaviours, such as prolonged sitting, increases the risk of developing metabolic disorders, including type 2 diabetes. This unfavourable consequence can be attributed, at least in part, to the detrimental effects of physical inactivity on skeletal muscle health and function.

Skeletal muscle facilitates bodily movement and exhibits high sensitivity to levels of physical activity. Furthermore, it serves as a major site of energy expenditure and the storage of ingested food, particularly carbohydrates. Insulin, a hormone involved in the storage of nutrients, primarily targets skeletal muscle and facilitates the clearance of carbohydrates (i.e., glucose) from the bloodstream following meals. Hence, maintaining the metabolic health of skeletal muscle is crucial for ensuring stable blood sugar levels over time.

Extended periods of sedentary behaviour diminish the responsiveness of skeletal muscle to insulin, a phenomenon known as peripheral insulin resistance. If left unchecked, this condition represents a critical step towards the development of type 2 diabetes. Moreover, periods of inactivity lead to losses in skeletal muscle mass and strength, which can hasten functional decline in vulnerable populations, such as the elderly and those in critical care.

On the other hand, exercise proves to be an effective approach to preserve or enhance skeletal muscle metabolism, size, and function. Exercise induces changes within skeletal muscle that promote the activation of various molecules (e.g., proteins) responsible for nutrient transport, muscle building, and gene regulation, among other critical processes. Nevertheless, there is still much to uncover regarding the molecular mechanisms underlying the benefits of exercise on skeletal muscle. Greater understanding of these mechanisms could inform personalised exercise recommendations and uncover novel avenues for drug discovery to improve human health.

Additionally, how physical activity is distributed across the day is an important factor to consider, and there is growing interest in incorporating short-duration physical activity breaks, often referred to as ‘exercise snacks’, to interrupt prolonged sitting. In controlled settings, such as laboratory-based studies, breaking up sedentary behaviours with low-to-moderate intensity physical activities (e.g., walking, stair-climbing, or bodyweight exercises) has shown improvements in blood glucose and lipid responses compared to uninterrupted sitting. Exercise snacks represent a convenient strategy for enhancing metabolic wellbeing. However, there is need to evaluate the benefits of breaking sedentary time using strategies that better represent real-world scenarios to provide actionable public health guidelines.

With these points in mind, the objectives of this thesis were as follows: (1) Evaluate the effectiveness of interrupting sedentary time in individuals engaged in their normal daily

routines, (2) Characterise the gene expression response of skeletal muscle to different types of exercise in both untrained and trained states within healthy individuals and those with metabolic impairments, including type 2 diabetes, and (3) Investigate the roles of specific molecules involved in skeletal muscle metabolism and adaptation in the context of physical activity.

Study I demonstrated that even a small addition of ≈750 steps dispersed throughout the day, equivalent to ≈10 minutes of extra walking time, improved glucose control in individuals with obesity and insulin resistance. Notably, those engaging in higher levels of physical activity while interrupting sedentary time experienced the most benefits, suggesting that more breaks from sedentary behaviour yield better metabolic health outcomes. However, adherence to the intervention, consisting of 3-minute activity bouts every 30 minutes between 08:00-18:00, was lower than anticipated. This raises questions about the long-term feasibility of such approaches in isolation from other modifiable lifestyle factors, like changes in dietary habits or structured exercise routines.

In **Study II**, a comprehensive analysis of multiple studies was conducted to compare the gene expression responses of skeletal muscle to a single bout of aerobic or resistance exercise, as well as after aerobic and resistance exercise training (i.e., multiple bouts over time), and periods of physical inactivity. This analysis revealed that a single bout of exercise in the untrained state elicits a distinct gene response in skeletal muscle compared to that observed after training. Intriguingly, the gene response exhibited more similarities between a single bout of aerobic or resistance exercise than either did to exercise training. This highlights how the adaptive response of skeletal muscle becomes more refined over time with dedicated training focused on a specific type of exercise.

Study II also identified nuclear receptor subfamily 4 group A member 3 (NR4A3) as a gene that shows increased expression in skeletal muscle after exercise, but decreased expression in response to physical inactivity. **Study III** further investigated the role of NR4A3 and its impact on glucose and protein metabolism in skeletal muscle. Notably, NR4A3 plays a crucial role in muscle building processes (i.e., muscle protein synthesis) and its depletion leads to a reduction in skeletal muscle size and compromises the ability of skeletal muscle to utilise glucose for energy provision. Therefore, diminished levels of NR4A3 in skeletal muscle during physical inactivity may directly contribute to muscle atrophy and impaired metabolism in this tissue.

Furthermore, study II recognised that individuals with obesity and/or type 2 diabetes exhibit an altered gene expression response to exercise training compared to healthy individuals. In **study IV** we found that this includes a heightened inflammatory response during the recovery period after exercise in people with type 2 diabetes, attributed to an increased influx of immune cells into skeletal muscle tissue. This phenomenon potentially

facilitates communication or ‘crosstalk’ between different cell types residing in skeletal muscle tissue. Specifically, the cytokine stromal cell-derived factor 1 (CXCL12/SDF-1) can be produced by immune cells (i.e., macrophages) or vascular cells (i.e., endothelial) in response to signals released by skeletal muscle fibres or low oxygen levels, respectively. In turn, CXCL12 can activate skeletal muscle progenitor or ‘satellite’ cells, which could be important for adaptive remodelling following exercise.

In summary, the research presented in this thesis underscores the central role of physical activity in improving human health, with a particular focus on exercise's potency to induce adaptations in skeletal muscle. These adaptations, in turn, contribute to the metabolic fitness of skeletal muscle and of the human body as a whole.

Abstract

Sedentary lifestyles, characterised by a lack of physical activity and prolonged periods of sitting, have been linked to reductions in whole-body metabolic flexibility and the increased risk of metabolic diseases, including type 2 diabetes. This can be attributed, at least partially, to the direct negative effects of physical inactivity on skeletal muscle insulin sensitivity, oxidative capacity, and overall metabolic health. In addition, sedentary behaviour can lead to anabolic resistance, resulting in losses of skeletal muscle mass and strength, which can further contribute to conditions like sarcopenic obesity, impairing physical performance and overall quality of life.

Conversely, physical activity plays a crucial role in maintaining and improving skeletal muscle health. Exercise is associated with various adaptations in skeletal muscle that enhance tissue oxidative capacity, substrate handling, insulin sensitivity, as well as skeletal muscle mass and strength. These positive changes in skeletal muscle contribute to improvements in systemic metabolic wellbeing.

The molecular mechanisms underlying skeletal muscle adaptation to the perturbations caused by physical activity are complex and involve intrinsic processes within the muscle fibre itself, as well as communication between different cell populations in composite skeletal muscle tissue. However, our understanding of the intricate details of these mechanisms remains incomplete. Gaining deeper insights into the regulation of skeletal muscle adaptation could not only facilitate personalised exercise recommendations but also uncover novel opportunities for drug discovery, ultimately leading to improvements in human health.

Despite the well-known benefits of exercise, physical activity guidelines are often not met by the general population. Therefore, there is a pressing need for low-level entry paradigms that can promote physical activity and reduce sedentary behaviour for the betterment of individual and public health. One such approach is the incorporation of frequent activity breaks or ‘exercise snacks’ into daily routines, which involves short-duration physical activity breaks throughout the day to disrupt prolonged periods of sitting. These interventions have demonstrated efficacy for cardiometabolic health in controlled settings, such as laboratory-based clinical trials. However, it is essential to evaluate the benefits of breaking sedentary time using strategies that better mimic real-world scenarios to inform practical public health guidelines.

In this thesis, the following objectives were pursued: (1) To assess the translational efficacy of interrupting sedentary time in improving cardiometabolic health. (2) To investigate the skeletal muscle transcriptome following exercise or physical inactivity in the context of health and metabolic diseases. (3) To determine the metabolic effects of physical activity-responsive transcription factors and signalling molecules in skeletal muscle.

Study I revealed that even a minor addition of ≈750 steps dispersed throughout the day, equivalent to ≈10 minutes of extra walking time, improved dynamic glucose control in individuals with obesity and insulin resistance. Notably, those who engaged in higher levels of physical activity while interrupting sedentary time experienced greater benefits, indicating that more breaks from sedentary behaviour lead to better metabolic health outcomes. Nevertheless, adherence to the intervention, which involved 3-min activity bouts every 30 min between 08:00-18:00, was lower than anticipated. This raises questions about the long-term feasibility of such approaches when considered in isolation from other modifiable lifestyle factors, including changes in dietary habits or structured exercise routines.

Study II employed a comprehensive meta-analytical approach to compare the skeletal muscle transcriptomic response to acute aerobic or resistance exercise, exercise training, and physical inactivity. This analysis revealed distinct gene signatures in skeletal muscle after a single bout of exercise in the naïve state, which differed from those observed after training of the same exercise modality. Interestingly, there was greater overlap in the skeletal muscle transcriptome between acute aerobic and resistance exercise than there was between acute exercise and exercise training. These findings highlight the refinement of the adaptive response in skeletal muscle over time through dedicated training to a specific exercise modality.

Study II identified the transcription factor nuclear receptor subfamily 4 group A member 3 (*NR4A3*) as a gene that is upregulated in skeletal muscle after exercise but downregulated in response to physical inactivity**. Study III** delved deeper into the role of NR4A3 in the context of physical inactivity and revealed its regulatory role in translation within skeletal muscle. Depletion of NR4A3 resulted in skeletal muscle atrophy and compromised glucose oxidation, instead favouring increased lactate production. Therefore, decreased levels of *NR4A3* during physical inactivity may directly contribute to muscle disuse atrophy and impaired skeletal muscle metabolism.

Furthermore, study II identified that individuals with obesity and/or type 2 diabetes exhibit an altered skeletal muscle transcriptional response to exercise training compared to healthy individuals. **Study IV** uncovered a heightened inflammatory response during the recovery period after exercise in individuals with type 2 diabetes. This response was attributed to an increased influx of immune cells into skeletal muscle tissue, potentially facilitating crosstalk between different cell types within the skeletal muscle interstitial space. Notably, the cytokine stromal cell-derived factor 1 (*CXCL12*/*SDF-1*) was found to be expressed by macrophages or endothelial cells in response to factors released by skeletal muscle fibres or hypoxia, respectively. CXCL12 activation, in turn, promoted the proliferation of skeletal muscle satellite cells, which could be integral for adaptive remodelling following exercise.

In conclusion, the research presented in this thesis emphasises the central role of physical activity in improving human health, with a specific focus on the ability of exercise to induce adaptations in skeletal muscle. The findings herein shed light on the intricate molecular mechanisms underlying skeletal muscle responses to physical activity, which contribute to the metabolic fitness of this tissue and of the human body as a whole.

List of scientific papers

I. **Smith JAB**, Savikj M, Sethi P, Platt S, Gabriel BM, Hawley JA, Dunstan D, Krook A, Zierath JR, Näslund E. Three weeks of interrupting sitting lowers fasting glucose and glycemic variability, but not glucose tolerance, in free-living women and men with obesity. Am J Physiol Endocrinol Metab. 2021;321:2, E203-E216. doi: 10.1152/ajpendo.00599.2020.

II. Pillon NJ, Gabriel BM, Dollet L, **Smith JAB**, Sardón Puig L, Botella J, Bishop DJ, Krook A, Zierath JR. Transcriptomic profiling of skeletal muscle adaptations to exercise and inactivity. Nat Commun. 2020;11:470. doi: 10.1038/s41467-019-13869-w

III. **Smith JAB**, Gabriel BM, Krook A, Zierath JR, Pillon NJ. Downregulation of NR4A3 During Inactivity Alters Glucose Metabolism and Impairs Translation in Human Skeletal Muscle. Unpublished, in manuscript.

IV. Pillon NJ, **Smith JAB**, Alm PS, Chibalin AV, Alhusen J, Arner E, Carninci P, Fritz T, Otten J, Olsson T, van Doorslaer de Ten Ryen S, Deldicque L, Caidahl K, Wallberg-Henriksson H, Krook A, Zierath JR. Distinctive exercise-induced inflammatory response and exerkine induction in skeletal muscle of people with type 2 diabetes. Sci Adv. 2022;8:36, eabo3192. doi: 10.1126/sciadv.abo3192

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1. Literature review

1.1 Obesity and type 2 diabetes are prevalent and interrelated metabolic diseases

1.1.1 Prevalence of obesity and type 2 diabetes

The World Health Organisation (WHO) estimated that in 2016 >650 million adults were living with obesity. This condition, characterised by greater adiposity, increases susceptibility to various noncommunicable diseases, including the metabolic syndrome and type 2 diabetes. Indeed, severity of the metabolic syndrome [1] and the occurrence of type 2 diabetes [2] scale linearly with body mass index (BMI, $kg.m^{-2}$), and even metabolically healthy obesity can covert to an unhealthy phenotype over time [3].

The interplay between type 2 diabetes and adiposity becomes further apparent when considering the global prevalence of this disease, which closely mirrors the escalating rates of obesity. The International Diabetes Federation's report in 2021 estimated that ≈537 million adults had diabetes, with projections that this number will rise to ≈783 million by 2045. Notably, >90% of all diabetes cases correspond to type 2 diabetes [4], implying that >483 million adults currently grapple with this condition and its associated comorbidities.

The impact of type 2 diabetes is emphasised by statistics on mortality and global health expenditure. In 2017 alone, ≈1 million deaths were attributed to type 2 diabetes and a further ≈349,000 with type 2 diabetes-related chronic kidney disease [5]. In attempts to combat this, considerable financial resources are allocated annually, with ≈966 billion United States dollars (USD) dedicated to addressing the complexities of diabetes. Estimates suggest that these costs will climb to ≈1.05 trillion USD by 2045.

Given the substantial health and socioeconomic challenges posed by obesity and type 2 diabetes, interventions to treat or prevent these diseases are at the forefront of scientific endeavour.

1.1.2 Aetiology and pathogenesis of type 2 diabetes

1.1.2.1 Homeostatic regulation of blood glucose levels

The homeostatic regulation of energy and glucose metabolism is coordinated by complex integration of central and peripheral cues involving the brain, gastrointestinal tract, liver, pancreas, adipose tissue, and skeletal muscle [6]. In healthy individuals, blood glucose is tightly controlled around 4-6 $mmol.L^{-1}$. This regulation is mainly achieved through the antagonistic actions of glucose-releasing hormone glucagon and storage-promoting hormone insulin in response to fasting and feeding, respectively. Fasting conditions, such as overnight sleep or longer periods between meals, stimulate α-cells of the pancreas to produce glucagon. Exactly how this occurs is poorly understood; however, it seems that low blood glucose (<4.4

mmol.L^{-1}) reduces insulin in the interstitial fluid surrounding α-cells, which alleviates the repression of glucagon secretion, promoting glucagon release [7]. Circulating glucagon primarily acts on G-protein coupled receptors in the liver, causing conformational changes, and subsequent activation of protein kinase A (PKA) and phospholipase C. This sequence of events inhibits glucose consuming processes, but potentiates gluconeogenesis and glycogenolysis, increasing hepatic glucose output and blood glucose levels [8].

Conversely, carbohydrate [9] and/or protein (i.e., amino acid) [10] containing meals stimulate the release of insulin from β-cells of the pancreas through overlapping but also discrete and complimentary mechanisms. Briefly, after carbohydrate ingestion, glucose enters the β-cell via glucose transporter 2 (GLUT2) and is phosphorylated by β-cell-specific glucokinase. High intracellular glucose concentrations activate transcription factors that promote transcription and translation of insulin, which is then stored in granules. Glucose within the β-cell is oxidatively metabolised, resulting in a greater adenosine triphosphate to adenosine diphosphate plus inorganic phosphate ([ATP]/[ADP][Pi]) ratio and subsequent closure of ATP-sensitive potassium channels. Reduced potassium efflux depolarises the β-cell, permitting calcium ion (Ca^{2+}) release from the endoplasmic reticulum. Ca^{2+} activates the translocation of insulin-containing granules to the plasma membrane, allowing insulin to enter the bloodstream [9]. Alternatively, protein-dependent insulin secretion occurs via direct (i.e., sodium symport) and indirect (i.e., partial oxidation) β-cell depolarisation, priming of insulin granules, and chlorine (Cl^-) efflux through volume-regulated anion (Cl^-) channels (VRAC) [10]. In both cases, insulin release is also potentiated by incretins, such as glucagon-like peptide 1 (GLP-1) [11], produced from L-cells of the small intestine after eating [12]. Circulating insulin then acts upon insulin-responsive tissues to facilitate systemic glucose and amino acid clearance, and further initiates intracellular anabolic and anti-catabolic processes, especially in skeletal muscle.

1.1.2.2 Insulin resistance, β-cell dysfunction, and type 2 diabetes

Type 2 diabetes is the endpoint of continuums of insulin resistance and β-cell dysfunction that synergistically deteriorate glycaemic control over time [13]. Progression through this continuum is exacerbated by obesity [14] and in particular visceral adiposity [15]. Insulin resistance is defined as reduced whole-body glucose uptake in response to physiological concentrations of insulin. Initially, maintenance of glucose homeostasis is achieved through compensatory secretion of more insulin from β-cells [16]. However, this cannot be sustained indefinitely and can lead to β-cell dysfunction in susceptible individuals if left untreated. Indeed, chronic hyperglycaemia causes progressive deterioration of β-cell metabolism in mice models of neonatal diabetes [17]. Acutely, glucose-dependent ATP production becomes impaired, preventing β-cell depolarisation, and subsequent insulin trafficking. Longer term consequences are glycogen accumulation, reduced autophagy, and

caspase-induced apoptosis of β-cells, which could contribute to declines in β-cell mass that are observed in people with type 2 diabetes over time [18].

As a result of reduced β-cell function and mass, the required amount of insulin can no longer be released, blood glucose levels rise, and type 2 diabetes is declared once a diagnostic threshold is reached. As of 2020, the American Diabetes Association's criteria for clinical type 2 diabetes are (in two separate samples, or in two test results from the same sample): a fasting plasma glucose ≥7 $mmol.L^{-1}$, a 2-h post-prandial glucose value ≥11.1 $mmol.L^{-1}$ during a 75 g oral glucose tolerance test, glycated haemoglobin (HbA1c) ≥ 48 $mmol.mol^{-1}$, and/or a random blood glucose reading ≥11.1 $mmol.L^{-1}$ [19].

1.1.3 Subtypes of type 2 diabetes

Despite clear diagnostic criteria, individuals with type 2 diabetes display considerable heterogeneity both in terms of symptomatic presentation [20, 21] and disease progression [21, 22]. Recently, four phenotypic clusters within type 2 diabetes have been recognised, with distinct metabolic disturbances: severe insulin deficient diabetes (SIDD), severe insulin resistant diabetes (SIRD), mild obesity-related diabetes (MOD), and mild age-related diabetes (MARD) [20]. These subtypes of type 2 diabetes exhibit varying degrees of whole-body and adipose-tissue insulin resistance [22], as well as different risk patterns for the development of diabetes-related comorbidities and complications [20-22]. However, the efficacy of employing this clustering approach for disease treatment has been questioned [21], and it remains unclear whether discrete subtypes of type 2 diabetes differ in their aetiology.

1.2 Genetics and lifestyle factors contribute towards metabolic disease

1.2.1 Genetic and epigenetic predisposition

Metabolic diseases including obesity, insulin resistance, and type 2 diabetes occur because of the interaction between inherited genetic/epigenetic factors (that predispose to the disease) and environmental stressors (which trigger the phenotype). This concept is termed 'genome-lifestyle divergence' [23], and is thought to result from a lack of selective pressure after the industrial revolution, leading to genomic enrichment of disease-predisposing alleles [24]. Inherited genetics can substantially increase the probability of developing type 2 diabetes over time. Having just one parent with type 2 diabetes raises the relative risk by 30% [25], and this increases to 60% [26] if both parents are affected. Monogenic mutations have been identified for obesity and type 2 diabetes, but these are rare. In obesity, such variants largely affect genes of the leptin-melanocortin axis, impacting brain systems that regulate adiposity [27]. Alternatively, severe insulin resistance and type 2 diabetes can occur from autosomal dominant inheritance of a large-effect mutation in the *AKT2* gene [28]. Nevertheless, most cases of obesity [29] and type 2 diabetes [30] are

considered polygenic traits, consisting of small-effect, single nucleotide polymorphisms (SNPs) in many genes. To date, 38 SNPs associated with type 2 diabetes have been discovered, in addition to nearly two dozen others linked with glycaemic traits. Still, the genetic variants identified thus far explain ≤10% of type 2 diabetes heritability [30], prompting suggestions that epigenetic programming, through environmental exposures, may account for much of the missing heritability. The nutritional status of both mother [31] and father [32] upon conception, and of the mother during pregnancy [31], can affect a child's susceptibility to disease in later life. For example, lower birth weights are correlated with higher incidence of insulin resistance and type 2 diabetes in adulthood [33]. This is further supported by retrospective studies of children exposed to gestational undernutrition during times of famine, such as the Dutch Winter Famine (1944-1945) and the Chinese Famine (1959-1961) [34]. Research in mice also suggests that high fat diet-induced maternal obesity leads to mitochondrial dysfunction in oocytes, which causes insulin resistance in pups, and that these epigenetic defects are passed through the maternal germline for ≥3 generations [31].

1.2.2 The obesogenic environment: higher caloric intakes and reduced physical activity

Humans have evolved under the selective pressures of regular physically activity [35] and periods of starvation, and the modern obesogenic environment antagonises genomic traits inherited to cope with such conditions [23]. Ultra-processed food products have become more plentiful and affordable worldwide [36]. These foods are energy-dense combinations of carbohydrate, fat, and salt that make them highly rewarding [37] and more likely to result in addictive-like eating behaviours [38]. An *ad libitum* diet of ultra-processed food increases energy intake by ≈500 kilocalories per day, and total bodyweight by ≈0.9 Kg after just two weeks. This is compared to an unprocessed diet, even when meals are matched for energy, macronutrient, sugar, sodium, and fibre contents [39]. Thus, ultra-processed foods appear to override satiety cues, promoting a chronically positive energy balance, and the development of obesity over time.

Similarly, technological advances have allowed lifestyles to become more sedentary and it is estimated that ≈35% of Europeans are physically inactive [40]. The detriments of physical inactivity were first described in 1950's, when greater incidences of coronary heart disease were reported among men in sedentary *versus* active jobs [41]. These findings have since been extrapolated to other noncommunicable diseases, including type 2 diabetes [42]. Indeed, secondary analysis of the Maastricht Study suggested that every additional waking hour spent in sedentary postures increased the risk of type 2 diabetes by ≈22% [43]. Furthermore, within several European countries, ≈40% of leisure time is now spent watching television [44] and the negative impact of television viewing on metabolic health may be exacerbated by unhealthy eating behaviours often associated with this sedentary behaviour [45].

1.2.3 Physical inactivity fosters insulin resistance

The adverse effects of inactivity on whole-body insulin response plays a causal role in these observations. Just five days of bed rest can induce insulin resistance, dyslipidaemia, and microvascular dysfunction in healthy people [46]. Such deleterious effects are also seen in less extreme models, where activity levels are reduced as opposed to completely removed. For example, ten days without formal exercise decreases insulin sensitivity in well-trained individuals [47], and restricting daily step count (from ≈10,500 to ≈1,300 steps) for two weeks causes peripheral insulin resistance in healthy young males, concomitant with decreased aerobic fitness ($\dot{V}O_{2\,max}$) and skeletal muscle atrophy of the legs [48].

The current environment affects both sides of the energy balance equation, promoting excess energy consumption, together with reduced energy expenditure. Inherited (epi)genetics can make some individuals more susceptible to obesity and type 2 diabetes when chronically exposed to such conditions. Furthermore, the deterioration of metabolic health is exacerbated by systemic loss of insulin sensitivity. As a primary tissue of insulin action [49, 50], deeper mechanistic interrogation of the critical regulators and cellular events orchestrating skeletal muscle insulin sensitivity may reveal therapeutic entry points for combatting insulin resistance and metabolic diseases, such as type 2 diabetes.

1.3 Skeletal muscle is a primary and critical site of insulin action

The human body consists of more than 600 skeletal muscles, composed of layers of connective tissue and fascicles (also called muscle bundles). Within fascicles reside intricate arrangements of muscle fibres, which are individual syncytial cells responsible for contraction. Each muscle fibre is encompassed by an endomysium (or basal lamina) that is anchored to the fibre membrane, known as the sarcolemma [51]. The complex architecture of skeletal muscle fibres (Figure 1) offers valuable insights into the intricate mechanisms governing muscle function and homeostasis.

1.3.1 Skeletal muscle fibre types

Three primary fibre types have been identified in human muscles of the torso and limbs: slow-oxidative MyHC type I (encoded by *MYH7*), fast oxidative-glycolytic (intermediate) MyHC type IIA (encoded by *MYH2*), and fast-glycolytic MyHC type IIX (previously known as type IID; encoded by *MYH1*) [52]. Type IIA and type IIX fibres are collectively termed 'type II fibres'. Each fibre type possesses unique contractile and biochemical properties that result from the expression of select excitation-contraction coupling and ATP-generating machinery, intricately tailored activities of the predominantly expressed MyHC ATPase [52, 53]. This coordinated expression is contingent upon the innervation of α-motor units and synchronised transcription from resident myonuclei [54, 55]. Intriguingly, myonuclei from type I and type II

fibres exhibit discrete chromatin accessibility and transcription factor motif enrichment profiles [54]. This suggests a crucial role for transcription factor-driven myonuclear programmes in fostering regionalised gene expression in muscle cells [54], as well as the co-expression of specific Ca^{2+}-handling, sarcomeric, and metabolic components [52-54]. Ultimately, this elegant matching allows for the alignment of contractile (also known as 'twitch') and metabolic characteristics across different fibre types.

Although most myonuclei within a given fibre display coordinated transcription of a single *Myh* isoform in mouse muscle, a minority of 'hybrid fibres' exist [54, 55]. These hybrid fibres contain myonuclei that express two or more *Myh* pre-mRNA genes either within the same nucleus or across different nuclei along the length of the fibre [54]. In the human vastus lateralis muscle, <10% [56, 57] to 40% [58] of skeletal muscle fibres may fall into the category of hybrid types. 'True' non-transitioning or non-regenerating hybrid fibres exhibit metabolic enzyme [56] and single-fibre contractile properties [59] that lie between those of their co-expressed MyHC isoforms. This adds further complexity to the slow-oxidative to fast-glycolytic fibre continuum, with the order of increasing contractile speed and glycolytic capacity being: type I → I/IIA → IIA → IIA/IIX → IIX [56, 59].

The properties of human skeletal muscle can vary markedly among individuals [60, 61], biological sexes [60], anatomical locations [62] and during ageing [61, 63]. Indeed, mitochondrial impairments, including those that occur with ageing, can force a glycolytic shift in muscle fibres without a corresponding change in MyHC [63]. This cautions against using MyHC as a strict marker of metabolism and vice versa. Additionally, human muscles are generally slower than most mammals [64] and several limb muscles of mice and rats are mostly comprised of fast-glycolytic MyHC isoform IIB (encoded by *Myh4*) [56, 64]. Type IIB fibres have the greatest peak power of all fibre types [64] but are not expressed in human muscle [52, 56, 63, 64]. Moreover, type IIA fibres are the most oxidative fibre type in rodents, whereas type I fibres are most oxidative in human muscle [56]. As such, these species differences should be considered when inferring human relevance from rodent physiology.

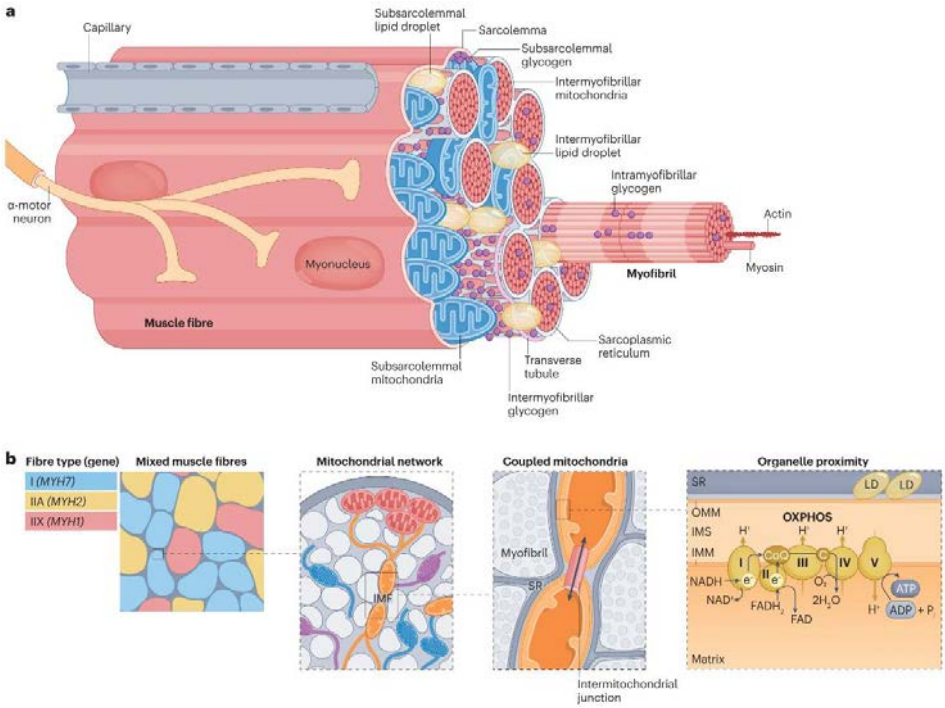


Figure 1. Skeletal muscle fibre ultrastructure. ***A. Energy stores within skeletal muscle fibres.*** Adenosine triphosphate (ATP) is crucial for sustaining muscle contraction. However, the availability of free ATP is limited in muscle (≈20–25 mmol per kg dry mass) and would be depleted within <2 seconds of all-out exercise [65, 66]. Thus, during short-duration maximal efforts, creatine phosphate (CrP) and glycogen are rapidly broken down and utilised to meet heightened ATP demands [65, 66]. The sarcoplasmic creatine kinase (CKM) reaction (not shown) plays a pivotal role by converting creatine phosphate into equimolar amounts of high-energy phosphate molecules that fuel skeletal muscle ATPases within milliseconds [67]. Glycogen granules (the storage form of glucose) are nonuniformly distributed among three specialised pools in skeletal muscle: the intermyofibrillar pool, subsarcolemmal pool, and intramyofibrillar pool [68]. The intermyofibrillar pool constitutes the majority of muscle glycogen (≈77–84%) and is strategically positioned between myofibrils, near mitochondria and the sarcoplasmic reticulum. In contrast, the subsarcolemmal pool is located just beneath the cell membrane, while the intramyofibrillar pool resides within the myofibril at the Z-line of the I-band [62]. During strenuous endurance [62], high-intensity interval [69], and resistance exercise [70] the mobilisation of intramyofibrillar glycogen takes precedence in fuelling myosin and sodium/potassium (Na^+/K^+)-ATPases. However, both intermyofibrillar and intramyofibrillar glycogen stores contribute to the energy requirements of myosin-ATPases and the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) [68]. Additionally, intramyocellular lipid (IMCL) utilisation peaks during moderate intensity exercise (≈60-65% $\dot{V}O_{2\ max}$) [71, 72]. IMCLs are stored in the hydrophobic core of lipid droplet ellipsoids at peripheral (subsarcolemmal, SS_{LD}) and central (intermyofibrillar, IMF_{LD}) regions within skeletal muscle fibres [73]. IMCLs are mostly deposited (>85%) in IMF_{LD} [73-75] but the distribution and characteristics of lipid droplet subpopulations varies depending on factors such as training status, body composition, and metabolic health [75]. ***B. Distinct mitochondrial subpopulations form a reticulum and share potential energy through electrically connected intermitochondrial junctions.*** Mitochondria play a crucial role in regenerating ATP during submaximal [76] and longer-duration high-intensity interval exercise [77], primarily through oxidative phosphorylation. Approximately 2–10% [78] of muscle volume is occupied by

subpopulations of subsarcolemmal (also known as peripheral) and intermyofibrillar mitochondria that exhibit positional, structural, and functional differences [78]. Subsarcolemmal mitochondria extend deep into the myofibrillar space and establish physical connections with adjacent intermyofibrillar mitochondria to form the mitochondrial reticulum [78] through electron-dense intermitochondrial junctions [79, 80]. Intermyofibrillar mitochondria interact with myofibrils [78, 80], the sarcoplasmic reticulum (SR), and intermyofibrillar lipid droplets [78, 79]. Notably, ≈20% of all intermyofibrillar mitochondria are connected to lipid droplets in oxidative mouse muscle, potentially facilitating more efficient ATP production and distribution [78]. Subsarcolemmal mitochondria are thought to specialise in generating membrane potential for subsequent transfer through the mitochondrial reticulum, into the intermyofibrillar mitochondrial network [80]. Intermyofibrillar mitochondria could then effectively utilise this potential energy to support rapid ATP production and fibre-wide diffusion to myofibrillar ATPases [78-80]. Hence, the mitochondrial reticulum may serve as a streamlined energy dispersal network, akin to an electrical power grid. Figure 1 is reproduced from Smith et al. [51] with permission.

1.3.2 Canonical insulin-mediated signalling events in skeletal muscle

1.3.2.1 Insulin-stimulated glucose transport

Insulin's effects are mediated by the binding of two insulin monomers to each insulin receptor [81] at the skeletal muscle cell surface. Insulin binding induces intracellular auto-phosphorylation and tyrosine kinase activity of the insulin receptor, which subsequently recruits insulin receptor substrate proteins (IRS). In skeletal muscle, insulin-stimulated pathways downstream of IRS-2 preferentially regulate lipid uptake and fat oxidation [82]. Alternatively, phosphorylation of IRS-1 leads to upregulation of phosphatidylinositol 3-kinase and the subsequent activation of two parallel, complimentary, cascades necessary for GLUT4 translocation and insulin-dependent glucose transport.

The first cascade involves phosphorylation of protein kinase B (AKT) by the mammalian target of rapamycin complex 2 (mTORC2) and phosphoinositide-dependent protein kinase 1 (PDK1), respectively [83]. AKT is a critical node in the canonical insulin signalling pathway and phosphorylation at both residues Ser^{473} and then Thr^{308} are essential for full kinase activation [84]. Active AKT phosphorylates and inhibits the Rab GTPase-activating protein TBC1 domain family member 4 (TBC1D4; also known as AS160), as well as glycogen synthase kinase 3 (GSK3). The second cascade comprises insulin activation of the Rho GTPase Ras-related C3 botulinum toxin substrate 1 (RAC1). Active RAC1 promotes dynamic cortical actin reorganisation via polymerisation by actin related proteins 2/3 (ARP2/3) and depolymerisation by cofilin [85]. Additionally, AKT might also support cortical actin remodelling by phosphorylating β-catenin to increase β-catenin/M-cadherin binding in response to insulin [86].

Collectively, the inhibitory phosphorylation of TBC1D4 and cycles of cortical F-actin remodelling facilitate the translocation and fusion of GLUT4-containing vesicles into the plasma membrane and transverse tubules (T-tubules) [85]. This facilitates glucose transport into the cell. Furthermore, AKT-dependent inactivation of GSK3 removes the repressive

phosphorylation of glycogen synthase (GS), increasing the rate at which intracellular glucose is stored as glycogen [83].

1.3.2.2 Insulin-dependent coordination of proteostasis

In addition to directing glucose and lipid metabolism, insulin action is central to skeletal muscle proteostasis via temporal coordination of anabolic and catabolic processes [87, 88]. Insulin concurrently stimulates amino acid uptake into skeletal muscle [89] and, through AKT, activates the mammalian target of rapamycin complex 1 (mTORC1). mTORC1 is a master regulator of cellular translation that permits myofibrillar protein synthesis and skeletal muscle hypertrophy [7] almost exclusively via the translation of messenger RNAs (mRNAs) containing pyrimidine-rich 5' terminal oligopyrimidine (5' TOP) motifs or 'TOP-like' motifs [90].

In response to insulin and amino acids, mTORC1 promotes translation through phosphorylation of two key effector substrates ribosomal protein S6 kinase beta-1 (S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), which govern distinct branches of protein synthetic signalling [91]. mTORC1 activation of S6K1 facilitates downstream translation initiation and improves the translational efficiency of spliced messenger RNAs (mRNA). Alternatively, mTORC1-phosphorylation of 4EBP1 dissociates 4EBP1 from eukaryotic initiation factor 4E (eIF4E) and enables 5' cap-dependent mRNA translation to proceed [91].

The integrity of the skeletal muscle proteome also requires balanced turnover of specific proteins [87]. Autophagy and the ubiquitin-proteasome system are fundamental to this process and both of these proteolytic pathways rely on the forkhead box protein O (FOXO) family of transcription factors (FOXO1, FOXO3, FOXO4) for the expression of atrogenes, such as f-box only protein 32 (or muscle atrophy F-box protein, MAFbx) and E3 ubiquitin-protein ligase TRIM63 (or muscle-specific RING finger protein 1, MuRF1) [92]. In response to insulin, AKT phosphorylates FOXOs at multiple residues to reduce their transcriptional activity through nuclear exclusion [93]. Furthermore, mTORC1 evokes repressive phosphorylation on serine/threonine-protein kinase ULK1 and transcription factor EB (TFEB) to dampen autophagic flux by interfering with 5' adenosine monophosphate-activated protein kinase (AMPK) upregulation of ULK1 and TFEB-dependent transcription of lysosomal biogenesis and autophagic machinery, respectively [91].

In humans, circulating insulin concentrations within the physiological postprandial range seems permissive (rather than obligatory) for muscle protein synthesis [88]. Thus, insulin's regulation of skeletal muscle mass occurs principally due to its anticatabolic effect in attenuating protein breakdown. The importance of this role of insulin in the healthy regulation of skeletal muscle mass is clearly observed in transgenic mice, where skeletal muscle specific deletion of the insulin receptor results in ≈20% reductions of skeletal muscle size and strength [93].

1.3.3 Skeletal muscle insulin resistance is a core feature underlying metabolic disease

Skeletal muscle accounts for ≈25% of postprandial glucose disposal and ≈85% of insulin-stimulated glucose uptake [49, 50]. When skeletal muscle is no longer sensitive to insulin, much of this glucose is diverted towards hepatic *de novo* lipogenesis and triacylglyceride synthesis, which increases plasma triacylglycerides and reduces high-density lipoprotein concentrations [94]. Similarly, branched-chain amino acid (BCAA) homeostasis is impaired in the skeletal muscle of people with type 2 diabetes and is further compromised following an oral glucose challenge in these individuals [95]. Indeed, the blunting of muscle protein breakdown by insulin is abrogated in those with insulin resistance [88] and defective proteostasis might underly the accelerated loss of skeletal muscle mass and strength in older adults with poorly controlled type 2 diabetes [96].

The development of adverse blood lipid profiles as a direct consequence of skeletal muscle insulin resistance, and in absence of secondary abnormalities associated with type 2 diabetes [94], suggests that skeletal muscle insulin resistance is a primary, casual factor in the pathogenesis of type 2 diabetes [97]. Furthermore, insulin resistance in skeletal muscle can propagate to other organ systems [98, 99] and often precedes β-cell dysfunction by decades [100]. As such, research devoted to role of skeletal muscle in the pathophysiology of type 2 diabetes provides a valuable entry point for strategies devoted towards the prevention and management of metabolic disease.

1.4 Physical inactivity impairs skeletal muscle metabolism and function

Skeletal muscle is a primary hub for nutrient storage, locomotion, and energy turnover and is thus central to the impact of physical activity on human health [51]. Periods of inactivity rapidly diminish skeletal muscle strength [101, 102], insulin sensitivity, and oxidative capacity [103]. These changes contribute toward declines in quality of life [104] and whole-body metabolic flexibility [105], and associate with increased risk of non-communicable diseases like type 2 diabetes [106]. These phenomena highlight the importance of regular physical activity in maintaining optimal metabolic function and overall health.

The pathogenesis of skeletal muscle insulin resistance is multifactorial and complex. Our group, and others, have identified perturbations within the insulin cascade that contribute to aberrant transduction in the skeletal muscle of people with insulin resistance and type 2 diabetes. This includes defective signalling through IRS1 [107], AKT (at Thr^{308}), AS160 [108], and RAC1 [109] proteins, and lower activity of PI3K [107] and GS [110]. Indeed, phosphoproteomic analysis of skeletal muscle cells for human donors with type 2 diabetes suggests that insulin resistance presents alongside cell-autonomous alterations at numerous proximal and distal sites in the insulin transduction pathway [111]. Such impairments decrease insulin-stimulated translocation of GLUT4 to the sarcolemma and T-tubules [112],

with subsequent decrements in glucose transport [112] and intracellular storage [110]. Reduced GLUT4 exocytosis is the definitive characteristic of insulin resistance and it is noteworthy that this occurs in the absence of changes in total GLUT4 protein expression [113].

Many factors could contribute to, or exacerbate, the aberrant signalling seen in the skeletal muscle of individuals with insulin resistance. Physical inactivity quickly reduces skeletal muscle mitochondrial content [114], and a smaller number and size of mitochondria are seen in the skeletal muscle of people with type 2 diabetes [115]. This may contribute to the impaired biochemical efficiency of insulin resistant skeletal muscle, as indicated by a ≈30-40% reduction in mitochondrial substrate oxidation [116] and electron transport chain activity [115], respectively. Attenuated oxidative capacity is thought to accelerate the progression of insulin resistance by promoting the accumulation of inter- and intra- muscular lipids ([103] and [102]). Bioactive lipid species from both inter- and intra- muscular lipid compartments can impede insulin signalling ([103] and [117]) and mitochondrial function [118]. In turn, chronically elevated production of reactive oxygen species (ROS) from dysfunctional mitochondria can inflict damage upon cellular components [119], and upregulate proteolytic pathways [119, 120] while dampening AKT-mTORC1 signalling [120], accelerating the loss of skeletal muscle mass [119, 120].

Physical inactivity favours signalling and transcriptional programmes that promote sustained periods of negative protein balance and skeletal muscle degradation. Indeed, detectable skeletal muscle atrophy occurs within just four days of disuse [101] and involves a complex molecular interplay. mTORC1 acts in tandem with ribosomal content (among other processes) to regulate muscle translation [121], and detriments in both mTORC1 [122] and ribosomal abundance [123] are observed in human skeletal muscle with inactivity. The downregulation of translational machinery, alongside insulin- and amino acid- resistance [101, 122, 124, 125], drives initial losses in muscle mass through dampened myofibrillar protein synthetic responses [101, 125]. Alternatively, the contribution of protein degradation systems towards disuse muscle atrophy is unclear in humans [101, 125].

Intermuscular lipid depots also secrete factors, such as inflammatory cytokines, that foster insulin resistance [117]. Cross-sectional and prospective studies have noted increased concentrations of reactants related to the innate immune system in the blood of people with type 2 diabetes [126, 127], including interleukin-6 (IL-6), alpha-1-acid glycoprotein (AGP), sialic acid, c-reactive protein (CRP), interleukin-1 beta (IL-1β), and tumour necrosis factor alpha (TNFα). Furthermore, circulating levels of CRP, IL-6, and IL-1β may predict future development of type 2 diabetes [127, 128], implicating systemic low-grade inflammation in the progression of this disease. TNFα, IL-6 and IL-1β can initiate stress-receptive intracellular pathways through IκB kinase beta (IKKβ) and cJUN N-terminal kinase (JNK) [129], which are also sensitive to ROS [130]. IKKβ and JNK stimulate nuclear factor κB (NFκB), and ETS Like-1

protein Elk-1 (ELK1), activating transcription factors (ATFs), and cJUN, respectively [129]. These transcription factors upregulate pro-inflammatory cytokines that both reinitiate IKKβ and JNK signalling and enter circulation, where they can negatively impact systemic insulin sensitivity [129]. In support of this, mice with genetic ablation of JNK1 ($JNK1^{-/-}$) are protected against obesity-induced insulin resistance [131], and 48 weeks of treatment with the IKKβ inhibitor [132] salicylate improves glycaemic control, and reduces triacylglycerides and inflammation in people with type 2 diabetes [133].

In addition, individuals with insulin resistance often exhibit a decrease in the abundance of type I muscle fibres and an increase in the expression of type II fibres [113]. This switch in fibre type distribution correlates with reduced peripheral insulin action and might be influenced by the reduced expression of glucose handling machinery within type II fibres [113]. However, both muscle fibre composition [57] and insulin sensitivity [134] are influenced by physical activity levels. Hence, the observed association between muscle fibre composition and insulin action in people with insulin resistance could be attributed to environmental factors as opposed to an underlying cause.

In summary, a notable interplay between exogenous factors, muscle intrinsic signalling, and metabolic events act synergistically and temporally to impair skeletal muscle insulin sensitivity. The use of 'omics technologies is enabling systems level characterisation of pathways contributing to the deterioration of skeletal muscle metabolic health. Indeed, >4,500 genes related to metabolism, inflammation, and sarcoplasmic reticulum stress were changed after nine days of bed rest inactivity [135]. Furthermore, exercise training and physical inactivity are not mere opposing ends of a linear spectrum, but rather multifaceted processes with distinct causal mechanisms [136]. Understanding the dichotomy between exercise and inactivity necessitates a deeper interrogation of the common and distinct signalling and transcriptional networks affected by these physical activity paradigms [137].

1.5 Physical activity improves skeletal muscle and human health

1.5.1 Different exercise modalities

Physical exercise can be broadly categorised into resistance, cardiorespiratory, balance, and flexibility-based activities. However, in this subsection, the focus will be on variations of resistance and cardiorespiratory exercise that are most often the primary areas of interest in exercise physiology studies.

1.5.1.1 Resistance exercise

Traditional resistance exercise involves performing repetitions of dynamic concentric (muscle-shortening) and eccentric (muscle-lengthening) contractions against external load.

This form of exercise effectively increases skeletal muscle mass and strength [138, 139]. The total amount of exercise performed (referred to as volume), frequency of training, and intensity of the exercise are interconnected variables that influence skeletal muscle adaptation and performance. When it comes to resistance exercise, volume is commonly measured by the number of times or sets a specific muscle group is trained per week. This serves as the main stimulus for muscle hypertrophy [140]. Generally, performing 12-20 sets per week is sufficient to maximise muscle mass accrual [141], whereas the frequency of resistance exercise can be adjusted to distribute training volumes according to individual preference [142].

Intensity in resistance exercise is typically expressed as a percentage of the maximum load an individual can lift, known as the one-repetition maximum (1 RM) [143]. Another way to gauge intensity is by measuring how close an exercise set is taken to momentary muscular failure, which occurs when the concentric portion of a movement cannot be completed [144]. Similar gains in muscle mass can be achieved regardless of load-intensity, within the range of <30% of 1 RM to ≥80% of 1 RM), so long as sets are taken close to failure [144]. This implies that increasing the number of repetitions against a fixed load or increasing the load for a fixed number of repetitions are both effective methods for promoting muscle hypertrophy [145]. On the other hand, high-load resistance training is more advantageous for improving absolute strength (1 RM) [143], tendon stiffness [146], and running economy (the metabolic cost of submaximal running at a given velocity) [147]. Therefore, implementing various loading strategies could be the optimal approach for achieving a wide range of muscle-related adaptations through resistance training.

1.5.1.2 Cardiorespiratory exercise

Cardiorespiratory exercise encompasses various subtypes that are associated with improvements in the maximum rate of oxygen consumption ($\dot{V}O_{2\ max}$) brought about by training [148, 149]. Endurance exercise, also known as aerobic exercise, usually involves continuous activity performed at low (<50%), moderate (≈50-79%), or high intensities (≥80%) of $\dot{V}O_{2\ max}$ [148]. High-intensity cardiorespiratory exercise can be further divided into high-intensity interval exercise (HIE) and sprint interval exercise (SIE), both involving periods of higher-intensity effort interspersed with low-intensity active recovery periods. HIE is conducted at intensities ≥80% of $\dot{V}O_{2\ max}$, while SIE is performed at supramaximal or 'all-out' intensities. Comparisons between cardiorespiratory exercise types suggest that training within the high-intensity range is either more [148] or equally [149] effective at increasing $\dot{V}O_{2\ max}$, while requiring less training volume than moderate intensities. HIE and SIE training also produce greater improvements in endothelial function, whereas moderate intensities are more beneficial for long-term glycaemic control [148]. Furthermore, there are distinct differences between subtypes of interval training. For instance, eight weeks of HIE training enhanced in cardiac stroke volume, $\dot{V}O_{2\ max}$, and 3 Km running performance. Conversely, an

equivalent period of SIE training led to improvements in anaerobic capacity and 300 m sprint performance [150].

Considering the complimentary physiological outcomes associated with different types of exercise, the diversification of training regimens is recommended to maximise both health and performance benefits.

1.5.2 Physical activity maintains and promotes skeletal muscle metabolic health

1.5.2.1 Exercise increases skeletal muscle and whole-body energy expenditure

Total daily energy expenditure (TDEE) reflects the sum metabolic demand of daily living and is a critical component of human health. Chronic positive energy balance results in obesity [151] and increased risk of associated comorbidities, such as type 2 diabetes [152]; whereas prolonged negative energy availability is associated with symptoms of Relative Energy Deficiency in Sport (RED-S) [153].

TDEE largely corresponds to organ-tissue metabolic activities [154, 155], particularly fat-free mass [154]. The resting thermogenesis of skeletal muscle is lower than most critical organs per unit of weight [155] because myosin is maintained in disordered-relaxed and super-relaxed conformations [156] (Figure 2). These myosin states are characterised by slow-to-extremely-slow ATP-kinetics, respectively [156, 157]. However, upon contraction, mechano-sensing rapidly initiates a myosin conformational switch from relaxed-to-active [158] and ATP-turnover can increase dramatically during short-duration, exhaustive exercise [65]. Therefore, muscle contributes substantially more to TDEE during physical activity to support continued myosin-actin cross-bridge cycling and active ion transport that accounts for ≈50-70% and ≈30-50% of ATP turnover during contraction, respectively [159].

During acute athletic events, from 800 m to marathon races, greater skeletal muscle metabolic activity contributes towards whole-body energy expenditures that can exceed basal rates (BMR) by ≈15-19-fold [160]. Yet, there appears to be a curvilinear decrease in metabolic scope (TDEE/BMR) over time that constrains habitual TDEE within a narrow range [160, 161]. A sustainable cap of ≈2.5-fold BMR has been proposed for TDEE and ≤2-fold BMR is a common observation in humans [160]. Furthermore, females and males typically compensate for ≈28% of calories burned through activity [162] and compensation may be exacerbated during negative energy balance [163]. The underlying causes of metabolic constraint/compensation are unclear but might include reduced non-exercise activity thermogenesis (NEAT; e.g., fidgeting, posture, etc.) and downregulated metabolism in organ systems [161, 162, 164]. Along with an increased drive to eat with long term exercise interventions [165], these findings might in part explain why physical activity alone is often inefficient for weight loss, but aids in weight maintenance [166] and improves many aspects of metabolic health.

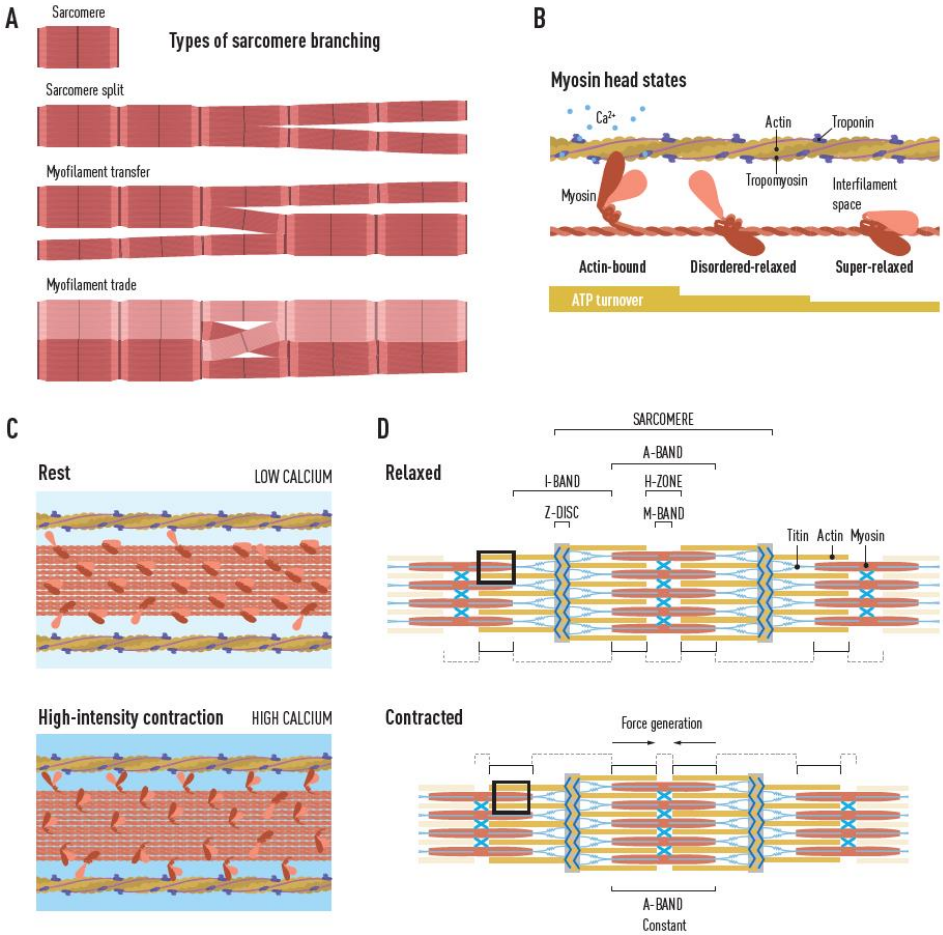


Figure 2. Myofibril organisation and skeletal muscle contraction. ***A. Three types of branching between sarcomeres.*** Myofibrils form a nonlinear matrix of sarcomeres [167-169] connected across the width and length of the muscle cell through three branching subtypes [167]: (1) Sarcomere splitting, where myofilaments from one sarcomere separate into two different myofibrils; (2) Myofilament transfer, in which myofilaments from a single sarcomere segregate to join a distinct, adjacent sarcomere; and (3) Myofilament trade, where myofilaments are shared between two neighbouring sarcomeres. This nonlinear network of sarcomeres provides an elegant mechanism for both longitudinal and lateral force transmission from muscles to bones through tendons [167]. Linked configurations of myofibrils could also minimise the impact of localised sarcomere damage and increase the robustness of contractile machinery across an entire skeletal muscle fibre. ***B. The conformational states of myosin heads.*** Individual sarcomeres contain organised arrangements of parallel thin (actin), thick (myosin), and elastic (titin/connectin) filaments. Under basal conditions, myosin heads are maintained in disordered-relaxed or super-relaxed conformations, with slow- to extremely-slow ATP turnover kinetics [156, 157] which contribute to the lower relative metabolic rate of muscle at rest [157]. In the disordered-relaxed state, free myosin heads protrude into the inter-myofilament space and can readily engage in contraction. However, tropomyosin sterically prevents their association with actin at resting sarcoplasmic calcium (Ca^{2+}) levels. Alternatively, in the super-relaxed state myosin binding protein C [170], regulatory light

chains [157, 171], and essential light chains [171] may act to anchor both myosin heads to the thick filament backbone, away from actin. Super-relaxed myosins could be further suppressed by direct head-to-head contact (that is, the 'interacting-heads' motif) that inhibits myosin ATPase [171]. ***C. Excitation-contraction coupling and myosin filament activation.*** Upon neuromuscular transmission, action potential propagation along the sarcolemma induces a voltage-dependent alteration of dihydropyridine receptor tetrads in the transverse tubule, which allosterically opens ryanodine receptor 1 on the sarcoplasmic reticulum to stimulate Ca^{2+} release [172]. Ca^{2+} binding of troponin exposes sites on actin that permits interaction with the head domain of formerly disordered-relaxed myosin after ATP hydrolysis [173]. This initial step imposes mechanical strain on thick filaments in response to high external load and critically initiates super-relaxed myosin binding with actin [173], which is necessary for maximal force production [173, 174]. Panels in Figure 2c correspond to the areas within black boxes on adjacent sarcomeres in Figure 2d. ***D. The sliding filament model of contraction.*** After strongly binding with actin, myosin undergoes a conformational change, releasing inorganic phosphate and shortening the sarcomeric I-band and H-zone regions to generate force (that is, the 'power stroke'), while A-band filaments remain relatively constant. The liberation of ADP from myosin post power stroke causes transient rigor until a new ATP molecule enters the myosin head nucleotide-binding pocket. This high-energy myosin-ATP state reduces affinity of myosin for actin and promotes dissociation of the myosin-actin crossbridge. Detached (disordered-relaxed) myosin then hydrolyses bound ATP and is again primed to interact with actin, repeating the cycle [175]. Figure 2 is reproduced from Smith et al. [51] with permission.

1.5.2.2 Contraction promotes insulin -independent and -dependent glucose disposal

Bouts of exercise sensitise skeletal muscle to hormones and nutrients in a manner specific to the contracted musculature [176]. During a single exercise session, skeletal muscle glucose uptake increases dramatically, reaching levels ≈50-fold higher than baseline upon the cessation of exhaustive endurance exercise at ≈100% of $\dot{V}O_{2\ max}$ [177]. This upregulation of glucose transport is primarily attributed to the insulin-independent exocytosis and fusion of GLUT4-containing vesicles into the sarcolemmal membrane and T-tubules [178]. The translocation of GLUT4 is governed by a complex interplay of various factors, including Ca^{2+} transients, metabolic stimuli, and mechano-transduction. These converging pathways activate calcium/calmodulin-dependent protein kinase II (CaMKII), AMPK and RAC1 [179]. Interestingly, redundancy between these pathways has been observed [180], suggesting a robust and intricate regulatory network. Notably, the role of RAC1 in contraction-induced glucose uptake potentially necessitates downstream ROS production from NADPH oxidase 2 (NOX2) [181] but is independent of AMPKα_2 [180]. Indeed, the catalytic α subunit of AMPK appears dispensable for exercise-stimulated glucose transport *in vivo* [182, 183].

While AMPKα might not directly influence contraction-stimulated glucose uptake, it exerts notable effects on post-exercise substrate metabolism [184] and insulin sensitivity [183]. AMPKα activation upregulates pyruvate dehydrogenase kinase 4 (PDK4) to promote the phosphorylation and inactivation of pyruvate dehydrogenase (PDH) [184]. Fatty acid oxidation might also be enhanced by AMPKα-dependent assembly of the RAS-related protein RAB8A-perilipin 5 (PLIN5) lipid droplet-to-mitochondrial tethering complex [185]. Additionally, AMPKα inhibits TBC1 domain family member 1 (TBC1D1) to extend insulin-independent glucose permeability of skeletal muscle following exercise [182]. Alternatively,

the inactivating phosphorylation of TBC1D4 by AMPKα augments GLUT4 translocation in response to insulin [183].

Skeletal muscle insulin sensitivity and glucose uptake after exercise are further improved through a combination of greater insulin-stimulated perfusion of muscle capillaries [186] and the redistribution of intramuscular GLUT4 into insulin-responsive storage vesicles [187]. Refined vesicular trafficking and subsequent sarcolemmal enrichment of GLUT4 in the post exercise period increases skeletal muscle membrane permeability to glucose by ≈36-fold after insulin stimulation, more than double that of non-exercised muscle (≈17-fold) [188]. The magnitude of this exercise-induced potentiation in skeletal muscle insulin sensitivity could be intensity-dependent, with activities performed at higher percentages of $\dot{V}O_{2\ peak}$ having a stronger effect [189].

1.5.2.3 Skeletal muscle protein synthesis

The stress imposed by a single bout of resistance exercise upregulates amino acid transporters and sensors in skeletal muscle. This response appears to be mediated, at least in part, by activating transcription factor 4 (ATF4) [190] and synergises with the contraction-induced migration of the mTOR–lysosomal complex to focal adhesions [191] and microvasculature [192] at the sarcolemma. Here, mTOR associates with RAS homologue enriched in brain (RHEB) and eukaryotic translation initiation factor 3 (EIF3) [192]. This molecular interaction subsequently triggers downstream target ribosomal protein S6 (RPS6) [191]. The concerted action of mTOR–EIF3 and the $Ser^{240/244}$ phosphorylation of RPS6 at the periphery of skeletal muscle fibres could facilitate the amino acid-sensitising effects of resistance exercise on mTORC1 activation [193]. As such, dietary protein intakes of ≥1.2–1.6 g per kg body mass daily modestly complement the hypertrophic response to resistance training [194].

1.5.2.4 Skeletal muscle lipid metabolism and storage depots

Within skeletal muscle, the liberation of non-esterified fatty acids (NEFAs) from intramuscular lipid droplets heavily relies on the coordinated action of adipose triacylglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) [195]. Exercise upregulates HSL in an intensity-dependent manner [196], achieved through the additive effects of contraction-induced events involving Ca^{2+} and protein kinase C (PKC) [197], as well as adrenaline-mediated pathways that activate cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) [198]. These signalling mechanisms promote the translocation of HSL to lipid droplets [199] and thereby enhance rates of muscle lipolysis [200].

Exercise-induced changes in NEFA utilisation also involve the sarcolemmal enrichment of long-chain fatty acid transporters [201]. The exocytosis of fatty acid translocase (FAT; also known as CD36), and possibly other transporters, appears to be independent of AMPK [202] but could involve calcium-calcium/calmodulin-dependent protein kinase kinase (CaMKK)

[203] and dual specificity mitogen-activated protein kinase kinase 1 (MAP2K1 or MEK1) and MAP2K2 (MEK2) signalling pathways [204]. These mechanisms contribute to the preferential breakdown of NEFAs entering exercising muscle, rather than their re-esterification and storage [205].

Interestingly, total intramyocellular lipid (IMCL) concentrations are similar between endurance-trained athletes and adults with type 2 diabetes [75, 206]. Referred to as the 'athlete paradox' [206] this phenomenon might be partially explained by distinct properties of lipid droplets [73, 75]. Individuals with type 2 diabetes tend to have a higher number of extremely large subsarcolemmal lipid droplets (SS_{LD}) in type II muscle fibres [73, 75], accompanied by lower subsarcolemmal mitochondrial contents [73]. This leads to a greater relative contribution of SS_{LD} to the overall IMCL pool [75] and fewer mitochondria-SS_{LD} contacts [73]. Spatially, the presence of SS_{LD} could interfere with insulin signalling and negatively impact peripheral insulin sensitivity in type 2 diabetes [75]. Conversely, endurance-trained athletes exhibit approximately twofold more IMCLs in type I muscle fibres. Additionally, they demonstrate increased abundance of ATGL and PLIN5 proteins [75] associated with enhanced turnover of lipid droplets [185]. Notably, the IMCL content in trained individuals is primarily stored in smaller intramyofibrillar lipid droplets (IMF_{LD}) [75], which are preferentially depleted during prolonged endurance exercise [74].

An eight-week program of high-intensity interval training has been found to reduce SS_{LD} size, increase the subsarcolemmal mitochondria-to-lipid droplet ratio, and redistributed IMCLs into small IMF_{LD} in type II fibres [73]. Consequently, lipid droplet profiles became similar among adults with varying BMIs or type 2 diabetes following the training intervention, irrespective of baseline differences [73]. These findings suggest that consistent exercise may somewhat alleviate skeletal muscle insulin resistance by promoting turnover[200] and remodelling of lipid droplets [73, 75].

1.5.3 Breaking sedentariness to promote whole-body metabolic health

In addition to formal exercise, research on the therapeutic impact of breaking-up sedentary behaviour with frequent, short intervals of physical activity (or 'exercise snacks') has grown. Sedentary time is implicated with higher risk of mortality, regardless of participation in moderate-to-vigorous physical activity [207]. Similarly, individuals in the highest quartile for number of breaks in sedentary time per week tend to have smaller waist circumferences, and lower 2-h plasma glucose levels after an oral glucose tolerance test, than those in the lowest quartile [208]. On average, these breaks were of light intensity and lasted <5 min. A greater number of prolonged (1-2 h) periods of sedentariness is also associated with the metabolic syndrome, even in individuals habitually walking ≈11,000 steps per day [209]. This suggests that sedentary behaviour is in an independent risk factor for obesity and type 2 diabetes, and that the way in which sedentary time is accumulated is also of importance.

In support of this, after consumption of a liquid meal (≈800 Kcal; ≈13 g protein, 75 g carbohydrate, and 50 g fat), interrupting 5 h of postprandial sitting with 2 min of light (≈3 $Km.h^{-1}$) or moderate (≈6 $Km.h^{-1}$) intensity treadmill walking, every 20 min, reduced glucose and insulin excursions by ≈24-30% and ≈23%, respectively, compared to uninterrupted sitting [210]. However, results from more ecologically valid trials are equivocal [211]. Furthermore, sedentary time is rarely standardised between intervention and control groups. Such conditions do not enable the benefits of breaking-up sedentary time to be objectively delineated from the overall reduction in sedentary behaviour, or from the increase in total physical activity.

Interrupting sedentary time potentially offers a practical and easy to interpret public health message. As such, translatable clinical trials are needed to better assess the efficacy of breaking-up sedentary time for the improvement of cardiometabolic health.

1.6 The importance of cellular crosstalk in skeletal muscle remodelling

During exercise, circulatory and microvascular responses work in tandem to maintain relatively constant plasma-to-interstitial glucose concentrations [212]. This not only ensures stable glucose levels but also results in enlargement of the area available for nutrient exchange to occur [213]. Such events further facilitate the release of discrete biologically active molecules from skeletal muscle [214]. These secreted molecules are thought to promote many of the favourable adaptations associated with physical activity [215] via autocrine or paracrine signalling within the skeletal muscle niche (Figure 3) or through endocrine actions.

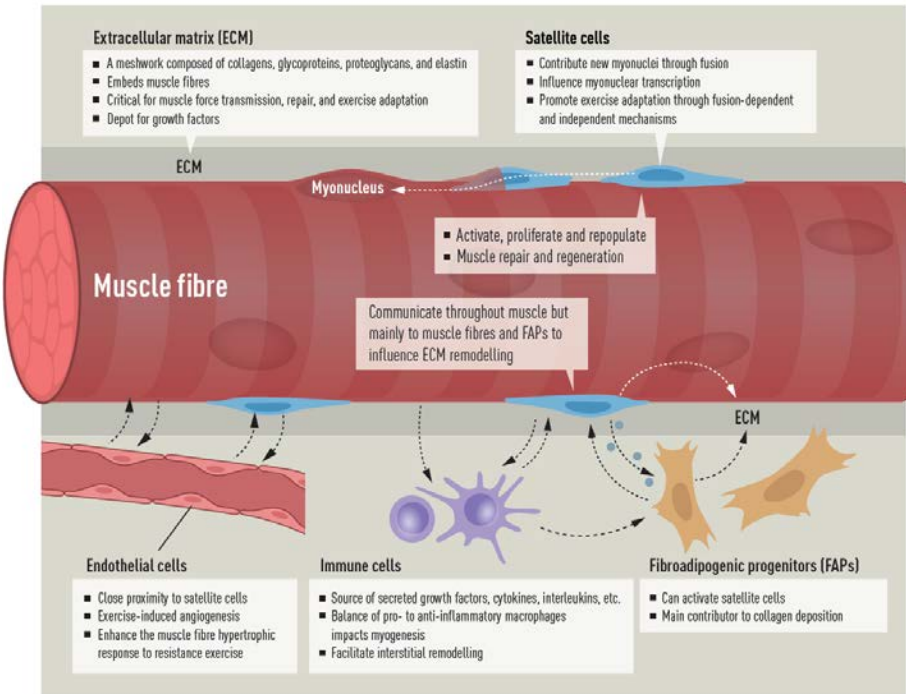


Figure 3. Skeletal muscle is a composite tissue. Approximately 40-45% of all cells in human vastus lateralis muscle are interstitial [216] and communication between these mononuclear cell populations, satellite cells, and muscle fibres is essential for skeletal muscle integrity, regeneration, and exercise adaptation. Figure 3 is reproduced from Smith et al. [51] with permission.

Interleukin 6 (IL-6) is a prime example of an exercise-stimulated factor secreted by skeletal muscle [217]. During endurance-type exercise, elevations in circulating IL-6 might contribute to short-term energy allocation by transiently inhibiting inflammatory processes, such as the production of tumour necrosis factor (TNF) by monocytes. Simultaneously, IL-6 promotes the preferential delivery of liberated non-esterified fatty acids (NEFAs) to working muscles [218]. Moreover, higher-intensity exercise, including sprints and resistance training, result in increased rates of muscle glycolysis and greater plasma lactate levels compared to moderate-intensity endurance exercise [219]. Muscle-derived lactate initiates a secretion axis involving adipose tissue-transforming growth factor β2 (TGFβ2), which facilitates improvements in murine glucose tolerance following 11 days of voluntary wheel running [220]. Additionally, lactate exiting muscle can be transformed into N-lactoyl-phenylalanine (Lac-Phe) through the action of CNDP2-expressing cells, such as macrophages and epithelial cells [221]. Intraperitoneal delivery of Lac-Phe caused appetite suppression and weight loss in obese mice. However, the concentrations of Lac-Phe observed in human plasma post-exercise were considerably lower than those administered in the murine experimental model [221].

This necessitates further investigation into the potential hunger-suppressing effects of Lac-Phe after acute exercise.

Mechanical overload induces a transient increase in fibroadipogenic progenitors (FAPs) within muscle [222], potentially through the migration of FAP-like adipose stromal cells from subcutaneous adipose tissue [223], followed by resident FAP proliferation [222, 223]. Ligand-receptor interactions are predicted to occur between FAPs and myogenic cells during early muscle differentiation [224]. Furthermore, the release of thrombospondin-1 (TSP1) by FAPs plays a role in activating satellite cells upon mechanical loading [222]. Activated satellite cells, in turn, communicate with FAPs [225, 226], muscle fibres [227], and other cells in the microenvironment [225] via secreted factors, such as myomiR-206-containing extracellular vesicles [225-227], facilitating appropriate transcriptional responses [225, 228] and promoting physiological extracellular matrix (ECM) deposition.

In cases of inflammatory muscle damage, the repair process in skeletal muscle appears to be coordinated between infiltrating immune cells, such as macrophages, and satellite cells [229, 230]. A similar interplay between macrophages and satellite cells might also contribute to muscle recovery and adaptation to physical activity. Following acute resistance exercise, macrophages exist along a pro- to anti-inflammatory (M1-to-M2) immunomodulatory spectrum in human muscle [231]. Conditioned medium from M1 or M2 macrophages enhances *in vitro* myoblast proliferation and differentiation, respectively [232]. Accordingly, imbalances in the M1-M2 macrophage ratio impairs murine muscle regeneration and results in excessive collagen accumulation [233]. Downhill running [234] exercise stimulates metoerin-like (*Metrnl*)-expressing macrophages to produce insulin-like growth factor 1 (IGF1) [235] and tumour necrosis factor-α (TNF-α) [234], promoting satellite cell-mediated repair [235] and FAP apoptosis [234] to restore muscle morphology [234, 235], function, and ECM integrity [234] in mice. Reciprocally, muscle can signal to macrophages through leukaemia inhibitory factor (LIF) [236] and protonated succinate [237] to encourage type I collagen turnover [236] and neuromuscular adaptations [237].

Exercise training leads to increased muscle capillarisation, which is partly driven by activating transcription factor 3/4 (*Atf3/Atf4*)-positive endothelial cells enriched near oxidative muscle fibres [238]. Endothelial cell expansion could be triggered by apelin (APLN) [239] released from skeletal muscle during exercise [240]. Vascular endothelial growth factor (VEGF) and secreted phosphoprotein 1 (also known as osteopontin, OSTP), produced by muscle in a peroxisome proliferator-activated receptor-γ coactivator 1α isoform 1 ($PGC1\alpha_1$)-dependent manner, further contribute to angiogenesis [241]. Increased vascularization enhances the hypertrophic response of skeletal muscle to resistance training [242], particularly of type II fibres in older individuals [231, 243, 244], by promoting closer association of satellite cells with capillaries [245] and facilitating endothelial-to-muscle fibre communication mediated by canonical Notch signalling [246]. Numerous other exercise-

responsive secretory factors regulated by PGC1α are thought to signal from muscle [247], including neurturin (NRTN), which coordinates slow-oxidative muscle fibre and slow-twitch motor neuron property transitions in mice. Notably, *NRTN* mRNA is upregulated in human vastus lateralis ≈72 hours after sprint interval exercise [248].

Collectively, cell-to-cell interactions and inter-organ signalling holds an important role in the local and global effects of exercise. However, in this area, further study is warranted regarding how the altered state of skeletal muscle and the perturbed systemic milieu interact in the context of metabolic disease. This investigation could reveal discrete effectors of cellular communication with actionable therapeutic implications.

1.7 Exercise training sustains and improves human metabolic health

Exercise training adaptations in muscle (Figure 4) collectively contribute towards improvements in gross performance measures such as $\dot{V}O_{2\,max}$ [134, 249], peak power output [250], ballistic power [251], and strength [134]. Furthermore, endurance [252] and resistance [253] training independently improve insulin sensitivity, glucose homeostasis, and other risk parameters associated with type 2 diabetes. Despite overlap, these exercise modalities pose unique physiological challenges [254] and promote distinct adaptations that most potently improve cardiometabolic health when performed concurrently [255]. Improved insulin sensitivity appears to be a localised and, somewhat, tissue specific response. GLUT4 content increased only in type I muscle fibres after two weeks of low-intensity, muscular-endurance-like exercise (a stimulus that would preferentially target type I fibres) [256]. Furthermore, 11 weeks of moderate-high intensity aerobic exercise improved insulin-stimulated glucose uptake in skeletal muscle, but not adipose tissue [257].

The insulin sensitising effects of exercise on skeletal muscle are likely enhanced by weight loss. Indeed, regular endurance exercise combined with fat loss over six months decreased markers of systemic inflammation, particularly plasma CRP [258]. This associated with reduced subcutaneous and visceral adiposity, as well as improved insulin sensitivity[258]. As such, fat loss and regular exercise could synergistically benefit skeletal muscle and whole-body insulin sensitivity through augmented signal transduction, intrinsic adaptive remodelling, and attenuated systemic inflammation.

Accordingly, several studies have demonstrated that many cases of type 2 diabetes are preventable through the modulation of dietary habits and physical activity levels. The Diabetes Prevention Program study allocated 3,234 individuals with impaired glucose tolerance into one of three groups: standard care (i.e., written dietary and activity guidelines, with a yearly follow-up) plus placebo tablets twice per day, standard care with metformin treatment twice per day (850 mg), or intensive lifestyle modification (≥150 min of moderate

intensity physical activity per week plus ≈7% weight loss throughout the trial) [259]. After 2.8 years, both the metformin and lifestyle intervention groups successfully reduced incidence of type 2 diabetes compared to placebo. However, lifestyle changes were more effective than metformin treatment (-58% versus -31%) [259]. Similar observations of the efficacy of exercise and/or diet in the prevention of type 2 diabetes were also made in other population-based trials, including the Da Qing Diabetes Study [260] and the Finnish Diabetes Prevention study [261].

Together, exercise training is an effective means for improving metabolic health. Yet, knowledge of the contributing mechanisms is still insufficient. A global approach to interrogate the peri-exercise skeletal muscle transcriptome could provide a more comprehensive understanding of the health-promoting cellular events, as well as the underlying molecular regulators. Understandings derived from such analysis could move exercise prescription towards improved personalised/targeted interventions.

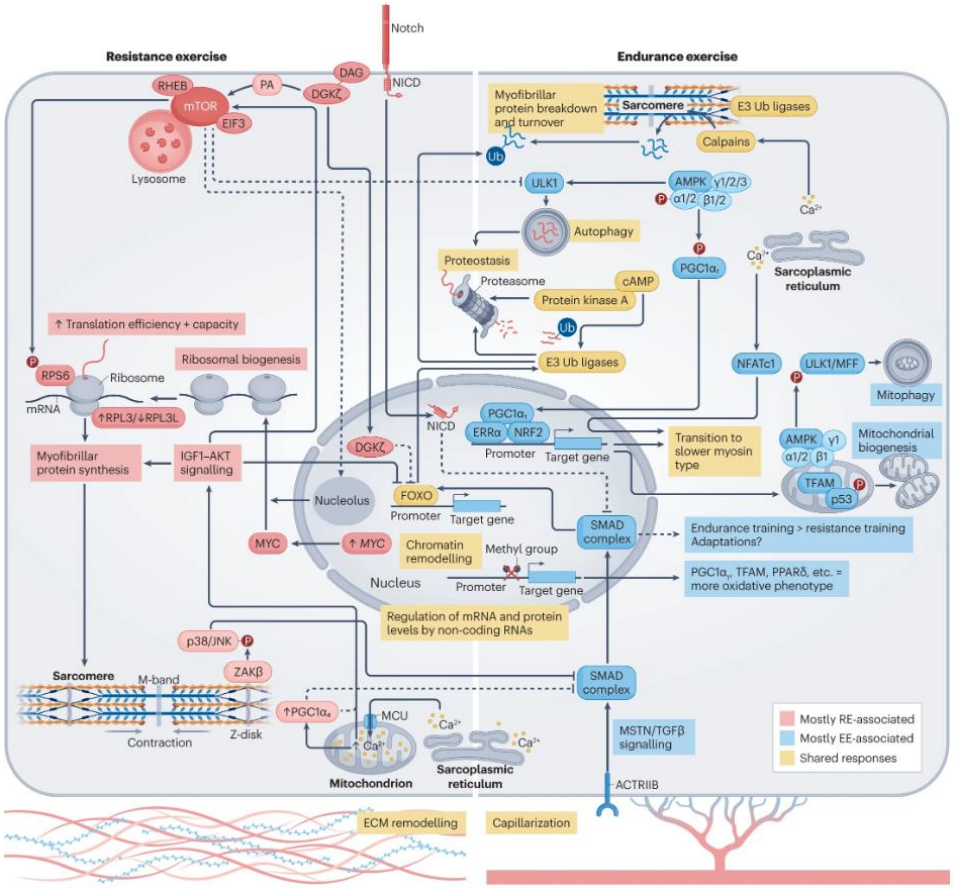


Figure 4. Molecular regulation of skeletal muscle adaptations to exercise. Acute exercise disrupts the delicate balance of systemic [254, 262] and local homeostasis [77]. This initiates a cascade of interconnected metabolic, hormonal, growth factor-related, inflammatory, and mechanosensitive processes that converge to orchestrate the temporal response of skeletal muscle adaptation to exercise. Exercise-induced perturbations trigger substantial posttranslational modification of the muscle proteome [219, 263] and DNA accessibility [264, 265]. Collectively, this drives transcription factor-dependent [266] alterations in gene networks [267], alongside microRNAs [268] and long-non-coding RNAs [269] that hone the molecular responses to exercise. While endurance exercise (EE) and resistance exercise (RE) are often perceived as distinct stimuli, primarily effecting oxidative *versus* hypertrophic muscle adaptations, they share enrichment of signalling cascades [219] and transcriptional networks [137] in the acute post-exercise period in the untrained state. For instance, coordinated proteolysis is a common feature after acute exercise, irrespective of modality [219]. This process is mediated by PKA and maintains protein quality control and physiological muscle remodelling [270, 271]. Additionally, both EE and RE induce AMPK activity [219] and total PGC1α mRNA expression [137]. AMPK phosphorylation of $PGC1\alpha_1$ [272] enhances angiogenic factors [273, 274] and mitochondrial bioenergetics in skeletal muscle [272, 273]. EE specifically augments mitochondrial AMPK-mediated mitophagy [275], as well as promoter hypomethylation and subsequent transcription of peroxisome proliferator-activated receptor-δ (PPARδ) and transcription factor A, mitochondrial (TFAM) [276]. However, it remains unclear if resistance exercise elicits similar responses. Although there are shared molecular events, post-exercise responses between EE and RE exhibit more distinctions than overlaps [137, 219]. After RE, rapamycin-sensitive substrates of mTORC1 are phosphorylated to greater extents

[219]. Mechanical overload during RE leads to translocation of the mTOR–lysosomal complex [192] and diacylglycerol (DAG) kinase-ζ (DGKζ) [277] to the sarcolemma. The colocalization of mTOR with RHEB, EIF3 [192], and phosphatidic acid (PA) produced by DGKζ [277] fully stimulate mTORC1-dependent translation of contraction-associated mRNAs. RE also modulates proteolytic signalling by attenuating ULK1 [278] and promoting nuclear DGKζ-mediated inhibition of FOXOs, thereby supporting muscle mass accrual by moderating myofibrillar protein breakdown [277]. Furthermore, muscle fibre contraction inhibits myostatin (MSTN)-transforming growth factor-β (TGFβ) signalling through ZAKβ-JNK [279, 280] and potentially Notch1 [281]. This represents one of many intracellular changes permitting resistance- over endurance- like adaptations [280]. Additionally, RE upregulates ribosomal biogenesis through MYC [121, 282] and a pool high ribosomal protein large 3 (RPL3) and low RPL3-like (RPL3L) ribosomes that may favour protein synthesis over translational fidelity [283]. Divergence between EE and RE becomes more pronounced at the transcriptomic level with repeated training exposure [137]. Endurance training increases genes associated with electron transport chain complexes [137], mitochondrial content, and skeletal muscle oxidative capacity [134]. In contrast, resistance training augments growth-related pathways [137], ribosomal abundance [282], and muscle mass [134]. This could be mediated, in part, by different isoforms of PGC1α. Nuclear PGC1α_1 and mitochondrial p53 are enhanced after high-intensity interval training [284], which might preserve mitochondrial content and function [285]. On the other hand, resistance training does not alter PGC1α_1 protein levels but instead enriches PGC1α_4 [286]. PGC1α_4 promotes muscle hypertrophy [287], associated with increased insulin-like growth factor 1 (IGF1)–AKT-mTORC1 signalling [288] and downregulation of *Mstn* mRNA [287] in mouse muscle. Unlike PGC1α_1, PGC1α_4 does not coactivate oestrogen-related receptor-α (ERRα) [287] or influence enzymes of oxidative phosphorylation [286, 287]. Considerable overlap in the initial stages of exercise training likely underlies the extent of adaptative similarity between EE and RE. Depending on individual predisposition, dedicated training towards a specific type of exercise potentially accentuates distinct differences in the adaptive response, leading to unique adaptations and the emergence of distinct skeletal muscle phenotypes over time [289]. There is little evidence supporting a blunting of muscle hypertrophy in humans through combined exercise training [290, 291]. However, concurrent training may impede gains in explosive strength [290]. Despite this, a combined exercise regimen might offer dual benefits for most individuals [134]. Figure 4 is reproduced from Smith et al. [51] with permission.

1.7.1 Acute exercise responses in the naïve versus exercise-trained state

The recurring stimulus of exercise training dampens select signalling [284] and transcriptomic [292] responses to an acute bout of physical activity. In particular, the directionality of altered genes contrasts substantially between exercise naive and trained murine muscle after thirty daily sessions of electrically induced mechanical overload [293]. At the 1-h sampling time point after exercise, ≈70% of ≈2,400 differentially expressed transcripts were upregulated on day two, whereas ≈83% (of ≈3,300 genes) were downregulated on day thirty [293]. This indicates that certain signalling mechanisms are sensitive to the modified intracellular environment that arises after a period of regular exercise. In combination with genetic predisposition [294, 295], the attenuated molecular responses observed post-exercise likely converge to limit the adaptive potential of muscle, slowing progress with advanced training experience. Nevertheless, some alterations might reflect a refined rather than impaired response. For example, pathways related to ribosomal biogenesis and protein synthesis show positive enrichment during the early stages of exercise adaptation in rat muscle. However, these pathways become downregulated in favour of metabolism-related processes once hypertrophy plateaus [293].

Given the diverse modifications observed, large-scale and high-throughput methods are required to investigate the modality-dependent temporal landscape of exercise adaptation in human skeletal muscle. Such approaches will enable the identification of specific changes in the acute exercise response that change consistent with training. Moreover, in-depth molecular profiling of skeletal muscle across a spectrum of metabolic health should enable better understanding of divergences in the adaptive response under conditions of disease.

2. Research aims

Physical inactivity impairs skeletal muscle health and function which, in turn, contributes towards the increased risk of developing obesity and metabolic diseases, such as type 2 diabetes. However, targeting skeletal muscle locomotion, in the form of exercise or ambulatory movement, is an effective measure to combat and manage whole-body metabolic impairments. This occurs, at least in part, because contraction improves skeletal muscle insulin sensitivity and substrate metabolism, and promotes remodelling within composite skeletal muscle tissue. Although physical activity counteracts many phenotypic manifestations of sedentariness, the mechanisms by which it does so are still not fully understood [136]. Indeed, exercise and physical inactivity differentially regulate similar pathways, yet there is little overlap at the level of individual genes [137]. Further insight into molecular coordination of the skeletal muscle transcriptome after exercise *versus* periods of inactivity should facilitate more precise exercise and pharmaceutical interventions for the betterment of metabolic health. Establishing accessible movement strategies, such as interrupting sedentary time, will compliment this process by informing entry-level guidelines so that the general public can refine their physical activity goals.

To contribute knowledge to these specific areas, the overall aims of this thesis were to:

- Assess the translational efficacy of interrupting sedentary time for the improvement of cardiometabolic health.

- Interrogate the skeletal muscle transcriptome after exercise or physical inactivity in the context of health and metabolic disease.

- Determine the metabolic effects of physical activity-responsive transcription factors and signalling moieties in skeletal muscle.

3. Materials and methods

In this section, select methods from the constituent papers of this thesis are presented, with the aim of providing a concise overview of their associated advantages and disadvantages. For a comprehensive account of all experimental procedures, readers are directed towards the methods section of the specific article of interest.

3.1 Continuous glucose monitoring

In **study I**, participants were equipped with continuous glucose monitors (CGMs) to assess glucose levels throughout each day of an intervention focused on investigating the impact of reducing prolonged sitting time on glycaemic control. CGMs have become an integral part of standard care for individuals with type 1 diabetes or type 2 diabetes receiving insulin therapy. Moreover, their practicality has led to increased utilisation in clinical trials targeting improvements in glycaemic control [296].

CGMs measure glucose concentrations in the interstitial fluid using a thin sensor filament inserted under the skin into the subcutaneous space. This enables the collection of real-time glucose readings, which are wirelessly transmitted to a receiver device at intervals of 1-5 min [296]. The receiver stores this information, allowing for the generation of glucose trend data related to glycaemic traits such as mean glucose levels, glucose level variation (%CV), or the time spent within, above, or below a specific target glucose range (e.g., 3.9-10.0 $mmol.L^{-1}$) [296]. Additionally, CGMs enable the use of calculations such as daily continuous net glycaemic action (CONGA), which assesses intraday glucose variation without relying on specific meal or exercise times [297].

Despite the practical advantages of CGMs, there are certain limitations to consider. These monitors are typically placed either on the abdomen or the upper arm. While there seems to be no discernible differences in sensor accuracy between anatomical locations [298], or discrepancies among different CGM brands [299], glucose readings from CGMs may exhibit inter-arm variations, with glucose levels potentially higher in the right arm compared to the left, irrespective of individual arm dominance [300]. This has important implications for sensor placement during clinical trials or when comparing results across studies. Furthermore, CGM sensors have a lifespan of up to two weeks before they need to be replaced [296], which may pose logistical challenges for trials of longer durations.

Overall, CGMs serve as a valuable method in the investigation of glycaemic control, allowing for real-time glucose measurements and the assessment of various glycaemic traits. However, considerations should be given to potential inter-arm variations in glucose readings and the limited lifespan of CGM sensors. By understanding the advantages and limitations associated with CGM, researchers can effectively utilise this method to advance our understanding of glucose dynamics and its impact on overall health.

3.2 Cell culture models

Cellular models play a pivotal role in the controlled study of biology, offering invaluable insights into the intricate molecular pathways and cellular responses that govern both healthy and pathological states. In **studies II-IV**, mouse C2C12 and/or primary human skeletal muscle myotubes were utilised as in vitro models to compliment investigations into skeletal muscle physiology.

3.2.1 Primary human and mouse C2C12 skeletal muscle cells

The immortalised cell-line of C2C12 myoblasts was originally isolated from mouse hindlimb muscle, whereas cultured primary myoblasts are extracted from human biopsies often obtained from the vastus lateralis muscle. Notably, both myoblast models possess the capacity to undergo differentiated, ultimately giving rise to uniform arrangements of multinucleated myotubes, resembling naïve skeletal muscle fibres. Indeed, the transcriptomes of skeletal muscle cells and tissues derived from the same species exhibit a strong correlation [301], underscoring the relevance of these model systems. Moreover, primary human skeletal muscle cells retain distinct characteristics associated with their respective donors, including features of insulin resistance in cells procured from individuals with type 2 diabetes [111]. However, the expression levels of genes encoding contractile proteins in both C2C12 and primary human myotubes are notably lower compared to those observed in fully matured skeletal muscle tissues [301], reflective of the limited maturity inherent to these culture models.

3.2.2 Metabolic responses of primary human and mouse C2C12 skeletal muscle myotubes

Unstimulated rates of glucose transport exhibit similarity between primary human and C2C12 myotubes. Nevertheless, C2C12 myotubes demonstrate greater rates of glucose oxidation under basal conditions, somewhat because primary human myotubes have a propensity to divert glucose away from oxidative pathways towards lactate production. Conversely, in response to insulin, primary human myotubes showcase higher rates of glucose incorporation into glycogen [301].

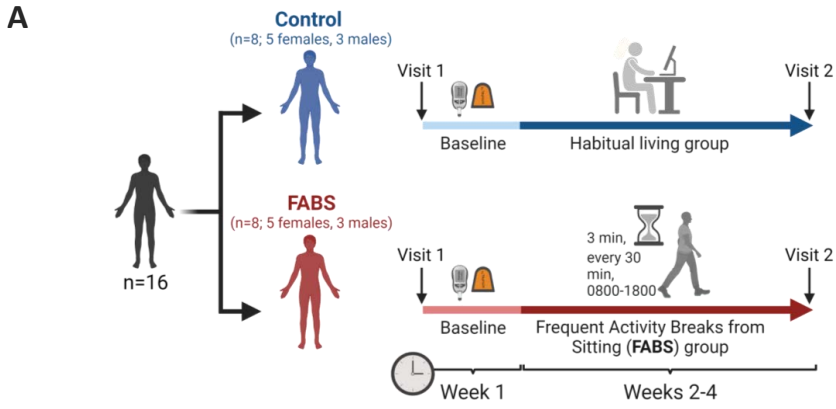
Furthermore, substantial disparities exist between primary human and C2C12 myotubes in terms of genes encoding sarcomeric proteins, with pathways associated with muscle development being especially enriched in C2C12 myotubes. Consequently, electrical pulse stimulation (EPS) elicits visible contractions in C2C12 myotubes cultures (15), contributing to greater EPS-stimulated glucose uptake. Nevertheless, primary human myotubes still display augmented glucose transport with this same stimulation paradigm [301].

In summary, primary human skeletal muscle myotubes and mouse C2C12 myotubes elicit metabolic and exercise-like responses akin to those observed in fully mature muscle tissue. However, the presence of unique differences in transcriptomic profiles, metabolic features, and contractile abundance highlights the context-dependent nature of selecting the most appropriate cellular system for addressing specific scientific inquiries.

4. Results

4.1 Study I: Three weeks of interrupting sitting lowers fasting glucose and glycaemic variability, but not glucose tolerance, in free-living women and men with obesity

In **study I**, we examined the impact of incorporating frequent activity breaks from sitting (FABS) on the glycaemic control of individuals with obesity, under free-living conditions. All participants underwent a comprehensive pre-trial assessment, which involved the placement of continuous glucose (CGM) and activPAL physical activity monitors. Block randomisation was then employed to allocate participants into either the no-intervention (Control) or FABS groups. The initial week of the trial (i.e., week 1) served as a baseline measurement period, during which participants maintained their habitual living patterns. Subsequently, participants were instructed to adhere to similar dietary behaviours for the duration of the trial. During weeks 2-4, the FABS group received notifications every 30 min, from 08:00-18:00, through a smartphone app connected to a smartwatch. These notifications were designed to prompt participants to engage in 3 min of low-to-moderate-intensity physical activity, such as walking, stair-climbing, or bodyweight squats etc. The app recorded a successful activity break when a minimum threshold of ≥15 steps was registered. In contrast, the Control group continued with their usual daily activity levels. At the end of week four, participants returned to the clinic and underwent the same procedures as their first visit, including anthropometric measurements, an oral glucose tolerance test (OGTT), and blood and skeletal muscle sampling. A schematic overview of the study design and select clinical characteristics are shown in Figure 5.



B **Visits 1 and 2:**

C

Select characteristics	Control	FABS
Age (years)	47 ± 5	49 ± 10
BMI (kg.m^{-2})	34.3 ± 3.9	33.4 ± 3.6
HbA_{1c} (mmol.mol^{-1})	37.4 ± 4.3	33.3 ± 6.2
HIRI	161.2 ± 41.3	55.9 ± 21 *
HOMA2-IR	2.2 ± 1.1	1.4 ± 0.8
HOMA2-%β	117.9 ± 19.8	100.7 ± 32.7
HOMA2-%S	52.9 ± 17	93.4 ± 54.7 †

Figure 5. Overview of the frequent activity breaks from sitting (FABS) intervention. ***A. Schematic summary of study design.*** Participants (n = 16) were randomised to Control (n = 8) or FABS (n = 8) groups. Activity and glucose continuous monitoring data were collected for 1 week of baseline and three weeks of intervention. ***B. Pre- (visit 1) and post- trial (visit 2) measurements.*** Anthropometric measures were made, blood and skeletal muscle samples were taken, and an oral glucose tolerance test (OGTT) was performed at clinical visits 1 and 2. ***C. Select baseline clinical characteristics of participants in the Control and FABS groups.*** BMI = body mass index; HbA1c = glycated haemoglobin; HIRI = hepatic insulin resistance index; HOMA = the homeostasis model assessment of: insulin resistance (HOMA2-IR), β-cell function (HOMA2-%β), and insulin sensitivity (HOMA2-%S). *p<0.0001 and † p=0.067, FABS vs. Control, unpaired Student's t test.

4.1.1 The FABS intervention marginally increased physical activity levels

Analysis of adherence to the breaking sitting protocol indicated high adherence in the FABS group during the first week of intervention (i.e., trial week 2). However, adherence levels declined towards baseline for six out of eight participants during weeks 3-4 (Figure 6A). Nevertheless, no statistical differences in adherence over time were observed in either group. Both groups displayed variations in the number of steps taken throughout the day, but the intervention had no discernible effect on 24-h stepping curves compared to baseline (Figure 6B). Despite this, FABS did lead to modest alterations in physical activity levels during the intervention weeks, as evidenced by a median increase of 744 steps per day, along with an additional 10.4 min spent walking (Figures 6C, D). Conversely, no changes in daily step count or walking time were observed in the Control group. Other physical activity behaviours, such as the number of postural transitions from sitting to standing, remained unchanged from baseline in both groups (Figure 6E).

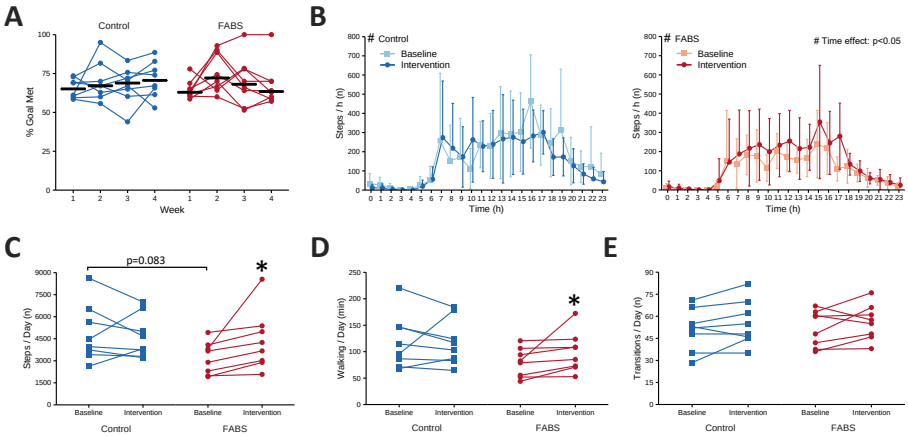


Figure 6. The FABS intervention marginally increased physical activity levels. A. weekly adherence to intervention protocol (%). Weeks 2–4 are intervention weeks, black lines indicate median group adherence for each week, and connecting lines represent patterns of adherence for each participant. **B.** Pattern of daily stepping activity during intervention weeks compared to baseline. Data are means (±SD) of median steps taken per hour. Paired mixed-design analysis of variance (time x intervention), #overall time affect ($p<0.05$). **C-E.** Median number of daily (**C**) steps taken, (**D**) minutes spent walking, and (**E**) postural transitions made from sitting to standing during intervention weeks compared to baseline. Wilcoxon signed-rank (within-group) and Mann–Whitney U (baseline between-group) tests, *$p<0.05$. Control group (blue, n = 8), FABS group (red, n = 8).

4.1.2 FABS had no effect on glucose tolerance but lowered fasting plasma glucose and interstitial glucose variability

At baseline, the Control and FABS groups exhibited insulin resistance, as evidenced by a homeostasis model assessment (HOMA) of insulin resistance (HOMA2-IR) >1.21 and a Matsuda Index <5 [302]. Notably, the FABS group demonstrated better hepatic insulin resistance index (HIRI) and HOMA2-%S scores, suggesting healthier liver and peripheral insulin sensitivity when commencing the study (Figure 5C). However, no changes in glucose tolerance or indices of insulin resistance/sensitivity were observed after the trial period in either group. As such, incremental areas under the curve (iAUC) for glucose and insulin excursions during an oral glucose tolerance test (OGTT) were unaltered after the intervention (Figures 7A, B). Nevertheless, post-trial fasting plasma glucose concentrations only displayed reductions in the FABS group (−0.34 $mmol.L^{-1}$), indicative of improved glycaemia (Figure 7C). This was further supported by continuous glucose monitoring, whereby within-daily variations of interstitial glucose (coefficient of variation, %CV) were consistently lowered in the FABS group (-2%) (Figure 7D).

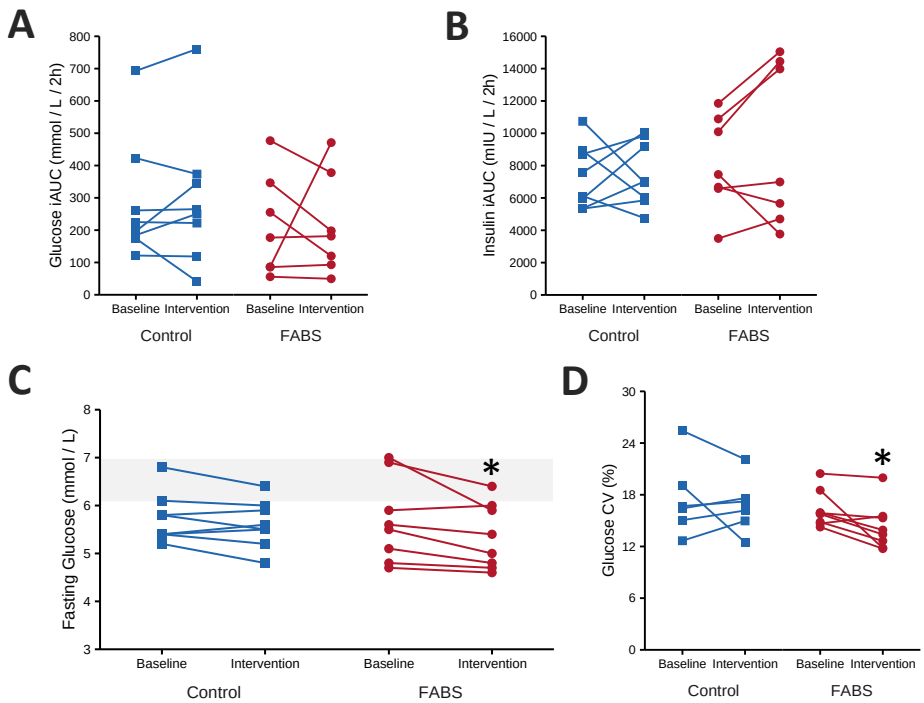


Figure 7. FABS had no effect on glucose tolerance but lowered fasting plasma glucose and interstitial glucose variability. A, B. Pre-to-post trial 2-h oral glucose tolerance test (OGTT) incremental areas under the curves (iAUC) for (**A**) glucose and (**B**) insulin in Control (blue, n = 8) and FABS (red, n = 7) groups. **C.** Pre-to-post trial changes in fasting plasma glucose levels for Control (blue, n = 8) and FABS (red, n = 8) groups. *p=0.037, paired Student's t test. Grey box = ADA range for impaired fasting glucose (≥6.1 to <7 mmol.L^{-1}). **D.** Pre-to-post trial effects on interstitial glucose coefficient of variation (%CV) for Control (blue, n = 6) and FABS (red, n = 8) groups. *p=0.039, paired Student's t test.

4.1.3 Greater volumes of FABS more consistently improved continuous glucose control

In light of the apparent variability of adherence to the intervention (Figure 6), we sought to determine the potential association between magnitude of improvements in glycaemic control and the amount of breaking sitting physical activities undertaken by participants in the intervention group. Accordingly, individuals in the FABS group were divided into high- (n = 4 females) and low- (n = 4, 1 female and 3 males) physical activity subgroups, as determined by the combined total number of steps and postural transitions performed per day during the intervention period (Figure 8A). Participants with higher levels of physical activity demonstrated a greater propensity to consistently and effectively reduce their glucose variability, as indicated by intervention and subgroup interactions across all parameters of dynamic glucose control. These interactions were primarily driven by baseline-to-intervention differences in the high-activity subgroup (SD, −0.20 mmol.L^{-1}, p = 0.014; %CV, −3.4%, p=0.016;

CONGA1, −0.21 mmol.L^{-1}, p=0.006; CONGA2, −0.24 mmol.L^{-1}, p = 0.013; CONGA4, −0.25 mmol.L^{-1}, p=0.040), which were not evident within the low-activity subgroup (Figures 8B-D).

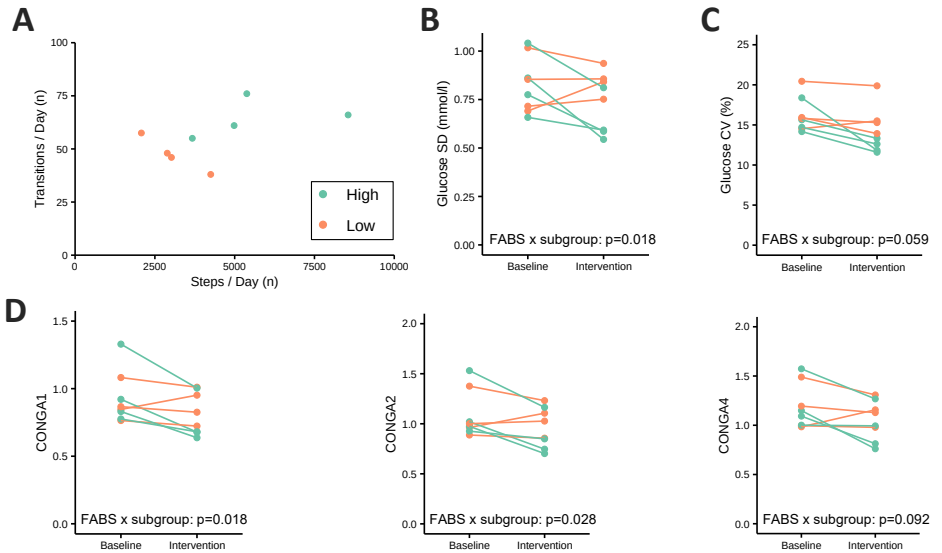


Figure 8. Greater volumes of FABS more consistently improved continuous glucose control. A. FABS group participants were separated into low- (orange) and high- (green) activity levels, based on the median number of steps and postural transitions taken per day during intervention weeks. **B-D.** Indices of dynamic glucose during intervention weeks compared with baseline, control colour-coded according to participant activity level in FABS group. Mean daily glucose (**B**) standard deviation (SD) and (**C**) coefficient of variation (%CV). **D.** Continuous overall net glycaemic action (CONGA) for 1-h (CONGA1), 2-h (CONGA2), and 4-h (CONGA4) intervals. Mixed-design analysis of variance (time x subgroup). Respective interaction effects are denoted within figures. Low-activity subgroup (orange; n = 4, 1 female and 3 males), high-activity subgroup (green; n = 4 females).

4.1.4 Study I discussion

In a real-world setting, reminders to interrupt prolonged sitting had a modest impact on stepping behaviour throughout the day. While this increase in activity did not improve glucose tolerance, it did result in lower fasting blood glucose levels and intraday glucose variability, which may have clinical relevance.

Interestingly, our study's findings regarding glucose tolerance differ somewhat from previous research [303]. This contrast could be attributed to the differences in the design of our free-living trial. Much of the existing literature on the benefits of interrupting sedentary behaviour comes from controlled laboratory interventions [303] that involve prolonged sitting in the control condition, compared to habitual levels [304]. However, even a single day of enforced sitting can negatively affect energy balance and decrease whole-body insulin sensitivity [305]. Therefore, previous trials may have inadvertently compared the effects of

interrupting sitting to a control group with impaired glucose tolerance, limiting the generalisability of their results to everyday scenarios.

Furthermore, many controlled laboratory trials have assessed the effects of activity breaks on glucose and insulin levels during the postprandial period (≥4 h) following mixed-macronutrient meals or drinks [210, 304, 306]. Alternatively, our study evaluated glucose tolerance the morning after the intervention period (without activity breaks) using only a glucose load, with measurements taken over a 2-h postprandial period. Previous research indicates that the glucose- and insulin- lowering effects of 17 sets of 2-minute intervals of light walking per day (every 20 min for a total of 7 hours; 34 minutes in total) does not accumulate over 3 days [306]. Therefore, daily repetition of breaking sitting protocols may be necessary to sustain any glycaemic benefit.

When comparing our study to other free-living trials conducted thus far, the baseline step counts were similar (≈3,000 to 4,500 steps per day) [307-309]. However, the interventions in these studies involved much larger amounts of physical activity (i.e., ≥2.5 hours standing plus ≥17,500 steps per day) to improve insulin sensitivity and blood lipid profiles [310]. In contrast, our intervention had a lower threshold activity requirement (≥15 steps every 30 min) and only increased daily steps by 744. This suggests that greater intensities, frequencies, and/or volumes of physical activity breaks from sitting may be necessary to have a lasting impact on insulin sensitivity, with volume potentially being the most critical factor for overall health [311].

Although there was no improvement in the oral glucose tolerance test response after the intervention, collective analysis of dynamic glucose measures [297] suggests that our intervention marginally reduces intraday variation in interstitial glucose levels compared to baseline. This effect seems to be more pronounced in participants with higher daily activity levels and may be partly attributed to improved blood flow to skeletal muscles. Inactivity promotes microvascular dysfunction [46], while light-intensity bodyweight exercises performed every 30 min enhance femoral artery dilation mediated by blood flow [312, 313]. These improvements in glucose dispersion within the skeletal muscle interstitium, and subsequent uptake, could contribute to increased 24-h carbohydrate oxidation when 5-min bouts of moderate-intensity walking are performed hourly over 9 h [314]. The mechanisms underlying the reduction in fasting glucose concentrations may also involve enhanced blood flow to skeletal muscles [312, 313] and attenuation of systemic inflammation. In fact, C-reactive protein (CRP) levels, which positively correlate with fasting glucose [315], were found to be lower in participants engaging in frequent activity breaks from sitting [316].

In conclusion, **study I** may represent the minimum effective dose for interrupting sedentary behaviour, and larger volumes of total activity may be required to achieve greater health benefits.

4.2 Study II: Transcriptomic profiling of skeletal muscle adaptations to exercise and inactivity.

At the time of Study II, >60 datasets had already been published, each examining the transcriptomic response of skeletal muscle to different modes of exercise in various populations. Given the common issue of underpowered human studies in the context of physical activity, these publicly available datasets presented an immense and untapped biological resource. Therefore, we adopted a meta-analytical approach to investigate these datasets, aiming to foster a deeper understanding of the molecular events that occur in skeletal muscle after exercise and physical inactivity. To this end, our analysis shed light on the contrasting transcriptomic responses of skeletal muscle to differing exercise modalities, physical inactivity, as well as disparities among phenotypically distinct individuals. An overview of the meta-analysis design and select participant characteristics within the different study groups are illustrated in Figure 9.

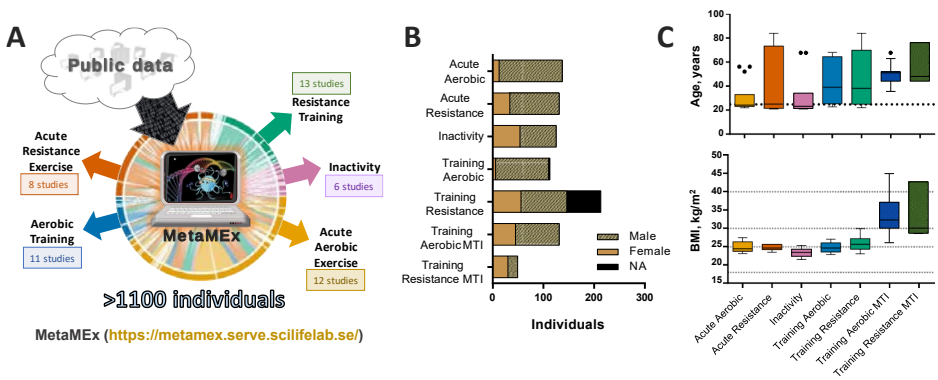


Figure 9. Overview of studies included to meta-analyse (MetaMEx) the human skeletal muscle transcriptomic response to exercise and physical inactivity. A. Schematic of the study design. The same methodology was applied to all studies: collection of raw data when available, quality control, annotation, normalisation, and calculation of statistics (fold-change; false discovery rate, FDR). The meta-analysis was then calculated using the restricted maximum-likelihood method, taking into consideration the average, standard deviation, and sample size for each study. **B.** Total number and sex of individuals within each study group. NA = annotation of sex not available. **C.** Age (top figure) and body mass index (BMI; bottom figure) of participants within study groups. Studies had a minority of female participants and varying age ranges. BMI was similar for individuals of normal health but higher in studies that included people with metabolic impairments (obesity and/or type 2 diabetes; MTI). Data are box-and-whisker plots with Tukey distribution.

4.2.1 Inter-array comparisons separate acute exercise from exercise training and physical inactivity

Utilising principal component analysis (PCA), we observed distinct clustering patterns of gene responses based on different intervention types (Figure 10A). Specifically, studies examining the effects of acute aerobic (i.e., endurance) and resistance exercise exhibited clustering patterns that were separate from those of studies investigating exercise training

and physical inactivity. Intriguingly, this suggests that acute bouts of endurance and resistance exercise elicit transcriptomic responses in skeletal muscle that are more similar to each other than to the stimulus of the same modality after a period of exercise training.

We next performed a comprehensive meta-analysis of all transcripts. By implementing restricted maximum likelihood, we calculated the fold-change and significance for each exercise- or physical inactivity- responsive gene on an individual basis. Following adjustment for multiple comparisons, we discovered a greater number of differentially expressed genes compared to what was obtained in individual studies, thereby highlighting the power of the meta-analysis approach. Notably, our analysis revealed that each intervention led to more discretely regulated genes than those that overlapped (Figure 10B). Indeed, the total number of responsive genes for each perturbation was: 897 for acute aerobic exercise, 2404 for acute resistance exercise, 1576 for physical inactivity, 82 for aerobic training, and 2049 for resistance training. Furthermore, acute aerobic and acute resistance exercise commonly regulated 360 transcripts, while aerobic training and resistance training displayed overlap for only 25 genes.

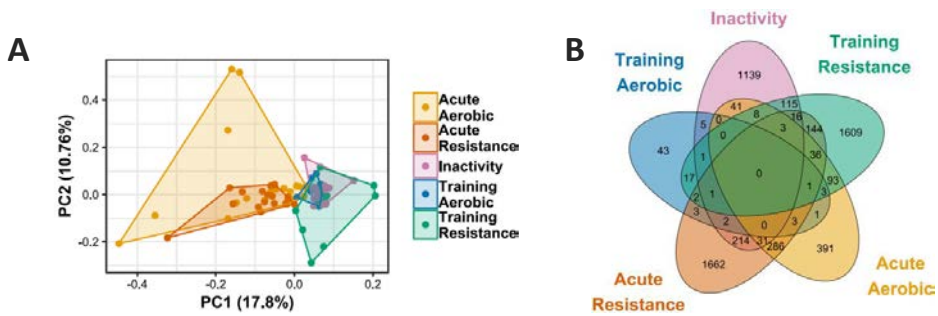


Figure 10. Inter-array comparisons separate acute exercise from exercise training and physical inactivity. A. Datasets of individuals with normal health were compared with using principle component analysis (PCA). **B.** Venn Diagram depicting overlap of differentially expressed genes (DEGs). FDR<0.01.

4.2.2 Transcriptomic pathways and select genes altered by exercise and physical inactivity

Gene ontology further highlighted shared and distinct transcriptional pathways regulated by different physical activity paradigms (Figure 11A). Both acute aerobic and resistance exercise upregulated pathways associated with the unfolded protein response, kinase activity, and metabolism-related processes, but only acute aerobic exercise altered the expression vascular development genes (i.e., 'extracellular structure organisation'). Additionally, whereas physical inactivity reduced the expression of genes involved in mitochondrial processes and oxidative ATP production, aerobic training had the opposite influence on these same pathways. These effects were further emphasised by the divergent impact of physical inactivity and aerobic training on genes encoding complexes of the

mitochondrial electron transport chain (Figure 11B). Alternatively, only resistance training augmented genes related to extracellular matrix remodelling (Figure 11A).

Local inflammation plays an integral role in skeletal muscle remodelling after exercise and our meta-analysis disclosed that acute exercise promotes the expression of numerous cytokine transcripts (Figure 11C). Acute aerobic exercise, in particular, had the strongest effect. However, the monocyte attractant C-C motif chemokine 2 (CCL2) was increased by both acute aerobic and resistance exercise. Conversely, exercise training evoked little perturbation of cytokines. This could be because skeletal muscle becomes more robust against contraction-induced damage with consistent exercise [317]. Additionally, biopsies in these training studies were typically collected 48 h following the final exercise bout. At this point, transient exercise-induced inflammatory responses had likely diminished. We also noted that exercise protocols commonly reduced levels of the negative regulator of skeletal muscle mass myostatin (*MSTN*). On the other hand, *MSTN* mRNA was elevated after the physical inactivity interventions (Figure 11C). Additionally, exercise interventions modulated transcripts of sarcomeric myosin heavy (MyHC) and light chains (Figure 11D), with most myosin isoforms being downregulated following exercise training. Both aerobic and resistance training reduced *MYH1* (encoding MyHC type IIX) and *MYH4* (encoding MyHC type IIB) mRNA. However, whereas aerobic training increased *MYH7* (encoding MyHC type I), this MyHC isoform was reduced by resistance training, as were the myosin light chain isoforms *MYL2* and *MYL3*. In contrast, inactivity was generally associated with an inverse MyHC profile compared to exercise training.

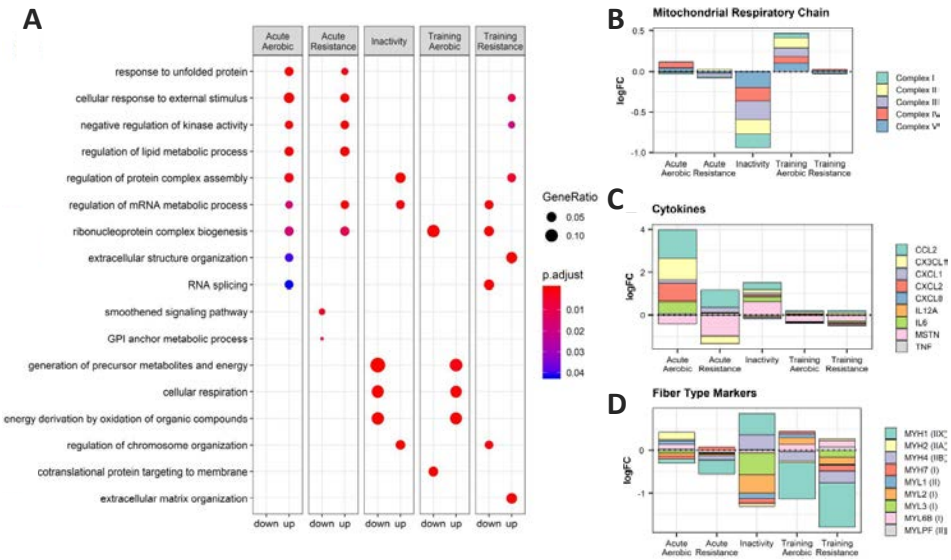


Figure 11. Transcriptomic pathways and select genes altered by exercise and physical inactivity. A. Gene ontology analysis of DEGs (FDR<0.01) in each group. **B-D.** Genes corresponding to proteins of interest were collected from the KEGG database and fold-changes were added to present the overall modification of enzymes involved in pathways related to (**B**) mitochondrial respiratory complexes (**C**), inflammation, and (**D**) muscle fibre type.

4.2.3 NR4A3 regulates the metabolic response to in vitro exercise

In response to muscle contraction, the transcription factor nuclear receptor subfamily 4 group A member 3 (*NR4A3*, also known as *NOR1*) is upregulated. This occurs predominantly via the calcium-dependent activation of cyclic AMP (cAMP)-responsive element-binding protein (CREB) [318]. There are several lines of evidence that NR4A3 is complicit in the metabolic response to exercise in skeletal muscle. Rats bred for aerobic fitness have skeletal muscle enrichment of NR4A3, which associates with higher mitochondrial abundance and increased running capacity [319]. Moreover, transgenic mice overexpressing of *Nr4a3* from a skeletal muscle-specific promoter experience remodelling towards a more oxidative phenotype [320]. This suggests that NR4A3 plays a causal role in the metabolic reprogramming of skeletal muscle.

Meta-analyses confirmed that *NR4A3* was upregulated in human skeletal muscle shortly following acute aerobic and resistance exercise (data not shown). Additionally, *NR4A3* responded to electrical pulse stimulation (EPS) in an intensity- and time- dependent manner in primary human skeletal muscle myotubes (data not shown). This indicated a conserved in vitro response of *NR4A3* to exercise-like stimuli and suggested primary human skeletal muscle myotubes were an appropriate study model. Therefore, we used small interfering RNA

(siRNA) to further explore the metabolic implication of NR4A3 in vitro. The results of these experiments are illustrated in Figure 12 and elaborated upon in the figure legend.

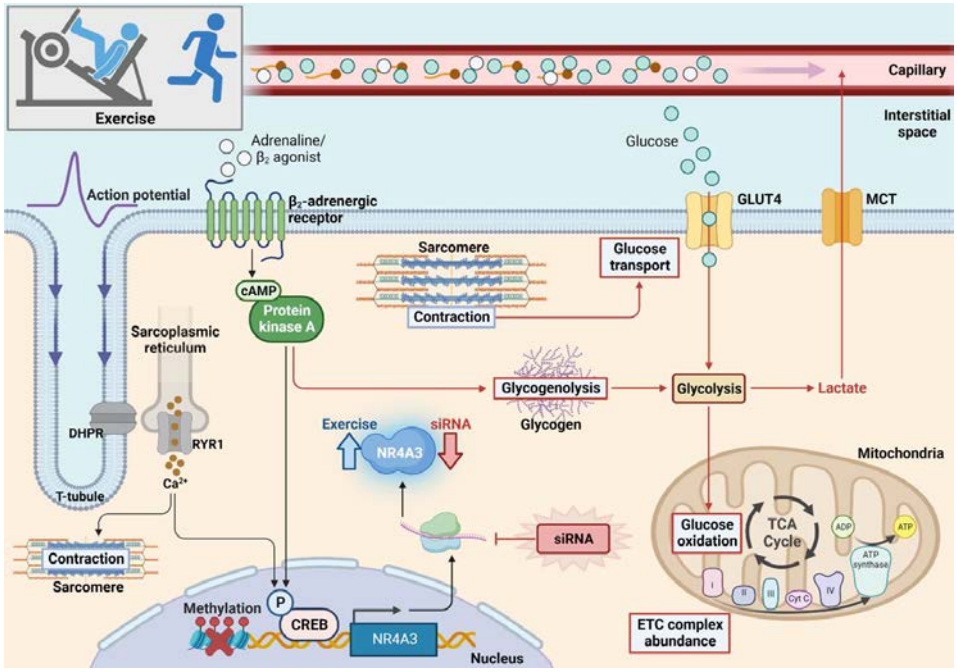


Figure 12. NR4A3 regulates the metabolic response to in vitro exercise. In response to exercise, a combination of calcium (Ca^{2+}) [318] and β_2 adrenergic signalling [321] converge to activate cyclic AMP (cAMP)-responsive element-binding protein (CREB). In combination with hypomethylation of its promoter [322], this induces *NR4A3* transcription. We found that small interfering RNA (siRNA)-mediated suppression of NR4A3 attenuated several exercise-like metabolic responses in primary human skeletal muscle myotubes. Specifically, *NR4A3* silencing blunted the electrical pulse stimulation (EPS)-dependent increase in glucose transport, the β_2-adrenergic receptor agonist salbutamol-induced upregulation of glycolytic flux, and diminished mitochondrial oxygen consumption rates, concomitant with reduced protein expression of electron transport chain (ETC) complexes. Red boxes and arrows indicate metabolic processes inhibited by siRNA downregulation of NR4A3. DHPR = dihydropyridine receptor tetrads, GLUT4 = glucose transporter 4, MCT = monocarboxylate transporter, RYR1 = ryanodine receptor 1, TCA cycle = tricarboxylic acid cycle, T-tubule = transverse tubule.

4.2.4 Differential response to exercise training in metabolically impaired individuals

We next used our curated database to compare the skeletal muscle transcriptomes of metabolically impaired individuals (with obesity and/or type 2 diabetes) with healthy counterparts after exercise training (Figure 13). Aerobic and resistance training studies were selected such that groups were matched for age. We also excluded studies with marked differences in training protocols employed or anatomical location of skeletal muscle biopsies. As such, our analysis included six studies of aerobic training and two studies of resistance. Intriguingly, principle component analysis separated aerobic and resistance training in

healthy individuals, but not in those with metabolically impairments (Figure 13A). Indeed, aerobic and resistance training induced distinct gene profiles in people with metabolic impairments versus healthy counterparts. This was clearly depicted by dissimilar pathway enrichments between groups when comparing the same exercise modality (Figure 13B). Nevertheless, the induction of classically exercise-responsive genes, including transcripts of mitochondrial complex assembly, lipid metabolism, and glycolysis, were largely similar between healthy and metabolically impaired individuals (data not shown). This is consistent with the propensity of exercise to improve skeletal muscle health in the context of metabolic disease.

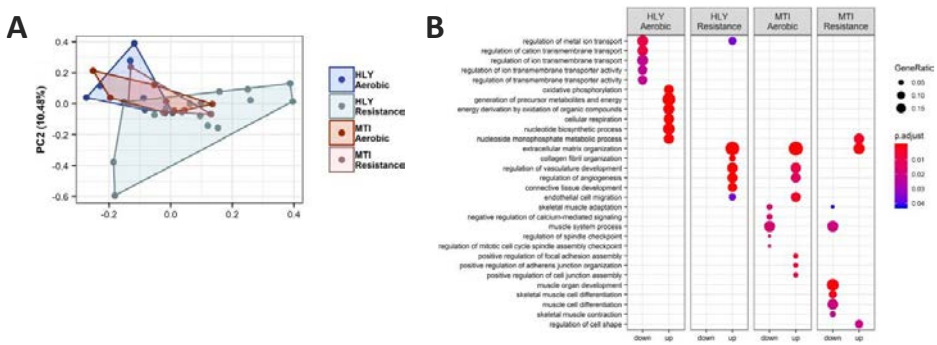


Figure 13. Differential response to exercise training in metabolically impaired individuals. A. Principle component analysis (PCA) comparing the exercise training response of individuals considered healthy (HLY) or metabolically impaired (MTI) individuals. **B.** Gene ontology analysis calculated based on genes with FDR<0.1 demonstrating a differential response of MTI to both aerobic (i.e., endurance) and resistance training protocols.

4.2.5 Study II discussion

In this study, we introduce a unique resource aimed at consolidating publicly available data to propel the formulation of new hypotheses and foster innovative discoveries. The MetaMEx database, accessible at https://metamex.serve.scilifelab.se/app/metamex, serves as a centralised repository of exercise transcriptomic studies, curated and comprehensively annotated based on age, sex, body mass index, and metabolic disease status. The establishment of this central hub offers numerous advantages, including the potential identification of knowledge gaps and the opportunity for initial hypothesis testing, ultimately leading to enhanced design of prospective intervention studies. This approach transcends the limitations of analysing individual arrays, thereby bolstering the statistical power to unearth novel biological mechanisms underlying skeletal muscle plasticity.

Through our meta-analytical approach, several previously unacknowledged or understudied pathways have been brought to light. Noteworthy among these findings are the divergent transcriptomic responses observed between different exercise modalities, physical inactivity, and phenotypically distinct individuals. For instance, our analysis revealed a greater

convergence of acute aerobic and resistance exercise responses when compared to exercise training. This aligns with the development of more refined transcriptomic patterns and the emergence of specific phenotypes over time with dedicated training towards a particular exercise modality [289]. Additionally, a targeted examination of genes encoding mitochondrial electron transport chain components and sarcomeric myosin heavy chain isoforms highlights the preferential increase in mitochondrial abundance induced by aerobic exercise training [249] and the gradual transition of skeletal muscle fibres towards a slower myosin type with consistent physical activity [57].

Mechanisms underlying the detrimental effects of physical inactivity differ from those conferring the benefits of regular physical activity [136]. Interestingly, we observed limited overlap between the individual genes regulated by aerobic or resistance training and those modified by inactivity. However, gene ontology analysis revealed that pathways associated with oxidative phosphorylation are downregulated during periods of inactivity and upregulated following aerobic training. This suggests that although distinct sets of genes are recruited in response to these divergent perturbations, they ultimately converge on the modulation of mitochondrial function.

Furthermore, we identified a specific subset of genes involved in transcriptional regulation, inflammation, and lipid transport that exhibited selective changes in healthy individuals while remaining unaltered in individuals with metabolic impairments. An in-depth exploration of these transcripts in the context of exercise may yield potential targets for optimizing exercise prescription in individuals with metabolic impairments.

In summary, **study II** provides an extensive analysis of skeletal muscle transcriptional responses to different modes of exercise and offers an intuitive online interface to seamlessly interrogate the MetaMEx database.

4.3 Study III: Downregulation of NR4A3 during inactivity alters glucose metabolism and impairs translation in human skeletal muscle

In study II, we identified *NR4A3* as one of only four transcripts inversely regulated by acute exercise and physical inactivity in human skeletal muscle. Furthermore, the gene expression profile of primary human skeletal muscle myotubes upon RNA interference of *NR4A3* was positively correlated with the *in vivo* human skeletal muscle transcriptome after physical inactivity. Therefore, in **study III**, we built upon these observations to gain further insight into NR4A3 in the context of skeletal muscle inactivity.

4.3.1 Downregulation of *NR4A3* early during inactivity correlates with transcripts involved in myogenic and metabolic pathways

Meta-analysis of eight published transcriptomic studies of the human skeletal muscle response to physical inactivity confirmed that *NR4A3* mRNA was reduced by 27% (Figure 14A). However, this decrease was not evident in all studies. Grouping based on the duration of inactivity demonstrated that skeletal muscle *NR4A3* expression was transiently repressed during the first days of disuse before returning to baseline levels after two weeks (Figure 14B). Correlation of *NR4A3* with all other transcripts present in the eight studies allowed for the characterisation of pathways co-regulated with *NR4A3* during physical inactivity (Figure 14C). Gene set enrichment analysis showed that *NR4A3* was positively associated with mitochondrial function and negatively associated with pathways related to cytoskeleton organisation, chromatin regulation, protein synthesis and degradation. Furthermore, combined analysis of two datasets exploring the restoration of ambulatory behaviour after physical inactivity showed that reloading restored *NR4A3* mRNA to pre-inactivity levels (Figure 14D), consistent with its role of as a contraction-responsive transcription factor in skeletal muscle

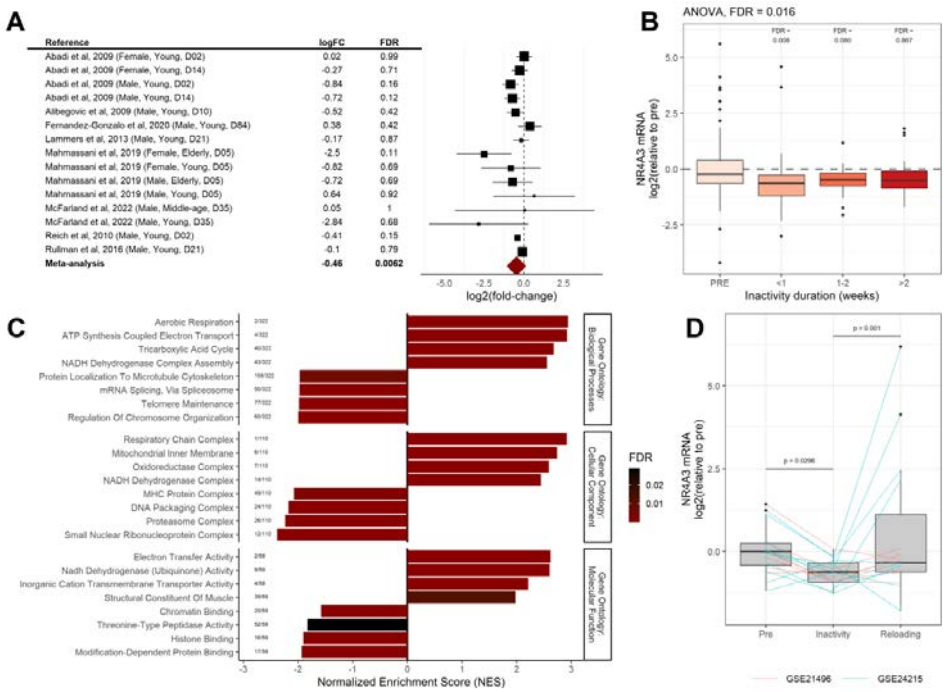


Figure 14. Downregulation of *NR4A3* early during inactivity correlates with transcripts involved in myogenic and metabolic pathways. A. Meta-analysis of transcriptomic inactivity studies in human skeletal muscle. **B.** Transcriptomic studies were pooled by the duration of inactivity protocols: less than one week, one-two weeks or more than two weeks. **C.** Gene set enrichment analysis with the Gene Ontology dataset was performed on genes ranked on Spearman correlation with *NR4A3* across all transcriptomic studies of inactivity. **D.** The two studies that explored effects of reloading after inactivity were merged and analysed as described in methods.

4.3.2 *NR4A3* silencing in primary human skeletal muscle myotubes attenuates glucose oxidation by diverting glucose towards lactate production.

To mimic the decreased levels observed in human skeletal muscle after inactivity, NR4A3 was experimentally downregulated using RNA interference in differentiated primary human skeletal muscle cells. Silencing of *NR4A3* in human myotubes was associated with modest compensatory increases in other NR4A family members *NR4A1* (*NUR77*) and *NR4A2* (*NURR1*) (Figure 15A). NR4A3 protein was almost exclusively localised to the nucleus in myotubes and silencing efficiently reduced nuclear protein abundance (Figure 15B). This depletion of NR4A3 lowered basal and FCCP-stimulated (uncoupled) glucose oxidation (Figure 15C) independent of glucose transport (data not shown). Rather, lactate production and release into culture medium was augmented upon *NR4A3* silencing (Figure 15D), indicating a shift in glucose fate that was also reported in mouse C2C12 myotubes [321]. Accordingly, mRNA expression of the alpha isoform of lactate dehydrogenase (*LDHA*) increased after *NR4A3* interference, while expression of the beta isoform decreased (*LDHB*) (Figure 15E). This

modification of LDH isoform composition suggests preferential production of lactate from glycolysis and/or less conversion of lactate to pyruvate [323]. Thus, the attenuation of glucose oxidation in NR4A3-deplete myotubes appears predominantly a consequence of greater diversion of glucose towards lactate, which would reduce glucose-derived acetyl-CoA entry into the tricarboxylic acid (TCA) cycle.

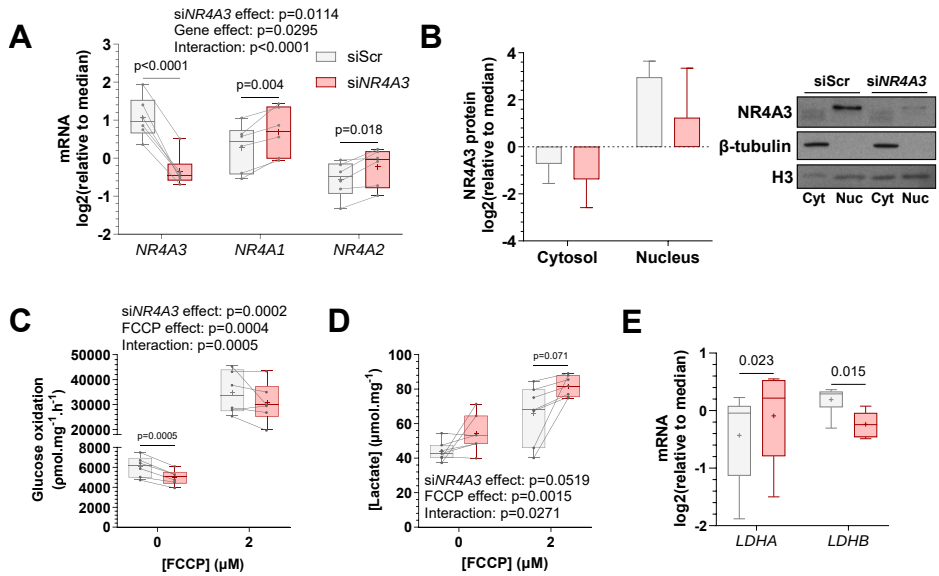


Figure 15. NR4A3 silencing in primary human skeletal muscle myotubes attenuates glucose oxidation by diverting glucose towards lactate production. Primary skeletal muscle cells were exposed to a control scramble sequence (siScr) or a silencing RNA targeting *NR4A3* (si*NR4A3*). **A.** mRNA expression of NR4A family members measured by RT-qPCR. n = 6, 2-way ANOVA (silencing x gene) with Šidák correction. **B.** Protein level of NR4A3 in cytosolic and nuclear fractions assessed by immunoblot analysis. Results are mean ± SEM, n = 2. **C.** Rates of radiolabelled glucose oxidation under basal and (2 µM) FCCP-stimulated conditions over 4 h. n = 6, 2-way ANOVA (silencing x FCCP) with Šidák correction. **D.** Lactate concentration in cell supernatant measured after 48 h of basal or (2 µM) FCCP-stimulated conditions. n = 7, 2-way ANOVA (silencing x FCCP) with Šidák correction. **E.** mRNA expression of lactate dehydrogenase (*LDH*) isoforms measured by RT-qPCR. Results are box-and-whisker plots with Tukey distribution. n = 6, paired t-tests with FDR correction.

4.3.3 NR4A3 depletion impairs protein synthesis, resulting in attenuated size of primary human skeletal muscle myotubes.

We observed that silencing of *NR4A3* consistently reduced the total protein and RNA abundance of cultured human myotubes (data not shown). Over 80% of cellular RNA is ribosomal (rRNA) and rates of translation are proportional to total RNA content in skeletal muscle [324]. These observations, combined with the association of reduced *NR4A3* levels during bed rest and limb immobilisation inactivity (Figure 14), led us to explore the putative effects of *NR4A3* silencing on protein synthesis. Relative rates of translation can be measured

in vitro by the surface sensing of translation (SUnSET) method, which detects puromycin incorporation into nascent peptide chains by immunoblot analysis [325]. Using this technique, we observed reduced puromycilation of proteins upon *NR4A3* silencing both at baseline and after insulin plus leucine stimulation (Figure 16A), signifying impaired protein synthesis with NR4A3 downregulation.

The efficiency and total capacity for translation in skeletal muscle is determined by mTORC1 signalling and ribosomal content, respectively [121, 123]. In vitro, AMPK impedes mTORC1 activity through phosphorylation of tuberous sclerosis complex 2 (TSC2) [326] and raptor [327]. Consistent with AMPK upregulation as a consequence of impaired glucose oxidation (Figure 15C), the ratio of phosphorylated-to-total protein of AMPK target sites on TSC2 (Ser^{1387}) and raptor (Ser^{792}) were increased by NR4A3 depletion (Figure 16B), indicative of AMPK interference of mTORC1. Furthermore, *NR4A3* silencing reduced the protein content of raptor, mTOR, and ribosomal protein S6 (RPS6) (Figure 16B). These collective AMPK-related and unrelated changes likely coalesced to inhibit $mTOR^{ser2448}$ phosphorylation and activation of downstream mTORC1-substrates, including $RPS6^{ser235/236}$ phosphorylation, with negative consequences for skeletal muscle protein synthesis following NR4A3 depletion (Figure 16A). Gene expression analyses also confirmed that *NR4A3* RNA interference decreased mRNA abundance of *RPS6*, as well as eukaryotic translation initiation factor 4E (*EIF4E*) and *45S* pre-ribosomal RNA (data not shown). Thus, our results imply that NR4A3 downregulation impedes both translational efficiency (i.e., mTORC1-signalling) and capacity (i.e., ribosomal abundance).

Attenuated rates of protein synthesis appear to be the primary contributor towards early losses of skeletal muscle mass during disuse atrophy in humans [101, 125]. Therefore, we assessed how diminished translation induced by *NR4A3* silencing impacted upon primary human skeletal muscle myotube size and contractile apparatus. Immunostaining for fast-type myosin heavy chain isoforms (MYH1/2; MyHC type-IIX/type-IIA) showed a striking decrease in myotube size. This difference was driven by a reduction in myotube area (Figure 16C) without any change in the number of nuclei or the ability of myoblasts to fuse into myotubes. Thus, the temporal downregulation of *NR4A3* during physical inactivity (Figure 14B) might contribute towards skeletal muscle disuse atrophy by impairing protein synthetic processes.

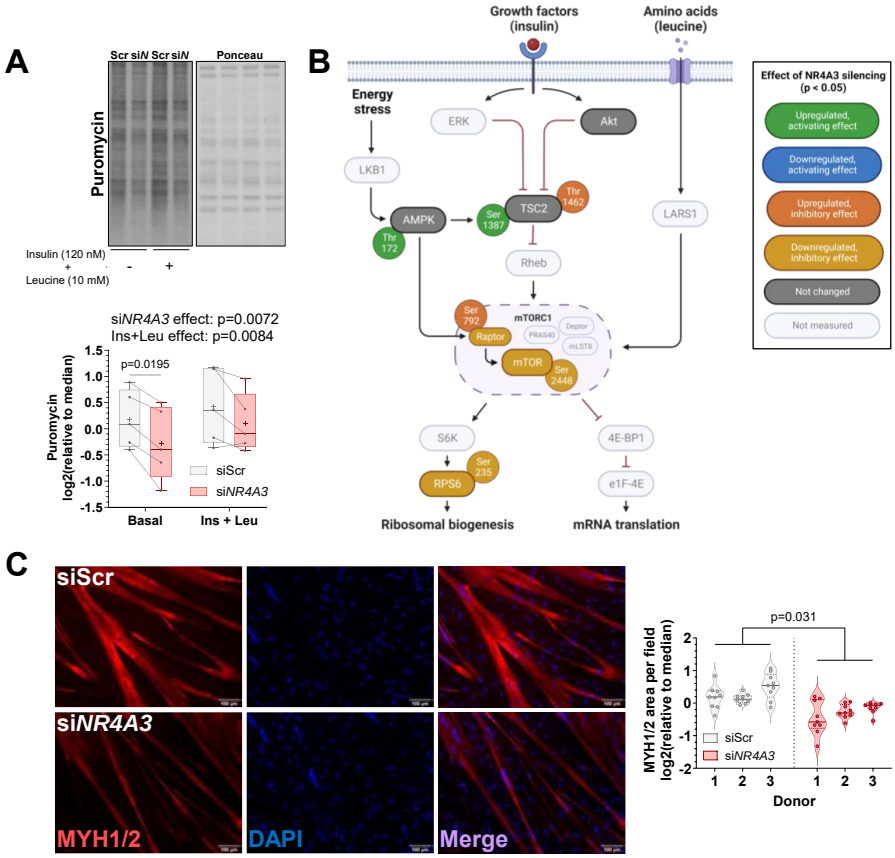


Figure 16. NR4A3 depletion impairs protein synthesis, resulting in attenuated size of primary human skeletal muscle myotubes. A. Representative immunoblot and quantification of cellular protein synthesis assessed by protein puromycilation (i.e., SUnSET method). n = 5, 2-way ANOVA (silencing x insulin + leucine) with Šidák correction. **B.** Schematic representation of immunoblot analysis. Signalling events depict positive and negative regulation of mTORC1. n = 5, 2-way ANOVA (silencing x insulin + leucine) with Šidák correction. **C.** Immunocytochemistry of fast myosin heavy chain isoforms (MYH1/2, red) and nuclei (DAPI, blue) from a representative donor, alongside quantification of MYH1/2 area. Scale bar = 100 µm. Results are violin plots with median and interquartile range. Circles represent measurements from different fields of view across three technical replicates per donor. n=3, nested paired t-test.

4.3.4 Overexpression of the canonical NR4A3 isoform increases muscle protein synthesis and partially restores glucose oxidation in NR4A3-deplete myotubes

The *NR4A3* gene produces four transcripts encoding three isoforms of the NR4A3 protein. The RNA interference method described in previous figures targeted an exon common to all isoforms, precluding the analysis of differential effects of select NR4A3 variants. Thus, we explored lentiviral overexpression of the canonical NR4A3 isoform (NR4A3-203; NM_006981.4) in primary human skeletal muscle myotubes. Overexpression of the

NR4A3-203 (*NR-203*) transcript by >140-fold increased total NR4A3 protein levels by >4.5-fold without altering the abundance of other NR4A family members (Figure 17A). Consistent with the effects of *NR4A3* silencing, NR4A3-203 overexpression enhanced basal protein synthesis, with no further stimulatory effect of insulin plus leucine treatment (Figure 17B). Furthermore, overexpression of the canonical NR4A3 isoform in the presence of siRNA targeting all *NR4A3* variants (Figure 17C) partially restored glucose oxidation (Figure 17D). Hence, the NR4A3-203 isoform appears to majorly underly the negative metabolic outcomes associated with NR4A3 downregulation.

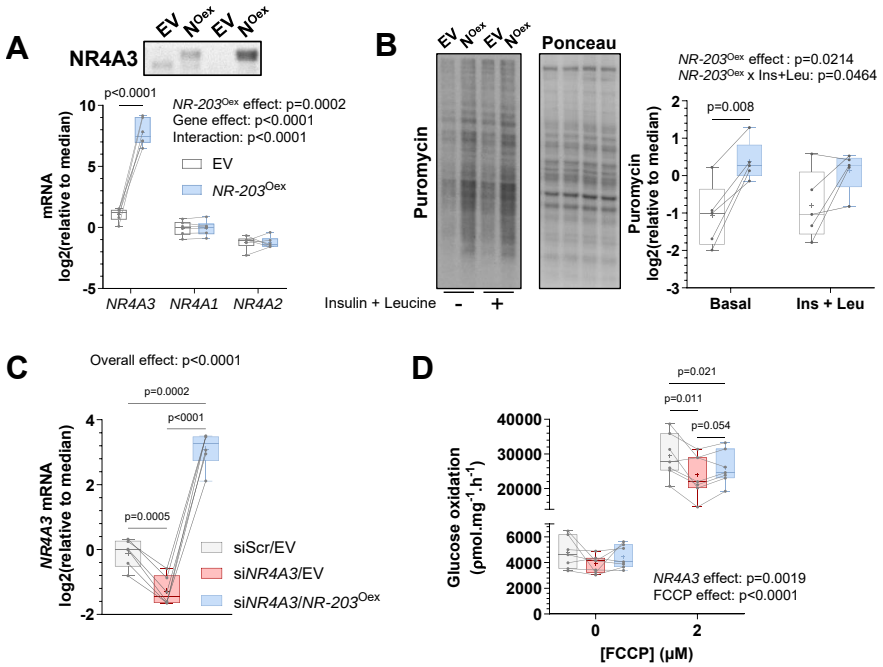


Figure 17. Overexpression of the canonical NR4A3 isoform increases muscle protein synthesis and partially restores glucose oxidation in NR4A3-deplete myotubes. Primary skeletal muscle cells were transduced with an empty vector control plasmid (EV) or a plasmid containing variant *NR4A3-203* (*NR-203*Oex). **A.** mRNA expression of NR4A family members measured by RT-qPCR. n = 6, 2-way ANOVA (overexpression x gene) with Šidák correction. Also, protein level of NR4A3 from two indicative donors. **B.** Representative immunoblot and quantification of cellular protein synthesis assessed by protein puromycilation (i.e., SUnSET method). n = 5, 2-way ANOVA (overexpression x insulin+leucine) with Šidák correction. **C.** *NR4A3* mRNA measured by RT-qPCR. n = 6, 1-way ANOVA with Tukey correction. **D.** Rates of radiolabelled glucose oxidation under basal and (2 µM) FCCP-stimulated conditions over 4 h. n = 7, 2-way ANOVA (overexpression x FCCP) with Fisher's LSD post-test.

4.3.5 Study III discussion

The induction of *NR4A3* expression following a single exercise bout, but not after exercise training, has been well-documented [137]. Similarly, NR4A3 exhibits transient downregulation during reduced physical activity and rapid upregulation upon reloading, but remains unchanged after prolonged immobilization (>2 weeks). Notably, the onset of muscle fibre atrophy occurs swiftly during periods of disuse [101], with the most aggressive decline observed within the initial two weeks of unloading [328], corresponding to the lowest levels of *NR4A3* mRNA. This distinctive expression pattern suggests a critical role for NR4A3 in regulating muscle function during acute transition phases characterised by intense tissue remodelling. Specifically, the first two weeks of unloading are associated with reduced postprandial [124] and total daily [122] rates of myofibrillar protein synthesis. In this study, we present compelling evidence implicating NR4A3 in directly influencing translation in muscle. Furthermore, downregulation of NR4A3 in skeletal muscle cells recapitulated the adverse effects observed in skeletal muscle tissue during periods of inactivity, including impaired glucose metabolism, reduced protein synthesis, altered expression of contractile apparatus, and diminished myotube size. Thus, our findings shed light on the involvement of NR4A3 in the control of skeletal muscle protein synthesis and metabolism during disuse atrophy.

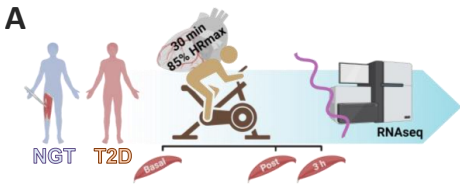
Our data provide mechanistic insights into the profound impact of NR4A3 downregulation on substrate utilisation, leading to a shift in metabolism towards increased lactate production, while decreasing glucose oxidation. Additionally, our findings corroborate previous studies in mouse C2C12 skeletal muscle myotubes [329] showing that NR4A3 depletion inhibits the mTORC1 signalling complex, a major molecular node in protein synthesis regulation. AMPK activation was found to phosphorylate TSC2 and raptor, thereby dampening mTORC1 transduction. However, we also observed decreased protein abundance of mTOR and RPS6, indicating that the activation of AMPK alone is unlikely to trigger this phenomenon. Notably, rates of translation and ribosomal biogenesis were attenuated upon *NR4A3* RNA interference, suggesting that the reduction in NR4A3 impairs cellular metabolism by impeding cell-wide anabolic pathways. In support of this, rescuing protein synthesis through the overexpression of the canonical NR4A3 isoform restored glucose oxidation in *NR4A3*-silenced myotubes.

Study III establishes a link between lower levels of NR4A3 and inactivity paradigms, underscoring the adverse effects of NR4A3 attenuation on protein synthesis and metabolic responses in human skeletal muscle. Considering that muscle mass and strength are important predictors of mortality in intensive care settings [330], NR4A3 may represent a pivotal molecular transducer whose downregulation contributes to the deleterious health consequences associated with sedentary lifestyles.

4.4 Study IV: Distinctive exercise-induced inflammatory response and exerkine induction in skeletal muscle of people with type 2 diabetes.

In Study II, notable discrepancies were identified in skeletal muscle after exercise training among healthy individuals and those with obesity or type 2 diabetes. This included dysregulation of inflammatory genes, indicating potential involvement of the immune system in the divergent adaptive response. Consequently, in **study IV**, we employed a combination of transcriptomic and biochemical assays in both human skeletal muscle and cell culture models to delve into the intricate role of noncanonical exercise-responsive inflammatory processes. By elucidating these pathways, we aimed to gain further insights into the interplay between exercise, inflammation, and metabolic disorders. The study design, as well as certain baseline clinical characteristics, are shown in Figure 18.

A



B

Select characteristics	NGT	T2D
n	17	20
Age (years)	60 ± 6	60 ± 5
BMI (kg.m^{-2})	27.5 ± 3.0	28.3 ± 2.7
HbA_{1c} (mmol.mol^{-1})	35.4 ± 3.4	47.1 ± 8.4 *
$VO_{2\ peak}$ (mL.Kg^{-1}.min^{-1})	36.9 ± 8.5	31.6 ± 8.3 †
Maximum heart rate (bpm)	165 ± 9	166 ± 16

Figure 18. Overview of the intervention. A. Schematic summary of study design. Seventeen males with normal glucose tolerance (NGT) and 20 males with type 2 diabetes (T2D) were recruited, matched for age, body mass index (BMI), and aerobic fitness (peak oxygen consumption, $\dot{V}O_{2\ peak}$). A first biopsy was taken in the basal (i.e., resting) state. Thereafter, participants performed a 30-min bout of cycling at 85% of maximum heart rate, and skeletal muscle biopsies were collected immediately (post) and 3 h after exercise (recovery). The transcriptome of skeletal muscle was analysed by RNA sequencing. **B.** Select baseline clinical characteristics of participants with NGT or T2D. HbA_{1c} = glycated haemoglobin. *p<0.001 and † p=0.078, T2D vs. NGT, unpaired Student's t test.

4.4.1 Enhanced inflammatory response to exercise in skeletal muscle of males with type 2 diabetes

Differential patterns of gene expression in skeletal muscle were observed between the post-exercise and recovery time points, particularly in individuals with type 2 diabetes. Following exercise, a substantial increase in skeletal muscle gene expression was noted during the recovery phase in males with type 2 diabetes, with 3185 genes uniquely responsive in this group (Figure 19A). Although both groups exhibited a similar overall response post-exercise, males with type 2 diabetes displayed a quantitatively greater transcriptional response in skeletal muscle during the recovery phase. Notably, numerous selectively upregulated genes were associated with inflammatory gene ontology pathways (Figure 19B). This indicated that exercise led to an amplified inflammatory response in individuals with type 2 diabetes as compared to those with normal glucose tolerance. This observation was corroborated by immunoblot analysis and immunohistochemical staining for indicators of immune cells in skeletal muscle. The abundance of CD206 (a sign of infiltrating macrophages) and CD86 (a

marker for all macrophages) were elevated in skeletal muscle lysates from males with type 2 diabetes after the recovery period (Figure 19C). Correspondingly, the number of cells expressing the general immune marker CD11b increased in those with type 2 diabetes following the recovery period (Figure 19D).

To further characterise the transcriptomic signature of human M1 and M2 macrophage states, a curated database of M1 and M2 blood-derived macrophages was generated. Overrepresentation analysis utilising M1 and M2 signatures demonstrated a heightened enrichment of M2-associated genes in males with type 2 diabetes at the recovery time point (Figure 19E). This suggests that exercise induced an M2-like immune response, which is linked to skeletal muscle hypertrophy and tissue remodelling. Notably, stress kinases such as extracellular signal–regulated kinase (ERK) and Janus kinase (JNK) exhibited phosphorylation immediately post-exercise, although the response did not between groups (Figure 19C). Furthermore, the canonical nuclear factor κB (NF-κB) pathway remained unaltered (i.e., phospho-p65), confirming that the recruitment of immune cells was a consequence of sterile inflammatory processes.

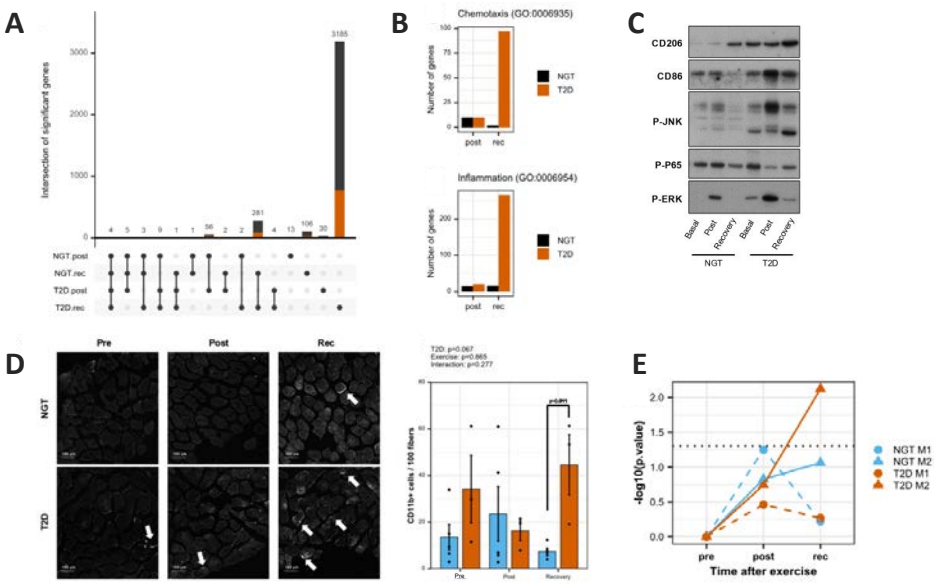


Figure 19. Enhanced inflammatory response to exercise in skeletal muscle of males with type 2 diabetes. A. Number of genes regulated by exercise (FDR < 0.01) and their intersection. Orange = genes annotated with inflammatory processes in gene ontology. **B.** Number of genes associated with the gene ontology pathways 'chemotaxis' and 'inflammatory response' (FDR<0.01). **C.** Representative immunoblots of macrophage markers and inflammatory pathways. **D.** Representative images of skeletal muscle cross-sections labelled with CD11b and quantification per muscle fibre. Data are means ± SE and individual data points, n = 3 to 5. 2-way ANOVA (exercise x type 2 diabetes) and pairwise t tests. **E.** Overrepresentation analysis using Fisher's exact test on pro- (M1) and anti- (M2) inflammatory macrophage genes signatures.

4.4.2 Cytokine cross-talk in composite skeletal muscle tissue

Cytokines are essential factors for immune responses. Interestingly, the induction of most exercise-regulated cytokines was augmented in the skeletal muscle of individuals with type 2 diabetes (Figure 20A). As a composite tissue, skeletal muscle relies upon intercellular signalling for physiological adaptive remodelling. Thus, to gain deeper insights into the potential directionality of cytokine-mediated communication between different cell populations following exercise, we compared the mRNA expression of cytokine receptors in various cell types. Included in this analysis were skeletal muscle tissue, primary human skeletal muscle myotubes, monocyte-derived macrophages, peripheral blood leukocytes, and endothelial cells (Figure 20B). We observed that circulating immune cells exhibited high expression of most cytokine receptors, whereas the expression levels were lower in skeletal muscle biopsies and primary myotubes. However, an exception was noted for atypical chemokine receptor 3, also known as C-X-C chemokine receptor type 7 (*ACKR3*/*CXCR-7*), which displayed higher expression in myotubes compared to immune cells. ACKR3 is a receptor for stromal cell-derived factor 1 (CXCL12/SDF-1), indicating that CXCL12/SDF-1 could exert effects on skeletal muscle cells. Furthermore, conditioned media from electrically stimulated primary human skeletal muscle myotubes increased *CXCL12* levels in THP1 macrophages, implicating its involvement in the communication between skeletal muscle and immune cells. Adding another layer of complexity, analysis of publicly available data revealed that hypoxia induced several exercise-responsive cytokines in human endothelial cells, including the expression of *CXCL12* (Figure 20C).

Together, *in vitro* experiments conducted in skeletal muscle cells, macrophages, and endothelial cells support the notion that the post-exercise inflammatory cytokine signature of individuals with type 2 diabetes arises from intricate signalling between several cell types within the skeletal muscle niche. This occurs in response to various stimuli, including contraction and hypoxia. CXCL12 appears to be a potential mediator of this communication. Indeed, circulating concentrations of CXCL12/SDF-1α was found to be elevated immediately after exercise in males with normal glucose tolerance (Figure 20D). Alternatively, CXCL12/SDF-1β was abundant in skeletal muscle tissue, although levels were unchanged with exercise (Figure 20E).

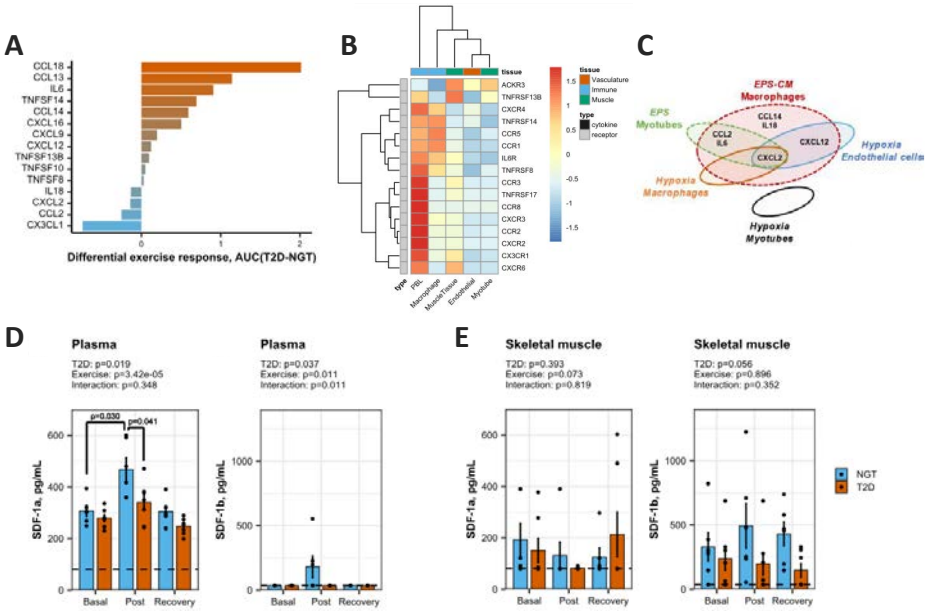


Figure 20. Cytokine cross-talk in composite skeletal muscle tissue. A. Differential response in skeletal muscle from males with T2D versus NGT for exercise-responsive cytokines (FDR<0.05). Area under the curve of mRNA to estimate the induction of genes relative to baseline values. **B.** Publicly available RNA sequencing data from bulk skeletal muscle tissue and peripheral blood leukocyte (PBL), as well as primary human skeletal muscle myotubes and monocyte-derived macrophages differentiated in vitro. **C.** Cytokines regulated by either electrical pulse stimulation (EPS) in primary human skeletal muscle myotubes (green), hypoxia in macrophages (orange), or hypoxia in endothelial cells (blue). Cytokines induced in human THP1 macrophages in response to conditioned media from electrical pulse–stimulated primary human skeletal muscle myotubes (red). No cytokines were induced in primary skeletal muscle myotubes exposed to hypoxia (black). **D, E.** CXCL12/SDF-1 measurement in plasma and skeletal muscle tissue lysate of males with T2D (n = 6) *versus* NGT (n = 7). Alpha and beta isoforms of CXCL12 were quantified using enzyme-linked immunosorbent assay (ELISA). Dotted lines represent the detection threshold of the assays. Data are mean ± SE and individual datapoints, n = 6 to 7, 2-way ANOVA (exercise x T2D) and pairwise t-tests.

4.4.3 CXCL12 alters skeletal muscle cell differentiation

CXCL12 was identified as a priority candidate for further characterisation due to its role as an exercise-responsive cytokine. Notably, this cytokine might be produced by non-myogenic cell populations to subsequently exert its effects on skeletal muscle cells through specific receptor interactions. In order to gain insights into its functional implications, gene set enrichment analysis was performed on genes ranked by Spearman's correlation. This analysis revealed a positive association between CXCL12 and 'muscle cell migration', while a negative association was observed with 'skeletal muscle contraction' (Figure 21A).

CXCL12 exerts its effects through the G protein–coupled receptors (GPCRs) C-X-C chemokine receptor type 4 (CXCR4) and ACKR3. To elucidate whether CXCL12 also signals through GPCRs in skeletal muscle, mouse C2C12 skeletal muscle myotubes were treated with recombinant CXCL12 protein. The inhibition forskolin-induced cyclic adenosine monophosphate (cAMP) production upon treatment, confirmed that CXCL12 signals through a GPCR in skeletal muscle (Figure 21B).

Because gene set enrichment analysis indicated a role for CXCL12 in skeletal muscle myogenesis (Figure 21A), we next exposed primary human skeletal muscle cells to CXCL12 during the differentiation process. In doing so, the beta isoform of CXCL12 increased the gene expression of the proliferative marker *MKI67*, as well as the myoblast markers myogenic factor 5 (*MYF5*) and paired box protein Pax-7 (*PAX7*) (Figure 21C). These findings collectively suggest that CXCL12 exerts a its effects on skeletal muscle myoblasts, through GPCR signalling, to activate their proliferation.

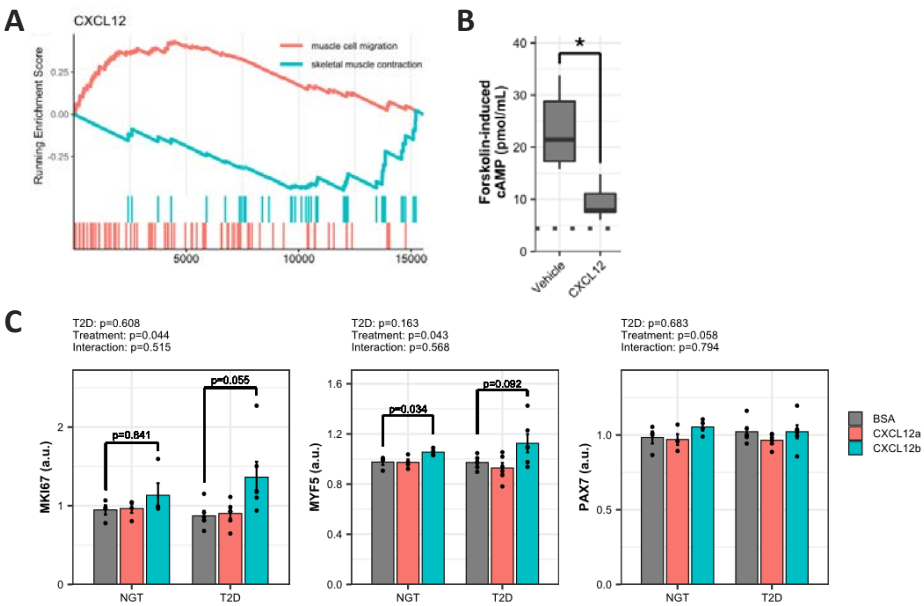


Figure 21. CXCL12 alters skeletal muscle cell differentiation. A. Gene set enrichment analysis of genes correlated with *CXCL12* in the RNA sequencing of skeletal muscle biopsies. The pathway 'muscle cell migration' was positively enriched, while 'skeletal muscle contraction' was negatively enriched. **B.** Inhibition of forskolin-induced cAMP production by CXCL12 (100 ng.mL^{-1}) measured in mouse C2C12 skeletal muscle myotubes. Data are box-and-whisker plots with Tukey distribution from five independent experiments. *p<0.05. **C.** Primary human skeletal muscle cells from individuals with type 2 diabetes (T2D) or normal glucose tolerance (NGT) were exposed to CXCL12a/SDF-1α or CXCL12b/SDF-1β (100 ng.mL^{-1}) during differentiation. mRNA expression of gene markers of proliferation (*MKI67*) and myoblasts (*MYF5* and *PAX7*) were measured by RT-qPCR. Data are means ± SE, n = 4-6 cultures from independent donors per group.

4.4.4 Study IV discussion

Inflammation is associated with various health issues including myopathies, metabolic disease, obesity, and cardiovascular diseases, but also plays a crucial role in myogenesis and the remodelling of skeletal muscle following exercise [331]. Our study delves into the exercise-inflammation signature found in individuals with type 2 diabetes, revealing that it differs markedly from those with normal glucose tolerance. Through transcriptomic analysis, we uncovered that the exercise-induced inflammation signature of people with type 2 diabetes may stem from inadequate oxygenation of active skeletal muscle, as evidenced by the production of hypoxia-responsive cytokines like CXCL12.

Notably, our research pinpointed CXCL12 as an exerkine liberated into the bloodstream post-exercise, with heightened mRNA induction observed in the skeletal muscle of males with type 2 diabetes. *CXCL12* was not induced by electrical pulse stimulation or hypoxia in primary human skeletal muscle myotubes. Instead, its production increased in endothelial cells subjected to hypoxia and macrophages exposed to conditioned medium from contracted primary myotubes. Consequently, we hypothesise that a cross-talk between these different cell types contributes to elevated CXCL12 expression in skeletal muscle tissue.

While whole-body deletion of *Cxcl12* impedes angiogenesis during skeletal muscle regeneration [332], muscle-specific knockout of *Cxcl12* in mice does not affect angiogenic properties [333]. This suggests that CXCL12 produced by non-myogenic cells holds greater relevance in skeletal muscle remodelling. We postulate that CXCL12 may be locally produced to stimulate resident tissue cells, such as muscle fibres, endothelial cells, and satellite cells, in a paracrine fashion.

Supporting this notion, CXCR4, the receptor for CXCL12, is expressed in satellite cells which are attracted and activated by CXCL12 [334, 335]. Further evidence emerges from studies on *Cxcr4*-deficient mice, which display impaired myogenesis [336], while injection of CXCL12 in a mouse model with skeletal muscle damage leads to increased fibre diameter and decreased fibrosis [337, 338]. In line with these findings, our experiments involving primary human skeletal muscle cells exposed to CXCL12 during the differentiation process resulted in heightened markers of myoblast proliferation. Collectively, our data, along with evidence from mouse models [336-338], suggest that the induction of CXCL12 in skeletal muscle of individuals with type 2 diabetes represents a beneficial response that promotes the recruitment and activation of satellite cells, ultimately driving skeletal muscle remodelling.

In summary, **study IV** sheds light on the intricate immunometabolic responses within skeletal muscle and the intricate interplay between exercise-induced and metabolism-induced inflammation. We present compelling evidence that a single bout of aerobic exercise elicits a distinct inflammatory response in the skeletal muscle of males with type 2 diabetes. Furthermore, we identify CXCL12 as a plausible exerkine and mediator of skeletal muscle

adaptation to exercise in individuals with type 2 diabetes. These findings have substantial implications for understanding the complexities of skeletal muscle physiology in the context of metabolic disorders.

5. Conclusions

Although the health benefits of exercise have been known since antiquity, deeper understanding of the molecular regulation of skeletal muscle adaptation to different exercise modalities in health and disease is necessary to facilitate more efficacious exercise prescription and translation into practice. Furthermore, despite heterogeneity in the adaptive response, the health promoting effects of exercise are experienced by all after an exercise intervention when a sufficient number of outcome variables are measured. As such, it is essential to establish entry-level physical activity guidelines so that those able to exercise can do so for the betterment of individual and public health.

In that regard, the work in this thesis adds important context to the existing literature. The specific conclusions of constituent papers in this thesis are:

- Study I: Even small amounts of low-to-moderate physical activity can benefit systemic glycaemic control when performed frequently to interrupt sedentary time. This probably occurs via increased perfusion of skeletal muscle tissue but needs to be performed consistently to sustain any positive effects. With that in mind, more practical strategies are needed to facilitate compliance to breaking sitting, with the goal of reducing sedentary behaviours.

- Study II: The skeletal muscle transcriptomic response to acute bouts of endurance and resistance exercise share greater overlap in the naïve versus exercise trained state. This likely contributes to common adaptations that diverge over time with dedicated training towards a given exercise modality. Physical inactivity and, in particular, endurance exercise training exhibit divergent regulation of transcriptomic pathways but there is little inverse overlap at the single-transcript level. Individuals with metabolic impairments have a divergent transcriptomic signature after exercise training compared to healthy counterparts, however response of classically exercise-induced genes is similar.

- Study III: Downregulation of the transcription factor NR4A3 contributed to skeletal muscle disuse atrophy through the attenuation of mTORC1-signalling and ribosomal biogenesis, which combine to impair translational efficiency and capacity. Restoring NR4A3 levels during periods of inactivity could benefit skeletal muscle metabolism by re-establishing rates of protein synthesis.

- Study IV: Males with type 2 diabetes have an exacerbated inflammatory response in the 3-h recovery period after acute high-intensity endurance exercise. This includes induction of the cytokine CXCL12, produced by macrophages or immune cells, that could mediate cellular crosstalk within skeletal muscle. CXCL12 can act upon G-protein coupled receptors to activate the proliferation of skeletal muscle satellite cells (i.e., myoblasts). Through this mechanism, CXCL12 may contribute towards skeletal muscle remodelling after exercise.

6. Points of perspective

The work in this thesis utilises clinical, transcriptomic, and biochemical analyses to interrogate physiological responses to physical activity, with particular focus on skeletal muscle.

Study I found that even modest increases in ambulatory behaviour throughout the day can have positive effects on interstitial glucose variability and fasting plasma glucose levels in individuals with obesity and insulin resistance. These improvements may be attributed to enhanced blood flow to skeletal muscle [312, 313]. Additionally, the reduction in fasting glucose concentrations may also involve the attenuation of systemic inflammation. An analysis found that individuals who frequently took activity breaks from sitting exhibited lower levels of C-reactive protein (CRP) [316], which is known to correlate with fasting glucose levels [315]. Future trials should investigate the long-term impact of interrupting sitting on inflammatory markers, considering the potential relevance to the reduced risk of coronary heart disease in individuals with lower fasting glucose levels [339].

Study I also indicated that higher volumes of physical activity breaks from sitting consistently led to improvements in glycaemic control. It is essential for future studies to explore the relationship between the frequency, intensity, and volume of activity breaks, and how these factors interact with different demographic profiles across the metabolic health spectrum. Indeed, the duration and timing of activity breaks had varying effects on glycaemic control in individuals with type 2 diabetes, with 3-min breaks every 30 min influencing post-lunch glycaemia, and 6-min breaks every 60 min affecting nocturnal glycaemia [340].

Importantly, the activity data reported in study I question the ecological practicality of regular 3-min bouts of activity. Considering that formal exercise regimens offer distinct cardiometabolic benefits [310], it is necessary to explore the potential of combining activity breaks from sitting with exercise routines to achieve the most effective solution for improving public health. This approach aligns with the overarching movement advocating for individuals to ‘sit less, move more, and exercise’ [341].

In **Study II**, a meta-analytical approach was employed to investigate the transcriptomic response of skeletal muscle to exercise and physical inactivity. While transcriptomic studies offer detailed insights into changes in gene expression, it is crucial to consider other biological responses that contribute to the adaptive nature of muscle following exercise. This encompasses post-translational modifications of proteins, as well as epigenetic alterations involving histones, DNA, and non-coding RNAs, which collectively establish a new homeostatic state in skeletal muscle. To fully understand the exercise-induced adaptations of skeletal muscle in both healthy individuals and those with metabolic impairments, comprehensive multi-omics analyses coupled with functional assays are necessary. A

spatiotemporal approach, incorporating subcellularly compartmentalised biosensors at multiple time points, would provide unprecedented resolution and uncover the intricate integrated signalling underlying adaptive processes in skeletal muscle.

Furthermore, the annotation of datasets examined in study II highlighted the importance of studies encompassing broader participant demographics and a wider range of exercise types and modalities. Multicentre interventions that include individuals spanning different age groups, metabolic health statuses, chronotypes, ethnicities, biological sexes, and social genders will contribute to a deeper understanding of the dynamic changes that coordinate the benefits of exercise training. Moreover, such approaches will help define the role of muscle in the holistic response to exercise. The knowledge gained from these endeavours will not only aid in personalised exercise prescription but also open up new molecular avenues for drug discovery aimed at improving human health.

In the context of physical inactivity, **study III** aimed to investigate the metabolic role of the transcription factor NR4A3 in skeletal muscle. The downregulation of NR4A3 in human skeletal muscle coincides with the period of most severe muscle disuse atrophy, characterised by reduced rates of muscle protein synthesis. The in vitro effects of NR4A3 as a bonified regulator of translation in primary skeletal muscle cells further support the notion of NR4A3 playing a causal role in human skeletal muscle disuse atrophy. However, establishing a stronger link would require an in vivo model demonstrating that skeletal muscle-specific overexpression of Nr4a3 can preserve muscle mass during conditions of atrophy, or that loss of *Nr4a3* directly contributes to muscle loss.

Overexpression of the canonical NR4A3 isoform in study III resulted in a twofold increase in protein translation and was able to rescue glucose oxidation in the presence of NR4A3 silencing. Nevertheless, it did not have a direct impact on glucose or lipid metabolism, select gene expression, markers of ribosomal biogenesis, or signalling related to protein synthesis. Thus, it is unclear how the canonical NR4A3 isoform precisely influences protein synthesis. Unbiased transcriptomic analysis could shed light on this matter. Additionally, it is possible that NR4A3 may influence specific processes such as ribosome specialisation [342], which may involve alterations in ribosomal protein abundance [283] or ribosome posttranslational modifications (e.g., methylation). Ribosome profiling could be employed to assess the translational capacity of ribosomes under NR4A3 overexpression, while ribosome methylation could be detected through ribosome methylation-sequencing.

Additionally, in study III, the silencing of NR4A3 in primary skeletal muscle myotubes using RNA interference targeting a common exon among all transcript variants produced a stronger phenotype than overexpression of the canonical NR4A3 isoform alone. As such, exploring the individual effects of distinct NR4A3 isoforms in future studies will provide a better understanding of NR4A3's regulation of metabolic processes in skeletal muscle.

Study IV revealed distinct immune-related patterns in the skeletal muscle of males diagnosed with type 2 diabetes, in comparison to individuals with normal blood glucose levels. Considering the well-documented benefits of regular exercise on metabolism and skeletal muscle function [51], and the counteractive effects of nonsteroidal anti-inflammatory drugs on exercise-induced muscle remodelling [343, 344], the heightened inflammatory response observed in individuals with type 2 diabetes might serve as a beneficial reaction to acute exercise. Engaging in consistent exercise training could potentially normalise this acute immune response, while also improving insulin sensitivity and lowering the risk of cardiometabolic complications [345].

In the treatment of type 2 diabetes, metformin is commonly prescribed as a first-line medication, either alone or in conjunction with lipid-lowering drugs. Within study IV, 80% of individuals with type 2 diabetes were on metformin. Additionally, the use of statins, which have been associated with muscle pain and myopathy [346], was more prevalent in individuals with type 2 diabetes. It should be noted that while metformin impairs skeletal muscle hypertrophy in response to resistance training [347], it has no impact on mitochondrial respiration, oxidative stress, or AMP-activated protein kinase (AMPK) activation [348]. Prior to the exercise session, participants refrained from taking medication for 24 h, and we detected no discernible transcriptomic differences to statin or metformin usage in principle component analyses (data not shown). However, it is important to acknowledge that our study was not designed to evaluate the effects of medication on exercise responses, thus leaving open the possibility that statin or metformin use may have influenced our findings. Additional research is necessary to determine whether statins or metformin directly affect the immunometabolic response of skeletal muscle to a bout of exercise.

Furthermore, it is necessary to highlight that study IV focused solely on an acute examination of middle-aged men, both with and without type 2 diabetes. Therefore, we did not account for the impact of modifiers such as age, biological sex, or ancestry when assessing the transcriptomic and immunometabolic responses to acute exercise. Consequently, it remains uncertain whether our results can be extrapolated to broader demographic groups. Additional investigations are warranted to ascertain whether the effects of acute exercise on the immunometabolic response of skeletal muscle vary across different demographics.

In summary, the research presented in this thesis underscores the pivotal role of physical activity in enhancing human health. The discoveries offer valuable insights into the intricate molecular mechanisms that underlie skeletal muscle responses to physical activity, thereby contributing towards a better understanding of how specific paradigms facilitate the metabolic resilience of skeletal muscle tissue and, in turn, systemic wellbeing.

7. Acknowledgements

Two things go without saying.

Firstly, surviving the PhD process is a collaborative effort and I couldn't have done it without a huge amount of support along the way. If you feel you helped, you most likely did. Thank you. It means more than you'll ever know.

Secondly, and unsurprisingly (…?), I'm waaaay behind schedule and need to get this book to print!

Cheers!

J x

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