Targeting mitochondrial bioenergetics and NAD^+ metabolism during sarcopenia and regeneration of skeletal muscle

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Sometimes
the smallest things
take up the most room in your
heart.
— Winnie the Pooh

Abapapapa
bababa.
— Mara Blumenschein

To Felix

Acknowledgements

Sometimes the grandest adventure is not where you go, but who you're with.

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A bear, however hard he tries, grows tubby without exercise.

— A. A. Milne

Abstract

The progressive decline of muscle mass and function called sarcopenia is a multi-factorial process associated to frailty, disability, and low quality of life in the elderly. The co-factor nicotinamide adenine dinucleotide (NAD^+) becomes a limiting factor in the course of aging and other pathological conditions. It has emerged as a major regulator of cellular metabolism, which is modulated by dietary precursors of the vitamin B_3 family. So far, no direct link between sarcopenia and alterations in NAD^+ metabolism has been reported. In this thesis, I have taken a multi-disciplinary approach combining molecular profiling in humans and dietary and genetic manipulations in rodents to characterize the skeletal muscle NAD^+ metabolism in the context of muscle plasticity and aging.

We have performed a multi-center study to characterize the molecular signature of human sarcopenia compared to age-matched controls. Sarcopenic participants of three cohorts in Singapore, the United Kingdom and Jamaica presented a prominent transcriptional signature of mitochondrial dysfunction, which translated into functional bioenergetic deficiency with lower mitochondrial protein expression and activity. Furthermore, we established a concurrent decrease in NAD^+ content of sarcopenic muscle, which correlated to lower muscle strength and function in these patients.

Supplementation with the dietary NAD^+ precursor nicotinamide riboside (NR) was shown to improve pathological muscle conditions and revert age-related mitochondrial dysfunction and stem cell senescence in mice. To test whether boosting NAD^+ through NR can be beneficial in the context of sarcopenia, we investigated the acute response to NR and its metabolization in young adult and old sarcopenic rats. Acute NR treatment efficiently increased NAD^+ levels and normalized specific age-related gene expression signatures in old rats, in particular related to fibrosis. Interestingly, our transcriptional profiling also revealed that the molecular response to NR is partially blunted in aging and suggests that aged muscle could be in a state of partial NAD^+ resistance.

To investigate the role of endogenous NR as NAD^+ precursor in the muscle, we studied mice deficient for NR kinases 1 and 2 (NRK1/2 dKO), the rate-limiting enzymes for NR salvage. NRK1/2 dKO mice develop normally, but exhibit elevated salvage of the NAD^+ precursor nicotinamide (NAM) to maintain tissue NAD^+. Thus, our results demonstrate for the first time that an endogenous NRK-dependent flux actively converts NR into NAD^+ in healthy skeletal muscle. Moreover, we showed that NRKs are required for muscle regeneration,

as dKO mice failed to efficiently regenerate the NAD^+ pool during the active phases of myofiber remodeling, which coincided with a transient delay of myofiber maturation. Conversely, dietary NR improved the activation of muscle stem cells and accelerated recovery of NAD^+ levels in regenerating myofibers of wild-type mice.

Altogether, this work demonstrates that both in humans and preclinical models, sarcopenia is tightly linked to mitochondrial bioenergetics and NAD^+ metabolism. Dietary manipulation of NAD^+ levels is a promising therapeutic strategy for the management of age-related muscle dysfunction for which we uncovered important mechanistic insights linking metabolic fluxes through the NAD^+ pathway to physiological adaptations.

KEYWORDS: Sarcopenia; skeletal muscle; aging; NAD^+; nicotinamide riboside (NR); nicotinamide riboside kinase (NRK); mitochondria; transcriptomics; metabolomics; human; ethnic diversity; muscle atrophy; muscle regeneration; CTX

Résumé

La sarcopénie est un syndrome associé au vieillissement se caractérisant par la perte progressive et généralisée de masse et de fonction musculaire. Ce processus d'origine multifactorielle entraine un accroissement du taux de fragilité et d'invalidité chez les personnes âgées ainsi qu'une dégradation de leur qualité de vie. Le nicotinamide adénine dinucléotide (NAD^+) est une coenzyme jouant un rôle central dans la régulation du métabolisme cellulaire et dont l'action peut être modulée par des précurseurs appartenant à la famille de la vitamine B3. Il a été démontré que le vieillissement, ainsi que d'autres types de pathologies, sont associés à une diminution de la quantité de NAD^+ dans l'organisme. Dans le cadre de ma thèse, j'ai opté pour une approche pluridisciplinaire combinant un profilage moléculaire d'échantillons humains ainsi que des interventions nutritionnelles et génétiques sur les rongeurs afin de caractériser le métabolisme du NAD^+ au cours de la régénération du muscle squelettique et du vieillissement musculaire.

Dans le cadre du projet intitulé Multi-Ethnic Molecular Determinants of Human Sarcopenia (MEMOSA), nous avons entrepris une étude multicentrique afin d'analyser la signature moléculaire de patients sarcopéniques par rapport à des donneurs de même âge et provenant de plusieurs cohortes d'origines ethniques différentes. Les patients sarcopéniques issus de trois cohortes provenant de Singapour, du Royaume-Uni et de Jamaïque ont tous présenté une signature transcriptionnelle prononcée traduisant un dysfonctionnement mitochondrial. Ce dernier se manifeste sous la forme d'une déficience bioénergétique corrélée à une diminution de l'expression et de l'activité des complexes constituant la chaine de transport d'électrons. De plus, nous avons observé une diminution systématique des niveaux de NAD^+ dans les muscles de patients sarcopéniques associée à une perte de force et de fonction musculaire chez ces patients.

Il a été montré que l'apport en nicotinamide riboside (NR), un précurseur de NAD^+ présent naturellement dans certains aliments, améliore la fonction musculaire et atténue la perte de fonction mitochondriale due à l'âge ainsi que la senescence cellulaire des cellules souches du muscle. Afin de tester si la stimulation de la production de NAD^+ par NR peut être bénéfique aux muscles sarcopéniques, nous nous sommes penchés sur les effets d'un traitement de NR chez de jeunes rats adultes et chez des rats sarcopéniques. Même si l'apport en NR n'a pas permis de totalement inverser la tendance sarcopénique observée chez les rats âgés, nous avons tout de même pu constater une augmentation de la quantité de NAD^+ de même que le rétablissement de certains profils d'expression à des niveaux comparables à ceux des jeunes rats, notamment en ce qui

concerne les signatures moléculaires liées à la fibrose. Notre profilage transcriptionnel a également révélé que la réponse moléculaire au NR était affaiblie chez les rats sarcopéniques, ce qui suggère que le muscle âgé pourrait se trouver dans un état de résistance partielle au NAD^+.

Finalement, afin de mieux comprendre le rôle endogène du NR dans le muscle squelettique, nous avons conduit une dernière étude capitalisant sur un modèle de souris présentant une déficience au niveau des NR kinases 1 et 2 (NRK1/2 dKO), des enzymes limitantes dans le processus de récupération du NR. Ces souris se développent normalement mais possèdent un taux de récupération accru d'un autre précurseur du NAD^+, le nicotinamide (NAM), permettant ainsi de maintenir les niveaux de NAD^+ dans le muscle. Nos résultats démontrent qu'il existe un flux endogène dépendant des NRKs promouvant activement la conversion de NR en NAD^+ dans le muscle squelettique sain. Etant donné que l'altération du processus de régénération joue un rôle majeur dans la perte progressive de masse musculaire avec l'âge, nous nous sommes ensuite demandés si les NRKs devenaient un facteur limitant pendant la régénération musculaire. Nous montrons que l'incapacité des fibres musculaires endommagées à pérenniser la récupération de NAD^+ suite à une blessure musculaire entraine une chute provisoire de la quantité de NAD^+ dans le muscle. Les souris NRK1/2 dKO ne sont alors plus en mesure de reconstituer les réserves en NAD^+ durant les phases actives de remodelage des fibres musculaires. Cette diminution de la quantité de NAD^+ dans les muscles de souris NRK1/2 dKO coïncide avec une maturation différée des fibres musculaires, démontrant ainsi l'importance primordiale des NRKs lors de la régénération musculaire. Par ailleurs, l'apport nutritionnel en NR améliore l'activation des cellules souches du muscle et accélère le réapprovisionnement des réserves en NAD^+ dans les fibres musculaires en régénération des souris de type naturel.

Dans l'ensemble, ce projet a permis de démontrer que la sarcopénie est étroitement liée à la bioénergétique ainsi qu'au métabolisme du NAD^+, et ce à la fois chez des patients et dans des modèles pré-cliniques. La manipulation des niveaux de NAD^+ au travers d'une intervention nutritionnelle constitue une stratégie thérapeutique prometteuse contre le déclin musculaire dû à l'âge. Dans ce travail, nous avons mis en lumière de premiers éléments capitaux décrivant le mécanisme par lequel les flux de la voie métabolique du NAD^+ se traduisent au niveau physiologique. Pendant la régénération musculaire, la production de NAD^+ à partir d'apports complémentaires nutritifs prévaut largement sur le taux de réapprovisionnement endogène généré par la voie de sauvetage de NAM. Ainsi, une intervention nutritionnelle au niveau du NAD^+ est envisageable afin de mieux comprendre l'équilibre entre les différents flux de la voie métabolique du NAD^+ et leur activation respective suivant le contexte physiologique.

MOTS CLES : Sarcopénie ; muscle squelettique ; vieillissement ; NAD^+ ; nicotinamide riboside (NR) ; nicotinamide riboside kinase (NRK) ; mitochondrie ; transcriptomique ; métabolomique ; humain ; diversité ethnique ; atrophie musculaire ; régéneration musculaire ; CTX

Zusammenfassung

Ein charakteristisches Merkmal des Alterns ist Sarkopenie, der fortschreitende Abbau von Muskelmasse und -funktion. Sarkopenie ist ein multifaktorieller Prozess, der mit Gebrechlichkeit, Behinderung und niedriger Lebensqualität bei älteren Menschen in Zusammenhang steht. Der mit zunehmendem Alter in Konzentration abnehmende Co-Faktor Nicotinamid-Adenin-Dinukleotid (NAD^+) ist ein wichtiger Regulator des Zellstoffwechsels und wird darum zu einem limitierenden Faktor während des Alterns. Bisher wurde allerdings noch keine Verbindung zwischen altersbedingtem Muskelschwund und NAD^+ Gewebeverfügbarkeit berichtet. In dieser Doktorarbeit habe ich durch Untersuchungen in Mensch und Nagetier die Krankheit Sarkopenie auf molekularer Ebene charakterisiert und den muskulären NAD^+ Stoffwechsel untersucht.

Zunächst haben wir die transkriptionelle Signatur von Sarkopenie im menschlichen Muskel charakterisiert. In drei unabhängigen Patientengruppen verschiedener ethnischer Herkunft konnten wir zeigen, dass im sarkopenischen Muskel vor Allem die Expression mitochondrieller Gene massiv beeinträchtigt ist. Dies spiegelt sich in einer verringerten Präsenz und Aktivität der einzelnen Elektronenketten Komplexe wider. Des Weiteren konnten wir eine geringere Menge an NAD^+ feststellen, die mit verminderter Muskelkraft und -funktion einherging.

Mausstudien haben gezeigt, dass der Verlust von NAD^+ ein entscheidender Faktor für zelluläre Dysfunktionen darstellt und dass Nahrungsergänzung mit dem NAD^+ Vorgängermolekül Nicotinamid Ribosid (NR) erfolgversprechende Ergebnisse in der Behandlung von Muskelerkrankungen und Alterserscheinungen aufweist. Um zu prüfen, ob NR eine positive Wirkung in der Behandlung von Sarkopenie zeigt, haben wir die akute molekulare Zellantwort zu NR im Muskel, sowie dessen Verstoffwechselung im Körper untersucht. Junge und sarkopenische Ratten zeigten erhöhte NAD^+ Muskelgewebekonzentrationen nach Verabreichung von NR. Altersbedingte Veränderungen in der zellulären Genexpression wurden in NR-behandelten Ratten zum Teil wieder umgekehrt, dies betraf insbesondere Signalnetzwerke, die mit Fibrose in Zusammenhang stehen. Interessanterweise konnten wir eine reduzierte Erregbarkeit durch NR in sarkopenischen Ratten feststellen, was auf eine partielle altersbedingte Resistenz gegenüber NAD^+ hinweisen könnte. Dies könnte dadurch erklärt werden, dass der enzymatische Verbauch des gebildeten NAD^+ in erkranktem Muskel verändert ist. Des Weiteren konnten wir eine erhöhte Anreicherung des NR- und NAD^+-Stoffwechselprodukts Nicotinamid (NAM) im sarkopenischen Muskel feststellen.

Da die Wirksamkeit von NR von seiner Verstoffwechselung durch NR Kinasen (NRK) abhängig ist, haben wir den NAD^+ Stoffwechsel, Muskelatrophie und Muskelregeneration in Mäusen untersucht, die keine NRK herstellen können (NRK1/2 dKO). Wir konnten zeigen, dass NR einen wichtigen Anteil am NAD^+ Metabolom im Muskel bildet, da der Verlust von NR als NAD^+ Provitamin durch erhöhte Umwandlung von NAM zu NAD^+ ausgeglichen werden muss. Des Weiteren konnten wir eine wesentliche Rolle für NRK während der Muskelregeneration aufzeigen, welche durch weniger effiziente NAD^+ Regulierung verzögert wurde. Im Gegenteil dazu zeigten Wildtyp Mäuse, die vor und während des Regenerationsprozesses NR erhielten, eine schnellere Regeneration von NAD^+ und erhöhte Aktivierbarkeit der Muskelstammzellen.

Zusammengefasst habe ich mit dieser Dokorarbeit gezeigt, dass Sarkopenie in Mensch und Tier von einem beeinträchtigten mitochondriellen Energiestoffwechsel, als auch von verringerter NAD^+ Verfügbarkeit charakterisiert ist. Obgleich sarkopenischer Muskel eine geringere Reakton zu diätischer Ergänzung mit dem NAD^+ Provitamin NR zeigt, scheint es sich dennoch um eine erfolgversprechende Behandlungsstrategie für altersbedingte Muskelschwäche zu handeln. Schlussendlich konnte ich in dieser Thesis zeigen, dass bestimmte physiologische Prozesse (zum Beispiel Muskelverletzungen) eine Veränderung des gewöhnlichen NAD^+ Stoffwechsels hervorrufen, sodass NR zum entscheidenden NAD^+ Vorgängermolekül wird. Diese wichtigen Einblicke in die Funktion von NR und NRK bereiten den Weg für patientenbezogene Behandlungsstrategien in der Zukunft.

SCHLÜSSELWÖRTER: Sarkopenie; Skelettmuskulatur; Altern; NAD^+; Nicotinamid Ribosid (NR); Nicotinamid Ribosid Kinase (NRK); Mitochondrien; Transkriptomik; Metabolomik; Menschen; Ethnische Diversität; Muskelatrophie; Muskelregeneration; CTX

Contents

List of Figures

List of Tables

List of abbreviations

4EBP1	4E-binding protein 1
ACMS	α-Amino-β-carboxymuconate-ε-semialdehyde
ACMSD	ACMS decarboxylase
ADP	Adenosine diphosphate
ADPr	ADP ribose
ALMi	Appendicular lean body mass index
AMP	Adenosine monophosphate
AMPK	AMP kinase
ANOVA	Analysis of variance
AOX	Aldehyde oxidase
ART	mono-ADPr transferases
ATP	Adenosine triphosphate
AWGSOP	Asian Working Group on Sarcopenia in Older People
BAT	Brown adipose tissue
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index (kg/m^2)
ctrl	Control
CTX	Cardiotoxin
Cytc	Cytochrome c
CS	Citrate synthase
DMEM	Dulbecco's Modified Eagle Medium
DMD	Duchene's muscular dystrophy
DNA	Desoxyribonucleic acid
dKO	double knockout
dpi	days post injury
ECM	Extracellular matrix
ERRα	Estrogen-related receptor alpha
ETC	Electron transport chain
EWGSOP	European Working Group on Sarcopenia in Older People
FAP	Fibro/adipogenic progenitor
FBS	Fetal bovine serum
FDR	False discovery rate
FGF	Fibroblast growth factor
FoxO	Forkhead box O
Fum	Fumarate
G3P	Glyceraldehyde-3-phosphate
GAPDH	G3P dehydrogenase
GH	Growth hormone
GSEA	Gene set enrichment analysis

H6PDH	Hexose-6-phosphate dehydrogenase
HIF1α	Hypoxia-inducible factor 1 alpha
HSS	Hertfordshire Sarcopenia Study
GTP	Guanosine triphosphat
H4K16	Lysine 16 of histone H4
HDAC	Histone deacetylase
ICD-10	International classification of diseases 10
IDO	Indoleamine 2,3-dioxygenase
IGF	Insulin-like growth factor
IL	Interleukin
i.p.	Intra-peritoneal
JNK	c-Jun N-terminal protein kinase 1
JSS	Jamaica Sarcopenia Study
KO	Knockout
LAL	Limulus amebocyte assay
LPS	Lipopolysaccharides
MAFbx	Muscle atrophy F-box
MeNAM	Methyl-nicotinamide
Me2PY	1-methyl-2-pyridone-5-carboxamide
Me4PY	1-methyl-4-pyridone-5-carboxamide
MEMOSA	Multi-Ethnic Molecular determinants of Sarcopenia
MeXP	(Me2PY + Me4PY)
Mfn2	Mitofusin 2
MHC	Myosin heavy chain
MIBP	Muscle integrin binding protein
MRF	Myogenic regulatory factor
mRNA	Messenger RNA
MRP	Mitochondrial ribosomal protein genes
mtDNA	Mitochondrial DNA
mTORC	Mechanistic/mammalian target of rapamycin
mtUPR	Mitochondrial unfolded protein response
MURF1	Muscle ring finger 1
MuSC	Muscle stem cell
Myog	Myogenin
NA	Nicotinic acid
NAAD	Nicotinic acid adenine dinucleotide
NAD^+	Nicotinamide adenine dinucleotide
NADH	1,4-Dihydronicotinamide adenine dinucleotide, reduced NAD^+
NADK	NAD^+ kinase
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	1,4-Dihydronicotinamide adenine dinucleotide phosphate, reduced $NADP^+$
NAM	Nicotinamide
NAMN	Nicotinic acid mononucleotide
NAMPT	Nicotinamide phosphoribosyltransferase
NAPRT	Nicotinic acid phosphoribosyltransferase
NAR	Nicotinic acid riboside
NFκB	Nuclear factor kappa B
NIHS	Nestlé Institute of Health Sciences
NMNAT	Nicotinamide mononucleotide adenylyltransferase
NMJ	Neuromuscular junction

NR	Nicotinamide riboside
NRK	Nicotinamide riboside kinase
NSAID	Non-steroidal anti-inflammatory drug
OXPHOS	Oxidative phosphorylation
PARP	poly(ADP-ribose) polymerases
Pax7	Paired box protein 7, satellite cell marker
PBEF	pre-B cell colony-enhancing factor
PBS	Phosphate buffered saline
PCAF	P300/CBP-associated factor
PGC1α	Peroxisome proliferator-activated receptor gamma co-activator 1 alpha
PI3K	Phosphatidylinositol 3 kinase
PNP	Purine nucleoside phosphorylase
PPARα	peroxisome proliferator-activated receptor α
PRPP	5-Phospho-α-D-ribose 1-diphoshate
qPCR	Quantitative real-time polymerase chain reaction
QPRT	Quinolate phosphoribosyl-transferase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
S6K1	S6 kinase 1
SARM	Selective androgen receptor modulator
SDH	Succinate dehydrogenase
SIRT	Sirtuin
sKO	single knockout
SSS	Singapore Sarcopenia Study
STAC	Sirtuin activating compound
Suc	Succinate
TCA cycle	Tricarboxylic acid cycle
TDO	Tryptophan 2,3-dioxygenase
TFAM	Mitochondrial transcription factor A
TGFβ	Tumor growth factor beta
TNFα	Tumor necrosis factor alpha
Trp	L-tryptophan
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
wk	Week
WT	Wild-type

1 Introduction

1.1 Skeletal muscle - a highly plastic tissue

The muscular system of the human body is comprised of smooth, cardiac and skeletal muscle. In contrast to "involuntary" smooth and cardiac muscle, skeletal muscle contractions are under conscious control of the somatic nervous system. Voluntary contraction and relaxation of skeletal muscles permit locomotion and body posture as well as breathing, blood circulation, swallowing, defecation, and urination. Representing up to 40% of the human body weight, skeletal muscle is the largest protein reservoir and plays a major role in metabolic homeostasis and body temperature regulation. Due to its high energetic demands, skeletal muscle is a major regulator of whole body energy metabolism. As the primary consumer of ingested glucose, skeletal muscle relies on insulin signaling, which stimulates glucose disposal. Impaired insulin signaling and insulin resistance of skeletal muscle have been connected to obesity and type 2 diabetes (Samuel and Shulman, 2012). Moreover, through secretion of several myokines such as fibroblast growth factor 21 (FGF-21), brain-derived neurotrophic factor (BDNF), interleukins 6, 8, and 15 (IL-6, IL-8, IL-15), as well as irisin, kynurenine, and others, skeletal muscle exerts an endocrine function, i.e. regulating the systemic effects of exercise, thermogenesis and lipid utilization (Pedersen and Febbraio, 2012; Schnyder and Handschin, 2015).

The maintenance of skeletal muscle health and plasticity relies on the fine-tuned balance of protein synthesis and degradation (see Section 1.1.2) as well as efficient muscle repair after injury (see Section 1.1.3). Aging gradually disturbs signaling pathways that regulate muscle growth and energy metabolism, eventually leading to the pathological loss of muscle mass and function called sarcopenia (see Section 1.2).

1.1.1 Structure of skeletal muscle

Skeletal muscle is a type of striated muscle and, as the name suggests, most muscles are attached to at least one bone by tendons (Figure 1.1a). Each skeletal muscle is composed of multiple muscle fascicles that in

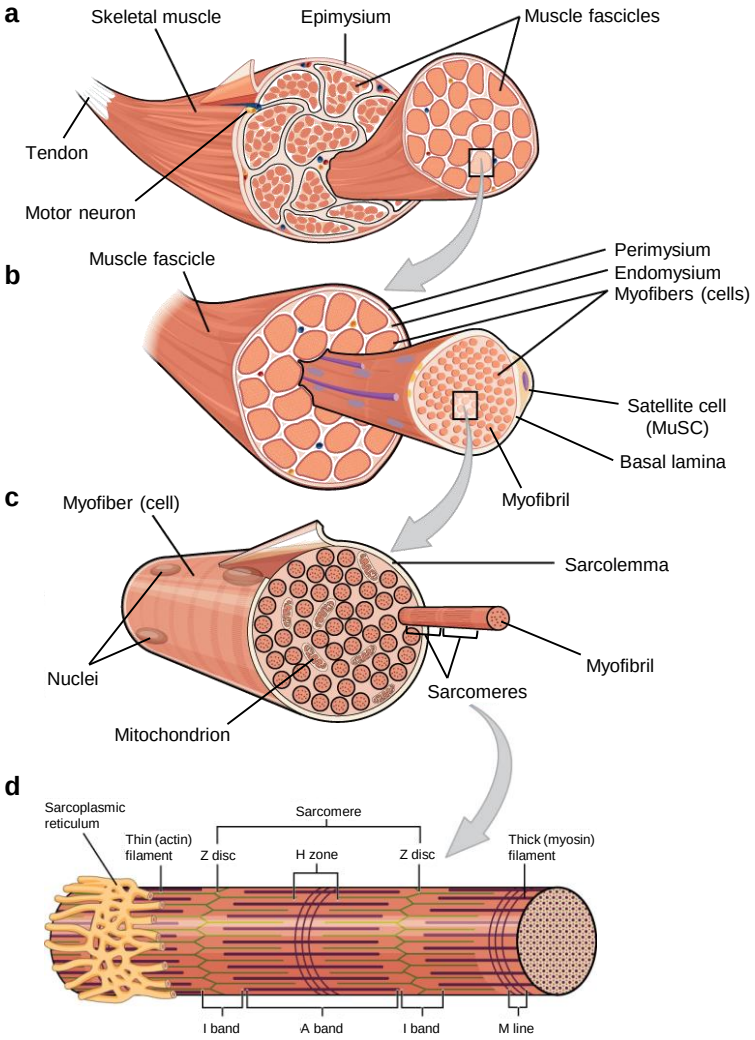

Figure 1.1: Structure of skeletal muscle. **(a)** Skeletal muscles are attached to bones by tendons and are composed of multiple muscle fascicles. **(b)** Each muscle fascicle consists of several myofibers, the single muscle cells. Myofibers are surrounded by the basal lamina, a network of extracellular matrix that provides a structural scaffold for myofibers and satellite cells, the resident muscle stem cells (MuSCs). **(c)** Myofibers derive from fusion of numerous myogenic progenitor cells and are thus multinucleated, elongated cells that contract longitudinally. **(d)** The contractile units of myofibers are myofibrils, a sequential construct of interleaved actin and myosin filaments that are arranged in structural units called sarcomeres. Calcium released from the sarcoplasmic reticulum is required for contraction of sarcomeres. Image adapted from *https://commons.wikimedia.org/wiki/File:1007_Muscle_Fibes_(large).jpg* and *https://commons.wikimedia.org/wiki/File:1022_Muscle_Fibers_(small).jpg*.

turn consist of several myofibers, the individual muscle cells (Figure 1.1b). Myofibers are surrounded by the basal lamina, a dense layer of extracellular matrix that enables embedding of the single myofibers, synaptic adhesion of motor neurons, and provides a niche for the resident muscle stem cells (MuSCs), also known as satellite cells (Mashinchian et al., 2018). Myofibers derive from fusion of such myogenic precursor cells and form long, multinucleated cells that can reach lengths of up to 35 cm (Zalewska et al., 2012). MuSCs further support muscle growth by fusing to existing myofibers. In the adult muscle, MuSCs reside in a quiescent state on the sarcolemma, the myofiber membrane. In case of muscle injury, MuSCs get activated and enter the

cell cycle to strongly proliferate and accommodate muscle regeneration (see Section 1.1.3). The contractile function of skeletal muscle is based on myofibrils, the contractile units of myofibers which are comprised of repetitive entities of interleaved actin and myosin filaments called sarcomeres (Hwang and Sykes, 2015) (Figure 1.1c,d).

The contraction of striated muscle occurs longitudinally and is a consequence of movement of the myosin heavy chain (MHC) head domain along the actin filaments. This process is induced by neuronal stimulation which triggers voltage- and calcium-dependent excitation-contraction coupling (Kuo and Ehrlich, 2015). In more detail, at the neuromuscular junction, acetylcholine is released by the motor neuron, binds muscle membrane receptors, and elicits a depolarization of the sarcolemma though sodium and calcium influx. The resulting action potential triggers the release of calcium from the sarcoplasmic reticulum which binds to troponin and releases it from actin to expose MHC binding sites. ATP hydrolysis induces conformational changes of the MHC head and provides the energy required for sliding of the filaments to drive muscle contraction (Kuo and Ehrlich, 2015).

Skeletal muscle is comprised of different muscle fibers, each expressing a specific type of MHC. Embryonic and neonatal MHCs (encoded by *MYH3* and *MYH8*) are expressed at high levels during development and re-expressed during muscle regeneration (D'Albis et al., 1988; Schiaffino et al., 2015) (see Section 1.1.3). These specific MHC isoforms are downregulated after birth and replaced by MHC1, MHC 2a, MHC2b, or MHC2x (encoded by *MYH7*, *MYH2*, *MYH4*, and *MYH1*, respectively), which characterize muscle fibers of type I, IIa, IIb, and IIx, respectively (Scott et al., 2001; Schiaffino et al., 2015). The different fiber types are categorized according to their contraction (twitch) speed and energy metabolism. Slow-twitch fibers of type I rely on mitochondrial oxidative metabolism which makes them resistant to fatigue. In contrast, fast-twitch fibers of type IIb rely on glycolysis to support high-intensity contractions during a short time. Fast-twitch fibers of type IIa and IIx have intermediate metabolic properties, with type IIa displaying oxidative and type IIx mixed metabolism (Schiaffino and Reggiani, 1994). The heterogeneous distribution of fiber types varies between different muscles and is dictated by the firing frequency of afferent motor neurons and therefore the contractile characteristics (Pette and Vrbová, 1985; Schiaffino and Serrano, 2002). The fiber type composition and size of skeletal muscle remains plastic throughout life and responds to different stimuli such as exercise and inactivity, hormones, injury, disease, and aging (Pette and Staron, 2001; Schiaffino and Serrano, 2002; Gundersen, 2011).

1.1.2 Regulation of skeletal muscle plasticity

Adult skeletal muscle is in general a post-mitotic tissue whose growth is governed by protein turnover in response to anabolic and catabolic signaling. Its plasticity relies on the fine-tuned regulation of those processes as well as its regenerative capacity. Anabolic stimuli can result from physical work or exercise and induce muscle hypertrophy, while states of inactivity, disease, and aging rather lead to catabolic metabolism and muscle wasting. The balance of protein synthesis and degradation is regulated by pathways that respond to mechanical stress (physical activity), growth factors and nutrient and energy availability (Sandri, 2008).

A primary regulator of skeletal muscle mass maintenance is mechanistic target of rapamycin complex 1 (mTORC1). This multi-protein complex acts as a sensor of intracellular nutrient availability and energy status and plays a fundamental role in protein turnover (Laplante and Sabatini, 2012). An upstream regulator of mTORC1 is insulin-like growth factor 1 (IGF1). Growth hormone (GH) controls secretion of IGF1 from the liver, but also muscle itself contributes specific IGF1 splicing products (Sandri, 2008). IGF1 and insulin activate the Akt signaling pathway via phosphatidylinositol 3 kinase (PI3K) (Bodine et al., 2001), which stimulates mTORC1 and promotes protein synthesis through inhibition of eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) and activation of S6 kinase 1(S6K1) (reviewed by Sandri (2008) and Schiaffino et al. (2013)). Moreover, the anabolic signaling inducing mTOR activity is further dependent on the availability of amino acids such as leucine (Tipton and Phillips, 2013; Hoppeler, 2016), which is sensed by Rag GTPases (Bar-Peled and Sabatini, 2014).
Skeletal muscle adaptation to exercise is majorly mediated by the transcriptional co-activator peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC1α). PGC1α is upregulated in response to exercise and induces the expression of mitochondrial genes and subsequently mitochondrial biogenesis and muscle oxidative capacity (Wu et al., 1999; Pilegaard et al., 2003). Other functions include regulation of glycogen storage and lipid metabolism during post-exercise refueling and of angiogenesis as a long term adaptation (reviewed in Chan and Arany (2014)). Long term adaptations to exercise are mediated by AMP-activated protein kinase (AMPK)-dependent phosphorylation of PGC1α and subsequent up-regulation of glucose uptake (Mounier et al., 2015). Other upstream effectors include calcium-calmodulin interaction (Norrbom et al., 2004), reactive oxygen species through nuclear factor kappa B (NFκB) activation (Gomez-Cabrera et al., 2005), vascular endothelial growth factor (VEGF) (Arany et al., 2008) and hypoxia-inducible factor 1 alpha (HIF1α) (Millet et al., 2016).

A major negative regulator of muscle growth is myostatin, a member of the transforming growth factor beta (TGFβ) superfamily that is produced by skeletal muscle. Signaling via myostatin and other TGFβ family members such as activin A induce activation of transcription factors Smad2 and Smad3. Nuclear translocation

and heterodimerization with Smad4 causes inhibition of muscle growth. Albeit the exact transcriptional targets of Smad2/Smad4 and Smad3/Smad4 remain to be identified, reduced anabolic signaling through Akt/mTORC1/S6K has been reported (Trendelenburg et al., 2009).

Muscle atrophy further results from increased proteolysis through the ubiquitin-proteasome system and the autophagy-lysosome pathway. These processes are normally suppressed through the inhibition of forkhead box O (FoxO) transcription factors by IGF1/PI3K/Akt, PGC1α or sirtuin 1 (SIRT1) (Stitt et al., 2004; Sandri et al., 2006; Lee and Goldberg, 2013), and stimulated by AMPK activity (Greer et al., 2007; Lee et al., 2019). FoxOs are major regulators of so-called atrogenes that encode for E3 ubiquitin-ligases such as atrogin-1/muscle atrophy F-box (MAFbx) and muscle ring finger 1 (MuRF1) (Bodine et al., 2001; Sandri et al., 2004). These ligases ubiquitinate a wide range of regulatory and structural proteins, destining them for proteasomal degradation. Moreover, through inhibition of mTOR signaling, FoxOs promote macroautophagy, which is a mechanism for the degradation and recycling of bulk cytoplasm and even organelles (i.e. mitophagy) (Sandri, 2012; Bonaldo and Sandri, 2013; Lee et al., 2019). Other transcriptional regulators of proteasome and autophagy are histone deacetylases (i.e. HDAC4 and 5) via myogenin (Moresi et al., 2010) as well as c-Jun N-terminal protein kinase 1 (JNK1) (Wei et al., 2008), HIF1α (Bohensky et al., 2007), and the pro-inflammatory transcription factor NFκB (Copetti et al., 2009). Indeed, inflammation, particularly the presence of cytokines tumor necrosis factor alpha (TNFα) and IL-6, is an important inducer of muscle atrophy (Peterson et al., 2011).

1.1.3 Skeletal muscle regeneration

Skeletal muscle regularly experiences injuries as a consequence of falls, fractures or excessive exercise which stimulate anabolic and regenerative pathways. The strong regenerative capacity of muscle tissue is largely dependent on the activation of MuSCs, typically expressing the satellite cell marker paired box protein 7 (Pax7) (Mauro, 1961; Seale et al., 2000; Kuang et al., 2007). Tissue damage stimulates these typically quiescent cells to enter the cell cycle and strongly proliferate (Figure 1.2). The myogenic program is controlled through sequential expression of myogenic regulatory factors (MRFs) which coordinate proliferation, commitment and differentiation of MuSCs (Rudnicki et al., 2008). Up-regulation of myogenic differentiation factor 1 (MyoD) commits MuSCs to the myogenic lineage (Sabourin et al., 1999; Cornelison et al., 2000). These now-called myoblasts are destined to undergo terminal myogenic differentiation, induced by down-regulation of Pax7 and up-regulation of myogenin (Zammit et al., 2002; Olguin and Olwin, 2004). Early differentiated myocytes start expressing embryonic MHC and permit tissue repair by fusing to damaged fibers or forming new ones (Kuang and Rudnicki, 2008). The resulting multinucleated fibers express desmin and MRF4 (Le Grand and Rudnicki, 2007), and embryonic MHC is replaced by neonatal and finally adult MHC isoforms (D'Albis et al., 1988; Schiaffino et al., 2015).

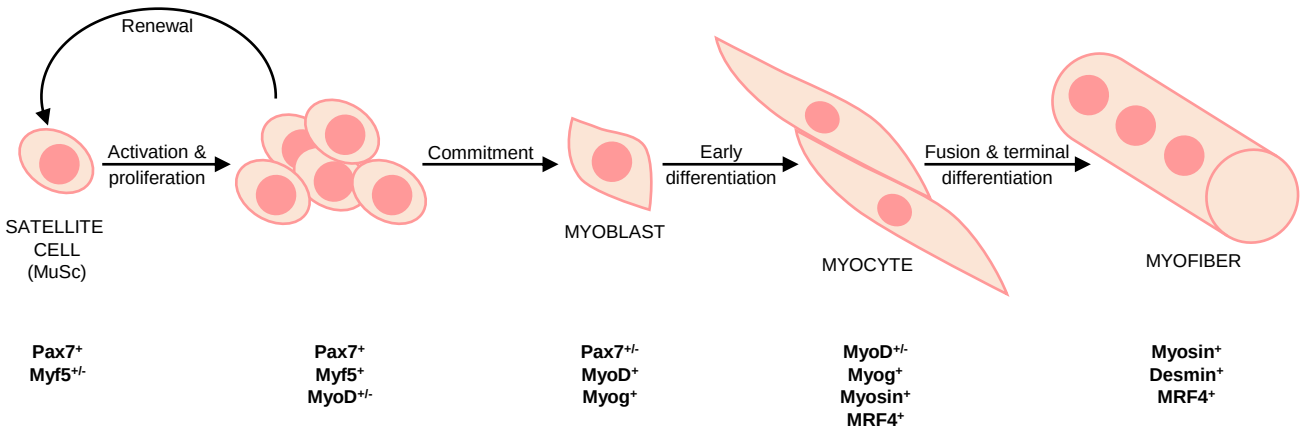

Figure 1.2: The myogenic program during muscle regeneration. Injury activates quiescent MuSCs to enter the cell cycle and strongly proliferate. A subset of activated cells returns to quiescence, while others commit to the myogenic lineage, forming myolasts. Myoblasts exit the cell cycle to undergo differentiation to myocytes, which fuse to repair damaged myofibers or to form new ones. Adapted from Tidball et al. (2014).

MuSCs display a high metabolic flexibility which allows them to smoothly transit between the different stages of their myogenic program (Folmes et al., 2012). The NAD^+ dependent deacetylase SIRT1 has been shown to be a master regulator of the metabolic reprogramming and gene expression that is associated to this transition. In quiescent MuSCs, SIRT1 interacts with MyoD via P300/CBP-associated factor (PCAF) to inhibit differentiation (Fulco et al., 2003). MuSC activation and proliferation leads to an increased energy demand which is addressed by the SIRT1-dependent induction of autophagy (Tang and Rando, 2014). Simultaneously, a metabolic switch from fatty acid and pyruvate oxidation to glycolysis reduces NAD^+ availability and thus SIRT1 activity, leading to increased histone acetylation which initiates transcription of myogenic genes (Fulco et al., 2003; Zhang et al., 2011; Ryall et al., 2015). Due to its central function in the myogenic program, loss of SIRT1 in muscle and MuSCs impairs muscle regeneration on different levels. The failure to upregulate ATP levels by autophagy leads to a delayed MuSC activation (Tang and Rando, 2014), and hyper-acetylation of H4K16 disturbs muscle gene expression leading to premature differentiation (Ryall et al., 2015).

A hallmark of tissue regeneration is the transient infiltration of the injured site by inflammatory cells. Pro-inflammatory macrophages (M1) arrive early at the site of damage to evacuate cellular debris and inhibit premature MuSC differentiation, thereby facilitating the adequate expansion of muscle progenitor cells (Segawa et al., 2008; Saclier et al., 2013; Varga et al., 2016). Subsequently, anti-inflammatory and pro-regenerative macrophages (M2) stimulate myogenic commitment and fusion by promoting myogenin expression(Arnold et al., 2007). At the same time, fibro/adipogenic progenitors (FAPs) expand and release pro-differentiation signals and are finally removed by the macrophages (Joe et al., 2010).

Another important process during muscle regeneration and recovery of tissue architecture is remodeling of

the niche through other muscle resident cells such as fibroblasts, endothelial cells or mesenchymal cells (Yang et al., 2011). These cells excrete essential components of the extracellular matrix (ECM) such as collagens, laminin, proteoglycans and glycoproteins, which support MuSC function and provide a structural scaffold for MuSC adhesion and fiber embedding through binding of surface receptors such as integrin $\alpha 7\beta 1$ and syndecan-4 (Bentzinger et al., 2013; Gattazzo et al., 2014). Especially during the initial phases of regeneration, M2 macrophages secrete ECM components such as fibronectin and collagen-VI, which boost MuSC function (Bentzinger et al., 2013; Lukjanenko et al., 2016). Furthermore, non-structural secreted matricellular proteins such as WISP1 participate in ECM signaling and promote MuSC adhesion and proliferation (Lukjanenko et al., 2019). Finally, the efficient re-organization of the basal lamina facilitates recovery of muscle innervation and formation of new neuromuscular junctions, a process imperative for fiber maturation, as the nature of neurogenic input determines the contractile characteristics of each newly-formed myofiber (Pette and Vrbová, 1985; Schiaffino and Serrano, 2002).

1.2 The aging muscle

The term sarcopenia, "poverty of flesh" (from the Greek *sarx* and *penia*), describes the progressive age-associated loss of muscle mass, strength and functionality. In the young body, lean muscle mass accounts for 30–35% of body mass, roughly 20% of which is lost at old age, and up to 50% with sarcopenia (Kyle et al., 2001; Denison et al., 2015). The concurrent decline in muscle strength and performance leads to a loss of independence and mobility in the elderly and an increased risk of falls and fractures (Janssen et al., 2002). Importantly, also skeletal muscles involved in respiration and excretion experience declines in function, which may lead to respiratory failure and incontinence (Kelley and Ferreira, 2017; Parker-Autry et al., 2017). This makes sarcopenia a strong predictor of hospitalization, disability, poor clinical outcome, and death (Fielding et al., 2011; Landi et al., 2013).

The prevalence of sarcopenia increases with age and ranges from 2–20% in community dwelling people aged older than 50 years and up to 33% among patients in long term care (Cruz-Jentoft et al., 2014). It was further estimated that up to 15% of people aged 65 years and above and up to 50% of individuals older than 80 years of age are affected by sarcopenia (Zembroń-Łacny et al., 2014). Based on our current demographic development, we can expect a dramatic increase of sarcopenic patients in the near future, which will pose a considerable health care burden.

Health care costs related to sarcopenia were estimated at USD $18.5 billion in the year 2000 (Janssen et al., 2004). More recently presented data for the year of 2014 estimated the cost of hospitalizations alone to be more than USD $40 billion, with almost half of the total cost attributable to individuals older than 65 years of age (Goates et al., 2019). Another study from the UK estimated that sarcopenia accounts for an annual excess

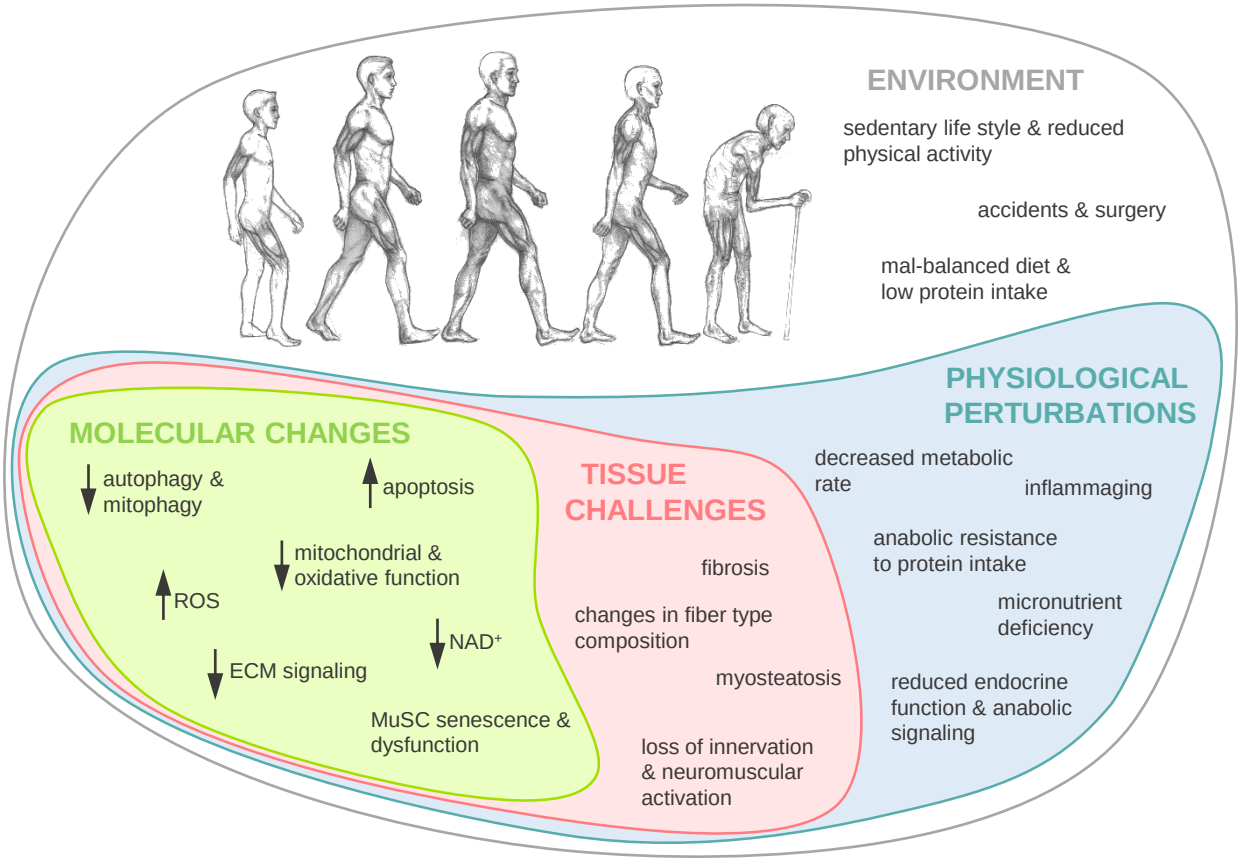

Figure 1.3: The multi-factorial causes of sarcopenia. The age-associated decrease of muscle mass, strength, and function is caused by a combination of environmental influences, systemic factors, and molecular changes.

cost of £2.5 billion (Pinedo-Villanueva et al., 2019).

To date, economical assessment of sarcopenia is still sparse, perhaps owed to the fact that a practical clinical definition of sarcopenia along with the recognition as a disease was still pending until just very recently. Since 2010, international expert working groups revisited the loose definition of sarcopenia to frame a consensus definition using muscle mass, muscle strength and physical performance as measurable parameters. Different cutoff values were specified for Caucasian and Asian populations, respectively, and are now being applied to diagnose sarcopenia (Cruz-Jentoft et al., 2010, 2019; Muscaritoli et al., 2010; Fielding et al., 2011; Morley et al., 2011; Chen et al., 2014; Studenski et al., 2014). As a result, sarcopenia was recognized as a disease and was awarded an International Classification of Diseases (ICD)-10 code in 2016 (Anker et al., 2016).

1.2.1 Pathophysiological and molecular changes contributing to sarcopenia

The physiological and molecular changes that result in sarcopenia are multi-factorial and underlie different environmental and systemic factors (Figure 1.3). A sedentary life style (oftentimes secondary to co-morbidity

or injuries) accelerates muscle decline in the elderly (Denison et al., 2015). Physical inactivity also reduces energy expenditure, and sarcopenic patients (compared to age-matched controls) exhibit a "malnurished" status which could be attributed to a generally decreased appetite in aged people, combined with a mal-balanced diet. In particular, low intake of proteins and lipids, and a diet deficient of micronutrients were correlated with low physical performance and the prevalence of sarcopenia (van Dronkelaar et al., 2018, 2019; Beaudart et al., 2019). An additional factor in this respect is anabolic resistance to protein intake in the elderly, which requires a higher relative protein intake compared to young individuals (Katsanos et al., 2006; Moore et al., 2015).

The aging body also experiences a change in endocrine function, which leads to decreases in anabolic hormones such as testosterone, estrogen, GH, and IGF1, and increases in catabolic mediators such as myostatin, glucocorticoid as well as the inflammatory cytokines tumor necrosis factor TNFα and IL-6 (reviewed by Sakuma and Yamaguchi (2012)). The observed systemic low-grade chronic inflammation, also called "inflammaging", has been correlated to functional decline and is therefore believed to be a major contributor to skeletal muscle wasting (reviewed by Dalle et al. (2017)).

At the tissue level, muscle quality is affected by increased inter- and intramuscular fat infiltration (myosteatosis), a process negatively correlated to muscle strength and function (Perkisas et al., 2018). Moreover, excessive extracellular matrix deposition leads to fibrosis and stiffening of the muscle tissue, which is thought to impair MuSCs function and regenerative capacity (Serrano and Muñoz-Cánoves, 2010). Another component that has been shown to affect the susceptibility of muscles to sarcopenia are changes in neuromuscular activation, which has been observed to decline specifically in sarcopenic muscles (Pannérec et al., 2016).

All the above presented factors contribute to an imbalance of anabolic and catabolic pathways in the sarcopenic muscle which leads to gradual changes in muscle characteristics. Muscle fibers decrease in number and size, and a shift in fiber type composition of sarcopenic muscle has been described (Ciciliot et al., 2013). Particularly type II fibers are lost during aging, as they atrophy faster, are susceptible to a transition from type II to type I MHC isoforms and are additionally affected by a decreased number of type II fiber MuSCs (Verdijk et al., 2014). Loss of proteostasis is a major contributor to declines in muscle mass and contractile function. Lower activation of anabolic IGF1/Akt/mTOR signaling coupled with impaired proteasome and autophagy activity causes a decline in quantity and quality of the muscle proteome (Coen et al., 2019). The accumulation of damaged proteins is further promoted by a decrease in redox homeostasis that leads to oxidative damage of cellular components (Capel et al., 2005; Lourenço Dos Santos et al., 2015). Increased levels of ROS are majorly generated by progressive accumulation of defective mitochondria, which can also trigger apoptotic signaling cascades (see Section 1.3). Oxidative stress further causes damage of DNA,

which leads to excessive activation of DNA repair processes, i.e. through NAD^+-dependent poly(ADP-ribose) polymerases (PARPs) and consequently depletion of cellular NAD^+ content. Finally, MuSC senescence and dysfunction paired with decreased ECM signaling (i.e. through exerkines or matrix factors) leads to reduced preservation and repair of myofibers in the aged muscle (Vinel et al., 2018; Lukjanenko et al., 2016, 2019).

1.3 The role of mitochondria in the aging muscle

Mitochondria are called the 'powerhouses of the cell' and their role in cellular energy production has been extensively studied. These organelles are derived of endosymbiontic origin and possess their own mitochondrial DNA (mtDNA), which is present in up to several thousands of copies per cell. During the evolution of the cellular-mitochondrial symbiosis most of those genes were transferred to chromosomes of the nuclear DNA and are now regulated by transcription factors such as PGC1α and estrogen-related receptor alpha (ERRα). Besides ATP generation, mitochondria play an important role in the metabolism of carbohydrates, nucleotides, amino acids, and lipids, as well as Fe-S-cluster synthesis, calcium homeostasis, and programmed cell death initiation (Zorov et al., 1997; Burger et al., 2003; van der Giezen and Tovar, 2005). Moreover, moderate ROS formation resulting from oxidative phosphorylation induces antioxidant gene transcription which leads to an adaptive hormetic response that can be protective in the context of aging (Musci et al., 2019).

1.3.1 Oxidative phosphorylation

Oxidative phosphorylation (OXPHOS) is the main metabolic pathway for the production of cellular energy (Figure 1.4). It is located at the inner mitochondrial membrane where a series of redox reactions convert dietary energy from organic acids and fats into utilizable energy in form of ATP. In a first step, complexes I-IV of the electron transport chain (ETC) generate a proton gradient across the inner mitochondrial membrane which is subsequently used by the F1F0-ATPase (complex V) for the phosphorylation of ADP to form ATP (Nelson and Cox, 2017).

The two electrons initially entering the ETC derive from the conversion of $NADH+H^+$ to NAD^+ at ETC complex I (NADH dehydrogenase) or of succinate to fumarate at complex II (succinate dehydrogenase). Both substrates are products of glycolysis and the tricarboxylic acid (TCA) cycle. The donated electrons are sequentially passed on to ubiquinone (oxidized) to form ubiquinol (reduced). Ubiquinol diffuses through the membrane and passes both electrons on to complex III (ubiquinol-cytochrome c oxidoreductase) where they are further transferred to cytochrome c. At complex IV (cytochrome c oxidase), cytochrome c is eventually oxidized with O_2 as final electron acceptor under formation of H_2O. During this series of redox reactions

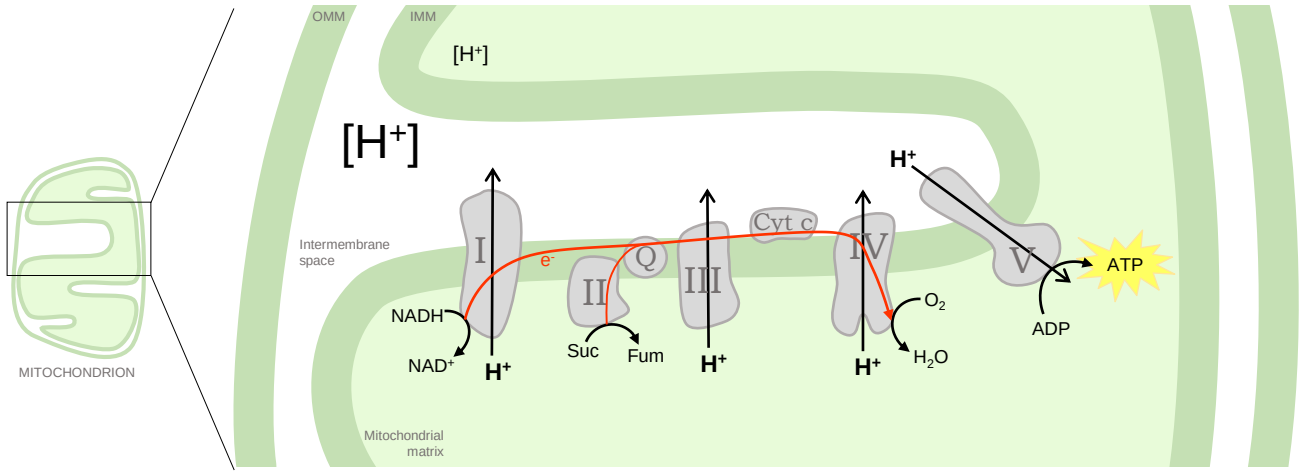

Figure 1.4: Oxidative phosphorylation in mitochondria. Complexes I to V are located at the inner mitochondrial membrane (IMM). Electrons (e^-, red arrows) are sequentially passed from complex I or II to complex IV via ubiquinone (Q), complex III, and cytochrome c (Cyt c). At complex IV, O_2 is oxidized as final electron acceptor under formation of H_2O. This series of redox reactions facilitates proton (H^+) pumping from the mitochondrial matrix into the intermembrane space through complexes I, III, and IV, resulting in a proton gradient and a high membrane potential. This potential energy is utilized by the F1F0-ATPase, where the reflux of protons into the matrix drives the binding and phosphorylation of ADP to form ATP. Fum = fumarate, OMM = outer mitochondrial membrane, Suc = succinate. For more details see Section 1.3.1.

protons are pumped from the mitochondrial matrix into the intermembrane space through complexes I, III, and IV, which forms a proton gradient and a high membrane potential. This potential energy is utilized by the F1F0-ATPase, where the reflux of protons into the matrix drives the binding and phosphorylation of ADP to form ATP (Nelson and Cox, 2017).

The individual complexes of the ETC have been observed to assemble into large macromolecular clusters, so-called supercomplexes or respirasomes (Wittig and Schägger, 2009).

1.3.2 Mitochondrial impairments related to aging and sarcopenia

Muscle aging has robustly been associated with decreases of mitochondrial mass and function in preclinical models as well as humans (Joseph et al., 2012; Johnson et al., 2013; Andreux et al., 2018). mtDNA and mitochondrial mRNA content is inversely correlated to age (Short et al., 2005). Abundance of mitochondrial proteins is decreased in old muscle compared to young, along with the enzymatic activity of TCA and ETC (Rooyackers et al., 1996; Conley et al., 2000; Short et al., 2005; Andreux et al., 2018). Hence, mitochondrial ATP production and oxidative capacity is compromised, which correlates to lower muscle function, i.e. gait speed (Coen et al., 2013).

Transcriptional regulation of mitochondrial genes relies mainly on the transcription factors PGC1α, ERRα, nuclear respiratory factor (NRF1/2), and their downstream target mitochondrial transcription factor A

(TFAM) (Scarpulla et al., 2012; Kupr and Handschin, 2015). Overexpression of PGC1α improves mitochondrial homeostasis in an ERRα-dependent manner and delays muscle aging in mice (Gill et al., 2019). Alternatively, it was shown that reduced NAD^+ levels in aged skeletal muscle induce a pseudohypoxic state through HIF1α accumulation, which disrupts nuclear-mitochondrial communication in a SIRT1-dependent manner and leads to loss of mitochondrial-encoded proteins in mice (Gomes et al., 2013). In addition to the decreased expression of mitochondrial proteins, accumulation of misfolded and damaged proteins can interfere with correct folding of other proteins and may cause destabilization of ETC protein complexes (Gómez and Hagen, 2012). Accordingly, NAD^+-mediated activation of the mitochondrial unfolded protein response (mtUPR) was shown to reverse MuSC mitochondrial dysfunction of aged mice (Zhang et al., 2016). Without sufficient activation of the mtUPR, a disbalanced stochiometric distribution of ETC complexes and supercomplex destabilization leads to impaired enzymatic function, inefficient electron transport, and thus lower ATP levels and elevated generation of ROS (Gómez and Hagen, 2012). The increased ROS burden further intensifies ETC dysfunction and contributes to an increase of oxidative (mt)DNA lesions that have been shown to drive myofiber loss in aging (Short et al., 2005; McKenzie et al., 2002; Herbst et al., 2016). Oxidative damage of mitochondrial as well as nuclear DNA is further associated to excessive PARP activation and thus leads to NAD^+ depletion (Pillai et al., 2005; Bai et al., 2011). This affects the regulation of mitophagy by NAD^+-dependent deacetylase SIRT1 as well as AMPK and promotes accumulation of dysfunctional mitochondria (Milan et al., 2015). The efficiency of mitophagy is further impaired by defective mitochondrial dynamics, i.e. fusion and fission, which has been connected to lower expression of mitofusin 2 (Mfn2) or Opa1 (Joseph et al., 2012; Sebastián et al., 2016).

Altogether, the above described factors form a continuum and lead to increased mitochondrial stress, which causes opening of the mitochondria transition permeability pore. In combination with decreased mitophagy, the accumulation of such defective mitochondria and leakage of their matrix content into the cytosol may cause nuclear DNA fragmentation, intitiation of apoptotic signaling pathways, and ultimately result in myofiber loss (Alway et al., 2017).

Results from rat studies (a commonly used model of sarcopenia) suggest that the above mentioned changes are intensified in sarcopenic versus protected muscles (Lowe et al. (2004); Ibebunjo et al. (2013); Pannérec et al. (2016) and own data, Chapter 3), but human data confirming this general conception was still missing. In our study presented in Chapter 2, we report that decreased mitochondrial regulation, gene expression and bioenergetics are major discriminating factors between sarcopenic patients and age-matched elderly that maintained normal muscle function.

1.4 Strategies to improve sarcopenia

The most effective strategy to prevent and ameliorate sarcopenia is exercise in the form of endurance, resistance and proprioceptive training (Landi et al., 2013; Phu et al., 2015; Musci et al., 2019). As a preventative intervention, regular exercise slows down the age-associated loss of muscle mass and strength and can further induce gain of same parameters, in addition to metabolic and cardiovascular improvements (Short et al., 2004; Konopka and Harber, 2014; Hoppeler, 2016).
The perhaps most critical mediators of exercise adaptation are AMPK and the mitochondrial regulator PGC1α (Weihrauch and Handschin, 2018). Activation of those key regulators induces mitochondrial biogenesis, function, and oxidative metabolism, and increases vascularization (Kupr and Handschin, 2015). Induction of the NAD^+ producing enzyme nicotinamide phosphoribosyltransferase (NAMPT) in response to exercise is suggested to further support increased mitochondrial activity (Costford et al., 2010; de Guia et al., 2019). Moreover, enhanced ETC supercomplex formation in response to exercise increases efficacy of mitochondrial ATP generation (Greggio et al., 2017). Lastly, exercise improves cellular proteostasis through stimulation of autophagy (Kim et al., 2013) and mitigates oxidative damage through mitohormesis (Musci et al., 2019).

Due to the malnourished status of sarcopenic patients, the effect of exercise can be further increased when paired with nutritional interventions. A diet rich in proteins and polyunsaturated fatty acids provides essential amino acids (particularly leucine) and building blocks to support muscle growth (Leuchtmann and Handschin, 2019). Moreover, adequate supply with micronutrients such as potassium, magnesium, phosphorus, iron, calcium, and especially with vitamin D is also recommended (Muir and Montero-Odasso, 2011; van Dronkelaar et al., 2018; Beaudart et al., 2019). It is still a matter of debate whether dietary interventions alone are sufficient to ameliorate sarcopenia (Cruz-Jentoft and Sayer, 2019). A promising dietary supplementation strategy emerged from the increasing body of preclinical evidence suggesting that dietary vitamin B_3 derivatives, precursor molecules of NAD^+, have the potential to boost energy metabolism and ameliorate age-related muscle defects (Connell et al., 2019). However, human data is still scarce and first published studies report ambiguous results (see Section 1.8.1).

The gastrointestinal peptide hormone ghrelin is a major regulator of appetite and food intake and thus an interesting pharmacological target. Ghrelin or ghrelin receptor agonist treatment restores food intake and leads to increases in muscle mass, presumably through stimulation of GH and IGF1 release. However, functional parameters did not benefit of ghrelin treatment in presented studies (Leuchtmann and Handschin, 2019). Due to the anabolic effects of GH and IGF1, replacement therapy has been investigated (Rudman et al., 1990; Philippou and Barton, 2014), but the possibility of adverse effects in non-deficient subjects (i.e. on glucose tolerance) limit their use (Liu et al., 2007). Similar results were reported for testosterone, wherefore

selective androgen receptor modulators (SARMs) are being explored as a safer treatment option (Dalton et al., 2011; Furrer and Handschin, 2019). A different approach are non-steroidal anti-inflammatory drugs (NSAIDs), which show a potential benefit in aged subjects when combined with exercise (Trappe et al., 2011, 2013). However, negative effects on muscle function were reported for young individuals (Lilja et al., 2018).

Given suboptimal success of pharmacological approaches, it is no surprise that alternative treatment strategies are being explored. Autologous stem cell therapy is an emerging field of tissue engineering, the efficacy and success of such intervention in aged patients is however limited, as the regenerative capacity of MuSCs strongly depends on systemic factors (i.e. growth factors), which are compromised with age (Delo et al., 2008). First preclinical studies have shown that overexpression of i.e. VEGF and PGC1α enhances the regenerative potential of injected muscle progenitor cells and improves muscle regeneration (Delo et al., 2008; Haralampieva et al., 2018). It might be intriguing to test whether, instead of genetic manipulation, implantation of those cells could also benefit of co-treatment with molecules that have been observed to boost stem cell function in mice, i.e. the exercise-induced myokine apelin (Vinel et al., 2018), the matricellular factor WISP1 (Lukjanenko et al., 2019), or the NAD^+ precursor nicotinamide riboside (NR) (Zhang et al., 2016).

1.5 NAD^+ as a key player in cellular metabolism and aging

More than a century ago, NAD^+ was discovered as a co-factor in yeast fermentation and therefore named "cozymase" (Harden and Young, 1906). Cozymase was later classified as a nucleoside sugar phosphate by v. Euler and Myrbäck (1929). It took 30 years after its initial discovery until Otto Warburg established the major function of NAD^+ to be the transfer of hydrogens in redox reactions (Warburg et al., 1935; Warburg and Christian, 1936). At the same time, Conrad Elvehjem discovered the importance of dietary NAD^+ precursors in the management of "black tongue" in dogs, a disease equivalent to pellagra in humans (Elvehjem et al., 1937). Pellagra used to be a common problem in areas with predominantly corn-based diets and was generally managed according to Joseph Goldberger's recommendation to enrich the diet in wheat and animal products (Goldberger et al., 1926). Today we know that the proposed diet (in contrast to corn) is a rich source of essential NAD^+ precursors such as nicotinic acid (NA), nicotinamide (NAM) and nicotinamide riboside (NR), commonly known as niacin or vitamin B_3.

As a redox co-factor, NAD^+ participates in reactions of glycolysis, mitochondrial TCA cycle and OXPHOS, and is therefore essential to enable efficient performance of cellular energy metabolism (see Section 1.7.2). Moreover, NAD^+ also serves as a limiting substrate for NAD^+-consuming enzymes implicated in calcium signaling (cyclic ADP-ribose synthases), DNA repair (poly(ADP-ribose) polymerases) and post-transcriptional regulation of cellular energy metabolism (sirtuins) (see Section 1.7.3). While redox reactions constantly

regenerate NAD^+ from its reduced form NADH, NAD^+ is cleaved to NAM and ADP-ribose when used as a substrate. This process generates a constant demand for NAD^+ biosynthesis, which is met either through *de novo* generation from tryptophan (Trp) or by salvaging the vitamin B_3 precursors of NAD^+ (see Section 1.6). NAD^+ levels as well as expression of the key biosynthetic enzyme NAM phosphoribosyl transferase (NAMPT) underlie the circadian clock and, more importantly, feeding rhythms (Nakahata et al., 2009; Mauvoisin et al., 2017). NAD^+ levels are increased during situations of energy deficit, such as fasting or calorie/glucose restriction (Fulco et al., 2008; Cantó et al., 2010) as well as exercise (Cantó et al., 2009, 2010; Costford et al., 2010). In contrast, states of high energy supply, for instance during high fat or high sugar feeding, result in diminished NAD^+ content in liver, skeletal muscle or brown and white adipose tissue (Yoshino et al., 2011; Cantó et al., 2012; Gariani et al., 2016). Of note, these energetic states are also characterized by increased and decreased sirtuin activity, respectively. SIRT1 is regulated by AMP activated protein kinase (AMPK) and requires NAD^+ as a co-substrate to perform its deacetylase activity on protein targets that are involved in the regulation of mitochondrial biogenesis and transcription of OXPHOS-related genes, i.e. PGC1α (Fulco et al., 2008; Cantó et al., 2010; Kulkarni and Cantó, 2015). In this regard it is not surprising that conditions that are characterized by metabolic dysfunction such as obesity and type 2 diabetes coincide with low NAD^+ availability and benefit from SIRT1-activating treatments.

It is well established that NAD^+ levels are also compromised during aging in virtually all organs and in many species from worms to humans (Braidy et al., 2011; Yoshino et al., 2011; Massudi et al., 2012; Gomes et al., 2013; Mouchiroud et al., 2013). A paucity of NAD^+ was further observed in adult stem cells of brain, muscle and skin (Stein and Imai, 2014; Zhang et al., 2016). The systemic loss of NAD^+ was largely attributed to reduced NAD^+ synthesis due to an age-associated decrease in NAMPT expression (Yoshino et al., 2011; de Guia et al., 2019) in combination with increased NAD^+ consumption by NAD^+ consuming processes such as DNA repair or CD38 activity (Mouchiroud et al., 2013; Camacho-Pereira et al., 2016). This phenomenon was linked to a decay in sirtuin activity (particularly SIRT1 and SIRT3), which plays a major role in the development of age-related metabolic dysfunction and diseases.

Sirtuin activating compounds (STACs) have been widely explored, but their specificity remains a matter of debate (see Section 1.7.3). As NAD^+ is a limiting factor for the enzymatic functionality of sirtuins, increasing its availability in the cell is a valid approach. In this respect, it has been established that boosting NAD^+ via exogenous supplementation of NAD^+ precursors may be the most promising and safe strategy in the management of both metabolic diseases as well as age-associated pathologies. As a consequence, research of NAD^+ precursor supplementation in preclinical and clinical settings has been gaining momentum and the current achievements of this rapidly evolving field will be presented in Section 1.8.1.

1.6 Biosynthesis of NAD⁺ in mammals

The enzymes involved in NAD⁺ biosynthesis and salvage are evolutionary conserved from bacteria, yeast, invertebrates to mammals (Magni et al., 1999; Rongvaux et al., 2003; Bieganowski and Brenner, 2004). Mammalian cells produce NAD⁺ either *de novo* from L-tryptophan (Trp) or via salvaging pathways that

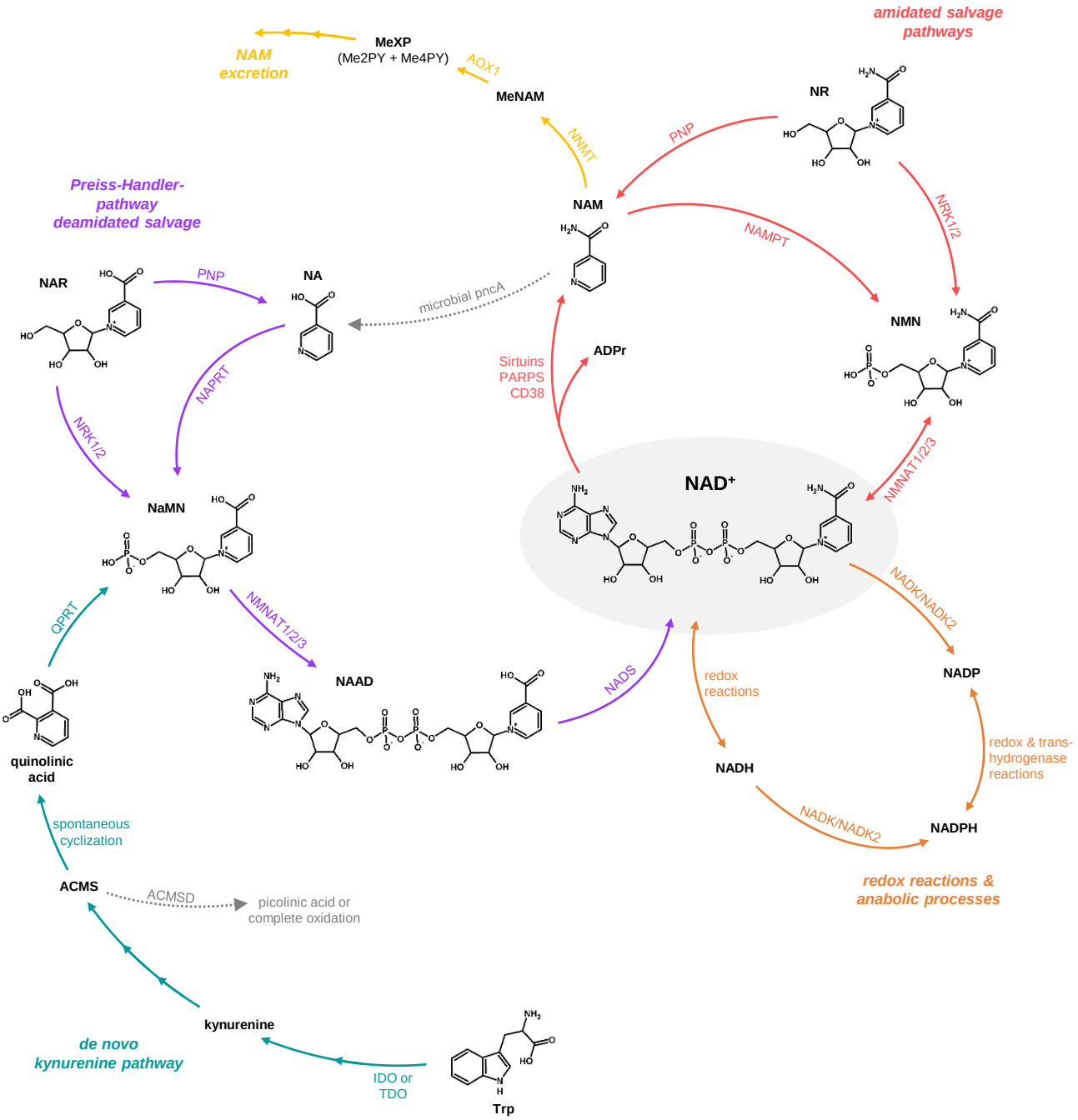

Figure 1.5: Mammalian biosynthesis and consumption of NAD⁺. NAD⁺ can be produced *de novo* from L-tryptophan or salvaged from vitamin B3 precursors NA, NAM and NR. NAD⁺ is reduced to NADH in redox reactions, phosphorylated to NADP⁺ to be used in anabolic processes, or consumed to NAM by NAD⁺ dependent enzymes. NAM can be salvaged back to NAD⁺ or methylated and oxidized to excretion products. *For abbreviations, see List of abbreviations p. xvii.*

utilize NAD^+ vitamin B_3 precursors or NAD^+ breakdown products that contain a pyridine ring (Figure 1.5). It is evident that different tissues have their preferred route of NAD^+ production. While only liver and kidney have been observed to synthesize NAD^+ *de novo*, other tissues greatly rely on salvage from NAM that is constitutively released by the liver (Liu et al., 2018). Generally, the biosynthetic pathways yielding into NAD^+ have been classified as 'deamidated' and 'amidated' (Mori et al., 2014). The former route comprises both *de novo* synthesis as well as NA salvage through the Preiss-Handler pathway, while the latter describes the NAM, NMN and NR utilizing route. The following sections provide an introduction to the biosynthetic pathways utilized by mammalian cells to produce NAD^+. Microbial NAD^+ synthesis and precursor salvage is by far more diverse (Gazzaniga et al., 2009) and may not be disregarded, especially in the context of a dietary supplementation regimen.

1.6.1 *De novo* pathway

De novo NAD^+ biosynthesis majorly takes place in the liver (Bender et al., 1982; Liu et al., 2018), where the essential amino acid Trp gets converted via the kynurenine pathway (Bender, 1983) (Figure 1.5). In a first rate-limiting step, Trp is catalytically converted to N-formylkynurenine either by tryptophan 2,3-dioxygenase (TDO) or by indoleamine 2,3-dioxygenase (IDO). The liver mainly relies on TDO which is induced by Trp itself as well as by glucocorticoids (Salter and Pogson, 1985; Comings et al., 1995). IDO, on the other hand, is rather expressed in extrahepatic tissues and activated by inflammatory stimuli (Konan and Taylor, 1996). This initial step is followed by a sequence of four enzymatic reactions that yield in α-amino-β-carboxymuconate-ε-semialdehyde (ACMS). ACMS can be further processed by ACMS decarboxylase (ACMSD), yielding in either picolinic acid or complete oxidization through the TCA cycle (Bender, 1983). However, at sufficient concentrations, ACMS can also undergo spontaneous cyclisation to form the NAD^+ precursor quinolinic acid (Ikeda et al., 1965). The ACMSD reaction serves as a gate-keeper for *de novo* NAD^+ synthesis, and it has been shown that pharmacological inhibition of ACMSD increases NAD^+ content (Katsyuba et al., 2018). In a second rate-limiting step, quinolate phosphoribosyl transferase (QPRT) converts quinolinic acid and 5-phospho-α-D-ribose 1-diphoshate (PRPP) to nicotinic acid mononucleotide (NaMN). Nicotinamide mononucleotide adenylyltransferases (NMNATs) subsequently catalyze the addition of the adenylyl moiety from ATP to form nicotinic acid adenine dinucleotide (NAAD) which is finally amidated to NAD^+ by glutamine-dependent NAD^+ synthetase (NADS) (Hara et al., 2003). NMNATs are the only enzymes common to all NAD^+ producing pathways, being able to transform both NaMN as well as NMN. NMNAT1 localizes to the nucleus, it is ubiquitously expressed and has been shown to be an essential enzyme in mice (Emanuelli et al., 2001; Conforti et al., 2011). NMNAT2 is primarily expressed in brain and localizes to the cytoplasm (including Golgi) (Raffaelli et al., 2002; Berger et al., 2005). Finally, NMNAT3 was detected in

cytosplasm and mitochondria (Zhang et al., 2003; Berger et al., 2005). Interestingly, loss of NMNAT3 does not impact mitochondrial NAD^+ levels in mice (Yamamoto et al., 2016).

1.6.2 Salvage pathways

Apart from liver and kidney, most mammalian tissues do not produce NAD^+ *de novo*, but rather salvage a variety of NAD^+ precursors obtained through the diet or NAD^+ consuming reactions.

Nicotinic acid

The deamidated salvage of NA was first described by Preiss and Handler (1958a; 1958b). This three-step pathway is initiated by nicotinic acid phosphoribosyltransferase (NAPRT), which produces NaMN from NA and PRPP (Figure 1.5). The following conversion of NAMN to NAAD and then NAD^+ overlaps with the *de novo* pathway (see Section 1.6.1). Interestingly, NAPRT activity is not affected by abundance of NAD^+, its regulation rather appears to be dependent on cellular energy homeostasis and ATP levels, with pyruvate activating and glycolytic metabolites inhibiting NAPRT activity (Hara et al., 2007; Galassi et al., 2012).

Nicotinamide

NAM plays a central role in the biosynthesis of NAD^+. The liver has a high turnover of NAD^+ and releases NAM into the bloodstream, thereby providing peripheral tissues with the salvageable NAD^+ precursor (Liu et al., 2018). Importantly, NAM is not only the main NAD^+ precursor utilized by the body, it also plays a major role in NAD^+ dependent reactions, e.g. as an inhibitor of sirtuins (see Section 1.7.3).
It takes two steps to produce NAD^+ from NAM (Figure 1.5). First, nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the condensation of NAM and PRPP to NMN (Rongvaux et al., 2002). Next, NMNATs convert NMN to NAD^+. In bacteria and yeast NAM can be hydrolyzed to NA by nicotinamidase *pncA* and *Pnc1*, respectively, and subsequently follow the Preiss-Handler pathway to form NAD^+ (Frothingham et al., 1996; Ghislain et al., 2002) (Figure 1.5). Even though mammalian cells do not express nicotinamidase, conversion of dietary NAM by intestinal microbiota is possible.
The essential nature of NAMPT activity for cellular metabolic fitness is becoming more and more evident. Intracellular NAMPT has been shown to be the rate-limiting enzyme in NAD^+ production (Revollo et al., 2004). Homozygous NAMPT knockout mice are embryonically lethal, and heterozygous mice appear to have lower NAD^+ levels in tissues that do not generate NAD^+ *de novo*, i.e. brown adipose tissue (BAT) (Revollo et al., 2007). But also liver tissue with active *de novo* pathway displays a 50% reduction in NAD^+ content in liver-specific NAMPT knockout mice (Dall et al., 2019). An even more dramatic 85% decline in muscular

NAD^+ was observed in muscle-specific NAMPT knockout mice, which exhibit significant fiber degeneration and impaired muscle function (Frederick et al., 2016). Decreased NAMPT expression and activity in human vascular smooth muscle cells was further connected to premature senescence (van der Veer et al., 2007). Interestingly, *mdx* mice, a model of Duchenne muscular dystrophy characterized by accelerated stem cell exhaustion, have reduced NAMPT expression, concurrent with increases in PARP activity and reduced muscle NAD^+ levels (Ryu et al., 2016). NAMPT expression is upregulated by states of high energy demand, such as fasting (Yang et al., 2007) and exercise (Koltai et al., 2010). Moreover, increased NAMPT expression leads to higher mitochondrial NAD^+ levels that are protective during genotoxic stress (Yang et al., 2007).
NAMPT is also excreted by cells and is then referred to as extracellular NAMPT (eNAMPT) (Revollo et al., 2007). eNAMPT was initially described as the cytokine pre-B cell colony-enhancing factor (PBEF) that pays a role in the immune response and maturation of B cell precursors (Samal et al., 1994). In another context, eNAMPT was described as the "visceral fat-derived hormone" visfatin and falsely attributed an insulin mimetic function (Fukuhara et al., 2005). Revollo et al. (2007) later identified eNAMPT/PBEF/visfatin as an essential factor for the regulation of β-cell function that is excreted by adipocytes, hepatocytes and cardiomyocytes. Very recently it has been reported that eNAMPT levels decline with age in mice and humans (Yoshida et al., 2019). More importantly, overexpression of NAMPT in adipose tissue led to increased circulating eNAMPT, improving health and life span in mice (Yoshida et al., 2019).

Nicotinamide riboside

Nicotinamide riboside (NR) was first discovered as a V-factor for growth in *Haemophilus influenzae* bacteria naturally deficient of the Preiss-Handler-pathway (Gingrich and Schlenk, 1944; Fleischmann et al., 1995). Bieganowski and Brenner (2004) established the highly conserved nature of NR kinases (NRKs) in fungi and humans and introduced NR as a nutrient found in milk.
NAD^+ synthesis from NR takes place in a two-step reaction: NRKs perform the first phosphorylation step to form NMN, which is then converted to NAD^+ by NMNATs (Figure 1.5).
NR has gained increasing attention since it was shown to promote lifespan in yeast, worms and mice (Belenky et al., 2007; Mouchiroud et al., 2013; Zhang et al., 2016). In addition to the NRK-dependent conversion of NR, yeast utilizes an alternative NR salvage pathway via Urh1/Pnp1/Meu1 nucleosidases that cleave NR to NAM, which is further converted by Pnc1 nicotinamidase to NA and then follows the Preiss-Handler pathway to form NAD^+ (Belenky et al., 2007). There is no known mammalian homolog to Pnc1. Nevertheless, NR may be converted to NAM by purine nucleoside phosphorylase (PNP) and subsequently yield in NAD^+ through NAMPT and NMN (Belenky et al., 2009) (Figure 1.5). Although the relevance of this pathway in mammals has not been investigated in depth, first studies report an NRK-independent conversion of exogenous NR in

NRK1/2 dKO mice, most likely to happen in plasma (Ratajczak et al., 2016).

Two NRK isoforms are known in mammals (Bieganowski and Brenner, 2004; Tempel et al., 2007). NRK1 and 2 (encoded by *Nmrk1* and *Nmrk2*, respectively) share 57% of sequence similarity (Bieganowski and Brenner, 2004). NRKs are redundant in their function of (exogenous) NR metabolization, and one isozyme may compensate the loss of the other in single KO studies (Fletcher et al., 2017). The ubiquitously expressed NRK1 was initially described as a tiazofurin kinase, enabling the first conversion step of this anti-cancer drug into a toxic NAD^+ analog (Cooney et al., 1983). J. Ratajczak (2017) demonstrated that NRK1-dependent utilization of endogenous NR is essential for the maintenance of metabolic homeostasis, as mice deficient of NRK1 exhibit altered glucose and lipid metabolism. Moreover, NRK1 is the rate-limiting enzyme for the efficient utilization of exogenous NR and NMN in hepatocytes (Ratajczak et al., 2016). Enzymatic activity measurements showed that NRK1 is highly active in kidney, liver and skeletal muscle, while other tissues (i.e. brain, heart, lung, spleen) display a comparatively low NRK1 activity (Zamporlini et al., 2014).

Muscle-specific NRK2 is a splice variant of muscle integrin binding protein (MIBP or ITGB1BP3) (Bieganowski and Brenner, 2004), which was originally described to regulate muscle cell differentiation and adhesion *in vitro* (Li et al., 1999, 2003). NRK2b is vital for zebrafish muscle development by regulating basal lamina assembly, laminin polymerization and paxillin concentration at the myotendinous junction (Goody et al., 2010). In mice, transcription of *Nmrk2* is strongly induced in response to ATP and NAD^+ depletion, presumably through an AMP-kinase and peroxisome proliferator-activated receptor α (PPARα)-dependent mechanism (Diguet et al., 2018). Up-regulation has been described in different pathophysiological settings, i.e. after traumatic lower limb muscle injury (Aguilar et al., 2015), during severe skeletal myopathy (after hexose-6-phosphate dehydrogenase (H6PDH) deletion) (Lavery et al., 2008) or in different models of cardiomyopathy (Xu et al., 2015; Diguet et al., 2018). Interestingly, despite its minimal expression levels in non-muscle tissues, transcriptional up-regulation of *Nmrk2* has also been observed in nerve cells after axonal damage (Sasaki et al., 2006). This highlights the relevance of endogenous NR in cellular responses to stress and energy depletion. Accordingly, results obtained in the context of this thesis suggest a role of NRKs during regenerative processes in skeletal muscle (see Chapter 4).

1.7 NAD^+ as a substrate and co-factor

1.7.1 Subcellular distribution of NAD^+ or "Where the cell is NAD^+?"

NAD^+ is present in all subcellular compartments. Importantly, all human NAD^+ biosynthetic enzymes (apart from mitochondrial NMNAT3) have been localized to cytoplasm or nucleus (Nikiforov et al., 2011). It is

widely accepted that NAD^+ can move freely between those two compartments, where it serves as a substrate for NAD^+ dependent enzymes (see Section 1.7.3), and also as a vital co-factor during glycolysis (see Section 1.7.2). The post-translational modifications performed by NAD^+ consuming enzymes play an important role in the regulation of cellular signaling and gene expression (Nikiforov et al., 2015). Particularly important in muscle is the regulation of calcium flux across membranes by cytosolic NAD^+ metabolites like ADPr, cyclic ADPr or O-acetyl-ADPr (Fliegert et al., 2007; Nikiforov et al., 2015).

The perhaps most important pool of NAD^+ is present in mitochondria, as they require NAD^+ and NADH as redox co-factors in oxidative metabolism (see Section 1.7.2). Accordingly, mitochondria maintain a rather distinct NAD^+ pool (Dall et al., 2019), which is even temporarily resistant to cellular NAD^+ depletion, i.e. by the NAMPT inhibitor FK866 (Pittelli et al., 2011). It was estimated that mitochondria obtain the highest absolute NAD^+ levels in the cell, with a high proportion of it present in the form of NADH (Sauve, 2008; Li et al., 2009). Therefore, the mitochondrial NAD^+/NADH ratio remains very low in comparison to the cytoplasm (<10 and 60-700, respectively) (Williamson et al., 1967; Li et al., 2009). It is evident that the inner mitochondrial membrane is impermeable for NAD^+ (Stein and Imai, 2012). Mammals appear to synthesize NAD^+ directly inside mitochondria, as there are no known mammalian homologs for mitochondrial NAD^+ (or NADH) transporters (in contrast to Ndt1and Ndt2 in yeast) (Todisco et al., 2006; Yang et al., 2007). However, little is known about mitochondrial NAD^+ generation, and NMNAT3 is the only NAD^+ producing enzyme reported to be localized in mitochondria to date (Raffaelli et al., 2002; Berger et al., 2005). Accordingly, its substrate NMN has been shown to be a cytoplasmic precursor of mitochondrial NAD^+ (Nikiforov et al., 2011), but mitochondrial importers for NMN remain to be identified. Similarly, cytoplasmic NAD^+ has been shown to maintain mitochondrial NAD^+ levels in HeLa cells (independent of NMN), albeit a mammalian homolog of a mitochondrial NAD^+ transporter has not yet been found (Cambronne et al., 2016).

NAD^+ was also detected in other subcellular compartments such as peroxisomes, endoplasmic reticulum (ER), and Golgi complex (Dölle et al., 2013). While it is known that peroxisomes utilize NAD^+ during β-oxidation and recycle the resulting NADH in redox reactions, a biological role for NAD^+ in ER and Golgi complex has not yet been elucidated (Nikiforov et al., 2015).

Finally, the role of extracellular NAD^+ is gaining increasing attention (Nikiforov et al., 2011). Albeit generally low, elevated extracellular NAD^+ can be the result of leakage after cell damage as well as active release (Zolkiewska, 2005). For instance, NAD^+ release during early inflammatory response in blood plasma was proposed to facilitate T cell expansion (Adriouch et al., 2007). Furthermore, NAD^+ was even described as a novel neurotransmitter and neuromodulator in smooth muscle (Breen et al., 2006; Mutafova-Yambolieva et al., 2007). Based on observations during muscle development and myocyte adhesion, an additional pool of membrane-proximal (intra- and extracellular) NAD^+ has been proposed for skeletal muscle (Goody and

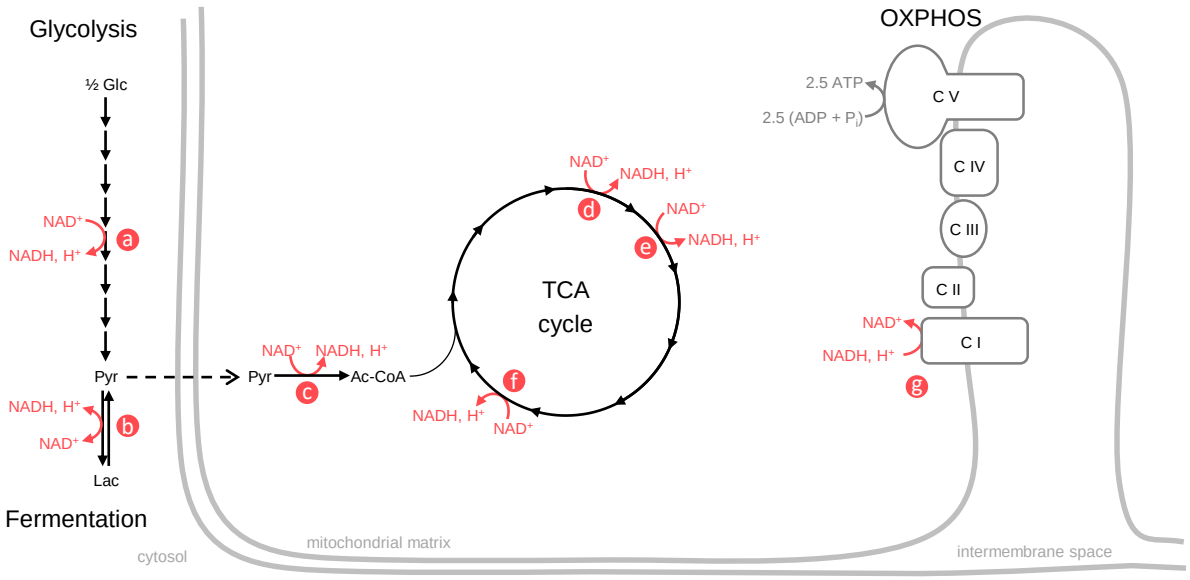

Figure 1.6: NAD$^+$ in redox reactions of glycolysis, TCA cycle and OXPHOS. (a) Glyceraldehyde-3-phosphate (G3P) to 1,3-bisphosphoglycerate by G3P dehydrogenase. **(b)** Pyruvate (Pyr) to lactate (Lac) fermentation by Lac dehydrogenase. **(c)** Pyr decarboxylation to acetyl-CoA (Ac-CoA) by Pyr dehydrogenase. **(d)** Isocitrate oxidation to oxalosuccinate by isocitrate dehydrogenase. **(e)** Oxidative decarboxylation of α-ketoglutarate to succinyl-CoA by α-ketoglutarate dehydrogenase. **(f)** Oxidation of malate to oxaloacetate by malate dehydrogenase. **(g)** Oxidation of NADH to NAD$^+$ by ECT complex I (C I). For a more detailed description of ECT and OXPHOS, see Section 1.3 and Figure 1.4.

Henry, 2018). This NAD$^+$ may be generated by membrane-located NRK2, which has been observed to be bound to the cytosolic tail of integrin α7β1 (Li et al., 2003). Extracellular NAD$^+$ (e.g. after muscle damage) may then be utilized by ecto-enzymes such as mono-ADPr transferases (ARTs) that mediate muscle cell adhesion via ADPr transfer onto integrin α7β1 (Zhao et al., 2005; Zolkiewska, 2005; Goody et al., 2010).

1.7.2 NAD$^+$ in redox reactions

The oldest known function of NAD$^+$ is that of a redox co-factor (Warburg et al., 1935). NAD$^+$ and NADH are central to cellular energy metabolism and essential for dehydrogenase or oxidoreductase reactions of glycolysis, TCA cycle and mitochondrial OXPHOS (Krebs and Veech, 1969) (Figure 1.6). In the first phase of glycolysis, one molecule of glucose is converted into two molecules glyceraldehyde-3-phosphate (G3P). G3P dehydrogenase (GAPDH) catalyzes the first NAD$^+$-dependent reaction, yielding NADH and forming 1,3-bisphosphoglycerate (Figure 1.6a). Pyruvate is the final product of glycolysis. Under anaerobic conditions (e.g. in strained muscles exhausted of oxygen) NAD$^+$ is regenerated by oxidation of NADH during the conversion of pyruvate to lactate by lactate dehydrogenase (Figure 1.6b). However, lactate fermentation can only be supported for short, intensive time periods of high energy demands. A more efficient utilization of glucose is achieved during aerobic conditions by decarboxylation of pyruvate to acetyl-CoA by pyruvate dehydrogenase

in mitochondria, again reducing NAD^+ to NADH (Figure 1.6c). Acetyl-CoA may then enter the TCA cycle that functions to provide important precursors of certain amino acids and to generate NADH from NAD^+ (reviewed in Akram (2014)). Three out of the ten reactions in the TCA require the reduction of NAD^+ (Figure 1.6d–f): Isocitrate oxidation to oxalosuccinate by isocitrate dehydrogenase, oxidative decarboxylation of α-ketoglutarate to succinyl-CoA by α-ketoglutarate dehydrogenase, and oxidation of malate to oxaloacetate by malate dehydrogenase (Stryer et al., 2002). The complete oxidation of one molecule of glucose thus leads to the generation of ten molecules of NADH. Mitochondrial NADH is used by complex I (NADH dehydrogenase or NADH/coenzyme Q oxidoreductase) to feed electrons into the ETC (Sazanov, 2015) (Figure 1.6g). Electron flux through complexes I/II to V enables proton pumping across the inner mitochondrial membrane that generates a proton gradient necessary for the synthesis of ATP by complex V (ATP-synthase) (see also Section 1.3 and Figure 1.4).

While NAD^+/NADH participate in catabolic reactions during energy production, their phosphorylated analogues $NADP^+$/NADPH act as redox equivalents in anabolic reactions. $NADP^+$ is produced from NAD^+ by NAD^+ kinase (NADK) (Figure 1.7a), which accounts for approximately 10% of total NAD^+ consumption in proliferating cells *in vitro* (Liu et al., 2018). Transhydrogenase activity of different enzymes leads to rapid conversion to NADPH. In contrast to NAD^+/NADH, intracellular levels of $NADP^+$/NADPH are comparably low and their ratio is maintained in favor of the reduced form. NADPH is involved in nucleotide synthesis in the pentose phosphate pathway (Kruger and von Schaewen, 2003) and an important co-factor in fatty acid and steroid synthesis (Ying, 2008). An additional role of NADPH is the detoxification of oxidative stress through reduction of glutathione, thioredoxin and peroxiredoxins (Tribble and Jones, 1990; Pandey and Flück, 2013).

1.7.3 NAD^+ as a consumed substrate

In more recent years, the importance of NAD^+ has been characterized beyond its classical role in redox reactions. Three main classes of NAD^+-consuming enzymes have been described: sirtuins (SIRTs), poly(ADP-ribose) polymerases (PARPs) and cyclic ADP-ribose synthases (Figure 1.7). As a co-substrate, NAD^+ is cleaved at the glycosidic bond, releasing NAM and ADP-ribose (ADPr) (Houtkooper et al., 2010).

Sirtuins

Sirtuins were described as deacetylases that consume NAD^+ to remove acetyl groups from lysine residues of histones and other proteins. Their enzymatic activity transfers those acetyl groups onto the ADPr moiety of NAD^+, releasing O-acetyl-ADPr, NAM, and the deacetylated protein (Sauve et al., 2006) (Figure 1.7b).

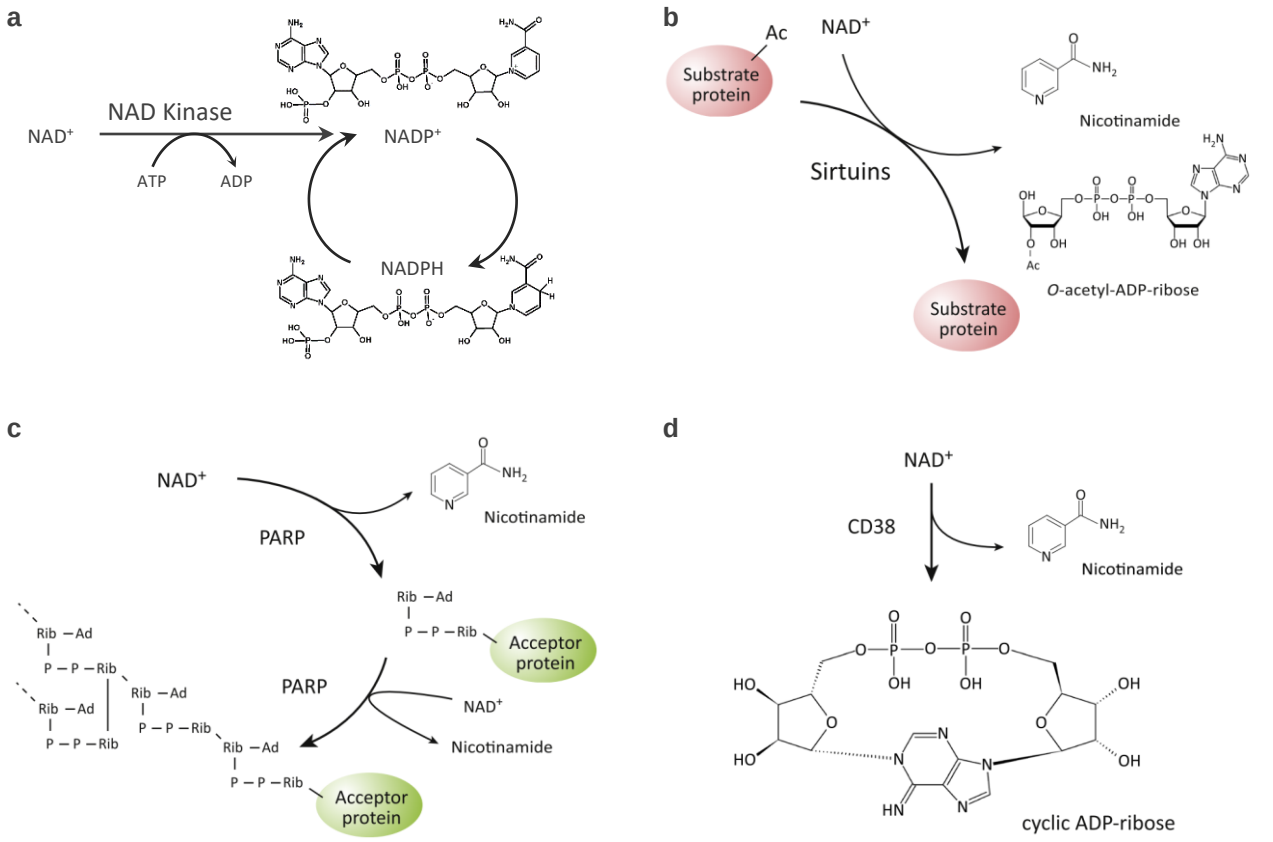

Figure 1.7: NAD$^+$ as a consumed substrate. **(a)** Formation of NADP$^+$ by NAD kinase removes NAD$^+$ from the cellular pool. **(b)** Sirtuins transfer acetyl moieties from substrate proteins onto the ADPr moiety of NAD$^+$, yielding O-acetyl-ADPr, NAM and the deacetylated substrate protein. **(c)** Repeated transfer of ADPr by PARPs leads to modification of acceptor proteins with long chains of ADPr homopolymers. **(d)** Cyclic ADP-ribose synthases like CD38 cleave NAD$^+$ and catalyze the cyclization of ADPr, yielding into cADPr and NAM. Adapted with permission from Imai and Guarente (2014).

Mammalian sirtuins are orthologues of the popular yeast silent information regulator Sir2, which promotes longevity in *Saccharomyces cerevisiae* through transcriptional silencing (Kaeberlein et al., 1999) and was proposed to be the key mediator of calorie-restriction-induced life extension in yeast (Howitz et al., 2003), worms (Tissenbaum and Guarente, 2001) and flies (Rogina and Helfand, 2004). Seven mammalian sirtuin homologs have been described: SIRT1–7 localize to distinct subcellular compartments and albeit sharing a conserved catalytic core, they display specialized enzyme activities, such as deacetylation (SIRT1, 2, 3, 5), mono-ADP-ribosyl transferase activity (SIRT2, 3, 4, 6) and removal of succinyl and malonyl groups (SIRT5) (Houtkooper et al., 2010; Anderson et al., 2014). Nuclear and cytoplasmic SIRT1 is the best studied sirtuin. No less important for cellular function are the mitochondrially localized SIRTs 3, 4, and 5, key regulators of mitochondrial metabolism (Dölle et al., 2013). The following paragraph provides a brief overview on SIRT1. For a more detailed description of sirtuin biology and the less extensively studied SIRTs 2–7, the reader is referred to excellent reviews from the recent years, i.e. Dölle et al. (2013); Imai and Guarente (2014); van de

Ven et al. (2017); Fang et al. (2019).

SIRT1 shuttles between cytoplasm and nucleus and acts as a transcriptional regulator through its deacetylase activity (Tanno et al., 2007). Deacetylation of histones H1, H3 and H4 leads to chromatin condensation and gene silencing (Toiber et al., 2011). Moreover, SIRT1 targets a wide range of signaling proteins and transcription factors (Gomes et al., 2016), such as PGC1α, the FoxO family, NFκB, p53, and MyoD (reviewed by Feige and Auwerx (2008)). SIRT1 is regarded as an energy sensor as it is activated during energetic stresses induced by fasting, caloric restriction and exercise (Rodgers et al., 2005; Fulco et al., 2008; Cantó et al., 2010), all of which are also characterized by increased NAD^+ levels. Due to its prominent role in metabolic regulation, SIRT1 is the most studied of the mammalian sirtuins (reviewed in Boutant and Cantó (2014)). In the liver, SIRT1 activation was shown to improve oxidative metabolism, cholesterol homeostasis and lipid catabolism, and protect against oxidative stress. Similarly, SIRT1 action in skeletal muscle leads to enhanced mitochondrial biogenesis, lipid oxidation and insulin signaling. A growing body of evidence thus highlights SIRT1 activation as a potent therapeutic approach in the fight against diet and age-related metabolic disorders (reviewed in Lavu et al. (2008); Boutant and Cantó (2014); Strycharz et al. (2018)).

The search for sirtuin activating compounds (STACs) identified a multitude of candidates, with the most prominent being resveratrol or quercetin (Howitz et al., 2003). Also synthetic compounds, such as imidazothiazole SRT1720, have been developed (Milne et al., 2007). However, the specificity of STACs is rather ambiguous as their mode of action appears to rely mainly on indirect mechanisms, e.g. through regulation by AMPK (Fulco et al., 2008; Cantó et al., 2009; Kulkarni and Cantó, 2015). Due to the strong dependency of sirtuins on NAD^+ availability, modulation of intracellular NAD^+ levels is a promising approach. Accordingly, inhibition of PARPs, sirtuins' main competitors for NAD^+, has shown to increase NAD^+ availability and sirtuin activity (Bai et al., 2011; Pirinen et al., 2014). Alternatively, increasing NAD^+ biosynthesis through precursor supplementation showed great potential in activating sirtuins (Cantó et al., 2012; Cerutti et al., 2014; Ryu et al., 2016) (see also Section 1.8).

PARPs

Poly(ADP-ribose) polymerases (PARPs) are best known for their important role in DNA damage repair. Homology searches and *in silico* characterization proposed the expression of up to eighteen putative PARP family members in humans (Amé et al., 2004; Otto et al., 2005). PARPs1 and 2 are the best studied and have been described to cleave NAD^+ and transfer its ADPr moiety onto nuclear proteins (Hassa et al., 2006) (Figure 1.7c). The resulting generation of long chains of ADPr homopolymers makes PARP1/2 one of the main consumers of cellular NAD^+, accounting for about one third of the total NAD^+ turnover under basal conditions (Liu et al., 2018). PARPs are activated by DNA strand breaks and by HSP70 after heat stress (Cantó

et al., 2015). Moreover, PARPs were also found to be activated through phosphorylation by ERK1/2 (which in turn is activated by oxidative stress) (Kauppinen et al., 2006). In times of overt DNA damage, PARPs become the dominant NAD^+ consumer in the cell, depleting up to 60–80% of cellular NAD^+ (Skidmore et al., 1979; Pillai et al., 2005). High PARP activation is thus limiting NAD^+ availability for other metabolic processes, e.g. leading to sirtuin inhibition (Pillai et al., 2005; Bai et al., 2011). In addition to their more "classical" role, PARPs can act as transcriptional co-activators and as such have been implicated in inflammation and aging (Altmeyer and Hottiger, 2009), as *Parp1* KO mice were protected from inflammatory and neurodegenerative disorders (Shall and de Murcia, 2000; Hassa et al., 2006). Finally, PARP1 has been reported to inhibit the rate-limiting glycolytic enzyme hexokinase, which may lead to energetic collapse and mitochondrial defects (Andrabi et al., 2014).

Cyclic ADP-ribose synthases

Cyclic ADP-ribose synthases utilize NAD^+ (and $NADP^+$) for the generation of second messenger molecules that are implied in cellular calcium mobilization (Guse, 2015). The most prominent members of this group are the plasma membrane proteins CD38 and CD157. These ectoenzymes cleave NAD^+ and release ADPr or further catalyze the cyclization of the ADPr moiety to form cyclic ADPr (cADPr), consuming a vast amount of NAD^+ in the process (Houtkooper et al., 2010) (Figure 1.7d). Another product of cADPr synthases is the second messenger nicotinic acid adenine dinucleotide phosphate (NAADP), that is generated from NADP by base exchange of the NAM ring with NA (Dölle et al., 2013). The regulation of intracellular calcium by CD38 regulates cell activation and proliferation, muscle contraction, immune responses and hormone secretion (Malavasi et al., 2008). However, it is assumed that CD38 is one of the main NAD^+ consumers in tissue, and elevated CD38 levels during aging were connected to NAD^+ depletion in liver of aged mice (Camacho-Pereira et al., 2016). Accordingly, CD38 deficient mice exhibit dramatically increased NAD^+, up to 30-fold compared to wild-type mice, along with enhanced SIRT1 activity (Aksoy et al., 2006; Barbosa et al., 2007).

1.8 The importance of NAD^+ precursors in health and disease

We naturally obtain NAD^+ precursors from our diet. The essential nature of NA, NAM and NR is emphasized by their general denotation as vitamin B_3. While it is commonly accepted that supplementation of all those precursors leads to induction of NAD^+ (albeit with different efficiencies), and decreased levels impair NAD^+ generation, their distinct roles at physiological concentrations is only starting to be understood.
Albeit most tissues do not produce NAD^+ *de novo*, the essential amino acid Trp has been reported to be the preferred substrate for NAD^+ biosynthesis in the liver (Bender et al., 1982; Liu et al., 2018). However,

Trp has different fates in the cell, i.e. as an important building block for proteins or as a precursor to neurotransmitters, such as serotonin and melatonin (Oxenkrug, 2010). Moreover, oxidation of ACMS, an intermediate of the NAD+ *de novo* pathway yields in Acetyl-CoA which can be used in the TCA cycle. Therefore it is not surprising that Trp is a rather poor NAD+ precursor and only produces NAD+ at high concentrations that exceed the enzymatic activity of ACMSD (see also Section 1.6.1).

De novo NAD+ production in the liver is of major importance, as liver constantly releases NAM, which is the primary NAD+ precursor in most peripheral organs (Liu et al., 2018). However, NAM is also the product of NAD+ consuming reactions and as such inhibits NAD+ cleaving enzymes, i.e. sirtuins (Bitterman et al., 2002; Avalos et al., 2005). NAM can be salvaged back to NAD+, but may also be oxidized and excreted. First studies are now reporting a physiological relevance of enzymes involved in NAM excretion such as Nicotinamide N-methyltransferase (NNMT, methylates NAM to MeNAM) and aldehyde oxidase (AOX1, oxidizes MeNAM to Me2PY and Me4PY) (Figure 1.5). NNMT was observed to be upregulated during aging, leading to decreased NAM salvage (Giresi et al., 2005; Neelakantan et al., 2019). In contrast, higher levels of MeNAM might also be beneficial, as AOX1 activity involves the generation of reactive oxygen species which in moderate levels is believed to promote longevity and metabolic health through mitohormesis (Ristow and Zarse, 2010; Schmeisser et al., 2013).

As explained above (Section 1.7.1), mitochondria supposedly possess their own NAD+ producing mechanisms that strongly rely on cytoplasmic NMN and NAD+ (Nikiforov et al., 2011; Cambronne et al., 2016). The recent discovery of a specific NMN transporter in intestinal cells suggests a tissue-specific preference for NMN as NAD+ precursor (Grozio et al., 2019). Moreover, NMN as a product of eNAMPT was shown to be a vital NAD+ precursor in the context of insulin secretion from the pancreas (Caton et al., 2011; Yoshida et al., 2019). Finally, extracellular NMN gets converted to NR (Ratajczak et al., 2016) and may also serve as a precursor to NR through activity of cytosolic 5'-nucleotidases (Kulikova et al., 2015). NR is a rather new NAD+ precursor in mammals, initially described by Bieganowski and Brenner (2004). Since then, it has been established that NR is a powerful, if not the most potent, exogenous NAD+ precursor (as discussed below). However, its role during homeostasis may be underappreciated. In healthy conditions, NA and NAM salvage appears sufficient to provide enough NAD+ for normal NAD+ metabolism and lack of NRKs can be efficiently compensated (Ratajczak et al., 2016; Fletcher et al., 2017). Interestingly, the effect of calorie restriction in yeast has been shown to be dependent on proper incorporation of endogenous NR (Lu et al., 2009). Likewise, metabolic challenges, disease states or aging demand for increased NAD+ turnover, and in muscle it has become evident that in those conditions the activity of NRK1 and NRK2 become essential, highlighting a distinct role of endogenous NR (Deloux et al. (2018) and own data, see Chapter 4). In line with this, human cells were found to release endogenous NR which can in turn serve as alternative NAD+ precursor for surrounding cells

(Kulikova et al., 2015).

1.8.1 Therapeutic strategies using NAD^+ precursors to boost NAD^+

As discussed above, NAD^+ and mitochondrial function are compromised in many metabolic disorders as well as during (pathological) aging (Sections 1.3 and 1.5). Exercise and calorie restriction are known for their beneficial effects on metabolic health and increasing NAD^+ and sirtuin activity through up-regulation of NAMPT (Costford et al., 2010; Smith et al., 2018; de Guia et al., 2019). Obviously, the applicability of such interventions may be limited by the severity of the condition or other factors. Therefore, different therapeutic strategies are being explored in an approach to increase cellular NAD^+ and sirtuin activity. First, reduction of enzymatic competition for NAD^+ by inhibition of the major NAD^+ consuming enzymes PARPs and CD38 (Bai et al., 2011; Haffner et al., 2015). Albeit potently increasing NAD^+ levels, the efficacy of this approach is under debate as inhibition of vital cellular processes such as DNA repair and calcium signaling might also have adverse implications. Second, stimulation of NAD^+ biosynthesis through overexpression of NAD^+ producing enzymes or by using specific activators. However, knowledge of allosteric activators of NAD^+ biosynthesis enzymes is scarce. Finally, increasing NAD^+ synthesis by providing exogenous precursors. While this is the most promising approach so far, more detailed knowledge about efficacy and metabolization in humans is still needed.
The following sections review the considerable body of evidence that has been published corroborating the conception that dietary supplementation of NAD^+ precursors is a valid approach to improve metabolic health.

NA & NAM

NA has a long history in the successful treatment of dyslipidemias and is one of the most effective approaches to ameliorate cardiovascular risk factors (Crouse, 1996). NA supplementation leads to reduced levels of circulating cholesterol and lipid deposition, and is even more effective when used in combination with statins and bile acid treatments (Altschul et al., 1955; Parsons and Flinn, 1959; Crouse, 1996). Orally administered NA and NAM both lead to increased NAD^+ levels in liver, blood, kidney and heart (Collins and Chaykin, 1972; Jackson et al., 1995), with NA being the superior NAD^+ precursor in comparison to NAM in these studies. However, the use of NA is limited by different important factors: NA has been observed to induce insulin resistance and elevate blood glucose levels, making it challenging to be used in individuals that already exhibit compromised glycemic control (i.e. type 2 diabetes) (Poynten et al., 2003). Moreover, 96 weeks of NA treatment was well tolerated in a large cohort of participants (Capuzzi et al., 1998). Unfortunately, NA

treatment was accompanied by a painful flushing response (Capuzzi et al., 1998), since it acts as an agonist of the G-protein-coupled receptor GPR109A (Wise et al., 2003).
Chronic NAM improved health span in mice and ameliorated glucose homeostasis in a high-fat diet study (Mitchell et al., 2018). NAM has been further shown to protect from axon degeneration (Wang et al., 2005). Nevertheless, the sirtuin-inhibiting properties of NAM limit its use as exogenous NAD^+ precursor (Avalos et al., 2005). Another important aspect to be considered is that certain types of cancers have been shown to predominantly rely on NAM salvage (Hasmann and Schemainda, 2003).

NMN & NR

Due to their independence of the rate-limiting NAMPT reaction, NR and NMN are powerful NAD^+ precursors *in vitro* and *in vivo*. Especially NR has received a lot of attention and has shown beneficial effects in a number of rodent disease models, and lately also in human clinical trials (see Table 1.1). It has been established that the route of administration of these precursors is critical for their assimilation into different tissues and their potential to boost NAD^+ (Frederick et al., 2016; Liu et al., 2018). For instance, intravenous administration delivered intact NR and NMN to muscle, kidney, and liver, while uptake after oral administration was mainly observed in the form of NAM (Liu et al., 2018).
NMN was reported to increase healthspan and promote physical activity in mice (Mills et al., 2016). Long-term (12 months) administration of NMN prevented age-associated gene expression changes and body weight gain, and improved age-related impairments of energy metabolism, insulin sensitivity and immune function (Mills et al., 2016). Similarly, NMN reduced glucose intolerance and enhanced hepatic insulin sensitivity in mouse models of type 2 diabetes induced by either high-fat diet or aging (Yoshino et al., 2011). The anti-diabetic effect of NMN may be attributed to the prominent role of eNAMPT in stimulating insulin secretion from the pancreas (Revollo et al., 2007). Depressed circulating levels of eNAMPT were observed during aging in mice and humans (Yoshida et al., 2019), and were also connected to inflammation and impaired β-cell function in a mouse model of fructose-rich diet-induced islet dysfunction (Caton et al., 2011). Supplementation of NMN lowered expression of pro-inflammatory cytokines and restored insulin secretion in a SIRT1 dependent manner (Caton et al., 2011).
While NMN serves as a substrate for circulating eNAMPT to stimulate β-cells, the effect of exogenous NMN in other organs appears to be dependent on its extracellular conversion to NR (Nikiforov et al., 2011; Ratajczak et al., 2016). Albeit the direct assimilation of exogenous NR into different tissues might be dependent on the route of administration (Frederick et al., 2016; Liu et al., 2018), it has been established that dietary supplementation increases tissue NAD^+ in a number of organs (Cantó et al., 2012; Liu et al., 2018). Co-supplementation with NR was protective in the context of diet-induced obesity (Cantó et al., 2012), diet-

Table 1.1: Therapeutic potential of NR supplementation. Selected preclinical studies reporting improved outcomes after treatment with NR and first clinical trials in humans. EtOH: ethanol, HFD: high-fat diet, HFHS: high-fat high-sugar, i.p.: intra-peritoneal, KI: knockin, KO: knockout, HKO: heart-specific KO, MKO: muscle-specific KO, LPS: lipopolysaccharides, NAFLD: non-alcoholoc fatty liver disease, SCs: stem cells, WT: wild type

Tissue	Model	Treatment	Reference
multiple	HFD-induced obesity (WT mice)	16 wks NR in HFD	Canto et al., Cell Metab, 2012
multiple	sepsis (mice injected with LPS)	single dose NR i.p.	Hong et al., Fr Rad Biol Med, 2018
brain	Alzheimer's disease (Tg2576 mice)	12 wks NR diet	Gong et al., Neurobiol. Aging, 2013
brain	Alzheimer's disease (APP/PSEN1 AD mice)	10 wks NR diet	Sorrentino et al., Nature, 2017
brain/neurons	noise-induced hearing loss (WT mice)	7-19 days NR i.p.	Brown et al., Cell Metab, 2014
heart	cardiomyopathy ($Trf1^{HKO}$ mice)	<15 days NR i.p.	Xu et al., Cell Rep, 2015
heart	dilated cardiomyopathy (SRF^{HKO} mice)	50 days NR diet	Diguet et al., Circulation, 2018
heart	doxorubicin-induced cardiotoxicity (WT mice)	single dose NR i.p.	Zhen et al., Clin Sci, 2019
liver	NAFLD (HFHS diet & $ApoE^{-/-}$ mice)	NR in HFHS diet	Gariani et al., Hepatology, 2016
liver	EtOH-induced liver injuries (WT mice)	16 days NR in EtOH diet	Wang et al., Redox Biol, 2018
liver	CCl_4 induced liver fibrosis (WT mice)	NR gavage	Jiang et al., Life Sci, 2019
muscle/brain	mitochondrial disease ($Sco2^{KO/KI}$ mice)	4 wks NR diet	Cerutti et al., Cell Metab, 2014
muscle	mitochondrial myopathy (Deletor mice)	16 wks NR diet	Khan et al., EMBO Mol Med, 2014
muscle/adult SCs	aging (WT mice)	6 wks NR diet	Zhang et al., Science, 2016
muscle	muscular dystrophy (mdx mice)	6 wks NR diet	Zhang et al., Science, 2016
muscle	NAD^+ deficiency ($Nampt^{MKO}$ mice)	6 wks NR water	Frederick et al., Cell Metab, 2016
muscle	muscular dystrophy (mdx mice)	12 wks NR diet	Ryu et al., Sci Transl Med, 2016
whole body	obesity & insulin resistance (humans)	12 wks oral NR	Dollerup et al., Am J Clin Nutr, 2018
whole body	humans	6 wks oral NR	Martens et al., Nat Comm, 2018
whole body	exercise & aging (humans)	single dose oral NR	Dolopikou et al., Eur J Nutr, 2019
muscle	humans	3 wks oral NR	Elhassan et al., Cell Rep, 2019

induced fatty liver disease (Gariani et al., 2016), as well as alcohol-induced liver injuries (Wang et al., 2018). In the heart, NR ameliorated the phenotype of transgenic mouse models of cardiomyopathy (Xu et al., 2015; Diguet et al., 2018) and prevented doxorubicin-induced cardiotoxicity (Zheng et al., 2019). NR has further shown promise in mouse models of Alzheimer's disease, where it restored cognition and prevented β-amyloid aggregation (Gong et al., 2013; Sorrentino et al., 2017). In muscle, enhanced tissue function and regeneration related to increased mitochondrial mass and oxidative metabolism in NR supplemented mice was observed in a wide range of disease models, i.e. mitochondrial disease (Cerutti et al., 2014), mitochondrial myopathy (Khan et al., 2014), muscular dystrophy and muscle regeneration (Ryu et al., 2016; Zhang et al., 2016), or severe NAD^+ deficiency (Frederick et al., 2016).

The beneficial effect of elevated NAD^+ levels has predominantly been attributed to enhanced SIRT1 activity, which mediates mitochondrial biogenesis and oxidative capacity in a PGC1α-dependent fashion Cantó et al. (2012); Gong et al. (2013); Wang et al. (2018). In turn, activation of mitochondrial SIRT3 was essential to prevent noise-induced hearing loss (Brown et al., 2014). Other studies reported that NR administration increased mitochondrial clearance through stimulation of autophagic/mitophagic flux and activation of the mitochondrial unfolded protein response (UPR^{mt}) (Khan et al., 2014; Gariani et al., 2016; Zhang et al., 2016; Sorrentino et al., 2017; Zheng et al., 2019). A comparable mechanism has been described for adult stem cells

in muscle, brain and skin of aged mice which displayed attenuated senescence and a rejuvenated phenotype after NR enriched diet (Zhang et al., 2016). Accordingly, aged mice treated with NR exhibited improved regenerative capacity of muscle and displayed increased lifespan (Zhang et al., 2016). Interestingly, NR did not elicit a therapeutic effect in mice with respiratory chain complex III deficiency, which was attributed to NAD^+-independent sirtuin activation caused by severe energy depletion (Purhonen et al., 2018).
These preclinical results highlight that NR has the potential to ameliorate metabolic disorders that are characterized by compromised sirtuin function and energy metabolism. Promising results in models of age-related pathologies encourage the exploration of NR as a preventative treatment in the context of aging.

NR in clinical studies

NR can be obtained as an over-the-counter supplement and has gained popularity as a "nutraceutical", supposedly promoting health and exercise performance among other benefits. Despite its widespread use as a dietary supplement and glorification in the media, it was only recently that NR —or its commercialized version *Niagen* (Chromadex)— has been tested and declared safe in humans (Trammell et al., 2016; Airhart et al., 2017; Dellinger et al., 2017; Martens et al., 2018; Conze et al., 2019). Currently, a number of clinical trials are underway investigating the efficacy of NR in the context of different pathological conditions (NCT02300740 (pharmacokinetics), NCT03151707 (redox homeostasis and bioenergetics), NCT02835664 (obesity and insulin resistance), NCT02812238 (immunity), NCT03432871 (mitochondrial diseases), NCT02942888 (mild cognitive impairment) and more still in the recruitment phase, see *ClinicalTrials.gov*; also reviewed in Connell et al. (2019)). A first published clinical trial with obese, insulin-resistant men showed that twelve weeks of dietary NR did not lead to adverse effects, but did also not improve investigated parameters such as insulin sensitivity, whole body glucose metabolism or resting energy expenditure (Dollerup et al., 2018, 2019). In contrast, a first study testing NR in old individuals reported positive effects on exercise performance and redox homeostasis by increasing blood NADH levels and lowering oxidative stress (Dolopikou et al., 2019). Interestingly, NR did not exert any improvements of investigated parameters in young participants, suggesting that the beneficial effect of NR is limited to individuals that already present compromised baseline NAD^+/NADH levels and elevated oxidative stress (Dolopikou et al., 2019). The most recently published clinical data show that three weeks of dietary NR supplementation of healthy aged subjects induced down-regulation of mitochondrial pathways in skeletal muscle but reduced circulating levels of inflammatory cytokines (Elhassan et al., 2019b). These first results show promise for the use of NR as a therapeutic agent, but also highlight that the investigative time frames used in animal models may simply be too short to make conclusive assumptions when applied to humans. Therefore, clinical studies investigating long-term safety and efficacy are highly anticipated.

1.9 General goals of this thesis

Sarcopenia is becoming an increasing health care burden in our aging society. Many studies have characterized the changes that affect skeletal muscle mass, strength, and function as humans age, yet studies uncoupling pathological from normal aging are sparse. In the first part of this thesis, we therefore characterized the molecular profile of human sarcopenic muscle compared to age-matched healthy controls (**Chapter 2**). Our results demonstrated exacerbated loss of muscle mitochondrial function and NAD^+ content during sarcopenia, which prompted us to further explore a therapeutic strategy targeting these aspects.

An increasing body of preclinical evidence suggests that boosting NAD^+ availability through precursor supplementation benefits mitochondrial function and mitigates disease pathology in a range of muscle conditions such as myopathies, dystrophies and aging. In the second part of this thesis, we therefore tested the effect of the NAD^+ vitamin B_3 precursor nicotinamide riboside (NR) in a rat model of sarcopenia (**Chapter 3**).

The potential of NR as an NAD^+ precursor is largely dependent on the enzymatic activity of NR kinases (NRKs), yet little is known about the contribution of NRKs to muscle NAD^+ homeostasis and their role in muscle physiology. Therefore, in the last part of this thesis, we studied the basal NAD^+ metabolism, muscle atrophy, and muscle regeneration in mice deficient for NRK1 and 2 (NRK1/2 dKO mice) (**Chapter 4**).

2 Loss of mitochondrial oxidative capacity with NAD^+ deficiency is the major molecular determinant of human sarcopenia across ethnicities

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Personal contribution

I designed, conducted and interpreted the experimental functional validation of our transcriptomics results and prepared the figures related to my work (Figures 4 and 5). I also assisted Eugenia Migliavacca with NAD^+- and mitochondria-related analyses of the transcriptomics data, and edited and approved the final manuscript.

Loss of Mitochondrial Oxidative Capacity with NAD$^+$ Deficiency is the Major Molecular Determinant of Human Sarcopenia across Ethnicities

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Aging causes a progressive loss of skeletal muscle mass and strength through lifestyle, endocrine, nutritional and cellular causes that are well-studied in healthy older populations[1,2]. In contrast, much less is known about sarcopenia, the pathological muscle wasting and weakness recently assigned an ICD-10 disease code due to its negative impact on physical function, quality of life and survival[3-5]. In the Multi-Ethnic Molecular determinants of Sarcopenia (MEMOSA) multi-centre study, we analysed genome-wide transcriptional changes of sarcopenic people vs age-matched healthy controls in muscle biopsies from 119 older men of different ethnic groups. Individuals with sarcopenia in Singapore, Hertfordshire UK and Jamaica reproducibly demonstrated a prominent transcriptional signature of mitochondrial bioenergetic dysfunction in skeletal muscle. This signature included down-regulation of a PGC-1α/ERRα transcriptional network, and reduced expression of oxidative phosphorylation and mitochondrial unfolded protein response genes. Gene expression changes translated functionally into fewer mitochondria and reduced expression and activity of mitochondrial respiratory complexes. Altered expression of NAD biosynthesis and salvage enzymes was observed, together with lower muscle NAD+ levels in older people with sarcopenia. This study provides the first integrated molecular profile of human sarcopenia across ethnicities, demonstrating a fundamental role of altered mitochondrial metabolism in the pathological loss of skeletal muscle mass and function in older people and paving the way for new therapeutic approaches to sarcopenia.

20 community-dwelling sarcopenic men of Chinese descent and 20 age-matched controls were recruited in Singapore (Singapore Sarcopenia Study, SSS, mean age 71.5 years); SSS findings were validated using existing cohorts in the UK (Hertfordshire Sarcopenia Study, HSS) and Jamaica (Jamaica Sarcopenia Study, JSS) (**Table S1**). Sarcopenia was defined based on harmonized consensus clinical definitions of the AWGSOP (SSS) or EWGSOP (HSS/JSS)[5], using skeletal muscle mass evaluation by DXA measurement of appendicular lean body mass index (ALMi), grip strength and gait

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speed (**Table S2**). Genome-wide transcriptome was profiled on vastus lateralis muscle biopsies using high coverage total RNA sequencing with >70 million reads per sample (**Table S3**).

Case-control analysis in SSS revealed a strong perturbation of muscle gene expression in sarcopenic participants where 179 genes encoding 150 proteins and 29 non-coding RNAs were altered in sarcopenic muscle with false discovery rate (FDR) <10% (**Fig. 1a**; **Table S4**). This sarcopenic signature was enriched in down-regulated genes out of which 133 genes were annotated under the mitochondrion gene ontology term (**Fig. 1b**, green ticks). Independent mRNA gene expression validation of 80 selected genes using nanoString nCounter demonstrated tight correlation with RNAseq data (**Fig. S1a**; **Table S5**), and confirmed lower expression of mitochondrial function genes in sarcopenic muscle (**Fig. 1c**). Network and gene ontology analysis of down-regulated genes distinguished several clusters linked to mitochondrial respiratory chain complexes, oxidative phosphorylation and mitochondrial translation (**Fig. 1d-e**). Mitochondrial alterations were also confirmed as the strongest signature in sarcopenic muscle in pathway enrichment analyses using CAMERA (**Fig. 2a-b**; **Table S6**). Mitochondrial respiratory chain, TCA cycle regulator, and oxidative phosphorylation gene sets were repressed in sarcopenic muscle with highly significant FDRs (<10E-10). Gene sets affected by age-related neurodegenerative diseases such as Alzheimer's were also down-regulated in sarcopenic muscle (**Fig. 2a**), but the enrichment of these gene sets was caused by regulators of mitochondrial function (**Fig. S1b**) that are also altered during neurodegeneration[6]. Mitochondrial function in skeletal muscle declines throughout the life course through impaired mitochondrial biogenesis, expression of mitochondrial respiratory complex subunits, mitochondrial respiration and ATP levels during aging[1,7,8]. To uncouple the pathological drivers of sarcopenia from the general effect of aging on mitochondrial function, we adjusted the genome-wide RNAseq analyses for age. Age-adjustment slightly decreased the statistical significance of the differentially expressed genes (**Fig. S1c**), but the rank of genes differentially expressed in sarcopenic muscle was highly conserved (**Fig. S1d,** r_s=0.95). Mitochondrial function and oxidative phosphorylation remained the strongest down-regulated hallmarks of sarcopenic muscle in age-adjusted pathway enrichment analyses (**Fig. S1e**). Thus, mitochondrial energy production is the strongest transcriptional signature of sarcopenia and pathological muscle dysfunction.

The SSS pathway enrichment analyses of sarcopenic muscle also revealed lower expression of the transcriptional networks regulated by the ERRα nuclear receptor (gene name *ESRRA*) and the PGC-1α transcriptional coactivator (*PPARGCA1*) (**Fig. 2a**), while the mRNAs of the energy sensor AMP-activated kinase (AMPK) and its downstream targets were not changed during sarcopenia (**Fig. S2a-c**). mRNA levels of PGC-1α and ERRα were reduced in sarcopenic muscle (**Fig. 2c**), and downstream targets including TFAM[9] were also downregulated (**Fig. 2c-d**). In addition, the promoters of the genes down-regulated in sarcopenic muscle were highly enriched in ERRα and NRF1 binding sites (**Fig. 2e-f**). The transcriptional regulators ERRα and NRF1 and their coactivator PGC-1α have been widely demonstrated to regulate mitochondrial gene expression in rodents and humans[9,10]. In particular, overexpression of PGC-1α or ERRα is sufficient to induce the expression of genes controlling mitochondrial activity and to trigger functional benefits on oxidative phosphorylation and ATP generation. Thus, reduced transcriptional activity of ERRα and of PGC-1α-dependent transcription factors in sarcopenic muscle contributes to the global mitochondrial alterations observed in sarcopenia.

Expression profiles of genes controlling mitochondrial dynamics through fusion and fission were lower in sarcopenic individuals, both through single gene and pathway enrichment analyses (**Fig. 2g**; **Fig. S2d**). In particular, Mitofusin 2 (*MFN2*) and *OPA1* were amongst the most strongly downregulated genes (**Fig. 2g**). The regulation of mitochondrial dynamics has been linked to the control of muscle metabolism and plasticity in pre-clinical models of muscle pathology and aging[11]. Interestingly, genetic loss of function of *Mfn2* and *Opa1* in mice is sufficient to cause sarcopenia[12-14], suggesting that the down regulation of these genes during human sarcopenia drives the loss of muscle mass and strength. Our protein association network analysis of genes with altered expression in sarcopenic muscle also revealed a particularly striking node containing mitochondrial ribosomal protein (MRP) genes (**Fig. 1d**). Many genes encoding both the small and large subunits of the mitochondrial ribosome were downregulated in sarcopenic muscle (**Fig. 2h**; **Fig. S2e**), demonstrating that sarcopenia associates with specific deficits of mitochondrial protein synthesis. MRPs are also important to balance mito-nuclear communication during aging and regulate a

protective mitochondrial unfolded protein response (UPRmt) important for the regulation of health span and longevity in preclinical models[15]. Interestingly, many genes controlling the UPRmt were strongly downregulated in the muscle of sarcopenic participants, including those encoding the mitochondrial heat-shock proteins, the protease ClpP and their transcriptional effectors UBL5, ATF4 and CHOP/Ddit3 (**Fig. 2i**; **S2f**). Thus, inefficient UPRmt activation during sarcopenia fails to compensate the lower production of mitochondrial proteins and their damage induced by oxidative stress[16,17].

The prevalence of sarcopenia differs by country[5], but potential differences in etiology across ethnic groups have been little studied. To confirm the prominent role of mitochondrial alterations in human sarcopenia observed in the SSS cohort, we used pre-existing cohorts of Caucasian (HSS, UK) and Afro-Caribbean (JSS, Jamaica) men with sarcopenia. Gene set enrichment analysis following high coverage RNA sequencing of sarcopenic and control muscle confirmed that mitochondrial bioenergetic dysfunction was a strong signature of sarcopenia in all cohorts/ethnicities (**Fig. 3a**), including lower oxidative phosphorylation, mitochondrial respiratory ETC and TCA cycle at FDR<10E-04 and 10% in HSS and JSS, respectively (**Fig. 3a**). Gene sets controlling mitochondrial function were also consistently depleted in participants with low ALMi (**Fig. 3b**) and low muscle function (**Fig. 3c**) in HSS and JSS cohorts. Together, transcriptomic analyses in the three independent cohorts confirmed a prominent contribution of genes controlling mitochondrial energy production and oxidative phosphorylation in maintaining muscle mass and function in older individuals of different ethnicity.

Beyond mitochondria, other signatures contributed to the molecular perturbations of human sarcopenic muscle. In particular, ribosome and translation ontologies were over-represented in the genes down-regulated in sarcopenia (**Fig. 2a**; **S2g**). Altered muscle protein synthesis has been widely associated with low muscle mass in older people, through a state of anabolic resistance where anabolic hormones and dietary amino acids fail to efficiently promote mTOR signalling and contractile protein synthesis in myofibers[18]. As a sign of altered protein anabolism, mTOR signalling was repressed in sarcopenic muscle (FDR=1.2E-02; **Fig. S2g**), but to a much lesser extent than oxidative phosphorylation (FDR<10E-10; **Fig. S1b**). Myofiber denervation and altered neuromuscular junction (NMJ) morphology have been proposed as cellular causes of muscle dysfunction during aging[19]. Strikingly, none of the transcriptional signatures of denervation detected in rodent models were observed in our study (**Fig. S2h**). Sets of genes controlling neuromuscular processes, NMJ structure and acetylcholine receptor signalling were not deregulated in sarcopenic muscle across the 3 cohorts (**Fig. S2i**; **S2k-l**), suggesting that neuromuscular dysfunction is not a major transcriptional mechanism of human sarcopenia. Chronic low-grade inflammation has also been proposed to contribute to sarcopenia through systemic cytokine changes and local targeted responses in skeletal muscle[20]. However, the transcriptional profiles of sarcopenic muscle did not detect inflammatory responses or perturbed signalling through typical pro-inflammatory signalling pathways like JAK/STAT and NFκB in SSS and HSS (**Fig. S2j-k**). JSS revealed a weak inflammatory response in sarcopenic muscle (**Fig. S2l**), suggesting that inflammation may associate only with a specific subset of sarcopenics.

To further understand the contribution of muscle mass and function, we performed a genome-wide association of muscle gene expression to ALMi, grip strength and gait speed as continuous variables (**Fig. S3a**). 318 genes encoding 276 proteins and 42 non-coding RNAs were associated with ALMi at FDR<10%. In contrast, muscle gene expression associations with grip strength and gait speed were weaker with only 7 and 9 genes, respectively, associated at a nominal p-value <0.001 but FDR>10%. A large number of genes regulated in sarcopenic muscle were also among the most associated with ALMi and grip strength, but not with gait speed (**Fig. S3a**, black dots). At the pathway level, mitochondrial function was the strongest biological process associated with muscle mass and muscle function as continuous variables (**Fig. S3b**). In particular, genes positively correlated with ALMi and grip strength were strongly enriched for TCA cycle, oxidative phosphorylation and mitochondrial respiratory chain gene sets (FDRs<10E-10). These gene sets related to mitochondrial bioenergetics were also modestly enriched in genes positively associated to gait speed (FDR<10%). Thus, genes controlling mitochondrial function positively associated with all continuous parameters of sarcopenia. Pathway enrichment analyses also confirmed positive association of ALMi and grip strength with ERRα and PGC-1α transcriptional networks (**Fig. S3b**, grey arrows), and with gene sets regulating branched-chain amino acid metabolism, ribosomal function and translation (**Fig. S3b**, white arrows). Together, these results demonstrate that all clinical parameters used to diagnose sarcopenia contribute to the molecular profiles of sarcopenic

muscle, with the strongest contribution from low muscle mass followed by grip strength and gait speed.

To further understand how the mitochondrial transcriptomic signature affects muscle bioenergetics during sarcopenia, the expression of all genes encoding the five mitochondrial respiratory complex subunits (**Fig. 4a**) was mapped in SSS sarcopenic vs control muscle. Mitochondrial complex genes were downregulated in sarcopenic muscle across the five complexes (**Fig. 4b**), translating to a global reduction in the protein expression of active subunits of mitochondrial respiratory complexes (**Fig. 4c**). NDUFA9, SDHa, UQCR2 and ATP5a, representing complexes I, II, III and V, respectively, were down-regulated by 44-51% in sarcopenic muscle (**Fig. 4d**). Enzymatic assays on mitochondria isolated from muscle biopsies demonstrated functional deficits of mitochondrial activity in sarcopenia (**Fig. 4e**). In particular, the enzymatic activity of complexes I-IV was lower in sarcopenic muscle (**Fig. 4e**) and the activity of all complexes correlated positively with ALMi (**Fig. S4a**). The activity of the two mitochondrial TCA cycle enzymes citrate synthase (CS) and succinate dehydrogenase was strongly reduced in sarcopenic muscle (**Fig. 4e**), confirming that a global alteration of oxidative metabolism and energy production is perturbed in human sarcopenic muscle. Reduced complex activity was linked to lower amounts of mitochondria as it was not affected when normalized to CS activity (**Fig. S4b**) or to CS or porin expression (data not shown). The expression of other mitochondrial proteins such as CS and porin 1 was also lower in sarcopenia (**Fig. 4f-g**), as expected from the down-regulation of the ERRα/PGC1α/NRF1/TFAM network which controls both the amount and bioenergetic activity of mitochondria[10]. Thus, sarcopenia arises from a general bioenergetic deficit which differs mechanistically from primary mitochondrial myopathies caused by decreased activity of a specific complex, and is important for functional impairment of sarcopenic people as contraction and muscle performance relies both on the amount and activity of mitochondria.

Intracellular levels of NAD+ have emerged as a major regulator of oxidative mitochondrial metabolism in health and disease[21], leading to the possibility that they could contribute to the mitochondrial signature of sarcopenic muscle. In gene set enrichment analysis, genes of the NAD metabolic process GO term were repressed in sarcopenia vs control in SSS, HSS and JSS (**Fig. 5a**). To confirm this signature, we measured NAD+ levels in muscle biopsies of SSS. Muscle NAD+ levels measured biochemically were decreased by 32% in sarcopenics vs controls (**Fig. 5b**), and were linear and 99% correlated using mass spectrometry (**Fig. S5a-b**). Muscle NAD+ levels also correlated positively with ALMi, grip strength, gait speed and complex I activity (**Fig. 5c**). To understand the mechanisms underpinning reduced muscle NAD+ in sarcopenia, we analysed the mRNA expression of the enzymes controlling NAD biosynthesis and salvage, and of NAD+ consuming enzymes (**Fig. S5c-f**). Sarcopenic muscle had reduced expression of NMNAT1 and NAMPT, two rate-limiting enzymes of NAD biosynthesis and salvage (**Fig. 5d & S5c**). In contrast, no increase in the expression of NAD consuming enzymes such as CD38 (**Fig. 5e-f**), PARPs (**Fig. S5e**) or sirtuins (**Fig. S5f**) was observed, suggesting that reduced NAD+ levels in human sarcopenia primarily result from inability to synthesize and recycle NAD. NAD+ decline during aging is well documented in preclinical models and has recently emerged in humans where skin and brain NAD+ decreases across the lifespan[22]. The reduction of skeletal muscle NAD+ levels in people with sarcopenia links for the first time NAD+ to an age-related pathology in humans and constitutes the first demonstration of NAD deficiency in human skeletal muscle. Although low amounts of mitochondria in human sarcopenia could contribute to reduced NAD+ levels given the high mitochondrial NAD+ concentration, mitochondria only represent 5-10% of myofiber volume[23] and NAD+ is also abundant in larger cellular compartments[24] which most likely also account for the sarcopenic NAD+ phenotype. In model organisms, low NAD+ is a causal mechanism for mitochondrial perturbations in age-related pathologies[25-27]. Reduced NAD+ levels in skeletal muscle of aged or Nampt deficient mice alters mitochondrial bioenergetics and impairs muscle mass, strength and endurance[25,26,28,29], suggesting that low NAD+ levels in sarcopenic people could contribute to impaired mitochondrial activity and sarcopenia progression in humans. Importantly, new therapeutic strategies have emerged to restore NAD+ levels in muscle by administration of dietary NAD+ precursors such as NR or NMN [22,29,30], and the enzymes converting these precursors to NAD+ were not affected by sarcopenia (**Fig. S5d**). Combined to the early human clinical testing of these interventions where they are safe and increase NAD+ levels[22], our study creates the mechanistic basis to test the clinical efficacy of NAD+ precursors on muscle strength and physical function in older people with sarcopenia. More broadly, our work also highlights that nutritional and

pharmacological mitochondrial therapeutics should be considered for the management of sarcopenia by stabilizing the mitochondrial machinery[31] or targeting energy sensors like AMPK, PPARs and sirtuins which converge on PGC-1α signalling[8]. The established benefits of physical activity on mitochondrial efficiency in aged skeletal muscle[1,23,32] also suggest that exercise programs to improve sarcopenia should maximize mitochondrial adaptations that enhance bioenergetic coupling. Collectively, the MEMOSA study results provide a genome-wide molecular resource of mechanisms and biomarkers of pathological muscle aging across ethnicities. Our work establishes loss of mitochondrial oxidative capacity as a major mechanism of sarcopenia which can be assessed non-invasively using 31P imaging as a biomarker[33], and provides a strong rationale for intervention trials targeting muscle mitochondrial bioenergetics to manage sarcopenia in older people.

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COMPETING INTERESTS

EM, TS, GC, AC, MPG, SK, SMo, SMe, JM, FR, PD and JNF are full-time employees of the Nestec SA. KMG and HPP have received reimbursement for speaking at conferences sponsored by companies selling nutritional products. KMG and SJB are part of academic research programmes that have received research funding from Abbott Nutrition, Nestec and Danone.

CONTRIBUTIONS

Contributor Role	Author initials
Conceptualization	JNF, KMG, NK, HP, TF, KAL
Methodology	AC, EM, JM, FR, GC, PD, NK, JNF, ESG, KMG, NK, KAL, AL, CM, SM, SN, AAS, TS, EA, HPP
Software	EM
Validation	EM, TS, GC, KN
Formal Analysis	EM, SJB, GC, JNF, FR, TS, PT, NK, GW, PFT, TP,YW
Investigation	EM, SM, AC, JM, FM, TS, GC, KN, ESG, EA, KAL
Resources	CC, LF, TF, COG, MKL, SCM, HPP, AAS, CYS, HES, SKHT, LW, PF-E, GN, LF
Data Curation	EM, CC, TF, NK,
Writing – Original Draft Preparation	EM, JNF, KMG, NK, KAL, HPP, AS, JSJ, CM
Writing – Review & Editing	all authors
Visualization	EM, NK
Supervision	PD, JNF, KMG, CM, NK
Project Administration	JNF, JSJ
Funding Acquisition	JNF, KMG, NK

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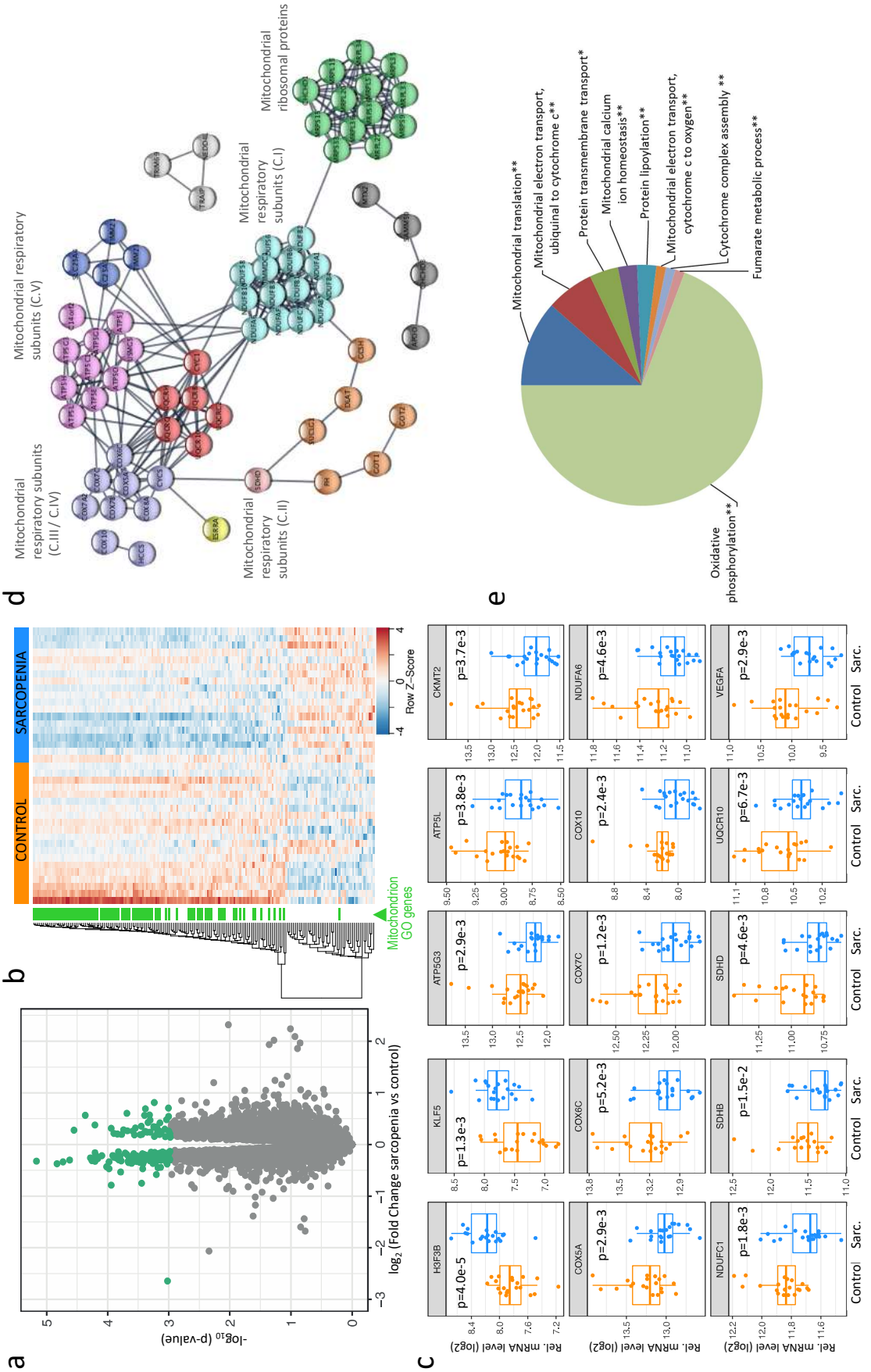
a
b
c
d
e
CONTROL
SARCOPENIA
Row Z-Score
Mitochondrion GO genes
-log10 (p-value)
log2 (Fold Change sarcopenia vs control)
Rel. mRNA level (log2)
Control
Sarc.
H3F3B
p=4.0e-5
KLF5
p=1.3e-3
ATP5G3
p=2.9e-3
COX7C
p=1.2e-3
ATP5L
p=3.8e-3
COX10
p=2.4e-3
CKMT2
p=3.7e-3
NDUFA6
p=4.6e-3
COX5A
p=2.9e-3
COX6C
p=5.2e-3
SDHD
p=4.6e-3
SDHB
p=1.5e-2
UQCR10
p=6.7e-3
VEGFA
p=2.9e-3
NDUFC1
p=1.8e-3
Mitochondrial respiratory subunits (C.III / C.IV)
Mitochondrial respiratory subunits (C.V)
Mitochondrial respiratory subunits (C.II)
Mitochondrial respiratory subunits (C.I)
Mitochondrial ribosomal proteins
Mitochondrial translation**
Mitochondrial electron transport, ubiquinol to cytochrome c**
Protein transmembrane transport*
Mitochondrial calcium ion homeostasis**
Protein lipoylation**
Mitochondrial electron transport, cytochrome c to oxygen**
Cytochrome complex assembly **
Fumarate metabolic process**
Oxidative phosphorylation**

Figure 1. RNA sequencing of human skeletal muscle in SSS. (a) Volcano plot of differentially expressed genes in skeletal muscle of sarcopenic vs age-matched healthy elderly people. P-values were calculated using moderated t-statistic. The 133 genes down-regulated and 46 genes up-regulated in sarcopenic muscle using a false-discovery rate (FDR) <10% are represented in green. **(b)** Heatmap showing the 179 genes differentially expressed from (A) with FDR<10%. Genes belonging to the cellular component GO term "mitochondrion" are labeled with a green tick. **(c)** Validation of gene expression changes in sarcopenic muscle of SSS for selected genes using quantitative mRNA profiling by nanoString nCounter; mRNA expression values are normalized to ten stable housekeeping genes. n=40 muscle samples analysed with a two sided t-test. **(d)** Network representation of the protein-protein interactions of genes differentially regulated in sarcopenic muscle at FDR <10% using STRING. Nodes with an interaction score > 0.9 are represented and colored by biological function. **(e)** Gene ontology enrichment of the genes regulated in sarcopenic muscle. Pie-chart represents the % of differentially expressed genes, *$p < 0.05$ and **$p < 0.01$. In a-b;d-e, n=39 SSS participant muscle samples.

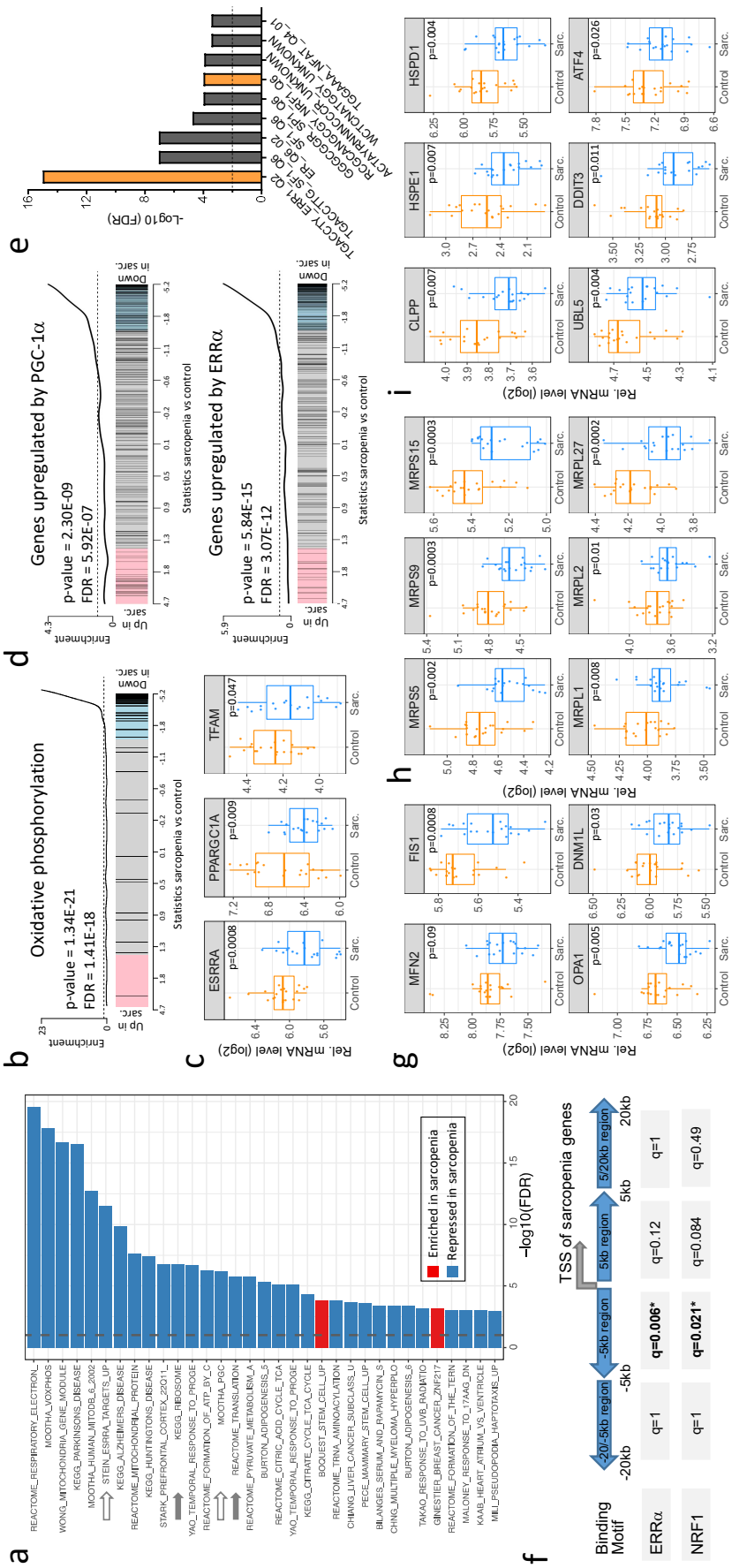
a
b
c
d
e
f
g
h
i
Oxidative phosphorylation
p-value = 1.34E-21
FDR = 1.41E-18
Genes upregulated by PGC-1α
p-value = 2.30E-09
FDR = 5.92E-07
Genes upregulated by ERRα
p-value = 5.84E-15
FDR = 3.07E-12
Enrichment
Up in sarc.
Down in sarc.
Statistics sarcopenia vs control
Rel. mRNA level (log2)
Control
Sarc.
ESRRA p=0.0008
PPARGC1A p=0.009
TFAM p=0.047
MFN2 p=0.09
FIS1 p=0.0008
OPA1 p=0.005
DNM1L p=0.03
MRPS6 p=0.002
MRPS9 p=0.0003
MRPS15 p=0.0003
MRPL1 p=0.008
MRPL2 p=0.01
MRPL27 p=0.0002
CLPP p=0.007
UBL5 p=0.004
HSPE1 p=0.007
DDIT3 p=0.011
HSPD1 p=0.004
ATF4 p=0.026
-Log10 (FDR)
-log10(FDR)
Enriched in sarcopenia
Repressed in sarcopenia
TSS of sarcopenia genes
Binding Motif
ERRα
NRF1
-20kb
-5kb
5kb
20kb
-20/-5kb region
-5kb region
5kb region
5/20kb region
q=1
q=0.006*
q=0.12
q=0.021*
q=0.084
q=0.49

Figure 2. Mitochondrial dysfunction is the major transcriptional change during sarcopenia in SSS. **(a)** Gene set enrichment analysis of sarcopenic vs control muscle using CAMERA and the C2 curated gene set collection from MSigDB. Gene sets are ordered according to the significance of their enrichment; only gene sets with an FDR <0.1% and a gene overlap < 75% are represented. White arrows highlight gene sets linked to the transcriptional regulation of mitochondrial function; gray arrows highlight gene sets linked to protein synthesis. **(b)** Enrichment plot for the oxidative phosphorylation gene set "Mootha_VOxPhos". **(c)** mRNA expression of transcriptional regulators of mitochondrial function in SSS sarcopenic vs control muscle. **(d)** Enrichment plot for PGC-1a target genes and ERRa target gene set ("Mootha_PGC1a" and "Stein_ESRRa_Up"). **(e)** Transcription factor binding site enrichment of the 4kb promoters of genes regulated in sarcopenic muscle at FDR q-value <0.05. X-axis represents the MsigDB transcription factor gene sets that passed the significance. **(f)** ERRa and NRF1 binding motif in the proximal and distal regions flanking the transcriptional start site (TSS) of the genes regulated in sarcopenic muscle at FDR q-value <0.05. Benjamini Hochberg-corrected median-adjusted q-values were computed by performing 1000 hypergeometric test permutations. **(g-i)** mRNA expression of genes regulating mitochondrial dynamics (g), mitochondrial ribosomal protein genes (h) and UPRmt genes (i) in SSS sarcopenic vs control muscle. In c;g-i nominal p-values of the moderated t-statistic are reported. For all panels, n=39 SSS participant muscle samples.

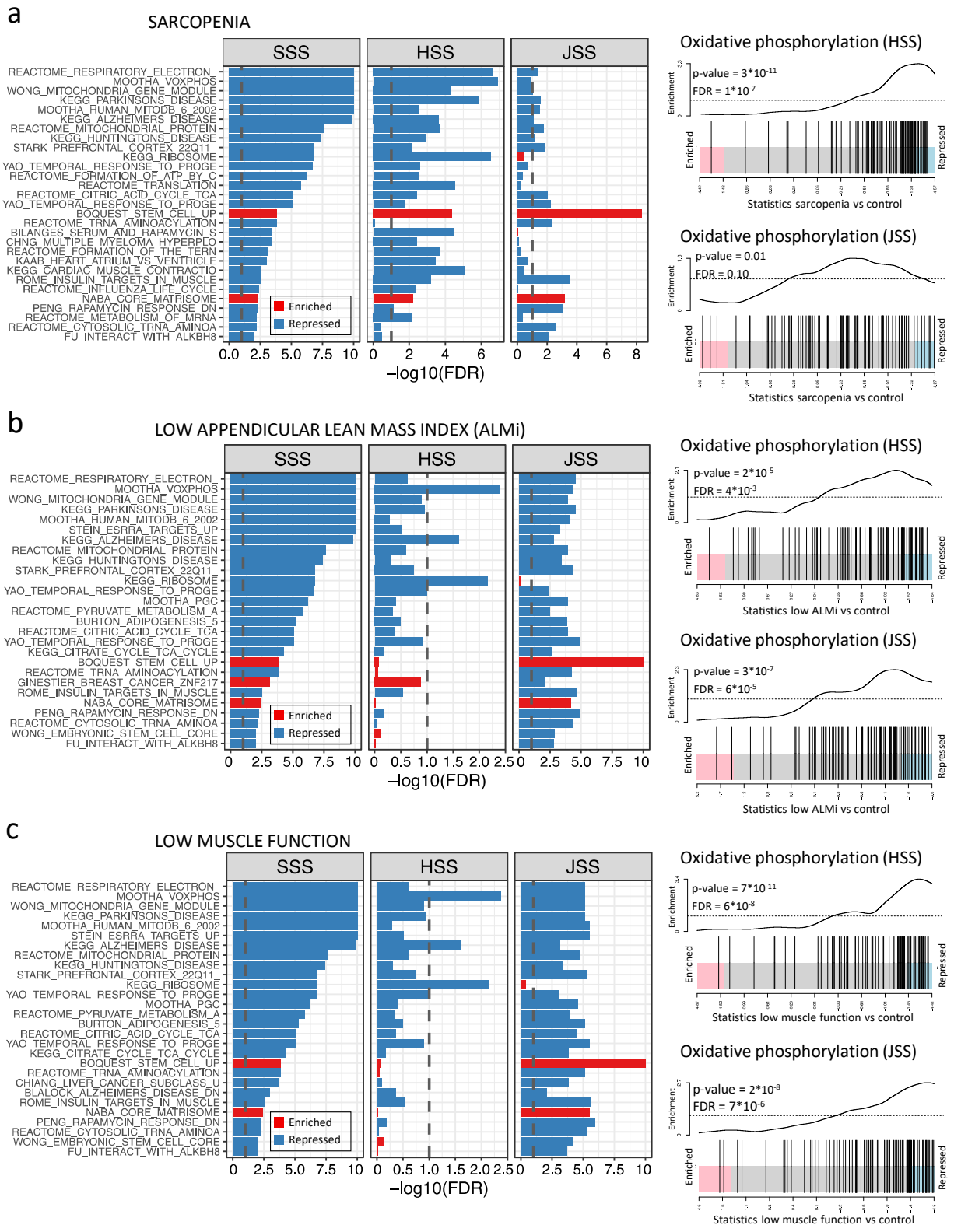

Figure 3. HSS and JSS cohorts confirm the transcriptional downregulation of mitochondrial function in the muscle of people with sarcopenia and low physical function from different ethnicity. Gene set enrichment analysis on muscle RNA expression in the discovery cohort (SSS) and 2 replication cohorts of different ethnicity (HSS, JSS) using CAMERA and the C2 curated gene set collection from MSigDB. **(a)** Sarcopenia vs control in SSS, HSS and JSS cohorts. **(b)** Low appendicular lean mass index vs control in SSS, HSS and JSS cohorts. **(c)** Low muscle function (grip strength or gait speed)

Figure 3 (continued). vs control in SSS, HSS and JSS cohorts. In the left panels, gene sets are ordered according to the significance of their association in the SSS cohort; only gene sets with an overlap between sets < 75% and an FDR < 1% in SSS and at least one other cohort are reported. The significance threshold of 10% FDR is represented by dashed grey lines and FDRs smaller than 10E-10 are trimmed. Right panels represent the enrichment plots for the "Mootha VOXPHOS" oxidative phosphorylation gene sets in the HSS and JSS cohorts. For all panels, n=39 (SSS, JSS) and n=40 (HSS) muscle samples per cohort were stratified in the different phenotypes as described in Table S2.

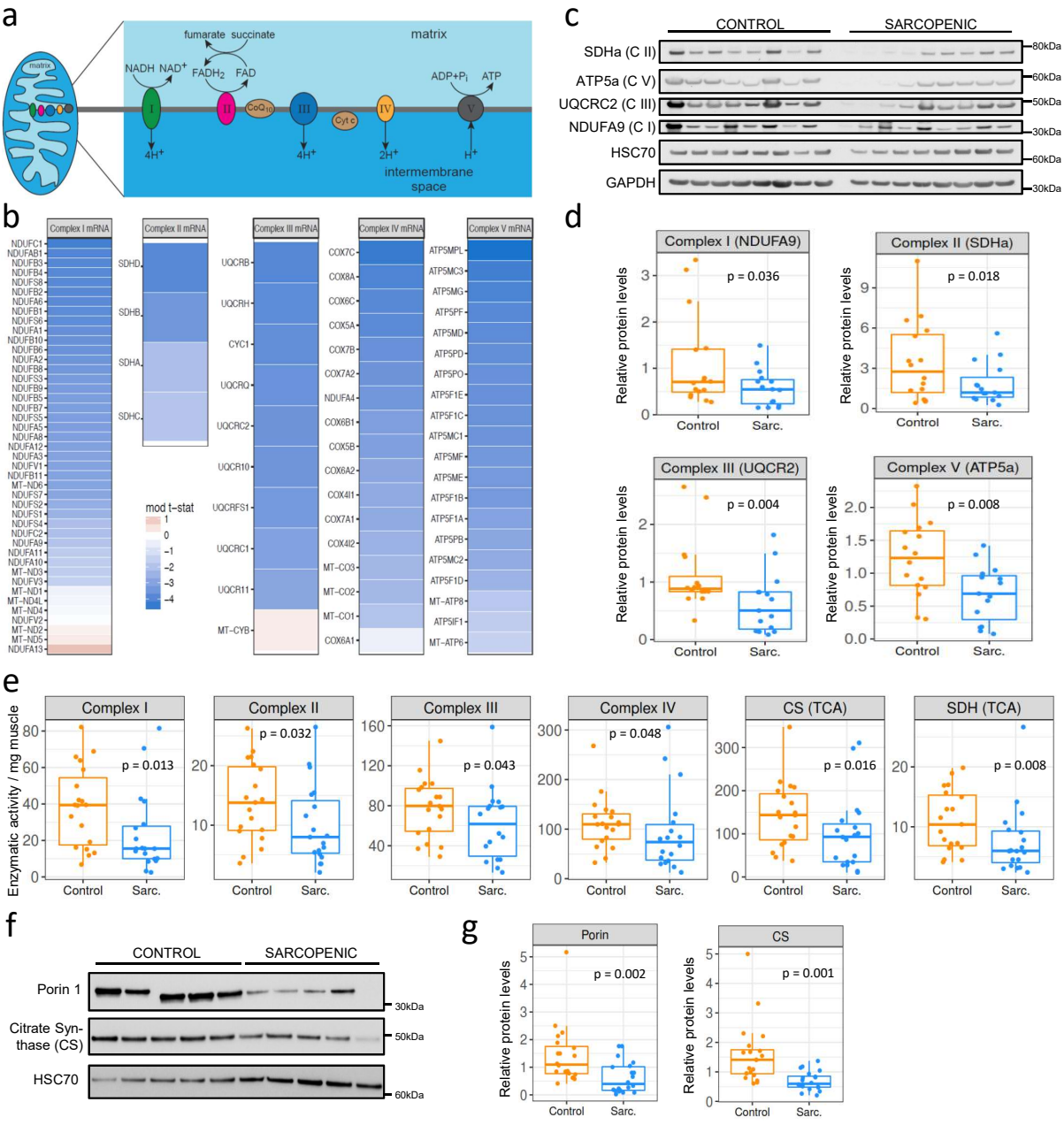

Figure 4. Mitochondrial bioenergetic activity is decreased in Sarcopenic muscle. (a) Schematic representation of a mitochondrion and the five respiratory complexes of the electron transport chain. **(b)** Gene expression change in sarcopenic vs control muscle of mRNAs encoding the subunits of the 5 mitochondrial respiratory chain complexes, color coded according to moderated t-statistics (SSS cohort; n=39). **(c-d)** Muscle protein expression of representative subunits of mitochondrial respiratory chain complexes and mitochondrial proteins measured by western blot. (c) Representative blots from one gel, with GAPDH and HSC70 included as house-keeping normalization controls. (d); Quantification of protein levels relative to GAPDH and HSC70 in all remaining samples analysed (SSS cohort; n=35); p-values based on a Wilcoxon test. **(e)** Enzymatic activity per mg muscle tissue of mitochondrial complexes I, II, III and IV, Citrate Synthase (CS) and Succinate Dehydrogenase (SDH) measured on mitochondrial extracts from remaining muscle biopsies of control and sarcopenic participants (SSS cohort; n=38); p-values based on a Wilcoxon test. **(f-g)** Muscle protein expression of porin 1 and CS by western blot (SSS cohort; n=35; p-values based on a Wilcoxon test).

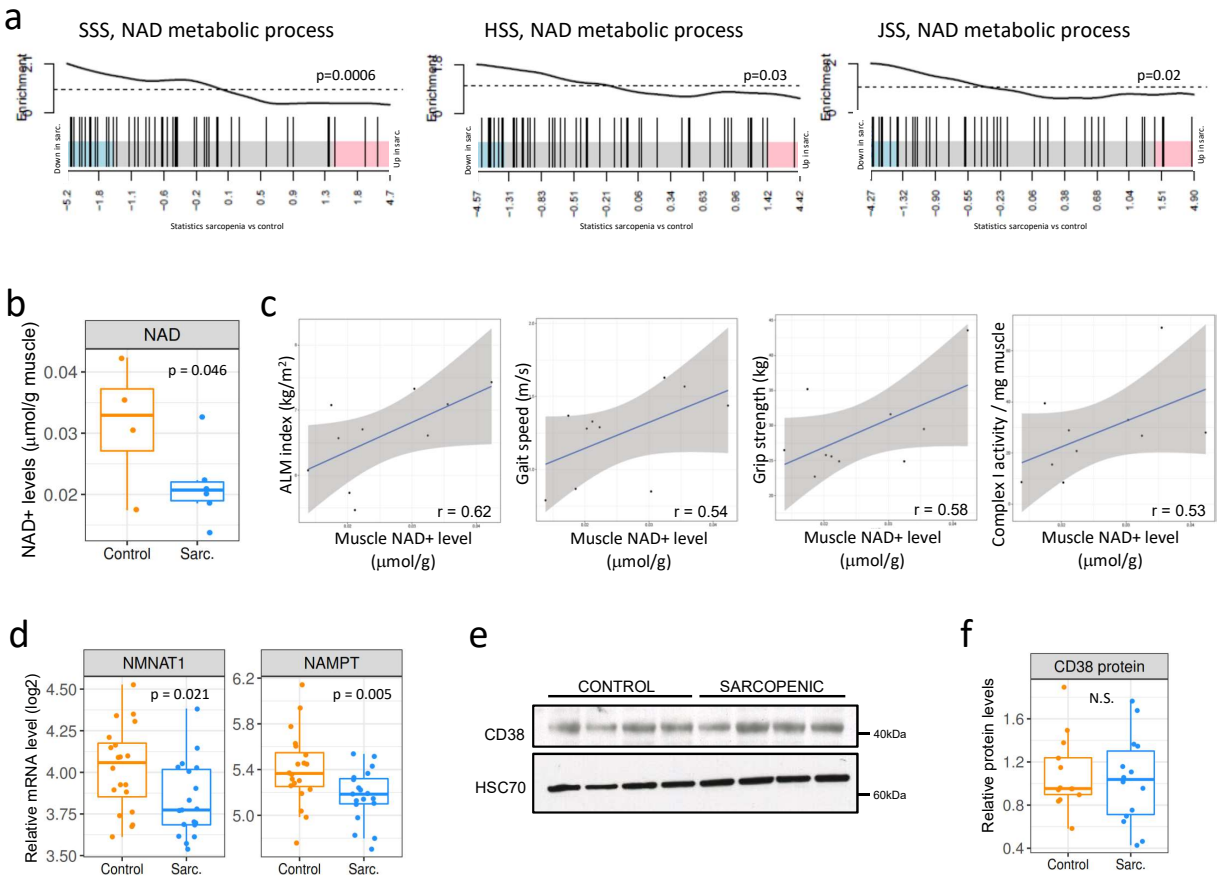

Figure 5. NAD⁺ levels are low in Sarcopenic muscle. (a) Gene set enrichment plot of the "NAD metabolic process" on sarcopenic vs control muscle mRNA in the discovery cohort (SSS) and 2 replication cohorts of different ethnicity (HSS, JSS). (**b**) NAD⁺ levels in remaining muscle biopsies of control and sarcopenic participants (SSS cohort, n=10); p-values based on t-statistics. (**c**) Muscle NAD⁺ levels positively correlate to ALM-index, grip strength, gait speed, and mitochondrial complex I activity. n=10 SSS muscle samples. **(d)** Muscle mRNA of NAD⁺ biosynthesis enzymes Nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1) and Nicotinamide phosphoribosyltransferase (NAMPT) (SSS cohort; n=39); p-values based on moderated t-statistics. **(e-f)** Muscle protein expression of CD38 by western blot (SSS cohort; n=35; p-values based on a Wilcoxon test).

SUPPLEMENTARY MATERIAL

METHODS

Singapore Sarcopenia Study (SSS). 20 Chinese male participants aged 65-79 years and 20 control participants of the same age/ethnic group without a diagnosis of sarcopenia were recruited from two studies on healthy community-dwelling older men in Singapore (Singapore Sarcopenia Group and Aging in a Community Environment Study [ACES]). The National Healthcare Group Domain-Specific Research Board (NHG DSRB) approved the study, reference number 2014/01304, and each participant gave written informed consent. Self-reported ethnicity was collected during the inclusion visit and weight and height were measured to the nearest 0.1kg and 1cm respectively. Total lean mass was measured through DXA scanning (APEX Software version 4.0.1, Discovery Wi DXA system). A standardised protocol was used to measure isometric hand grip with Jamar hand-held dynamometer and the mean of 3 three attempts from the dominant hand was used as the final measure. Gait speed was calculated from a timed 6 meter walk. The diagnosis of sarcopenia was based on AWGSOP definition[1] that was defined as the total appendicular lean mass normalized for height ≤7.00 kg/m^2, evidence of either low physical performance based on gait speed ≤0.8 m/sec OR low muscle strength based on hand grip <26 kg. Semi-open muscle biopsies of the vastus lateralis muscle were collected using a BioPince™ (Angiotech) 16G full core biopsy needle with 3 adjustable stroke lengths (13mm, 23mm, 33mm) from the 20 male participants and 20 aged matched controls, snap frozen in liquid nitrogen and stored at -80°C until further analysis.

Hertfordshire Sarcopenia Study (HSS). 105 healthy community dwelling older men, 68-77 years old, who participated in the UK Hertfordshire Cohort Study (HCS)[2] were prospectively recruited into this present study (HSS) as previously described[3]. Inclusion criteria for the HSS included the availability of birth records detailing birth weight and weight at one year. Men were excluded if they had a diagnosis of active ischaemic heart disease, myopathy or neuromuscular conditions affecting the legs or a history of diabetes. The Hertfordshire Research Ethics Committee approved the study under approval number 07/Q0204/68 and each participant gave written informed consent. Weight was measured once to the nearest 0.1 kg with floor scales (SECA, Hamburg, Germany). 40 Caucasian participants from HSS were randomly selected for MEMOSA inclusion after stratification based on their sarcopenia phenotype using the EWGSOP. Height was measured to the nearest 0.1cm. Total lean mass was calculated from body composition analysis by DXA (Hologic Discovery, software version 12.5). A standardised protocol was used to measure isometric grip strength with a Jamar dynamometer (Promedics, Blackburn, UK)[4]. Gait speed was calculated from a timed 3 meter walk. The diagnosis of sarcopenia was based on the EWGSOP definition[5] that was defined as the total appendicular lean mass normalized for height ≤7.23 kg/m^2, evidence of either low physical performance based on gait speed ≤0.8 m/sec or low muscle strength based on hand grip <30 kg. Semi-open muscle biopsies with a Weil-Blakesley conchotome were obtained from participants after an overnight fast, as previously described[3], snap frozen in liquid nitrogen and stored at -80°C until further analysis.

Jamaica Sarcopenia Study (JSS). 40 male Afro-Caribbean participants aged 63-89 years were recruited through community-based (churches, community centers, senior citizen clubs) screening using the snowballing method for referrals. The University of West Indies Research Ethics Committee approved the study under approval number 180,10/11, and each participant gave written informed consent. All participants included in this study were from African origin based on self-report of at least three grandparents of African origin. Weight was measured once to the nearest 0.1 kg with floor scales (SECA, Hamburg, Germany). Height was measured to the nearest 0.1cm with a stadiometer (SECA, Hamburg, Germany). Total lean mass was calculated from body composition analysis by DXA (GE Lunar Prodigy) Advance, Software: Encore 2011, Version 13.60.033). A standardised protocol was used to measure isometric grip strength with a Lafayette hand dynamometer (Lafayette Instrument Company, Lafayette, Indiana). Gait speed was calculated from a 6 minute walk test. The diagnosis of sarcopenia was based on the EWGSOP definition[5] as described above for HSS. Vastus lateralis muscle biopsies were obtained using a 5 mm Bergstrom needle (Stille-Werner, Ronkonkoma, NY) after participants had been characterized. Biopsies were obtained from the mid vastus lateralis muscle, about 12 cm above patella on the anterolateral thigh, following a 12 hour overnight fast, and after 24-36 hours of any structured exercise exposure. Muscle samples were separated, frozen in cooled isopentane, and stored at –80°C until analysis.

RNA extraction. Total RNA was extracted from muscle biopsies of HSS using the *mir*Vana miRNA Isolation Kit (Life Technologies) and from muscle biopsies of SSS and JSS using the QIAzol Lysis Reagent (Qiagen) followed by miRNAeasy purification Kit (Qiagen). Frozen muscle samples were placed into 600 µl Lysis/Binding buffer and homogenised using a Dispomix Homogeniser until all visible clumps were dispersed. The isolation procedure was then performed according to manufacturer's instructions using the total RNA isolation protocol. RNA quantity was measured with Ribogreen (Life Technologies) and RNA quality was checked using the Standard Sensitivity RNA Analysis Kit on a Fragment Analyzer (Advanced Analytical Technologies). All RNA samples were homogeneous and passed quality control with 260/280nm ratio >1.8 and RIN scores >7.

RNA sequencing. For each sample, 250 ng of total RNA was employed as starting material for library preparation. Sequencing libraries were prepared using the TruSeq Stranded Total RNA HT kit with the Ribo-Zero Gold module (Illumina), followed by 13 cycles of PCR amplification with the KAPA HiFi HotStart ReadyMix (Kapa BioSystems). Libraries were quantified with Picogreen (Life Technologies) and size pattern was controlled with the DNA High Sensitivity Reagent kit on a LabChip GX (Perkin Elmer). Libraries were then pooled at an equimolar ratio and clustered at a concentration of 7 pM on paired-end sequencing flow cells (Illumina). Sequencing was performed for 2 x 101 cycles on a HiSeq 2500 (Illumina) with v3 chemistry. The generated data were demultiplexed using Casava. Reads were aligned to the human genome (hs_GRCh38.p2) using STAR[6], and the number of reads mapped within genes was quantified by HTSeq[7] (version HTSeq-0.6.1p1, mode union, strand reverse, quality alignment greater than 10). SSS samples had a sequencing depth of 75-104 million reads per sample, of which 34-77 million reads where uniquely

mapped. HSS and JSS had a sequencing depth of 51-110 and 55-88 million reads per sample, respectively, out of which 38-84 million and 39-69 million were uniquely mapped.

mRNA differential expression and pathway enrichment analyses. All statistical analyses data were performed using R version 3.3.3 and relevant Bioconductor packages (e.g. limma 3.30.13, edgeR 3.16.5). Unless otherwise stated, 40 samples from each cohort were analyzed. For differential expression analysis, all samples with more than 35 million uniquely mapped reads were included. One sample (from the SSS cohort) which did not reach this threshold because of an abnormally low percentage of uniquely mapped reads was excluded from the analysis. Differentially expressed genes between control and sarcopenic samples were defined using the limma package[8]. Briefly, after removing genes with a mean expression lower than 20 reads, data were normalized by the trimmed mean of M-values (TMM) method as implemented in the edgeR function calcNormFactors[9], and the voomWithQualityWeights function was applied to model the mean-variance relationship and estimate the sample-specific quality weights[10]. P-values were corrected for multiple testing using the Benjamini-Hochberg method. The same procedure was applied when characterizing the associations between gene expression and the continuous or categorical parameters (ALMi, grip strength and walking speed) used to define sarcopenia. Pathway enrichment analysis was performed using CAMERA[11], a competitive gene set test querying whether a set of genes annotated in the Molecular Signatures Database (MSigDB)[12] is enriched in differentially expressed or continuously associated genes. MSigDB v5.2 collections H (hallmark gene sets), C2 (curated gene sets) and C5 (GO gene sets) were used to perform pathway analyses[13]. To circumvent the absence of a mammalian UPRmt GO category, we have created a custom mammalian UPRmt gene set using the lower organism UPRmt GO:0034514 category and manual curation of the mammalian UPRmt homologs based on published reviews[14-16]. To circumvent the fact that several gene sets are redundant in public databases, we have removed gene sets with a gene overlap > 75% from figure visualization (although these gene sets were still considered for computation of FDRs). The overlap between 2 gene sets of different sizes was defined as OL=2*c/(n+m)*100, where n and m are the size of the 2 gene sets and c is the genes in common, and the threshold of 75% was selected from a range from 50-90% to maximize biological diversity while minimizing overlaps).

Network and gene ontology analyses. Protein interaction networks were generated with the 149 protein coding genes differentially regulated in sarcopenia using STRING version 10 (http://string-db.org/)[17], using all data sources, a confidence score of 0.9, and the maximum number of interactors shown in the first shell set to 5. The interaction network was colored manually based on biological function of the proteins and the network connectivity was enriched at a p-value < 1.0e-16 when compared to a random sampling of 149 proteins. In addition, the unique identifiers of differentially expressed genes were used as an input for functional analyses using Cytoscape (version 3.5.1)[18]. Genes differentially expressed were used for functional enrichment analysis to decipher functionally grouped gene ontology and biological process using ClueGO[19]. pV correction was estimated using a Bonferroni stepdown method. Results are presented as pie charts in Figure 1E.

Transcription factor binding enrichment analysis. Molecular Signature Database (MsigDB, http://software.broadinstitute.org/gsea/msigdb/), was used to identify the transcription factor target gene sets significantly associated with the 179 differentially expressed genes identified in Fig 1A at an FDR q-value <0.05. Under MsigDB, the C3 sub-collection TFT (transcription factor targets) containing 674 motif gene sets was used for this analysis. To investigate the enrichment of ERRA and NRF1 binding sites in distal and proximal regulatory regions of the 178 genes (excluding chr M) associated with sarcopenia, we extracted their DNA sequence via UCSC table browser using the hg38 human genome assembly. Sequence lengths interrogated included 5kb and 5-20Kb upstream/downstream of the transcriptional starting site (TSS). Transcription factor binding site/ motif enrichment analysis was performed as previously described[20] using the findMotifs.pl tool embedded within Homer, a tool used for motif discovery and next generation sequencing analysis. Input sequences were randomly scrambled and used as background sequences for enrichment analysis. To ensure robustness of results, hypergeometric test was repeated 1000 times to compute Benjamini Hochberg corrected median adjusted p-value/q-values.

Gene expression validation by nanoString nCounter. mRNA expression of 70 genes of interest were selected based on up- or down-regulation in sarcopenic muscle or on biological relevance was validated using a customized nanoString nCounter panel (Table S5), a method orthogonal to sequencing, based on the binding of probes directly to the mRNA. Each target gene was detected using a pair of reporter and capture probes. Reporter probes carry a unique color code that enables the molecular barcoding of the genes of interest. The expression level of a gene is measured by counting the number of times the color-coded barcode for that gene is detected. The experiment was performed from 100 ng total RNA, strictly following the manufacturer's recommendations. Ten genes, stably expressed in skeletal muscle, were selected based on their low coefficient of variation in the RNAseq profiles of this study, and used as housekeeping genes (HKG) for normalization. Primary analysis was performed with the dedicated nSolver software (nanoString).

Mitochondrial enzymatic activity. Mitochondrial enzyme and respiratory chain complex activities were measured on mitochondrial fractions isolated from 15-30mg of frozen muscle biopsies as previously described[21,22], for all biopsies where sufficient material remained after transcriptomic experiments. Briefly, tissues were homogenized in 10mM potassium phosphate buffer (pH 7.4) and mitochondrial-enriched fractions collected after a 800g centrifugation were used for enzymatic assays. Complex I activity was assessed by measuring rotenone-sensitive coenzyme Q1-dependent NADH reduction [21]. SDH and complex II activities were assessed by measuring 2,6-Dichlorophenolindophenol (DCPIP) reduction either in the absence or in presence of coenzyme Q, respectively[23]. Complex III activity was measured by cytochrome C reduction[24]. Complex IV activity was measured by assessing cytochrome C oxidation[25]. Citrate synthase activity was assessed by measuring DTNB reduction at 412nm in the presence of Acetyl-CoA and oxaloacetate[26]. Enzymatic activities were normalized to the amount of muscle analyzed for all samples with remaining biopsy material (n=38).

Western blot. Western blots were performed using protein extracts remaining from the enzymatic assay preparations. Protein concentration was determined by a bicinchoninic acid (BCA) assay (Pierce #23227) and samples were prepared with 4x LDS sample buffer (Novex #NP007). 30µg protein per sample were resolved by standard western blot procedure on 4-12% Bis-Tris protein gels (Novex #WG1403BX10), and then transferred to PVDF membranes using the semi-dry system from Life Technologies/Invitrogen. Membranes were cut based on molecular ladder size to detect proteins of different size from the same membrane with different antibodies. Detection was achieved using an OXPHOS antibody cocktail (Abcam #ab110412, 1:1000), or antibodies against Porin1 (Abcam #ab15895, 1:1000) , citrate synthase (Abcam #ab96600, 1:1000), CD38 (R&D System #MAB24041, 2µg/ml), GAPDH (Abcam #ab37168, 1:5000) and HSC70 (Santa Cruz, sc-7298; 1:50000), with the relevant secondary antibodies and using enhanced chemiluminescence (ECL; Pierce #321016) detected by standard autoradiography with exposure time adjusted to the expression of each protein. Complexes I, II, III and V were correctly detected in all samples but complex IV was only detected in less than 20% of samples and was excluded from the analysis. Films were scanned and quantified using Image J.

NAD+ quantification. NAD+ levels were measured in human muscle biopsies of the MEMOSA study as previously described[27], for all biopsies where sufficient material remained after transcriptomic experiments. Briefly, 5mg muscle tissue from remaining biopsies was lysed in 200µL 0.6M perchloric acid and the supernatant was diluted 250-fold in 100mM Na2HPO4 pH 8.0. 100µL of diluted sample was combined with 100µL reaction mix (100mM Na2HPO4 pH 8, 2% ethanol, 90U/mL alcohol dehydrogenase, 130mU/mL diaphorase, 10µM resazurin, 10µM flavin mononucleotide, 10mM nicotinamide), and the fluorescence increase (Ex 540 nm/Em 580) was measured over 10 min. NAD+ content was calculated from a standard curve and normalized to tissue weight.

For optimization of NAD+ measurements and benchmarking using mass spectrometry, frozen muscle biopsies (~2-50 mg) independent of the study were extracted in 1300 µL of a cold mixture of methanol:water:chloroform in 5:3:5 (v/v). Extracts were spiked with 60 µL of [U]-13C-NAD+ labelled biomass from home-made yeast as internal standard, and kept cold throughout the procedure. Muscle extracts were homogenized with 3 mm tungsten carbide beads using a tissue mixer (Qiagen TissueLyser II) for 3 min at 20 Hz, followed by 20 min 1,500 rpm shaking at 4°C in a thermo-shaker (Thermomixer C, Eppendorf). Samples were then centrifuged 10 minutes 15,000 rpm at 4°C, and the upper polar phase was dried overnight in a vacuum centrifuge at 4°C and 5 mbar, and then stored at -80°C, before analysis. Dry samples were reconstituted in 50 µL 60% (v/v) acetonitrile:water, centrifuged for 2 minutes at 15,000 rpm, and the supernatant was transferred into a glass vial for hydrophilic interaction ultra high performance liquid chromatography mass spectrometry (UHPLC-MS) analysis, in a randomized order. The UHPLC consisted of a binary pump, a cooled autosampler, and a column oven (DIONEX Ultimate 3000 UHPLC+ Focused, Thermo Scientific), connected to a triple quadrupole spectrometer (TSQ Vantage, Thermo Scientific) equipped with a heated electrospray ionisation (H-ESI) source. 2 µL of each sample were injected into the analytical column (2.1 mm x 150 mm, 5 µm pore size, 200 Å HILICON iHILIC®-Fusion(P)), guarded by a pre-column (2.1 mm x 20 mm, 200 Å HILICON iHILIC®-Fusion(P) Guard Kit) operating at 35°C. The mobile phase (10 mM ammonium acetate at pH 9, A, and acetonitrile, B) was pumped at 0.25 mL/min flow rate over a linear gradient of decreasing organic solvent (0.5-16 min, 90-25% B), followed by re-equilibration for a total run time of 30 min. The MS operated in positive mode at 3500 V with multiple reaction monitoring (MRM) and in each scan event: scan width was 1 m/z and scan time was 0.05 s, peak with for Q1 was 0.25 FWHM and for Q3 0.70 FWHM. The sheath gas was 20 arbitrary units, and the auxiliary gas was kept 15 arbitrary units. The temperature of vaporizer was 280°C and the temperature of the ion transfer tube was 310°C. The tube lens voltage and collision energy were individually optimized for each fragment ion of NAD+ (664 > 428, 524). The software Xcalibur v4.1.31.9 (Thermo Scientific) was used for instrument control, data acquisition and processing. Positive ion mode extracted chromatograms using the MRM trace of NAD+ were integrated. A calibration curve of NAD+ (Sigma) with 10 data points between 0.62 and 40 µM was used for quantification of NAD+ in biological samples, normalized to internal standard. Dehydrated polar extracts were independently re-extracted for enzymatic NAD+ quantification using perchloric acid extraction as described above.

Data access & statistics. The unprocessed transcriptomic data of this study have been deposited in the Gene Expression Omnibus under accession numbers GSE111006, GSE111010, GSE111016 and integrated in the series GSE111017. These entries can be consulted for review purposes using the reviewer token wnebykuefdejnkv at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE111017. Statistical methods for transcriptomic experiments using 2-tailed statistics and correction for multiple testing are reported above. Data distributions were plotted as box-plots representing the 25th percentile (1Q), the median, and 75th percentile (3Q), with whiskers extending from the 1Q to the smallest value within 1.5*interquartile range (IQ=3Q-1Q) and from the 3Q to larger value within 1.5*IQ. Functional validation of hypotheses on mitochondrial function generated from the transcriptomic results were analyzed using one-tailed non-parametric (mitochondrial complex expression and activity) or parametric (NAD) statistics after assessing the distribution of the variables with a Shapiro-Wilk normality test. The datasets analysed during the current study are available from the corresponding authors on reasonable request. Due to ethical concerns, supporting clinical data cannot be made openly available. The MEMOSA team can provide the data on request subject to appropriate approvals, after a formal application to the Oversight Group of the different cohorts through their respective corresponding author.

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SUPPLEMENTARY TABLES

Table S1. Participant Characteristics.

	SSS (n=40*) **Mean** +/- **SD**	**HSS** (n=40) **Mean** +/- **SD**	**JSS** (n=40**) **Mean** +/- **SD**
Ethnicity	Chinese	Caucasian	Afro-Caribbean
Gender	Male	Male	Male
Age (years)	71.48 +/- 4.28	72.93 +/- 2.44	75.28 +/- 7.70
Height (m)	1.65 +/- 0.06	1.72 +/- 0.06	1.70 +/- 0.08
Weight (kg)	60.34 +/- 6.33	80.63 +/- 13.69	61.94 +/- 13.91
BMI (kg/m^2)	22.08 +/- 1.43	27.29 +/- 3.62	21.47 +/- 4.35
Total Lean body mass (DXA) (kg)	41.44 +/- 4.63	54.61 +/- 7.22	47.05 +/- 6.29
Fat Mass (DXA) (kg)	15.56 +/- 3.80	22.10 +/- 7.25	11.45 +/- 9.32
ALMi (kg/m^2)	6.81 +/- 0.75	7.93 +/- 0.90	7.48 +/- 0.81
Gait speed (m/s)	1.24 +/- 0.30	1.10 +/- 0.19	1.05 +/- 0.26
Grip strength (kg)	30.01 +/- 6.86	37.08 +/- 7.17	30 +/- 8.64

SSS: Singapore Sarcopenia Study, HSS: Hertfordshire Sarcopenia Study, JSS: Jamaica Sarcopenia Study.
BMI: Body mass Index, m/s: metres per second, ALMi: appendicular lean mass index.
*: one sample was discarded from RNAseq data analyses after quality control (fraction of uniquely mapped reads < 0.5)
**: one sample was excluded because of inadequate amount of RNA and RNAseq was not performed

Table S2. Case/control stratification of clinical variables in the 3 cohorts.

SSS	Control[1] (n=20)			Sarcopenic[2] (n=20)			
	Mean	+/-	SD	Mean	+/-	SD	p-value
Age (years)	70.20	+/-	4.10	72.75	+/-	4.18	N.S.
BMI (kg/m^2)	22.70	+/-	1.01	21.47	+/-	1.54	5.3E-03
Lean body mass (kg)	44.53	+/-	3.86	38.36	+/-	3.01	2.2E-06
Fat Mass (kg)	15.39	+/-	3.67	15.73	+/-	4.02	N.S.
ALMi (kg/m^2)	7.42	+/-	0.39	6.20	+/-	0.48	1.4E-08
Grip strength (kg)	35.11	+/-	5.93	24.91	+/-	2.56	2.0E-08
Gait speed (m/s)	1.40	+/-	0.23	1.09	+/-	0.29	6.3E-04

HSS	Control[3] (n=28)			Low Muscle Mass[4] (n=9)				Low Muscle Function[5] (n=7)				Sarcopenic[6] (n=4)			
	Mean	+/-	SD	Mean	+/-	SD	p-value	Mean	+/-	SD	p-value	Mean	+/-	SD	p-value
Age (years)	72.60	+/-	2.34	73.03	+/-	2.67	NS	74.91	+/-	2.69	NS	74.28	+/-	3.59	NS
BMI (kg/m^2)	27.93	+/-	3.84	24.89	+/-	1.71	2.5E-03	27.31	+/-	2.44	NS	26.43	+/-	1.37	NS
Lean mass (kg)	56.68	+/-	6.55	46.77	+/-	3.47	3.4E-06	51.52	+/-	7.96	NS	46.13	+/-	4.27	6.9E-03
Fat Mass (kg)	22.83	+/-	7.80	19.77	+/-	4.74	NS	22.94	+/-	6.05	NS	23.41	+/-	4.37	NS
ALMi (kg/m^2)	8.24	+/-	0.78	6.82	+/-	0.26	8.2E-10	7.44	+/-	0.95	NS	6.74	+/-	0.25	1.9E-06
Grip strength (kg)	39.93	+/-	5.12	30.78	+/-	6.74	3.2E-03	27.14	+/-	7.38	2.9E-03	25.50	+/-	6.61	0.019
Gait speed (m/s)	1.15	+/-	0.14	1.00	+/-	0.25	NS	0.92	+/-	0.28	NS	0.91	+/-	0.36	NS

JSS	Control[3] (n=15)			Low Muscle Mass[4] (n=14)				Low Muscle Function[5] (n=20)				Sarcopenic[6] (n=9)			
	Mean	+/-	SD	Mean	+/-	SD	p-value	Mean	+/-	SD	p-value	Mean	+/-	SD	p-value
Age (years)	75.60	+/-	7.81	76.64	+/-	7.10	NS	74.75	+/-	8.23	NS	76.78	+/-	7.85	NS
BMI (kg/m^2)	22.72	+/-	3.60	17.94	+/-	1.96	1.9E-04	21.57	+/-	4.82	NS	18.29	+/-	2.23	1.2E-03
Lean mass (kg)	49.54	+/-	7.42	42.62	+/-	3.82	4.4E-03	46.03	+/-	5.51	NS	42.04	+/-	4.44	5.2E-03
Fat Mass (kg)	12.4	+/-	10.4	6.30	+/-	5.18	NS	12.46	+/-	9.10	NS	7.28	+/-	6.09	NS
ALMi (kg/m^2)	8.07	+/-	0.62	6.66	+/-	0.37	1.1E-07	7.29	+/-	0.85	3.5E-03	6.58	+/-	0.42	5.6E-07
Grip strength (kg)	36.47	+/-	8.44	26.14	+/-	6.84	1.2E-03	24.60	+/-	5.92	1.0E-04	22.78	+/-	6.24	1.80E-04
Gait speed (m/s)	1.15	+/-	0.21	1.03	+/-	0.28	NS	0.95	+/-	0.28	0.021	0.98	+/-	0.30	NS

1: ALMi>7kg/m^2 and GripStrength≥26kg and GaitSpeed>0.8m/s
2: ALMi≤7kg/m^2 and (GripStrength>26kg or GaitSpeed≤0.8m/s) - AWGSOP consensus definition[1]
3: ALMi>7.23kg/m^2 and GripStrength>30kg and GaitSpeed>0.8m/s
4: ALMi≤7.23kg/m^2
5: GripStrength≤30kg or GaitSpeed≤0.8m/s
6: ALMi≤7.23kg/m^2 and (GripStrength≤30kg or GaitSpeed≤0.8m/s) – EWGSOP consensus definition[5]

Table S3- Table of RNAseq quality control in the SSS, HSS and JSS cohorts.

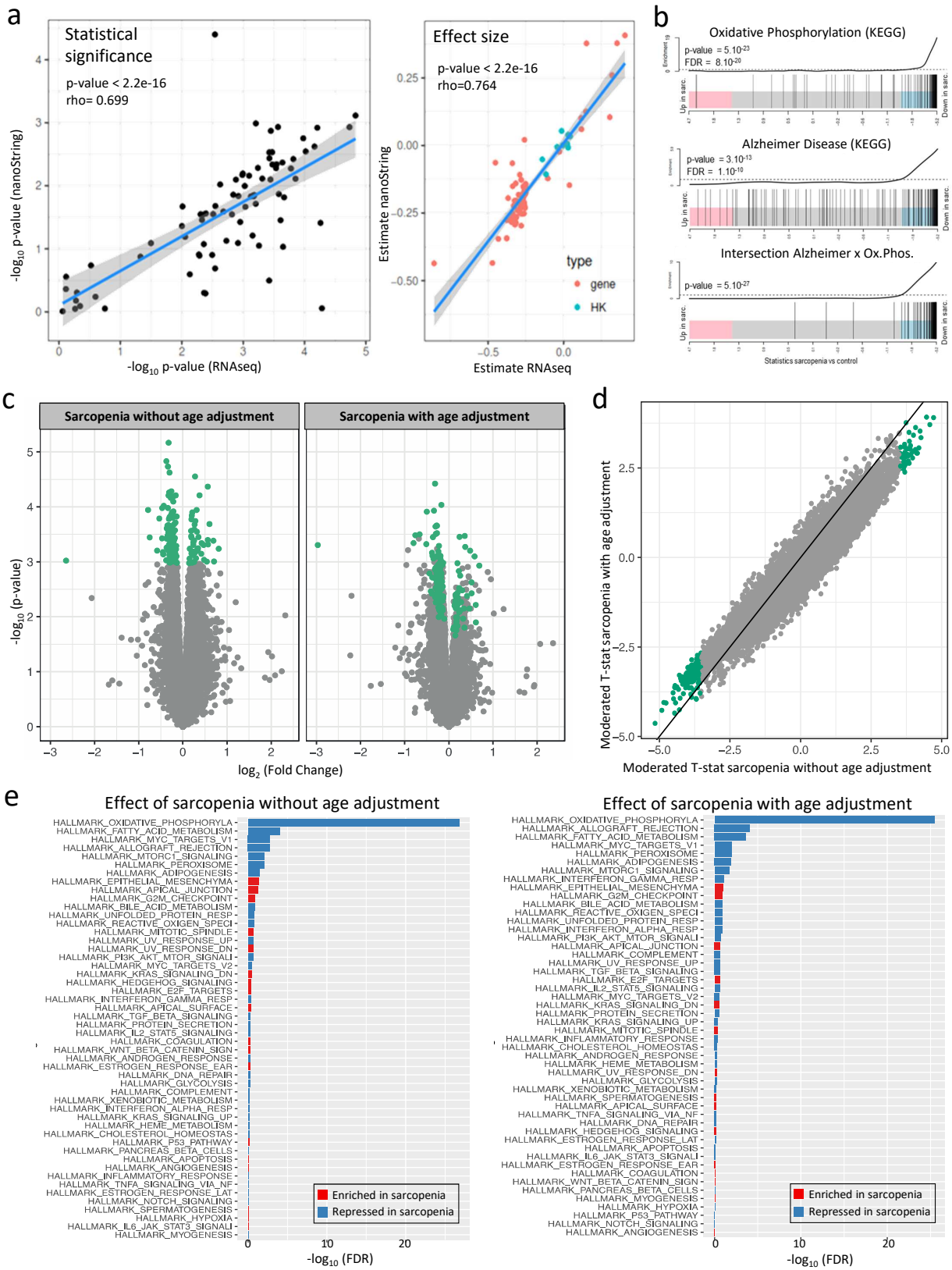
Table S4- List of individual gene expression by RNAseq in SSS. Different tabs represent the sarcopenic vs control differential expression, and the continuous association of gene expression with ALMi, grip strength and walking speed.

Table S5- List of nanoString vs RNAseq gene expression in SSS for genes selected for validation.

Table S6- List of individual gene set enrichment in the SSS pathway enrichment analysis. Different tabs represent the sarcopenic vs control enrichment using CAMERA, and the enrichment of the genes correlated with ALMi, grip strength and walking speed.

SUPPLEMENTARY FIGURES

Figure S1. Mitochondrial dysfunction is the major transcriptional signature of human sarcopenic muscle after age adjustment and with independent technical replication. (a) Correlation of RNA sequencing results with independent nanoString nCounter validation for the 80 genes measured with both technologies in human skeletal muscle. Statistics were computed using Spearman rank correlation. **(b)** Gene set enrichment plot of sarcopenic vs control muscle in SSS for KEGG oxidative phosphorylation, Alzheimer's disease and their intersection. The intersection of the 2 gene sets was extracted to demonstrate that the overlap between the transcriptional signatures of neurodegeneration and sarcopenia is driven by oxidative phosphorylation genes. **(c)** Volcano plot of differentially expressed genes in sarcopenic vs age-matched muscle for models without and with age adjustment as a covariate. p-values were calculated using moderated t-statistic. Genes with a FDR lower than 10% in the model without age adjustment are represented in green in both plots. **(d)** Scatter plot of the moderated t-values for each gene in the models without and with age adjustment. Genes with a FDR lower than 10% in the model without age adjustment are represented in green and the identity line is represented in black. **(e)** Gene set enrichment analysis of sarcopenic vs control muscle using models without and with age adjustment as a covariate, performed using CAMERA and the Hallmark gene set collection from MSigDB. Gene sets are ordered according to the significance of their enrichment. For all panels, n=39 SSS participant muscle samples.


a
Statistical significance
p-value < 2.2e-16
rho= 0.699
-log10 p-value (nanoString)
-log10 p-value (RNAseq)
Effect size
p-value < 2.2e-16
rho=0.764
Estimate nanoString
Estimate RNAseq
type
gene
HK
b
Oxidative Phosphorylation (KEGG)
p-value = 5.10-23
FDR = 8.10-20
Alzheimer Disease (KEGG)
p-value = 3.10-13
FDR = 1.10-10
Intersection Alzheimer x Ox.Phos.
p-value = 5.10-27
Up in sarc.
Down in sarc.
Statistics sarcopenia vs control
c
Sarcopenia without age adjustment
Sarcopenia with age adjustment
-log10 (p-value)
log2 (Fold Change)
d
Moderated T-stat sarcopenia with age adjustment
Moderated T-stat sarcopenia without age adjustment
e
Effect of sarcopenia without age adjustment
Effect of sarcopenia with age adjustment
HALLMARK_OXIDATIVE_PHOSPHORYLA
HALLMARK_FATTY_ACID_METABOLISM
HALLMARK_MYC_TARGETS_V1
HALLMARK_ALLOGRAFT_REJECTION
HALLMARK_MTORC1_SIGNALING
HALLMARK_PEROXISOME
HALLMARK_ADIPOGENESIS
HALLMARK_EPITHELIAL_MESENCHYMA
HALLMARK_APICAL_JUNCTION
HALLMARK_G2M_CHECKPOINT
HALLMARK_BILE_ACID_METABOLISM
HALLMARK_UNFOLDED_PROTEIN_RESP
HALLMARK_REACTIVE_OXIGEN_SPECI
HALLMARK_MITOTIC_SPINDLE
HALLMARK_UV_RESPONSE_UP
HALLMARK_UV_RESPONSE_DN
HALLMARK_PI3K_AKT_MTOR_SIGNALI
HALLMARK_MYC_TARGETS_V2
HALLMARK_KRAS_SIGNALING_DN
HALLMARK_HEDGEHOG_SIGNALING
HALLMARK_E2F_TARGETS
HALLMARK_INTERFERON_GAMMA_RESP
HALLMARK_APICAL_SURFACE
HALLMARK_TGF_BETA_SIGNALING
HALLMARK_PROTEIN_SECRETION
HALLMARK_IL2_STAT5_SIGNALING
HALLMARK_COAGULATION
HALLMARK_WNT_BETA_CATENIN_SIGN
HALLMARK_ANDROGEN_RESPONSE
HALLMARK_ESTROGEN_RESPONSE_EAR
HALLMARK_DNA_REPAIR
HALLMARK_GLYCOLYSIS
HALLMARK_COMPLEMENT
HALLMARK_XENOBIOTIC_METABOLISM
HALLMARK_INTERFERON_ALPHA_RESP
HALLMARK_KRAS_SIGNALING_UP
HALLMARK_HEME_METABOLISM
HALLMARK_CHOLESTEROL_HOMEOSTAS
HALLMARK_P53_PATHWAY
HALLMARK_PANCREAS_BETA_CELLS
HALLMARK_APOPTOSIS
HALLMARK_ANGIOGENESIS
HALLMARK_INFLAMMATORY_RESPONSE
HALLMARK_TNFA_SIGNALING_VIA_NF
HALLMARK_ESTROGEN_RESPONSE_LAT
HALLMARK_NOTCH_SIGNALING
HALLMARK_SPERMATOGENESIS
HALLMARK_HYPOXIA
HALLMARK_IL6_JAK_STAT3_SIGNALI
HALLMARK_MYOGENESIS
HALLMARK_ALLOGRAFT_REJECTION
HALLMARK_PEROXISOME
HALLMARK_MTORC1_SIGNALING
HALLMARK_INTERFERON_GAMMA_RESP
HALLMARK_REACTIVE_OXIGEN_SPECI
HALLMARK_UNFOLDED_PROTEIN_RESP
HALLMARK_INTERFERON_ALPHA_RESP
HALLMARK_PI3K_AKT_MTOR_SIGNALI
HALLMARK_APICAL_JUNCTION
HALLMARK_COMPLEMENT
HALLMARK_UV_RESPONSE_UP
HALLMARK_TGF_BETA_SIGNALING
HALLMARK_E2F_TARGETS
HALLMARK_IL2_STAT5_SIGNALING
HALLMARK_MYC_TARGETS_V2
HALLMARK_KRAS_SIGNALING_DN
HALLMARK_PROTEIN_SECRETION
HALLMARK_KRAS_SIGNALING_UP
HALLMARK_MITOTIC_SPINDLE
HALLMARK_INFLAMMATORY_RESPONSE
HALLMARK_CHOLESTEROL_HOMEOSTAS
HALLMARK_ANDROGEN_RESPONSE
HALLMARK_HEME_METABOLISM
HALLMARK_UV_RESPONSE_DN
HALLMARK_GLYCOLYSIS
HALLMARK_XENOBIOTIC_METABOLISM
HALLMARK_SPERMATOGENESIS
HALLMARK_APICAL_SURFACE
HALLMARK_TNFA_SIGNALING_VIA_NF
HALLMARK_DNA_REPAIR
HALLMARK_HEDGEHOG_SIGNALING
HALLMARK_ESTROGEN_RESPONSE_LAT
HALLMARK_APOPTOSIS
HALLMARK_IL6_JAK_STAT3_SIGNALI
HALLMARK_ESTROGEN_RESPONSE_EAR
HALLMARK_COAGULATION
HALLMARK_WNT_BETA_CATENIN_SIGN
HALLMARK_PANCREAS_BETA_CELLS
HALLMARK_MYOGENESIS
HALLMARK_HYPOXIA
HALLMARK_P53_PATHWAY
HALLMARK_NOTCH_SIGNALING
HALLMARK_ANGIOGENESIS
Enriched in sarcopenia
Repressed in sarcopenia
-log10 (FDR)

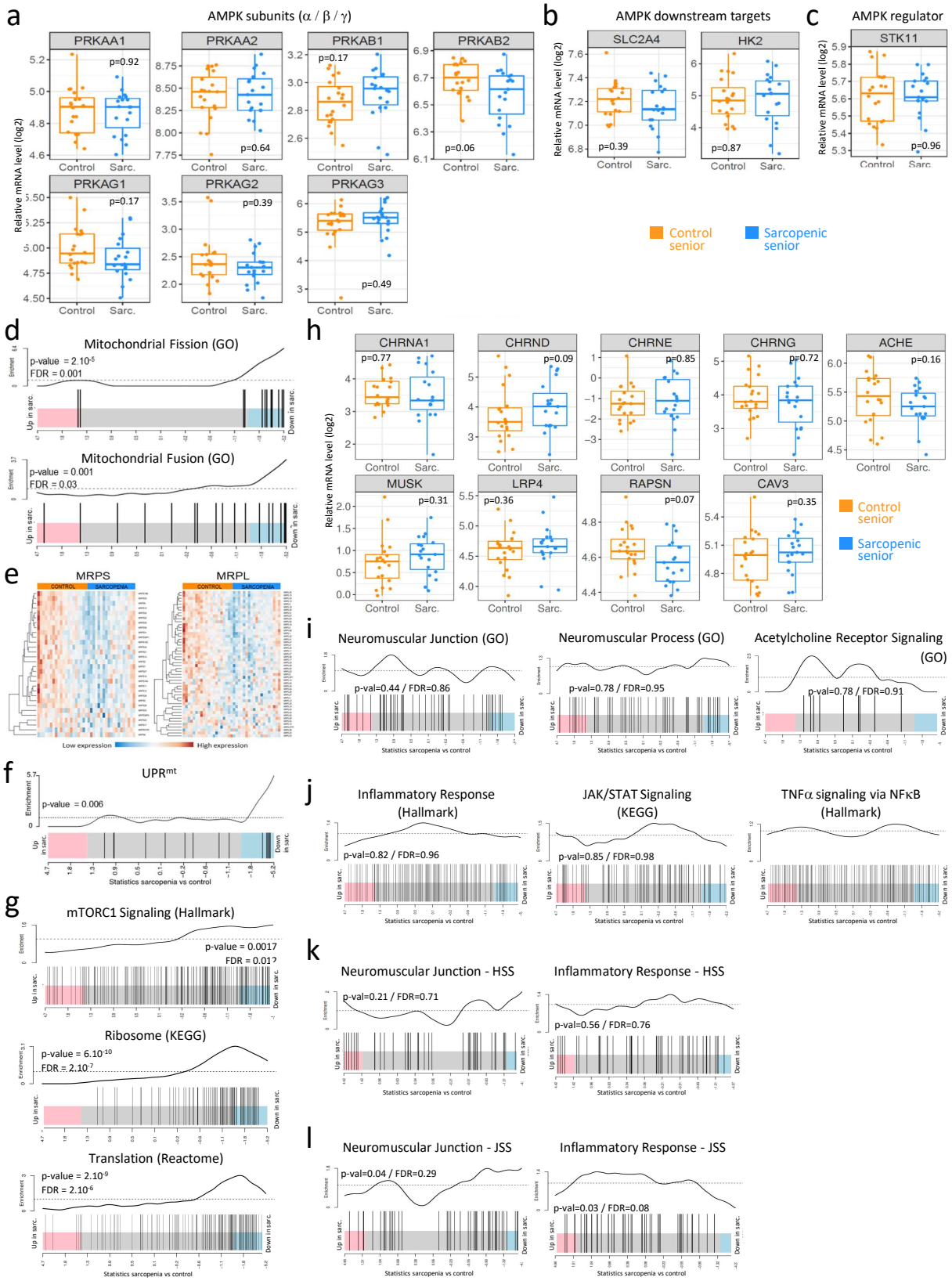


Figure S2. Muscle RNAseq reveals transcriptional changes in sarcopenia linked to mitochondrial function and protein synthesis, but does not detect transcriptional changes linked to neuromuscular dysfunction, denervation and inflammation. (a-c) mRNA expression of AMP-activated Protein Kinase (AMPK) subunits (a), of its downstream targets

Figure S2 (continued). Glut4/SLC2A4 and Hexokinase 2 (HK2) (b), and of its upstream regulator LKB1/STK11 (c), in SSS sarcopenic vs control muscle. Nominal moderated t-test p-values are reported. **(d;f-g;i-j)** Gene set enrichment plots of sarcopenic vs control muscle in SSS for mitochondrial dynamics (d), UPRmt (f), muscle protein synthesis (g), neuromuscular function (i) and inflammation (j). For panel f, a custom mammalian UPRmt gene set was used (see methods). **(e)** Heat maps of all genes coding small (MRPS) and large (MRPL) subunits of the mitochondrial ribosome in sarcopenic vs control muscle. **(h)** mRNA expression of genes controlling neuromuscular junctions in SSS sarcopenic vs control muscle. Nominal moderated t-test p-values are reported. **(k-l)** Neuromuscular junction and inflammatory response gene set enrichment plots of sarcopenic vs control muscle in HSS (k; n=32 HSS muscle samples) and in JSS (l; n=24 JSS muscle samples). In a-j, n=39 SSS participant muscle samples.

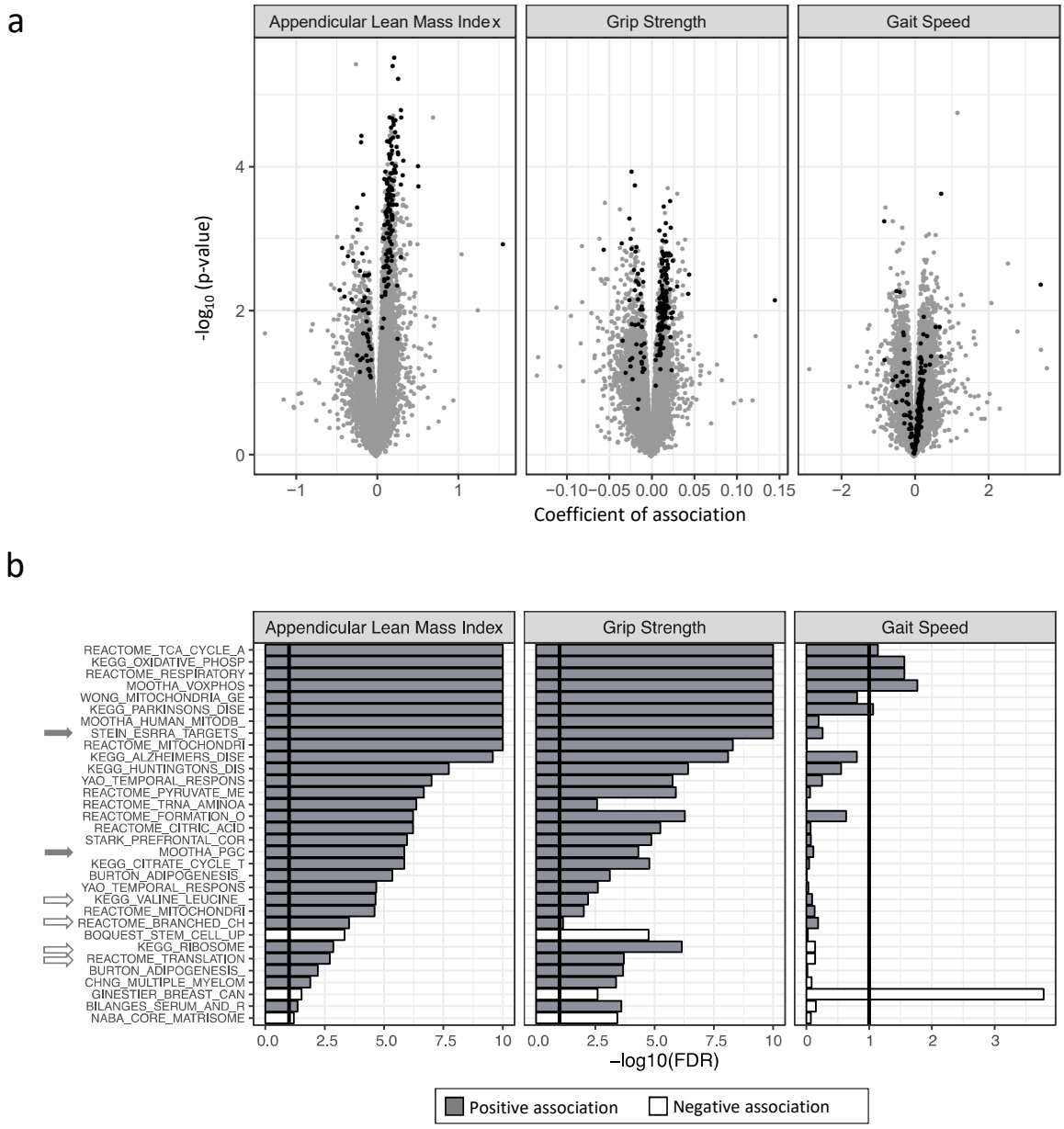


Figure S3. The transcriptional signature of sarcopenia in human muscle is mainly driven by the loss of appendicular lean mass index (ALMi) and grip strength. (a) Volcano plot of genes associated with ALMindex, grip strength and gait speed. p-values were calculated using moderated t- statistics and coefficients of association represent the $\log_2$ fold change of gene expression per unit of variable of interest. Genes which are differentially regulated with sarcopenia at a FDR<10% (Fig. 1a) are represented in black. **(b)** Gene set enrichment analysis of genes associated with ALMi, grip strength and gait speed using CAMERA and the C2 curated gene set collection from MSigDB. Gene sets are ordered according to the significance of their association with ALMi; only gene sets with an FDR<10% in at least one association and with a gene overlap <75% are represented. The significance threshold of 10% FDR is represented by black vertical lines and FDRs smaller than 10E-10 are trimmed at 10E-10. For all panels, n=39 SSS muscle samples.

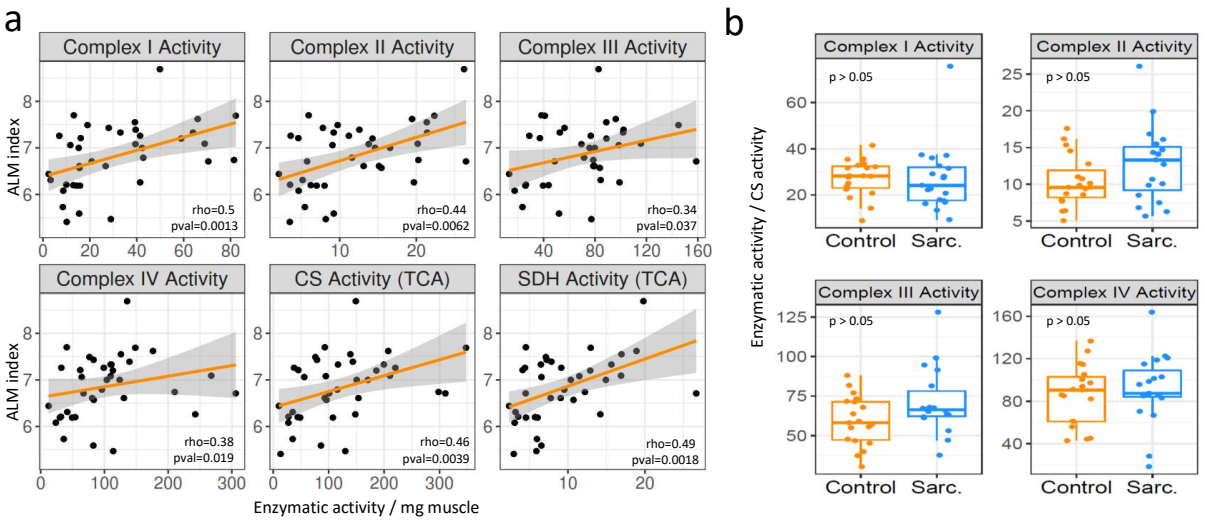


Figure S4. Mitochondrial activity in case/control and continuous measures of sarcopenia. (a) Mitochondrial enzymatic activities per mg of muscle are correlated to ALM index. Regression lines are represented in orange and their 95% confidence intervals in gray. n=38 SSS muscle samples. **(b)** Enzymatic activity of mitochondrial complexes I, II, III and IV normalized to Citrate Synthase (CS) on mitochondrial extracts from muscle biopsies of control and sarcopenic participants. n=38 SSS muscle samples.

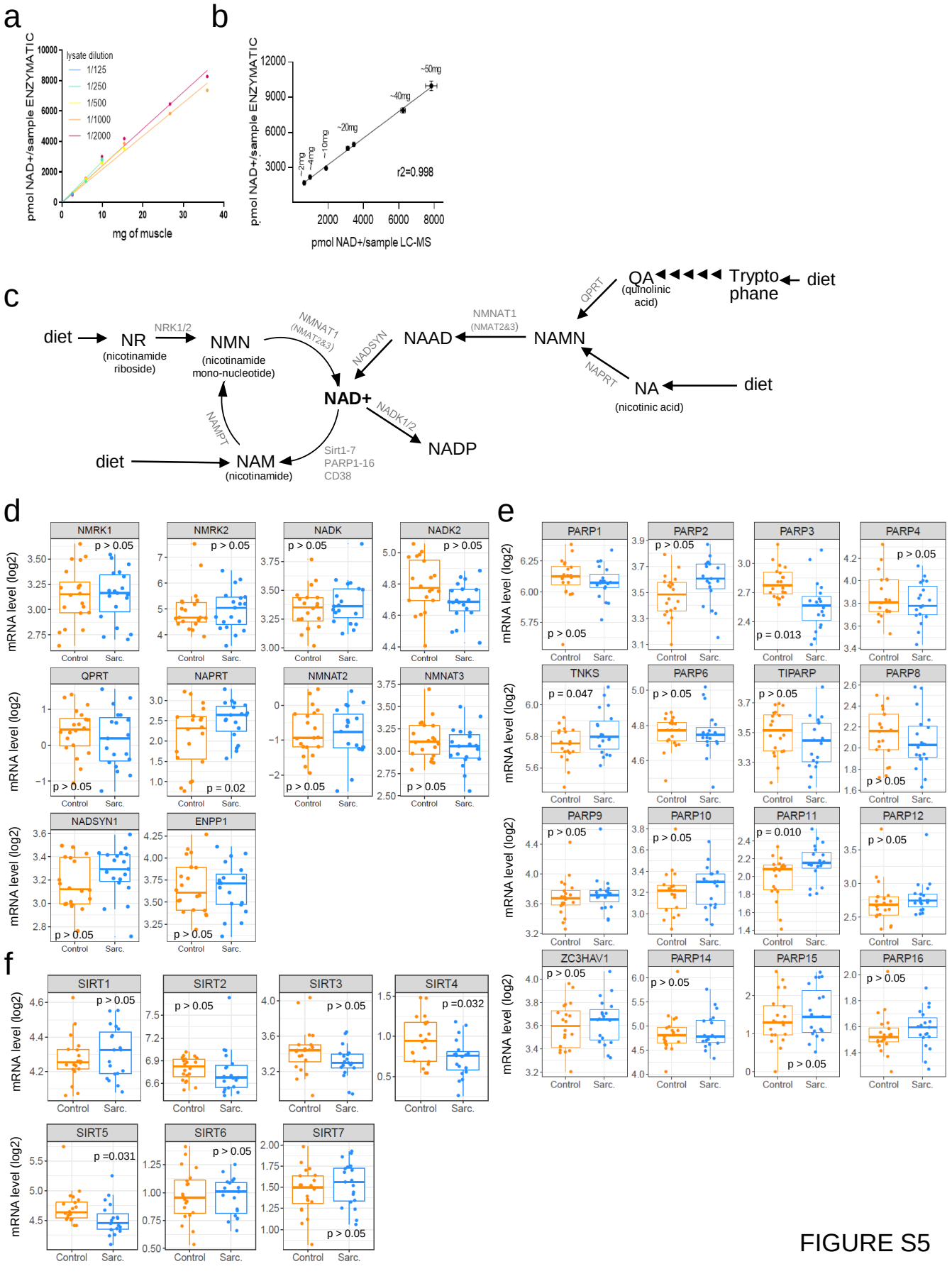

a
pmol NAD+/sample ENZYMATIC
lysate dilution
1/125
1/250
1/500
1/1000
1/2000
mg of muscle
b
pmol NAD+/sample ENZYMATIC
~2mg
~4mg
~10mg
~20mg
~40mg
~50mg
r2=0.998
pmol NAD+/sample LC-MS
c
diet
NR
(nicotinamide riboside)
NRK1/2
NMN
(nicotinamide mono-nucleotide)
NMNAT1 (NMAT2&3)
NADSYN
NAAD
NMNAT1 (NMAT2&3)
NAMN
QPRT
QA
(quinolinic acid)
Trypto phane
diet
NAPRT
NA
(nicotinic acid)
diet
NAD+
NADK1/2
NADP
NAMPT
diet
NAM
(nicotinamide)
Sirt1-7
PARP1-16
CD38
d
mRNA level (log2)
NMRK1
NMRK2
NADK
NADK2
QPRT
NAPRT
NMNAT2
NMNAT3
NADSYN1
ENPP1
p > 0.05
p = 0.02
Control
Sarc.
e
mRNA level (log2)
PARP1
PARP2
PARP3
PARP4
TNKS
PARP6
TIPARP
PARP8
PARP9
PARP10
PARP11
PARP12
ZC3HAV1
PARP14
PARP15
PARP16
p = 0.047
p = 0.013
p = 0.010
p > 0.05
f
mRNA level (log2)
SIRT1
SIRT2
SIRT3
SIRT4
SIRT5
SIRT6
SIRT7
p =0.032
p =0.031
p > 0.05
Control
Sarc.

FIGURE S5

Figure S5. NAD^+ pathway in case/control and continuous measures of sarcopenia. (a) The enzymatic quantification of NAD^+ is linear with different amounts muscle biopsy and different dilutions of muscle extracts. n=3 independent biopsies. **(b)** Enzymatic quantification of NAD+ tightly correlates to quantification of NAD^+ by liquid chromatography – mass spectrometry (LC-MS) on the same muscle extracts. n=3 independent biopsies. **(c)** NAD+ biosynthesis pathway. **(d-f)** mRNA expression of NAD^+ biosynthesis genes (d), PARPs (e) and sirtuin (SIRT, f) genes in SSS sarcopenic vs control muscle. Nominal moderated t-test p-values are reported using n=39 SSS muscle samples.

3 Nicotinamide riboside promotes benefits in sarcopenic muscle by overcoming a state of partial NAD^+ resistance

The following chapter describes the effect of aging on NAD^+ metabolism and transcriptional signaling, and reports technical challenges that emerged during sample analysis.

Personal contribution

I designed the acute NR study and conducted the *in vivo* work with the technical support of the animal research facility staff of the Nestle Research Center. I performed and interpreted all molecular experiments of this study and analyzed the results from the NR diet study performed by Paulina Cichosz (Figure 3.1), as well as the omics data produced by the NIHS genomics and metabolomics platforms. I wrote the manuscript under supervision of Jerome Feige.

Nicotinamide riboside promotes benefits in sarcopenic muscle by overcoming a state of partial NAD^+ resistance

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The co-factor nicotinamide adenine dinucleotide (NAD^+) is a major regulator of cellular metabolism that becomes a limiting factor in the course of natural aging. In mice, improving oxidative metabolism with the NAD^+ precursor nicotinamide riboside (NR) has been shown to revert age-related mitochondrial dysfunction and stem cell senescence in skeletal muscle. In this study, we investigated whether NR can be beneficial in the context of sarcopenia, the age-related loss of muscle mass and function. Chronic dietary supplementation and acute injection of NR efficiently increased NAD^+ levels in liver and skeletal muscle of young healthy and old sarcopenic rats. Metabolomics analyses revealed that old rats accumulated nicotinamide mononucleotide (NMN) in muscle tissue and exhibited increased levels of nicotinamide (NAM) excretion products after injection of NR. Transcriptional profiling of gastrocnemius muscle and liver revealed that the molecular response to NR was partially blunted in the old tissues. Nevertheless, NR was still effective in sarcopenic muscle where it normalized age-related changes in gene expression and reversed a transcriptional signature of pathological aging related to fibrosis. Altogether, our work demonstrates that aging leads to a state of partial NAD^+ resistance and uncovers novel transcriptional networks regulated by NR with potentially beneficial effects on sarcopenia.

KEYWORDS: sarcopenia; skeletal muscle; aging; nicotinamide riboside; NAD^+; fibrosis; transcriptomics; metabolomics

INTRODUCTION

Sarcopenia is the progressive age-related loss of muscle mass and function. Its pathogenesis is multifactorial, ranging from disuse to inflammation, oxidative stress, insulin resistance and loss of metabolic homeostasis, nutritional deficiencies, endocrine perturbations, and stem cell exhaustion (Lynch, 2011). Progressive atrophy and loss of motor units in combination with the infiltration of fat and non-contractile tissues lead to reduced muscle quality (Ryall et al., 2008). These changes in muscle architecture and fiber composition are caused by a multitude of cellular and molecular factors and impairments in energy metabolism and mitochondrial function have been proposed to play a key role (Short et al., 2005; Johnson et al., 2013).

NAD^+ is an essential molecule that participates in metabolic processes such as energy production, glycolysis, fatty acid and amino acid oxidation, where it acts as a co-factor or co-substrate for different classes of NAD^+-dependent enzymes, i.e. poly-ADP-ribose polymerases (PARPs), cADP-ribose transferases (i.e. CD38), and sirtuins (reviewed by Cantó et al. (2015)). Accordingly, NAD^+ deficiency has been connected to a number of conditions including aging and skeletal muscle loss (Braidy et al., 2011; Gomes et al., 2013; Khan et al., 2014; Zhang et al., 2016). In line with this, improving NAD^+ synthesis through supplementation of its vitamin B_3 precursor nicotinamide riboside (NR) was reported to ameliorate pathological muscle conditions, such as muscular dystrophy (Ryu et al., 2016; Zhang et al., 2016), mitochondrial myopathy (Khan et al., 2014), mitochondrial disease (Cerutti et al., 2014) or muscle stem cell (MuSC) senescence (Zhang et al., 2016). To test whether NR can be beneficial in the context of sarcopenia, we investigated the transcriptional regulation elicited by NR and its metabolization in sar-

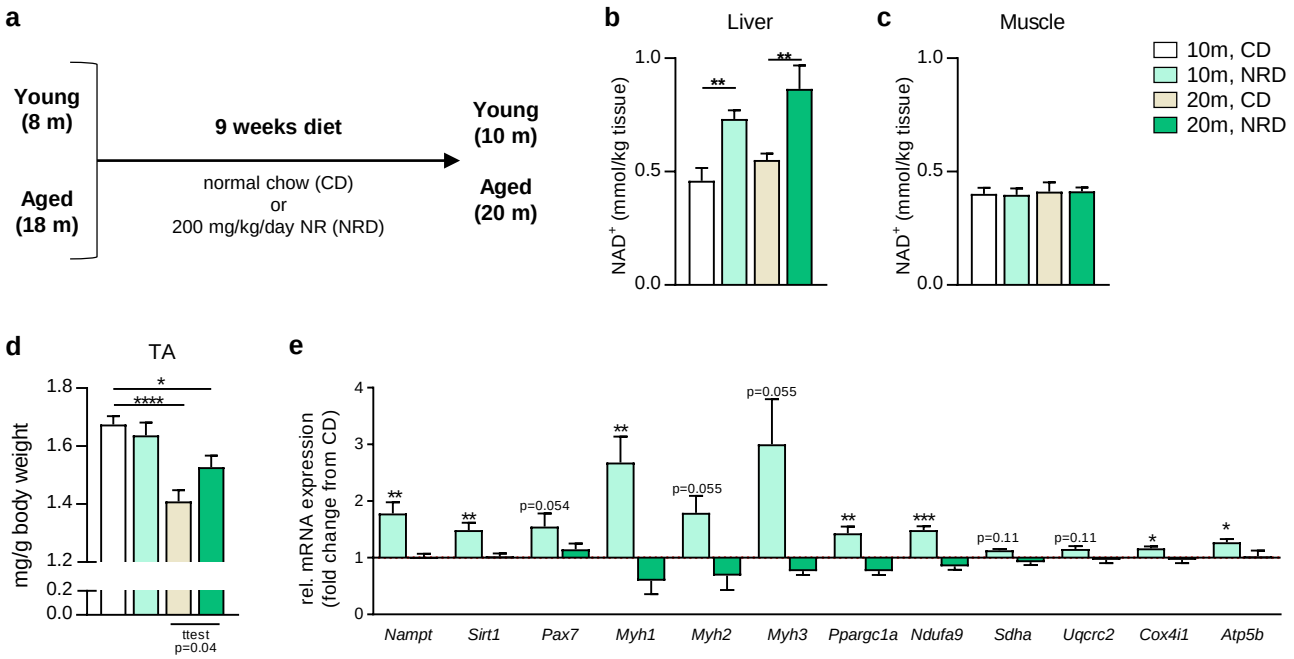


Figure 1: Tissue of aged rats is less responsive to dietary NR at the transcriptional level. **(a)** Rats were fed NR-supplemented chow for a duration of 9 weeks. NAD^+ values in **(b)** liver and **(c)** gastrocnemius muscle quantified by NAD^+ cycling assay. **(d)** Weight of tibialis anterior muscle normalized to whole body weight. **(e)** Relative mRNA levels expressed as fold change compared to respective control diet of each age group. Shown are selcted genes related to NAD^+ metabolism (*Nampt*, *Sirt1*), muscle physiology (*Pax7*, *Myh1*, *Myh2*, *Myh3*), and mitochondrial metabolism (*Ppargc1a*, *Ndufa9*, *Sdha*, *Uqcrc2*, *Cox4i1*, *Atp5b*). mRNA expression measured by qPCR relative to *Gapdh* as housekeeping gene. Results shown are mean +/-s.e.m. of n=9–11 rats. *p<0.05, **p<0.01, ***p<0.001 versus respective control diet of each age group, determined by **(b–d)** one-way ANOVA followed by Sidak's post-hoc test or **(d,e)** Student's t-test (gene expression changes in aged rats are all non-significant with p>0.2).

copenic rats.

RESULTS

Aging blunts the transcriptional response to NR

Young healthy (8 months old) and aged pre-sarcopenic (18 months old) male Wistar rats received 9 weeks of NR-enriched diet (**Fig. 1a**). At the end of the study, pre-sarcopenic rats did not display lower NAD^+ content of liver and skeletal muscle compared to young controls. The chronic supplementation of NR induced a similar extent of NAD^+ up-regulation in liver of young and aged rats (**Fig. 1b**). NAD^+ content of gastrocnemius muscle was not majorly affected by dietary NR (**Fig. 1c**), an effect that has already been observed in chronic long-term mouse experiments (Frederick et al., 2016). Nevertheless, we observed that rats from the NR-fed group were partially protected from muscle loss, while there was no effect on total body weight (**Fig. 1d** and data not shown). In young rats, dietary NR induced a transcriptional response with enhanced expression of several genes related to NAD^+ metabolism, muscle physiology, and mitochondrial function (**Fig. 1e**). As dietary NR reaches the muscle in form of NAM after conversion in the liver (Liu et al., 2018a), we observed up-regulation of NAM phosphorybosyl transferase *Nampt* in young animals (**Fig. 1e**). Albeit levels of NAD^+ remained unchanged, expression of the major NAD^+ consuming enzyme *Sirt1* was also induced (**Fig. 1e**). Regarding myogenic genes, expression levels of the stem cell marker *Pax7* as well as embryonic *Myh3* were elevated (**Fig. 1e**), in line with a previous study that reported increased number of MuSCs after 6 weeks of dietary NR (Zhang et al., 2016). Furthermore, up-regulation of *Myh1* and *Myh2* (encoding for MHC1 and MHC2a, respectively) suggests the induction of oxidative fibers (**Fig. 1e**), as those myosin heavy chain isoforms are predominantly expressed by fibers of type I (slow-twitch oxidative) and type IIa (fast-twitch oxidative-glycolytic), respectively. In line with this observation, in muscle of young rats we observed up-regulation of the transcriptional co-activator *Ppargc1a* (encoding for PGC1α) along with

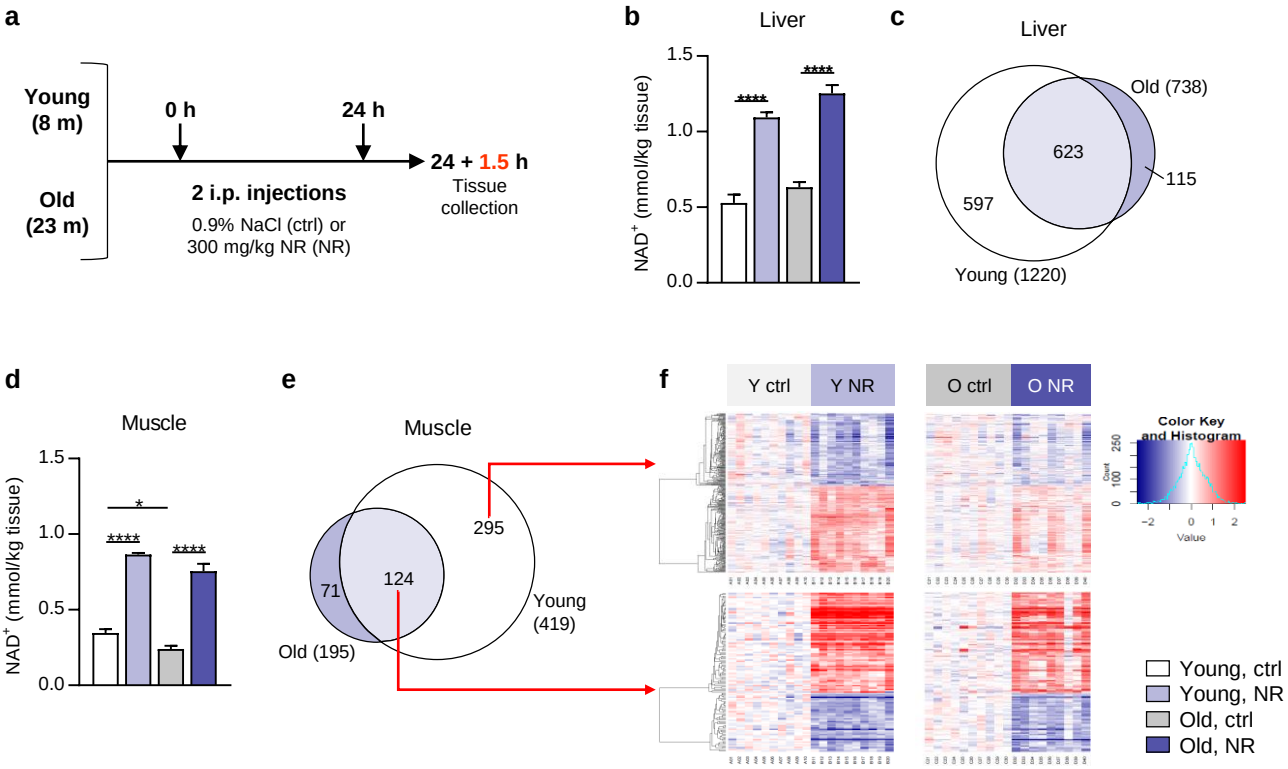
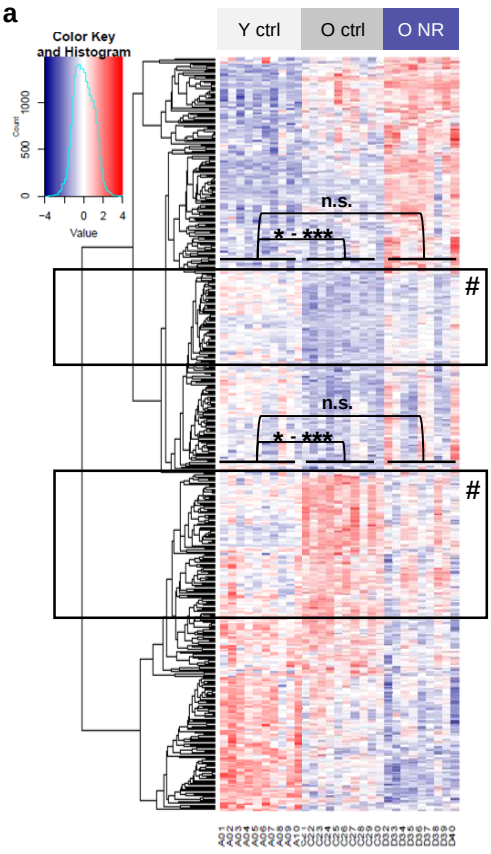


Figure 2: Aging does not impact the acute metabolization of NR to NAD$^+$, but old rats show a blunted response to injected NR. **(a)** Rats were injected i.p. 2 times within 24 h with either 0.9% NaCl (ctrl) or 300 mg/kg NR (NR). Tissues were collected 1.5 h after the second injection. **(b)** NAD$^+$ content of liver quantified by NAD$^+$ cycling assay. **(c)** Area-proportional Venn diagram showing genes differentially regulated after NR injection in liver of young (white) and old rats (purple). **(d)** NAD$^+$ content of plantaris muscle quantified by NAD$^+$ cycling assay. **(e)** Area-proportional Venn diagram showing genes differentially regulated after NR injection in gastrocnemius muscle of young (white) and old rats (purple). **(f)** Heatmap visualizing the fold change in young and sarcopenic muscle for 419 probe sets regulated by NR in young muscle only, centered to the respective control group. **(b,d)** Results shown are mean +/-s.e.m. of n=9–10 rats. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus young ctrl by one-way ANOVA followed by Sidak's post-hoc test. **(c,e,f)** mRNA expression measured by ILLUMINA microarray, genes differentially regulated based on FDR<5%, absolute log2FC>0.5, n=9–10

induction of representative genes of the five mitochondrial ETC complexes (complex I *Ndufa9*, complex II *Sdha*, complex III *Uqcrc2*, complex IV *Cox4i1*, and complex V *Atp5b*) (**Fig. 1e**). Notably, none of these genes were differentially expressed in muscle of aged rats receiving NR diet (p>0.2, **Fig. 1e**).

The absence of a transcriptional response of candidate genes to dietary NR in aged rats prompted us to test whether this observation may be linked to physiological adaptations during chronic treatment. Therefore, we performed an acute NR study and administered two intraperitoneal (i.p.) injections of NR within 24 hours to investigate the acute signaling induced in young healthy (8 months old) and old sarcopenic (23 months old) rats (**Fig. 2a**). Elevated NAD$^+$ levels in muscle and liver of NR-injected rats confirmed the systemic distribution of NR and its successful conversion to NAD$^+$ in the different tissues (**Fig. 2b,d**). Importantly, while old sarcopenic rats exhibited reduced muscle NAD$^+$ levels at baseline (**Fig. 2d**), age did not affect the conversion rate of NR to NAD$^+$ (**Fig. 2b,d**). In order to investigate the transcriptional response to NR injection, we performed microarray analyses on RNA extracted from total muscle and liver of these rats. Strikingly, the effect of NR was stronger in muscle and liver of young rats, with almost double as many probe sets differentially regulated compared to old animals (**Fig. 2c,e**). We observed that the majority of probe sets regulated in the old tissue overlapped with the response of young rats. To better understand our observations, we isolated the probe sets differentially expressed in young muscle only (419 gene sets, of which 124 overlap with old muscle) and visualized



b

GSEA gene set	size	FDR q-value
UP-regulated in SARCOPENIA (old vs. young)		
NEMETH_INFLAMMATORY RESPONSE LPS UP	73	0
JECHLINGER_EPITHELIAL TO MESENCHYMAL TRANSITION UP	61	0
PID_INTEGRIN1 PATHWAY	55	0
REACTOME_SIGNALING BY WNT	45	1.37E-05
LABBE_TGFB1 TARGETS UP	146	6.75E-05
KEGG_ECM RECEPTOR INTERACTION	64	7.89E-05
TIAN_TNF SIGNALING VIA NFKB	23	0.003
PID_ERBB1 DOWNSTREAM PATHWAY	78	0.003
PID_NOTCH PATHWAY	44	0.020
DOWN-regulated in SARCOPENIA (old vs. young)		
REACTOME_TCA CYCLE AND RESPIRATORY ELECTRON TRANSPORT	56	0.042
REACTOME_GLUCOSE METABOLISM	40	0.078

c

GSEA gene set	size	FDR q-value
UP-regulated in OLD NR (NR vs. ctrl)		
REACTOME_ACTIVATION OF NF KAPPAB IN B CELLS	43	2.87E-04
ST_ERK1 ERK2 MAPK PATHWAY	26	1.39E-03
ST_P38 MAPK PATHWAY	26	0.002
REACTOME_SIGNALING BY WNT	45	0.002
DOWN-regulated in OLD NR (NR vs. ctrl)		
KEGG_ECM RECEPTOR INTERACTION	64	1.09E-04
PID_INTEGRIN1 PATHWAY	55	0.002
PLASARI_TGFB1 SIGNALING VIA NFIC 10HR UP	39	0.002
REACTOME_TCA CYCLE AND RESPIRATORY ELECTRON TRANSPORT	56	0.004
JECHLINGER_EPITHELIAL TO MESENCHYMAL TRANSITION UP	61	0.060

Figure 3: Acute NR modulates the transcription of gene sets that are dysregulated in sarcopenic muscle. **(a)** Heatmap displaying hierarchical clustering results of all 490 probe sets with differential expression in gastrocnemius muscle of young and old rats after NR injection. Used algorithm: average linkage; distance: 1-correlation. n=9-10. # - Highlighted in the box are gene sets that show a change towards a healthy transcriptional phenotype in sarcopenic muscle treated with NR. #p>0.2 in old NR versus young ctrl. **(b-c)** Representative gene sets regulated in sarcopenic muscle (gastrocnemius) **(b)** before and **(c)** after NR injection. Gene set enrichment analysis of MSigDB curated gene sets c2v5.1.

the expression levels as a function of change from the respective control groups (**Fig. 2f**). Upon investigation of the overlapping subset of genes (124 gene sets, **Fig. 2f** bottom), we could appreciate that transcriptional changes induced by NR displayed the same directionality in young and old muscle. As for the 295 genes exclusively regulated in young muscle (**Fig. 2f** top), we observed that NR induced subtle regulation of the same genes in the old muscle, following a similar trend as seen in the young. However, the responsiveness of old muscle towards NR was comparatively low and rather heterogeneous, an observation that was not correlated to individual NAD^+ levels (data not shown). Of note, the 3 old rats featuring the strongest transcriptional responses were also the ones that displayed the highest gastrocnemius muscle mass within their group (data not shown).
Taken together, our data shows that aging does not affect the conversion of NR to NAD^+. However, aging leads to a state of partial NAD^+-resistance, since only part of the transcriptional response induced by NR is preserved in old rats.

Acute NR modulates the transcription of gene sets that are dysregulated in sarcopenic muscle

A central question we aimed to address was whether NR has the potential to revert sarcopenia-related changes in gene expression in muscle. 23 months old rats exhibited a sarcopenic phenotype in their hindlimb muscles, evaluated by lower muscle mass, lower muscle function, and transcriptional profiles of sarcopenia-specific genes (**Fig. S1a–c**).
At the gene expression level, we identified a subset of probe sets that was dysregulated in sarcopenic muscle and whose expression levels were rescued after NR administration (**Fig. 3a**). The heatmap in **Fig.**

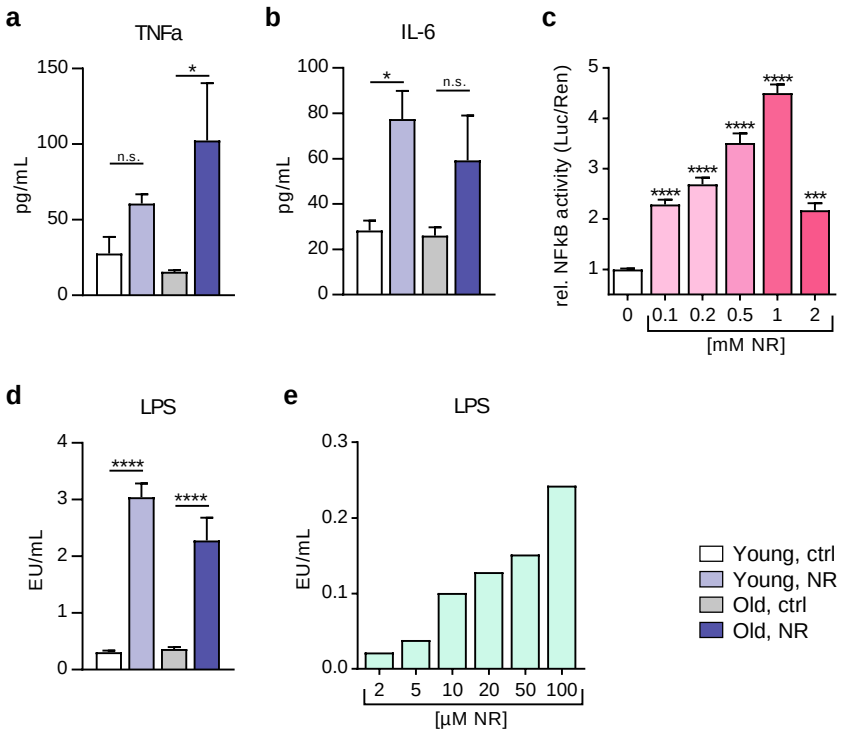


Figure 4: NR-induced activation of inflammatory response might be a consequence of endotoxin contamination. Cytokine levels of **(a)** TNFα and **(b)** IL-6 measured by ELISA in rat plasma. **(c)** Activation of NFκB transcriptional activity measured by gene reporter assay in C2C12 cells after 6 h of treatment. **(d)** Concentration of lipopolysaccharides in rat serum measured by endotoxin assay, expressed as endotoxin units (EU) per mL. **(e)** Concentration of lipopolysaccharides in a solution of NR in water measured by endotoxin assay, n=1 per dilution. Results shown are mean +/-s.e.m., *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by one-way ANOVA followed by Sidak's post-hoc test.

3a displays all 490 probe sets with differential expression in gastrocnemius muscle of young and old rats after NR injection. We established that approximately half of those genes (162 in young, 86 in old, of which 51 overlap) were also regulated in old sarcopenic compared to young healthy muscle. Highlighted are specific subsets of genes sets that NR regulated in sarcopenic muscle to approach expression levels similar to healthy untreated animals (expression old NR vs young ctrl not different with FDR q>0.2). This visualization indicates that NR treatment reverted certain sarcopenia-related expression signatures to the levels observed in healthy control animals (**Fig. 3a**).

Gene set enrichment analysis (GSEA) of microarray data from gastrocnemius highlighted that sarcopenic muscle exhibited an up-regulation of pathways mainly related to inflammation (i.e. "inflammatory response to LPS", "TNF signaling via NFκB") and fibrosis (i.e. "epithelial to mesenchymal transition", "integrin 1 pathway", "signaling by Wnt", "TGFB1 targets", "ECM receptor interaction" (Biressi et al., 2014; Stearns-Reider et al., 2017)). Down-regulated genes were enriched in pathways related to energy metabolism (i.e. "TCA cycle and respiratory electron transport", "glucose metabolism") (**Fig. 3b**). Importantly, NR injection inverted the age-related "dysregulation" of a subset of these pathways, particularly gene sets related to fibrosis (**Fig. 3c**). To simplify our approach and reduce redundancy between gene sets, we performed an additional GSEA on the "Hallmark" gene set collection, a highly curated collection of 50 major pathways (Liberzon et al., 2015). This allowed us to identify up-regulation of superordinate pathways such as "DNA repair" and "Unfolded protein response (ER)", stress response pathways considered to be protective in sarcopenic muscle (Estébanez et al., 2018). Notably, NR injection intensified the expression of gene sets mapped to those pathways (**Fig. S1e,f**).

Overall, our data show that two doses of NR modulate –and even normalize– the transcription of gene sets observed to be deregulated during sarcopenia.

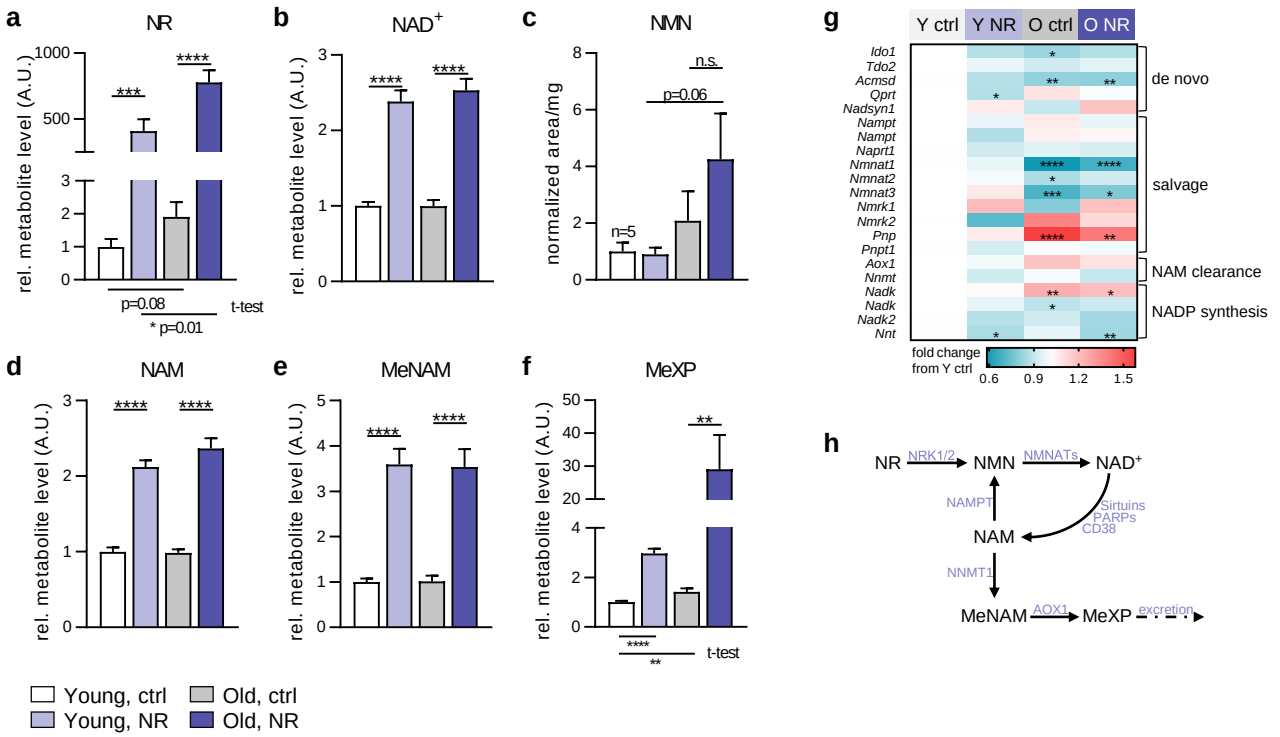


Figure 5: NR injection acutely elevates the skeletal muscle NAD$^+$ metabolome and enhances NAM excretion in old muscle. **(a-f)** Qualitative analysis of NAD$^+$ metabolites measured by LC MS/MS in gastrocnemius muscle (area normalized to an internal standard and mg protein per sample). **(g)** Heatmap displaying relative mRNA expression of NAD$^+$ pathway related genes measured by microarray. Colors indicate fold change from expression levels in young control, blue/red displays down-regulation/up-regulation. **(h)** Main NAD$^+$ biosynthesis and consumption pathways in skeletal muscle. Results shown are mean +/-s.e.m., *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus young ctrl by one-way ANOVA followed by Sidak's post-hoc test, n=10.

LPS contamination of NR activates inflammatory response

Interestingly, one of the most up-regulated transcriptional signatures in our microarray analyses following NR treatment was the NFκB signature, regardless of age or tissue (**Fig. 3c**). This was particularly intriguing as other studies had rather reported an anti-inflammatory effect of NR (Lee et al., 2015; Hong et al., 2018). Upon further investigation, we confirmed a strong induction of the pro-inflammatory cytokines TNFα and IL-6 in the plasma of injected rats (**Fig. 4a,b**). In order to test whether this effect was caused by activation of NFκB, we performed an *in vitro* luciferase gene reporter assay using C2C12 mouse myoblasts. The results from this assay illustrated that NR induced NFκB transcriptional activity in a dose-dependent manner (**Fig. 4c**). Based on all these observations, we proceeded to explore the possibility of a contamination with endotoxins or lipopolysaccharides (LPS), respectively, using a limulus amebocyte lysate (LAL) assay. Regrettably, serum of NR-injected rats tested positive for LPS (**Fig. 4d**). Finally, detection of LPS in NR diluted in endotoxin-free water confirmed that our observations were specific to NR and not NAD$^+$ dependent (**Fig. 4e**).

Importantly, we did not observe any signs of inflammation or sepsis, neither transcriptional up-regulation of NFκB when NR was administered through the diet (data not shown).

Enhanced abundance of NAM excretion products in old rats

In order to investigate whether aging affects the metabolization of NR and NAD$^+$, we next performed metabolomics analyses of NAD$^+$ metabolites extracted from muscle, liver and plasma of NR-injected rats. We found that NR injection acutely elevated a wide range of metabolites across tissues (**Fig. 5** and **S2**). At baseline, sarcopenic muscle tissue appeared to accumulate more NR compared to young muscle. NR injection resulted in a vast increase in NR (400-fold from ctrl group), with similar up-regulation in

both age groups (**Fig. 5a**). Increases in NAD^+ after injection confirmed that aging does not impact NR-induced up-regulation of NAD^+ (**Fig. 5b**), corroborating our previous results from enzymatic measurements (**Fig. 2d**). Muscle NMN levels were difficult to detect and approached the lower detection limit for young muscle samples. In aged muscle, NMN was more readily detected and increased after injection of NR (**Fig. 5c**). In line with these observations, our transcriptomics data showed age-related down-regulation of Nmnat1-3 which was not changed after NR injection (**Fig. 5g**), suggesting that NMN accumulates in aged muscle. We further found increased levels of NAM and its methylation product MeNAM in muscle of injected rats. Interestingly, MeXP (sum of Me2PY and Me4PY (1-methyl-2-pyridone-5-carboxamide and 1-methyl-4-pyridone-5-carboxamide), the final oxidation products of NAM) was exclusively elevated in old muscle after NR injection, a metabolic signature that we also observed in liver and plasma of old rats (**Fig. 5f** and **S2**).

Altogether, our data highlight that exogenous NR acutely elevates the skeletal muscle NAD^+ metabolome in young and old rats. Accumulation of NR and NMN in old muscle and transcriptional down-regulation of NMNATs suggests age-related alterations in NAD^+ salvage that might favor NAM excretion in old rats.

DISCUSSION

To better understand the physiological and therapeutic implications of boosting NAD^+ metabolism in sarcopenic muscle, we investigated the effect of NR supplementation on skeletal muscle of rats, an animal model that naturally develops a sarcopenic phenotype with aging (Ibebunjo et al., 2013; Pannérec et al., 2016).

Young adult and aged pre-sarcopenic rats received NR-supplemented chow for a duration of 9 weeks. First of all, we could detect that chronic NR supplementation induced NAD^+ levels in liver tissue, but muscle NAD^+ remained stable. Similar observations had been previously reported for chronically supplemented mice and it had been consequently shown that orally administered NR enters the muscle in form of NAM, a less potent NAD^+ precursor (Frederick et al., 2016; Liu et al., 2018a). Despite the absent influence of NR on muscle NAD^+ levels, we observed that NR supplementation led to a partial protection from age-associated muscle loss in the aged pre-sarcopenic rats. It would have been intriguing to investigate a potentially preventative effect of NR on additional physiological parameters in this study, but regrettably, the 20 months old rats did not yet display any defects in muscle function, fiber morphology or the transcriptional signature of sarcopenia markers (data not shown).

In muscle of young rats, chronic dietary supplementation induced up-regulation of genes related to oxidative metabolism and oxidative fiber type. This is in line with previous studies that reported increases in mitochondrial function and gene expression as the primary mechanism of action for NR in different animal disease models (Cantó et al., 2012; Cerutti et al., 2014; Zhang et al., 2016). As our data is restricted to enzymatic NAD^+ measurements, we cannot exclude that dietary NR effectuated a change in abundance of other NAD^+ metabolites, which may have the potential to modulate the cellular metabolism independent of NAD^+ itself.

Nevertheless, one striking observation from this experiment was that in contrast to young rats, gene expression in aged muscle was minimally affected by dietary NR, indicating a possible NR/NAD^+ resistance at the transcriptional level. Therefore, we next tested whether aged muscle indeed responds differently to NR. To overcome the limitations of chronic dietary NR supplementation on the NAD^+ boosting in muscle, we designed a study that investigated the acute transcriptional signaling induced by NR administered by intraperitoneal. injections in young adult and old sarcopenic rats. The acute setting helped us to verify that old rats are not deficient in upregulating NAD^+ after exogenous precursor administration, and microarray transcriptomics analyses confirmed our initial observations on a larger scale: young rats were more responsive to exogenous NR, regulating approximately double as many gene sets compared to old rats. Moreover, this observation was not restricted to skeletal muscle, as similar results were obtained for transcriptome analysis of the liver.

A key focus of this work was to determine whether NR has the potential to revert sarcopenia-related changes in gene expression. Gene set enrichment analysis (GSEA) of sarcopenic muscle from old rats revealed a signature of upregulated fibrosis, a key hallmark of skeletal muscle aging (Serrano and Muñoz-Cánoves, 2010). Despite the lower respon-

siveness of aged muscle, two injections of NR efficiently reverted this signature and induced downregulation of the respective pathways. Recently, NR was reported to protect from CCl_4- and also diet-induced liver fibrosis in mice (Jiang et al., 2019; Pham et al., 2019) Furthermore, it was shown that NR alleviates fibrosis and improves muscle regeneration through rejuvenation of MuSCs in old as well as *mdx* mice (model of Duchenne Muscular Dystrophy with increased fibrosis) (Zhang et al., 2016). Here we show that NR modifies fibrotic signaling on the whole-muscle level, a regulatory mechanism that could also positively influence the stem cell niche as previously reported. Interestingly, downregulation of the same pathways was also observed in muscle of young rats injected with NR, indicating that early treatment might have the potential to protect rats from fibrosis during aging.

Up-regulation of stress response pathways such as DNA repair and endoplasmatic reticulum (ER) unfolded protein response (UPR) is a consequence of cellular perturbations in aged tissue. DNA repair can be positively regulated during states of high NAD^+ availability through activation of NAD^+-dependent PARPs (Zhang et al., 2014). UPR(ER) has been reported to be activated during exercise (Estébanez et al., 2018), a state also characterized by increased NAD^+ levels (Cantó et al., 2010). Accordingly, NR injection further stimulated the activation of those pathways, highlighting the versatile action spectrum of NR.

Other pathways enriched in sarcopenic muscle demonstrated increased inflammation and decreased energy and mitochondria metabolism. To our surprise, NR injection intensified the transcriptional dysregulation of those pathways, led to increased inflammation and decreased energy metabolism. Interestingly, similar findings were obtained in a study in healthy rats that reported disturbed energy and redox metabolism and impaired exercise performance after NR supplementation (Kourtzidis et al., 2018). Likewise, a first study in humans reported downregulation of mitochondrial pathways, but anti-inflammatory effects of dietary NR (Elhassan et al., 2019). In hematopoietic stem cells, NR was observed to induce down-regulation of mitochondrial pathways through induction of mitochondrial clearance (Vannini et al., 2019). However, we did not observe any up-regulation of autophagy or mitophagy in our dataset and mitochondrial protein expression was not changed (data not shown). Moreover, this mechanism appears to be rather tissue specific and in contrast to our previous results and other studies in muscle that observed enhanced mitochondrial function upon NR treatment (Cantó et al., 2012; Khan et al., 2014). A perhaps more likely possibility would be excessive PARP activation in response to the acute increase in NAD^+. In fact, our transcriptomics data suggests up-regulation of different PARP family members during aging (data not shown). The higher baseline expression might prime the old tissue to favor NAD^+ utilization by PARPs. Moreover, PARPs were also reported to be activated in a DNA-independent manner through phosphorylation by ERK1/2 (Kauppinen et al., 2006), a pathway that is upregulated during oxidative stress and was also upregulated by NR in our GSEA. PARP1 has been reported to inhibit the rate-limiting glycolytic enzyme hexokinase, thus leading to energetic collapse and mitochondrial defects (Andrabi et al., 2014). A rapid acceleration of PARP catalytic activity would also deplete NAD^+ and increase NAM, which reduces SIRT1 activity and abolishes its beneficial effects on oxidative metabolism (Pillai et al., 2005; Bai et al., 2011).

Interestingly, PARP1 and SIRT1 are positive and negative regulators of NFκB, respectively (Yeung et al., 2004; Altmeyer and Hottiger, 2009). Induced PARP1 and reduced SIRT1 activity could explain an induction of inflammatory pathways after NR injection. However, the transcriptional signature of inflammation was very strong and detection of increased cytokine levels (TNFα, IL-6) as well as LPS in the blood of treated rats indicated that the response was a direct reaction to the injected compound itself. We further established that the LPS present in the NR induced NFκB transcriptional activity *in vitro*, and therefore cannot exclude the possibility that NR elicited a mild systemic inflammatory response in injected rats. Our blood analysis showed an approximately 10-fold induction of detected endotoxin units in treated animals. During acute sepsis, the increase in endotoxin concentrations can vary but is generally stronger than what we observed (up to several 1000-fold during extreme sepsis) (Pearson et al., 1985; Ikeda et al., 1987; Breuhaus and DeGraves, 1993). Nevertheless, we acknowledge the possibility that the inflammatory response elicited by NR injection might have affected results obtained from these animals, especially in regard of mito-

chondrial dysfunction, which is induced during activation of the inflammasome (Liu et al., 2018b) or can result from generation of nitric oxide during sepsis (Singer and Brealey, 1999; Arulkumaran et al., 2016). Most importantly, rats having received the same NR orally did not show any overt signs of inflammation and/or dysregulation of inflammatory or mitochondrial genes, suggesting that the endotoxin burden in this setting was marginal.

Similar to previous studies (Gomes et al., 2013) and our observations from humans (Chapter 2), we observed decreased muscle NAD^+ levels in sarcopenic muscle. Nevertheless, we could show that young and old rats are equally able to increase NAD^+ levels after NR administration. However, the extent at which rates of NAD^+ generation and consumption contribute to this increase might differ with age. Metabolomics analysis of muscle, liver and plasma showed that i.p. administered NR was distributed through circulation and that muscle experienced the highest increase in NR content. This is in accordance with NAD^+ tracer flux analyses that established that intravenously administered NR is readily taken up by skeletal muscle (and kidney) and disappears rapidly from circulation, as it quickly degrades into NAM, which is the preferred precursor for liver (Liu et al., 2018a). Accordingly, we observed that injected rats exhibited a strong increase in plasma NAM levels. Accumulation of NMN in sarcopenic muscle suggests that reduced NAD^+ might be a result of insufficient NMNAT activity. This could also explain the observed tendency to increased NR content in sarcopenic muscle, indicating that during aging, NMNAT might become a rate-limiting enzyme for NAD^+ biosynthesis. A reduced rate of NMN salvage might further stimulate NAM excretion as a means to limit NAM accumulation (and the consequent inhibition of NAD^+ consuming enzymes).

Recent literature suggests that NAM excretion is an important factor to be taken into account for new therapeutic strategies of boosting NAD^+ (Pissios, 2017). First human data shows that dietary NR increases the abundance of NAM excretion products in skeletal muscle, blood and urine (Trammell et al., 2016; Dollerup et al., 2018; Conze et al., 2019; Elhassan et al., 2019). In aged human muscle, *Nnmt* mRNA expression (Nictotinamide N-methyltransferase, methylates NAM to MeNAM) was reported to be up-regulated (Giresi et al., 2005). Increased NNMT protein was further observed in a recent mouse study proving that treatment with a NNMT inhibitor improves regenerative activity of aged MuSCs through modulation of cellular NAD^+/NADH redox states (Neelakantan et al., 2019). Albeit this mechanism might play a role during muscle regeneration, in unchallenged rats we did not observe transcriptional differences in Nnmt expression in any of our groups. Furthermore, increases in MeNAM were similar in young and old mice. However, one of our major observations from the metabolomics analysis was the pronounced increase in the MeNAM oxidation product MeXP exclusively in tissues of old rats treated with NR, suggesting either a defect in MeXP excretion or increased NAM clearance. The oxidation of MeNAM to MeXP by AOX1 involves the generation of reactive oxygen species (ROS) (Schmeisser et al., 2013). Moderate activation of oxidative stress might be beneficial in the context of aging as it has been shown to increase life span in *C. elegans* and other organisms (Ristow and Zarse, 2010; Schmeisser et al., 2013). On the other hand, over-activation of AOX1 and excessive ROS generation might lead to increased oxidative damage of cellular components such as DNA, which in turn activates PARP activity (Altmeyer and Hottiger, 2009; Zhang et al., 2014). Based on all this evidence, it is tempting to speculate that NAM excretion might be favored in old animals at baseline but leads to excessive ROS generation acutely after NAD^+ boosting.

Collectively, our data show that aging leads to a state of decreased transcriptional sensitivity towards exogenous NR, suggesting a differential use of the generated NAD^+ in tissue of old animals. Nevertheless, NR was still effective in old muscle where it normalized age-related changes in gene expression and reversed a transcriptional signature of aging related to fibrosis. In conclusion, our work uncovers novel transcriptional networks regulated by NR with potentially beneficial effects on sarcopenia.

METHODS

Animal work. All animal experiments were carried out according to Swiss and EU ethical guidelines and approved by the local animal experimentation committee of the Canton de Vaud under license number VD 2764. Male Wistar rats were obtained from Janvier Labs (Le Genest-Saint-Isle, France). Animals were housed by two in standard type 4 cages in a standard temperature- and humidity-controlled environment with a 12:12 h light:dark cycle. Animals had access to nesting material and were provided with *ad libitum* water and standard animal chow diet unless otherwise specified. For the dietary supplementation study, pellets were prepared by mixing pow-

der diet (ResearchDiets, D12450JMi) with water alone (control diet, CD) or water containing NR (NR diet, NRD, Biosynth) as previously described (Feige et al., 2008). Rats received 200 mg NR per kg bodyweight per day, diet was changed every 2–3 days. For the acute NR study, rats were injected intraperitoneally (i.p.) with 500 mg NR per kg bodyweight. Two injections were timed at exactly 25.5 h and 1.5 h before tissue collection. Tissues were dissected free of fat, weighted and snap frozen in liquid nitrogen. Electromyography (EMG) was performed under isoflurane anaestesia as previously described (Pannérec et al., 2016). The measured compound muscle action potential (CMAP) was recorded and analyzed using the Keypoint software (Neurolite, Switzerland).

Blood analyses. Plasma levels of TNFa and IL-6 were measured using commercial ELISA kits following the manufacturer's instructions (abcam). Serum endotoxin levels were measured using a chromogenic endotoxin quant kit according to manufacturer's instructions (Pierce)

Gene expression. Total RNA was extracted from muscle and liver biopsies using the miRNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RNA quality was checked using the Standard Sensitivity RNA Analysis Kit on a Fragment Analyzer (Advanced Analytical Technologies). All cRNA targets were synthesized using the Illumina TotalPrep-96 RNA amplification Kit (Life Technologies) and fragmented according to the Affymetrix protocol, based on the Eberwine T7 procedure. Briefly, double-stranded cDNA was produced from 200 ng of total RNA, followed by *in vitro* transcription and cRNA labeling with biotin. 7.5 µg of cRNA were hybridized on an Affymetrix Rat 230 PM 96-Array in an Affymetrix GeneTitan device (Affymetrix, CA, USA) followed by staining and scanning in the same instrument. Affymetrix probes were normalized and summarized to probe sets using the Robust Multi-array Average (RMA) approach. We applied a non-specific filter to discard probe sets with low variability and retained probe sets with SD greater than median SD of all probe sets. Genes were tested for differential expression using Limma for age, intervention, and interaction term between age and intervention (Smyth, 2004). We defined probe sets with Benjamini-Hochberg adjusted p-value <0.05 as differentially expressed. Pathway enrichment analysis was performed using CAMERA (Wu and Smyth, 2012), a competitive gene set test querying whether a set of genes annotated in the Molecular Signatures Database (MSigDB) (Subramanian et al., 2005) is enriched in differentially expressed genes. MSigDB v5 collections H (hallmark gene sets), C2 (curated gene sets) and C5 (GO gene sets) were used to perform pathway analyses (Liberzon et al., 2015). Tables of GSEA results are available upon request.

For qPCR analysis, mRNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions. Expression of mRNA was then measured by qPCR using SYBR Green real time PCR technology (Roche). Reactions were performed in duplicate in a 384-well plate using the Light Cycler (Roche). Gene expression was normalized to *Gapdh* as housekeeping gene. Relative gene expression between groups or genotypes was assessed using the ΔΔCt method. Primers used: *ATP5b* (fw CATTTAGGGGAGAGCACCGT; rev ATTTTGATTGGTGCCCCCGA), *Cdkn1a* (fw GATATGTACCAGCCACAGGCAC; rev CAGGTCGGACATCACCAGGA), *Chrna1* (fw GGTGGCCATCAATCCGGAAA; rev AGACGCTGCATGACGAAGTG), *Cox4i1* (fw AAGAGCTACGTGTATGGCCC; rev TTCATGTCCAGCATCCGCTT), *Fst* (fw TCCGGATCTTGCAACTCCATC; rev AGGGAAGCTGTAGTCCTGGTC), *Hprt* (fw CTCCTCAGACCGCTTTTCCC; rev CACTAATCACGACGCTGGGAC), *Myh1* (fw AGCTTCAAGTTCAGACCCACG; rev TGAGCCTCGATTCGCTCCTT), *Myh2* (fw AGGCAGCTCTGATGCTGTAGA; rev TGGGGACATGACCAAAGGCT), *Myh3* (fw GTTTGGCATAGCTGCCCCTT; rev CCACCAGGGTCCTGTTGTCT), *Mylk2* (fw GAAGCTGTGTCAGACGAGGC; rev GGCACTGCTCAGCACTCATT), *Myogenin* (fw GGTCCCAACCCAGGAGATCA; rev GCGTCTGACACCAACTCAGG), *Nampt* (fw GCTACTGGCTCACCAACTGGA; rev TGCCGGCAGTCTCTTGTGAA), *Ndufa9* (fw CTTCCAATGTCACGTCCTGC; rev CCACAACTCCACTGACGGAT), *Pax7* (fw CTGGAAGTGTCCACCCCTCTT; rev TGAAGACCCCACCAAGCTGA), *Ppargc1a* (fw TCGCAACATGCTCAAGCCAA; rev AGTCGTGGGAGGAGTTAGGC), *Sdha* (fw AGCCTCAAGTTCGGGAAAGG; rev CCGCAGAGATCGTCCATACA), *Sirt1* (fw TCTGACTGGAGCTGGGGTTTC; rev GAAGTCCACAGCAAGGCGAG), *Sln* (fw TCTTTGCTTCTCTTCAGGACGTG; rev GTGGCATGGCCCCTCAGTAT), *Uqcrc2* (fw CCTCAAAGTTGCCCCAAAGC; rev TGGTAAACTCAAGTTCCTGAGGC).

NAD$^+$ measurements and metabolomics. For enzymatic assays, NAD$^+$ was extracted from 20 mg of frozen tissue and measured using the colorimetric EnzyChrom NAD$^+$/NADH assay kit according to manufacturer's instructions (BioAssay Systems).

Sample preparation for metabolomics analysis was based on Ratajczak et al. (2016). Biological samples (liver, 2-10 mg; muscle, 9-18 mg; plasma, 60 µL) were reconstituted in 200 µL of a solution of 100 mM ammonium acetate acetonitrile:water 85:15 (v/v). Liver and muscle extracts were homogenized with 5 mm tungsten carbide beads using a TissueLyser II (Qiagen) for 1.5 min at 20 Hz, followed by incubation on ice during 1 h, to allow for protein precipitation. Freshly thawed plasma was vortexed for 20 s. All samples followed 10 min centrifugation at 15.000 rpm at 4°C. The supernatants were either diluted 1:10 in 100 mM ammonium acetate acetonitrile:water 85:15 (v/v) (plasma), or directly transferred (liver, muscle) to glass vials for hydrophilic interaction chromatography (HILIC) ultra-high performance liquid chromatography mass spectrometry (UHPLC-MS) analysis. The UHPLC consisted of a binary pump, a cooled autosampler, and a column oven (Vanquish UHPLC+ Focused, Thermo Scientific), connected to an orbitrap mass spectrometer (Orbitrap Fusion Lumos Tribrid, Thermo Scientific) equipped with a heated electrospray (H-ESI) source. Samples were injected into an analytical column (2.1 mm x 150 mm, 5 µm pore size, 200Å HILICON iHILIC®-Fusion(P)), guarded by a pre-column (2.1 mm x 20 mm, 200Å HILICON iHILIC®-Fusion(P) Guard Kit) operating at 35°C. The mobile phase (10 mM ammonium acetate at pH 9, A, and acetonitrile, B) was pumped at 0.25 mL/min flow rate over a linear gradient of decreasing organic solvent (0.5-16 min, 90-25% B), followed by re-equilibration for a total running time of 30 min. On-the-fly alternating negative (3 kV) and positive (3.5 kV) ion modes was used for ionization and the following source parameters were applied: 20 sheath gas, 15 aux gas, ion transfer tube temperature 310°C, vaporizer temperature 280°C. MS acquisition was performed at 60k orbitrap resolution, in centroid mode, by scanning between the mass range 85 to 850 Da. The software Xcalibur v4.1.31.9 (Thermo Scientific) was used for instrument control, data acquisition and processing. Positive ion mode extracted chromatograms

using the corresponding accurate mass of NR, Nam, NA, NAD^+ and NMN were integrated and used for relative comparison. Retention time and mass detection was confirmed by authentic standards.

In vitro experiments. C2C12 mouse myoblasts were cultured in 1x Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% FBS (Gibco) and 1% Penicillin/Streptomycin (Gibco) under standard conditions (37°C, 5 % CO_2). To investigate NFκB transcriptional activation, we used a firefly luciferase reporter plasmid controlled by NFκB-responsive elements (pNF-κB-Luc plasmid, Agilent Technologies) and pE1F-Renilla (REF) as transfection control. Cells were transfected with 100 ng of plasmid DNA using Lipofectamine 3000 (Thermo Fisher) according to manufacturer's instructions. Treatments were performed 40 hours post-transfection with 0.5–2.0 mM NR in complete cell culture medium for 6 hours. Cell lysis and luciferase signal measurement was performed using a Dual Luciferase Reporter Assay System (Promega) according to manufacturer's instructions.

Statistical analysis. All genome wide statistical analyses were performed using R version 3.1.3 and relevant Bioconductor packages. GraphPad Prism Software version 7.02 for Windows was used for all other statistical analyses and preparation of graphs. Statistical significance was assessed as stated in the figure legends. Student's t-test was used for binary comparisons, comparison of more than 2 groups done by one-way ANOVA and Sidak's multiple comparisons post-hoc test. All data are expressed as mean value +/- s.e.m.

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AUTHOR CONTRIBUTIONS

T.S., A.P., and J.N.F. designed the study. T.S., P.C., and A.P. performed the experiments. Sy.M. and F.R. performed microarray experiments, of which E.M. performed statistical analysis. M.P.G. and So.M. performed metabolomics measurements and data curation. T.S. and J.N.F. analyzed data. T.S. and J.N.F wrote the manuscript and all authors approved the final manuscript.

SUPPLEMENTARY DATA

Figure S1 Sarcopenic phenotype and transcriptomic signature of rats after acute NR injection.

Figure S2 Higher abundance of metabolites deriving from NAM in old rats.

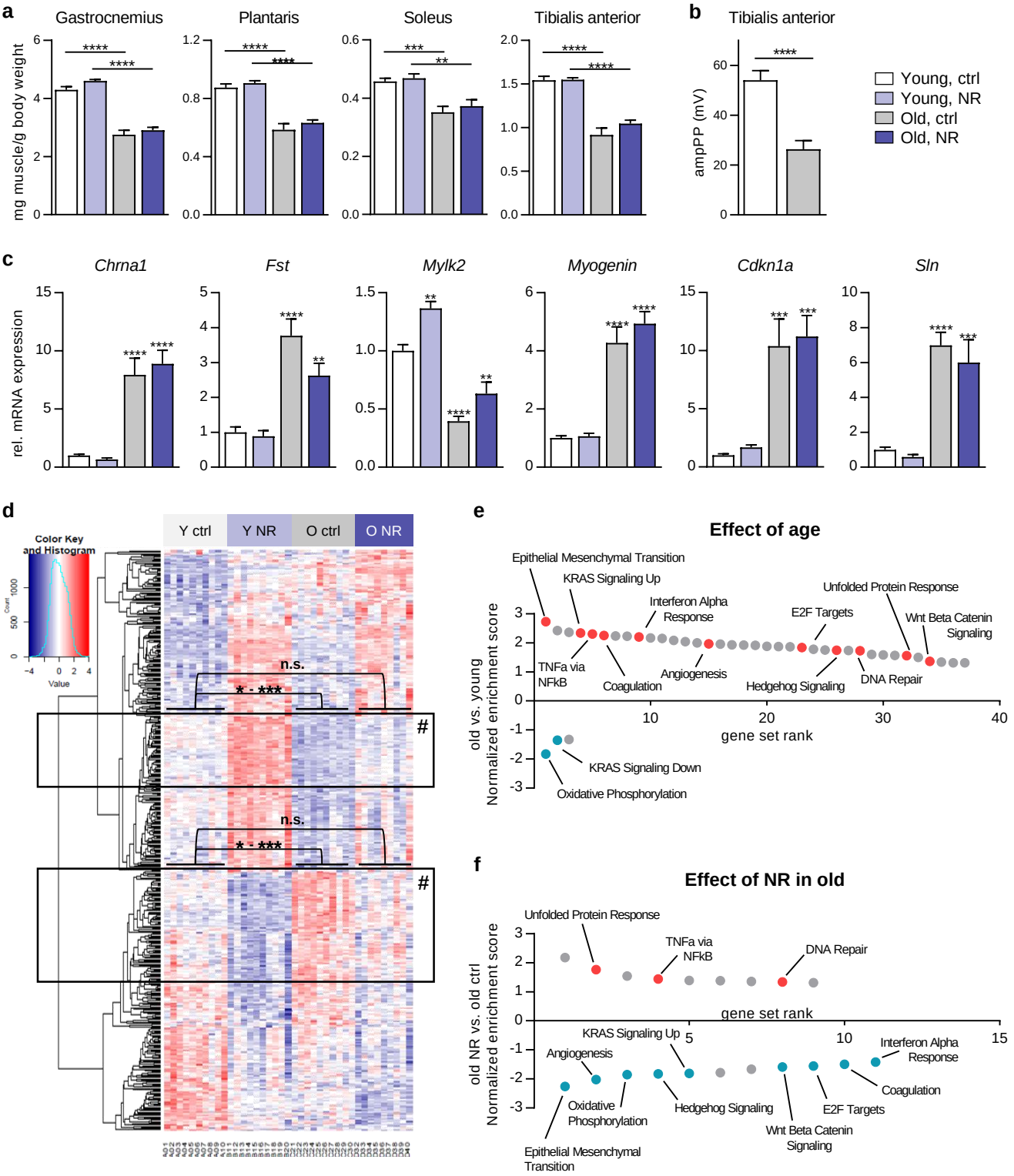

Figure S1: Sarcopenic phenotype and transcriptomic signature of rats after acute NR injection. **(a)** Weight of different hindlimb muscles normalized to whole body weight. **(b)** Compound muscle action potential (CMAP) measured by electromyography in tibialis anterior muscle before NR treatment. **(c)** mRNA expression of sarcopenia related genes: cholinic receptor nicotinic alpha 1 (*Chrna1*), follistatin (*Fst*), myosin light chain kinase 2 (*Mylk2*), *Myogenin*, p21 (*Cdkn1a*), and sarcolipin (*Slp*). Gene expression measured by qPCR relative to *Gapdh* as housekeeping gene. **(d)** Heatmap displayingall 490 probe sets with differential expression in gastrocnemius muscle of rats after NR injection. Used algorithm: av. linkage; distance: 1-correlation. n=9-10. # - Highlighted in the box are gene sets that show a change towards a healthy transcriptional phenotype in sarcopenic muscle treated with NR. **(e–f)** GSEA summary of "Hallmark" gene set collection, pathways differentially regulated in sarcopenic muscle (gastrocnemius) **(e)** before and **(f)** after NR injection. NOM p<0.06, FDR q<0.25. Results shown are mean +/-s.e.m., *p<0.05, **p>0.01, ***p<0.001, ****p<0.0001 versus young ctrl by **(b)** Student's t-test, n=12 or **(a,c)** one-way ANOVA followed by Sidak's post-hoc test, n=10.

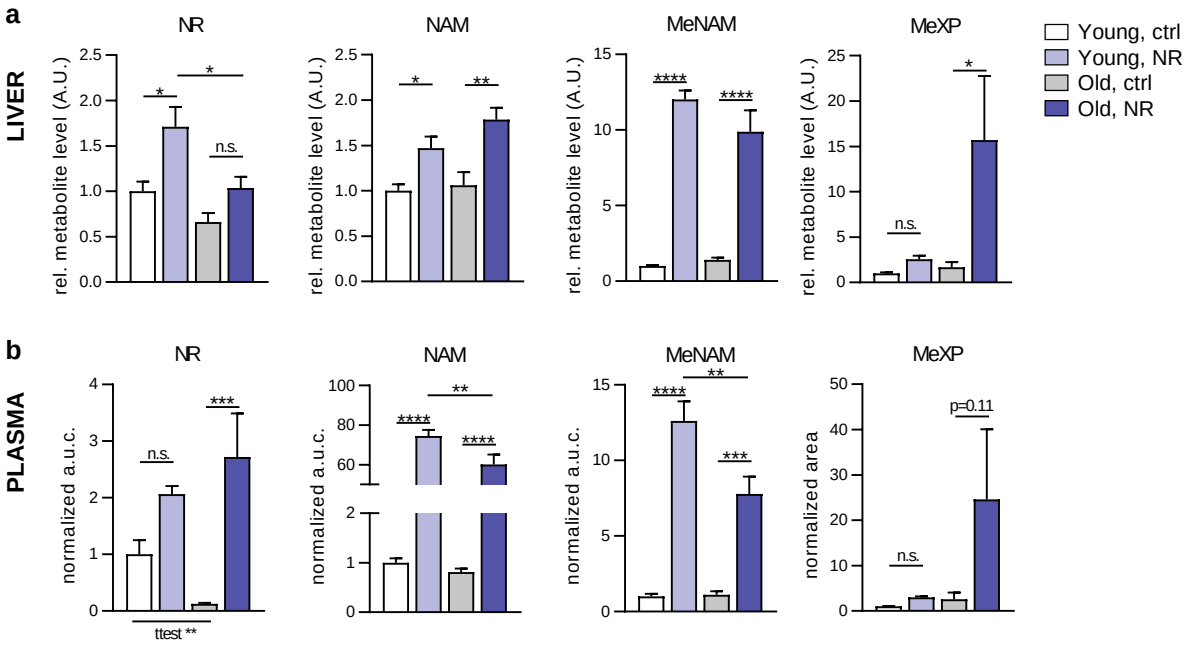

Figure S2: **Higher abundance of metabolites deriving from NAM in old rats**. Qualitative analysis of NAD^+ metabolites by LC MS/MS in **(a)** liver (area normalized to Y ctrl, internal standard and mg of tissue) and **(b)** plasma (area normalized to Y ctrl). Results shown are mean +/-s.e.m., *p<0.05, **p>0.01, ***p<0.001, ****p<0.0001 versus young ctrl by one-way ANOVA followed by Sidak's post-hoc test, n=6.

4 Nicotinamide riboside kinases are rate-limiting for skeletal muscle regeneration

The following chapter is reprint from the manuscript **Sonntag *et al.***, prepared for submission in Q4 2019.

Personal contribution

I designed the study, bred the mice and conducted the *in vivo* work with the technical support of José-Luis Sanchez Garcia. I performed and interpreted all molecular and histological analyses of this study. I further analyzed the results from the NR diet study performed by Paulina Cichosz (Figure 6), and wrote the manuscript under supervision of Jerome Feige.

Nicotinamide riboside kinases are rate-limiting for skeletal muscle regeneration

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Nicotinamide riboside (NR) is a potent NAD^+ precursor with beneficial effects in the context of muscle diseases and aging. NR kinases (NRKs) are essential for the conversion of NR to NAD^+, but little is known about their endogenous role in skeletal muscle. Full body NRK1/2 double KO (dKO) mice breed and develop normally, and exhibit normal muscle NAD^+ levels, as the loss of NRK-driven NAD^+ synthesis alters the flux through nicotinamide (NAM) salvage. We hypothesized that pathological muscle conditions could further alter the balance of NAD^+ salvage routes. In a model of neurogenic muscle atrophy we observed that dKO mice experience similar transcriptional regulation of muscle wasting compared to wild-type (WT) mice, but fail to upregulate NAD^+ levels in response to denervation. We discovered that this inability to utilize NR as NAD^+ precursor becomes a limiting factor in the context of muscle regeneration. Cardiotoxin injection leads to muscle damage and a transient loss of tissue NAD^+ in WT mice, which recovers with progressing tissue regeneration. We could show that NRK1/2 are the rate-limiting enzymes in this process, as dKO mice display delayed recovery of NAD^+ content which manifested in retarded maturation of regenerating muscle fibers. Dietary NR supplementation of WT mice, on the other hand, improved NAD^+ pool regeneration and myogenic activation after injury. For the first time, our results demonstrate that endogenous NR contributes to muscle NAD^+ homeostasis and becomes the critical NAD^+ precursor during pathological conditions.

KEYWORDS: Skeletal muscle; nicotinamide riboside; NRK; NAD^+; muscle atrophy; muscle regeneration; CTX

INTRODUCTION

Skeletal muscle is a very adaptive tissue that strongly responds to a multitude of stimuli such as exercise or injuries as well as disuse and disease. While the latter induce the shrinkage and atrophy of muscle fibers, exercise and injuries rather stimulate anabolic and regenerative pathways.

Nicotinamide adenine dinucleotide (NAD^+) plays a central role in muscle metabolism, both as a vital co-factor in redox reactions as well as a co-substrate for NAD^+ consuming enzymes such as sirtuins, which are involved in the regulation of mitochondrial function and muscle stem cell (MuSC) activation (Fulco et al., 2003; Tang and Rando, 2014; Ryall et al., 2015). Skeletal muscle NAD^+ production relies on the salvage of dietary vitamin B_3 precursors such as nicotinamide (NAM) and nicotinamide riboside (NR) (Frederick et al., 2016; Ratajczak et al., 2016; Fletcher et al., 2017). Muscle-specific NAM phosphoribosyltransferase (NAMPT) knock-out mice exhibit a dramatic 85% decline in muscle NAD^+ levels, highlighting the prominent role of NAM as NAD^+ precursor in muscle (Frederick et al., 2016). Nevertheless, supplementation of exogenous NR induces tissue NAD^+ more efficiently than exogenous NAM (Fletcher et al., 2017), and recent studies describe the beneficial effect of dietary NR supplementation on mitochondria and MuSC function in mice (Cerutti et al., 2014; Khan et al., 2014; Ryu et al., 2016; Zhang et al., 2016).

NRKs 1 and 2 phosphorylate NR to nicotinamide mononucleotide (NMN), which is subsequently converted to NAD^+ by NMN adenylyltransferases (NMNATs) (Bieganowski and Brenner, 2004; Trammell et al., 2016). This initial step is not only essential for the utilization of NR as NAD^+ precursor, but also

for dietary NMN salvage, as NMN needs to be converted to NR in order to enter the cell (Nikiforov et al., 2011; Ratajczak et al., 2016). NRK1 is ubiquitously expressed in all tissues, while NRK2 has been exclusively found in skeletal muscle (Li et al., 1999; Ratajczak et al., 2016; Fletcher et al., 2017). Expression of NRK1 and 2 (encoded by *Nmrk1* and *Nmrk2*, respectively) is increased during primary mouse myoblast differentiation *in vitro*, with *Nmrk2* mRNA expression peaking during time of myoblast fusion (Fletcher et al., 2017). A role of NRK2b in zebrafish muscle development was further described in regulation of basal lamina assembly, laminin polymerization and paxillin concentration at the myotendinous junction (Goody et al., 2010). In contrast to zebrafish, mice lacking either one or both NRKs develop grossly normal with no obvious muscle phenotype at baseline (Ratajczak et al., 2016; Fletcher et al., 2017; Deloux et al., 2018). Nevertheless, given the reported implication in muscle architecture as well as the observation that *Nmrk2* is a damage-inducible transcript in muscle and even non-muscle tissues (i.e. during neuronal injury) (Sasaki et al., 2006; Lavery et al., 2008; Aguilar et al., 2015; Xu et al., 2015; Diguet et al., 2018), we hypothesized that NRKs may be important in the context of muscle atrophy and regeneration.

Using NRK1/2 dKO mice, we demonstrate that NR is an endogenous NAD^+ precursor in the muscle and that loss of NRKs induces increased flux through NAM salvage. We further show that NRK1/2-mediated NAD^+ biosynthesis becomes rate-limiting in the regulation of muscle NAD^+ during pathological conditions such as atrophy and regeneration and that deficiency of NRK1/2 delays maturation of regenerating fibers. We finally highlight the potential of exogenous NR in dietary supplemented WT mice, which regenerate NAD^+ faster and have increased myogenic activation after muscle injury.

RESULTS

Mice deficient for NRK1 and NRK2 develop normally, but exhibit altered NAM salvage

The role of NRKs has been previously studied in mouse models of NRK1 or NRK2 single KO (sKO) (Ratajczak et al., 2016; Fletcher et al., 2017; Deloux et al., 2018). As activity of one NRK isozyme may compensate the loss of the other in sKO studies, we studied mice deficient of both NRKs 1 and 2 (full body NRK1/2 dKO). The previous reports on the inability of NRK1/2 dKO cells to efficiently convert exogenous NR into NAD^+ (Ratajczak et al., 2016; Fletcher et al., 2017) prompted us to analyze whether the NAD^+ metabolome is skewed in the absence of NRKs.

NRK1/2 dKO mice have normal body weight and muscle mass of different hindlimb muscles (**Fig. 1b,c**). As expected, expression of *Nmrk1* and *Nmrk2* mRNA was not detected in skeletal muscle of NRK1/2 dKO mice (**Fig. 1d**). Importantly, NRK1/2 deletion did not induce transcriptional compensation through *Pnp*, an alternative route of NR metabolism known from yeast (Bieganowski and Brenner, 2004) (**Fig. 1d**). In line with previous reports (Fletcher et al., 2017), NAD^+ levels measured both enzymatically and by LC-MS metabolomics were not altered in NRK1/2 dKO compared to control muscle (**Fig. 1e,f**), demonstrating that the production of NAD^+ under homeostatic conditions is either not dependent on NRKs or that the absence of NRKs is compensated by alternative fluxes in the NAD^+ salvage pathway. In support of the latter option, we used LC-MS/MS metabolomics to demonstrate that NR is enriched more than 10-fold in NRK1/2 dKO muscle (**Fig. 1g**). Interestingly, methyl-nicotinamide (MeNAM) was lower in NRK1/2 dKO muscle (**Fig. 1f**), indicating that the absence of NRKs alters salvage of NAM into the NAD^+ cycle at the expense of MeNAM, which is targeted for excretion (Pissios, 2017). Reduced MeNAM elimination from NRK1/2 dKO muscle cannot be explained by changes in expression of enzymes degrading NAM outside of the NAD^+ pathway like *Nmnt* or *Aox1* (**Fig. S1a**), but may be linked to higher flux into NAM salvage, as NRK1/2 dKO muscle presented mildly increased expression of *Nampt* and *Nmnat1*, two rate-limiting enzymes for NAD^+ salvage in skeletal muscle (**Fig. 1h**).

Altogether, our results demonstrate that deletion of NRK1/2 does not cause NAD^+ deficiency in skeletal muscle but skews NAD^+ production towards NAM salvage, thereby demonstrating that the conversion of endogenous NR to NMN is physiologically active under normal homeostatic conditions.

NRK1/2 dKO does not alter muscle wasting induced by denervation

Given our observation that deletion of NRK1/2 skews the NAD^+ flux in healthy skeletal muscle, we reasoned that pathological conditions could further alter the balance of NAD^+ biosynthesis routes

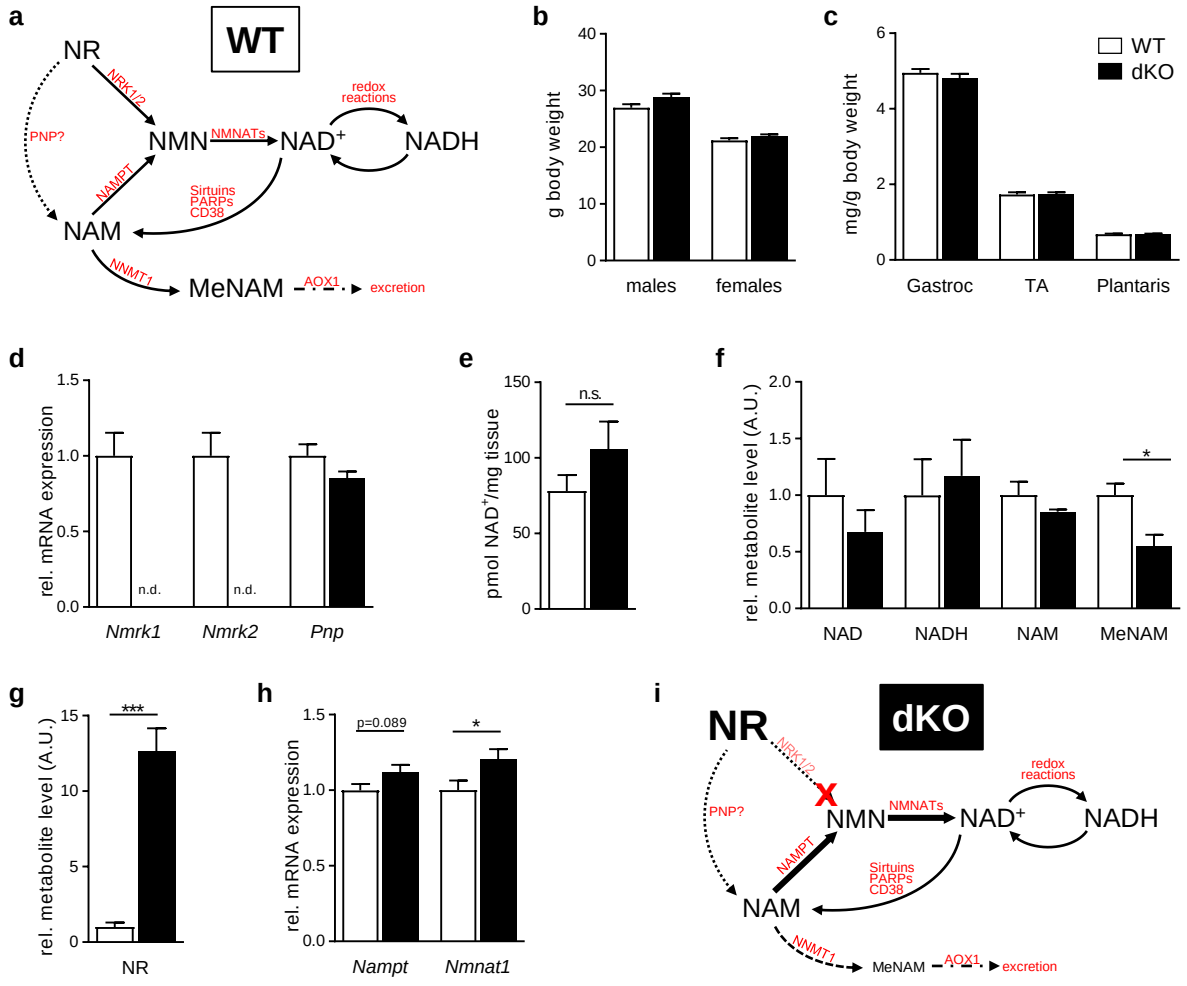

Figure 1: Mice deficient of NRK1 and NRK2 are grossly normal, but exhibit altered NAM salvage. **(a)** Main NAD^+ biosynthesis and consumption pathways in WT skeletal muscle. **(b)** Body weight of young WT and dKO mice. n=17–22. **(c)** Weights of hindlimb muscles gastrocnemius, tibialis anterior (TA) and plantaris of males, n=22. **(d)** mRNA expression levels of NR converting enzymes in TA muscle, n=16. **(e)** NAD^+ tissue levels in TA muscle measured by enzymatic cycling assay. n=16. **(f–g)** LC-MS/MS qualitative metabolomics targeted to **(f)** NAD^+, NADH, NAM, MeNAM, and **(g)** NR in gastrocnemius muscle. Shown are qualitatively measured metabolite levels relative to WT, peak area normalized to an internal standard and mg protein per sample, n=3. **(h)** mRNA expression levels of the major skeletal muscle NAD^+ biosynthesis enzymes in TA muscle, n=16. **(i)** Proposed mechanism for altered NAD^+ biosynthesis and flux in dKO muscle. NAM salvage to NAD^+ is increased at the expense of NNMT1- and AOX1-dependent NAM excretion. Results shown are mean +/-s.e.m. with *p<0.05, **p<0.01, ***p<0.001 versus WT, determined by Student's t-test. mRNA expression measured by qPCR relative to *Atp5b*, *Eif2a*, and *Psmb4* as housekeeping genes.

and may lead to more severe defects on NAD^+ metabolism and muscle physiology. To investigate this possibility, we induced muscle wasting by performing unilateral resection of the sciatic nerve in NRK1/2 dKO and WT control mice.

In WT mice, *Nmrk2* expression was strongly downregulated during denervation while *Nmrk1* remained expressed at normal levels (**Fig. 2a,b**). As expected, *Nmrk1/2* expression was not detected in NRK1/2 dKO muscle. Muscle NAD^+ levels increased in response to denervation-induced muscle atrophy in WT mice, however, NRK1/2 dKO mice failed to elevate NAD^+ levels, indicating that NRKs become rate-limiting to regulate NAD^+ levels after denervation (**Fig. 2c**). Despite this difference in NAD^+ metabolome, muscle wasting induced by denervation was similar in WT and NRK1/2 dKO mice, with both groups losing 20% of muscle mass compared to the contra-lateral innervated muscle (**Fig. 2d**). Gene expression analysis confirmed that the *Hdac4*/*Myogenin* transcriptional network activated upon denervation (Moresi et al., 2010) was induced

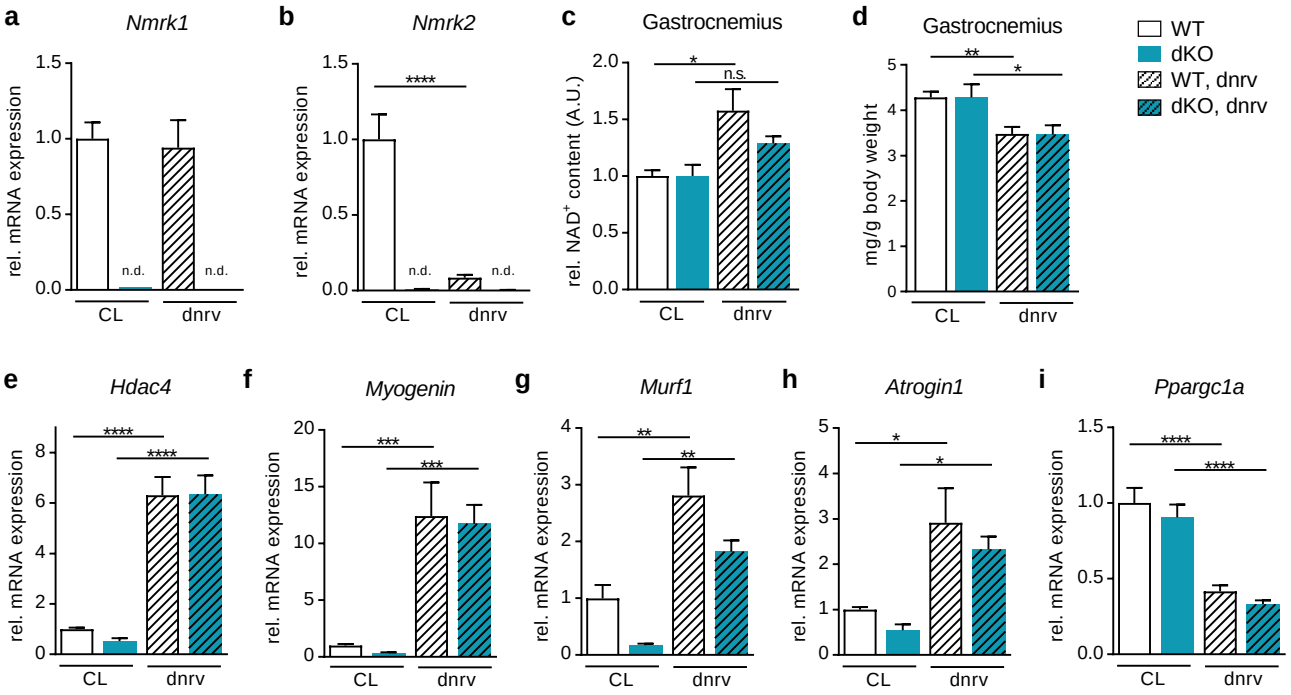

Figure 2: Loss of NRK1 and NRK2 does not affect the transcriptional regulation of denervation-induced muscle atrophy. Mice were subject to unilateral resection of the sciatic nerve, tissues were collected 1 week after denervation. **(a–b)** mRNA expression levels of **(a)** *Nmrk1* and **(b)** *Nmrk2*. **(c)** Relative NAD^+ content of gastrocnemius muscle quantified by NAD^+ cycling assay. **(d)** Weight of gastrocnemius muscle normalized to whole body weight. **(e–i)** mRNA expression levels of atrogenes. Gene expression of mRNA extracted from TA muscle measured by qPCR with *bActin* as housekeeping gene. Results shown are mean +/-s.e.m., *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus WT CL leg by one-way ANOVA followed by Bonferroni's post-hoc test, n=6.

at similar levels in both genotypes (**Fig. 2e,f**). In addition, downstream atrogenes such as *Murf1* and *Atrogin1* which encode E3 ligases mediating muscle protein breakdown were induced normally in the denervated muscles of NRK1/2 dKO mice (**Fig. 2g,h**), while down-regulation of *Ppargc1a* in response to denervation was also unaffected in NRK1/2 dKO muscle (**Fig. 2i**).

Altogether, our results demonstrate that NRK1/2 do not modulate muscle wasting induced by denervation and that modulation of NAD^+ metabolism by NRK1/2 only plays minor roles during loss of innervation.

NRK1/2 deficient mice display delayed regeneration of NAD^+ levels after injury

We then asked whether NRK1/2 could play more prominent roles in conditions where muscle fibers are injured and NAD^+ metabolism could be required either for muscle stem cell metabolism or regulation of NAD^+ levels when myofiber integrity recovers. To do so, hindlimb muscles of WT and NRK1/2 dKO mice were injected intramuscularly with cardiotoxin (CTX) and the regeneration process was monitored at 3, 7, and 14 days post injury (dpi).

In WT mice, muscle injury leads to a transient decline in NAD^+ levels at 3 dpi when many myofibers have died or have damaged membranes, and then rapidly recovers to normal levels as myofibers recover integrity during the regeneration process (**Fig. 3a**). Interestingly, regenerating NRK1/2 dKO muscle had lower NAD^+ levels at 3 and 7dpi, and was ultimately able to restore normal NAD^+ levels at 14dpi when regeneration has proceeded. At 7dpi, NAD^+ levels in injured muscles were 45% lower in NRK1/2 dKO mice compared to WT, demonstrating that NRK1/2 causes a transient failure for repairing myofibers to restore the NAD^+ pool (**Fig. 3a**). Gene expression analysis in WT mice demonstrated that NRK1/2 expression is not affected by muscle injury and remains stable during regeneration (**Fig. 3b,c**). In contrast, the rate limiting enzymes of NAD^+ salvage *Nampt* and *Nmnat1* are strongly downregulated after injury, and only recover during termi-

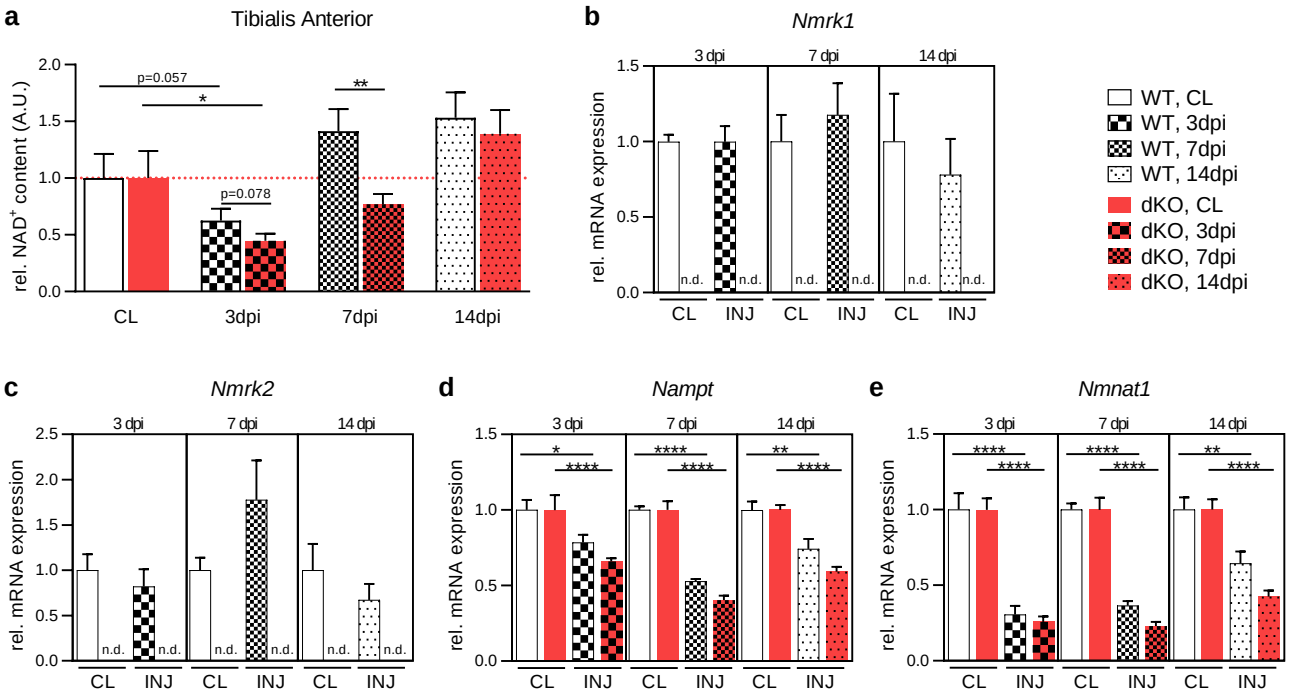

Figure 3: NRK1/2 deficient mice display delayed regeneration of NAD$^+$ levels after injury. Mice were subject to unilateral CTX injection into hindlimb muscle, tissues were collected 3, 7, and 14 days post injury (dpi). **(a)** Relative NAD$^+$ levels in TA muscle measured by cycling assay. **(b–e)** mRNA expression of NAD$^+$ salvage enzymes extracted from TA muscle and measured by qPCR relative to *Atp5b*, *Eif2a*, and *Psmb4* as housekeeping genes. Results shown are mean +/-s.e.m., *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus the respective CL leg of each genotype by **(a)** Student's t-test or **(b–e)** one-way ANOVA followed by Sidak's post-hoc test, n=8.

nal regeneration after the 14 dpi time point (**Fig. 3d,e**). Deletion of NRK1/2 abolishes NRK1 and NRK2 expression but does not influence the response of Nampt and Nmnat1 which are equally downregulated in WT and NRK1/2 dKO mice (**Fig. 3b–e**).

In summary, NRK1/2 dKO alters regeneration of the NAD$^+$ pool after muscle injury by blunting the conversion of NR into NAD$^+$ in regenerating myofibers which have not yet turned on NAM salvage. These results demonstrate that the balance between NR- and NAM-mediated NAD$^+$ salvage is modulated by physio-pathological conditions and that NR-mediated NAD$^+$ becomes rate-limiting in newly formed muscle fibers during muscle regeneration.

MuSC activation and inflammation are not affected in dKO muscle

Delayed recovery of NAD$^+$ levels during regeneration of NRK1/2 dKO muscle could either arise from delayed muscle stem cell activation in the first days after injury, which causes delayed regeneration of myofibers, or by a direct effect of NRKs on NAD$^+$ metabolism in newly formed fibers, which could subsequently alter regenerating myofiber maturation. To discriminate between these two options, we tested for early markers of muscle stem cell activation and myogenic commitment which are required for efficient tissue repair.

Expression of the muscle stem cell marker *Pax7* was induced normally at 3 dpi in NRK1/2 dKO mice, and the number and proliferation of Pax7$^+$ muscle stem cells measured by histology was also similar in WT and NRK1/2 dKO regenerating muscle (**Fig. 4a–c**). Myogenic commitment during regeneration was not delayed in NRK1/2 dKO mice, as the mRNA expression of myogenic transcription factors *MyoD1* and *Myogenin* was slightly more increased in NRK1/2 dKO than in WT muscle at 3 dpi, and the number of MyoD$^+$ muscle stem cells was the same in both genotypes (**Fig. 4d–f**). We also excluded that NRK1/2 dKO alters the inflammatory profile of immune cells that invade skeletal muscle in the first few days after injury as both the expression of macrophage markers (**Fig. S2a–c**) and of the cytokines TNFα, IL-1b, and IL-6 (**Fig. S2d–f**) were unchanged between WT

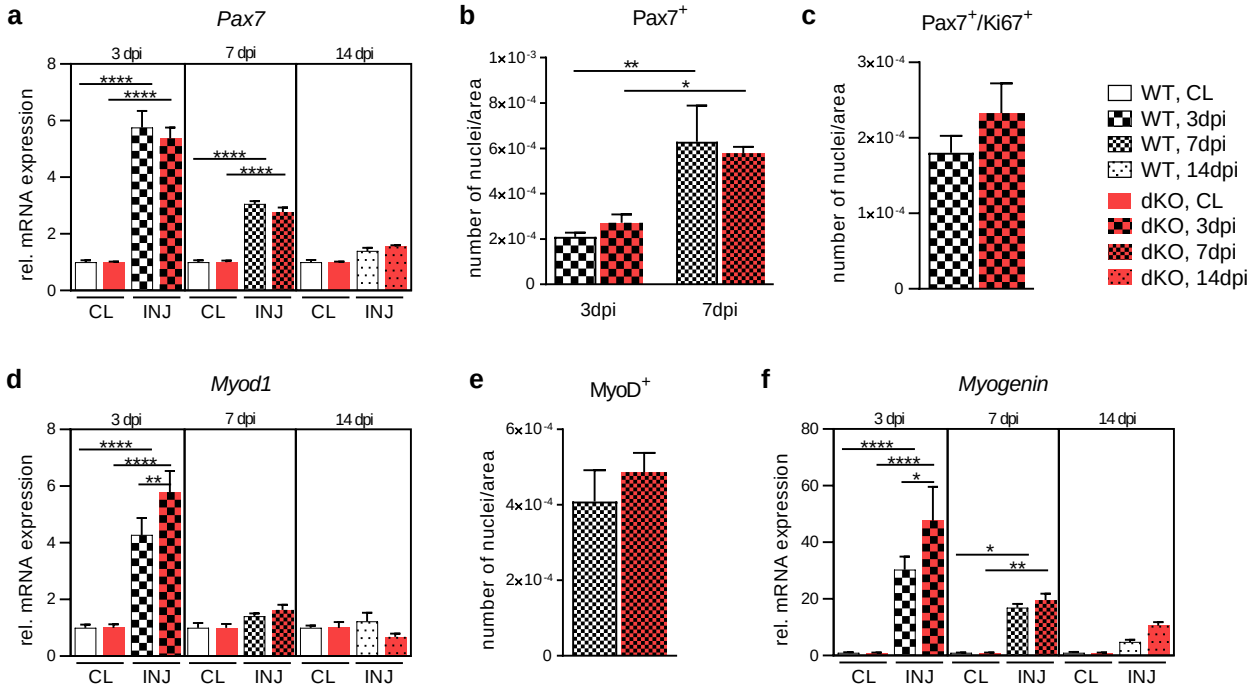

Figure 4: MuSC activation is not affected in dKO muscle. Mice were subject to unilateral CTX injection into hindlimb muscle, tissues were collected 3, 7, and 14 days post injury (dpi). **(a)** *Pax7* mRNA expression. **(b–c)**Histological quantification of **(b)** total and **(c)** proliferating MuSCs at 3 and 7 dpi. **(d)** *MyoD1* mRNA expression. **(e)** Histological quantification of committed MuSCs expressing MyoD protein at 7 dpi. **(f)** *Myogenin* mRNA expression. mRNA extracted from TA muscle and measured by qPCR relative to *Atp5b*, *Eif2a*, and *Psmb4* as housekeeping genes. Results shown are mean +/-s.e.m., *p<0.05, **p>0.01, ***p<0.001, ****p<0.0001 versus the respective CL leg of each genotype by **(a, d, f)** one-way ANOVA followed by Sidak's post-hoc test or **(b, c, e)** Student's t-test, n=6-8.

and NRK1/2 dKO mice.

These results demonstrate that NRK1/2 dKO mice have normal muscle stem cell activation and inflammatory response after muscle injury. Thus, deletion of NRK1 and 2 does not impair the early phases of muscle regeneration and most likely alters NAD^+ metabolism directly in regenerating myofibers.

Delayed maturation of newly formed fibers in dKO muscle

We next examined the maturation of muscle fibers during the time course of regeneration and focused on the transition from 7 to 14 dpi when NRK1/2 dKO muscle has impaired NAD^+ recovery (**Fig. 3a**). During this time frame, regenerating muscle fibers of WT mice switch their myosin heavy chain (MHC) composition from the transient expression of embryonic MHC *Myh3* that sustains the first steps of muscle maturation, to expression of the adult MHC *Myh4* when they terminally mature (D'Albis et al., 1988; Schiaffino et al., 2015).

Importantly, NRK1/2 dKO muscle failed to efficiently transition from embryonic to adult MHC isoforms during muscle regeneration, as embryonic *Myh3* expression remained high while the recovery of adult *Myh4* was delayed at 14 dpi (**Fig. 5a,b**). We confirmed the maturation defect of NRK1/2 dKO regenerating myofibers by demonstrating that the cross sectional area of newly formed myofibers with centralized nuclei is significantly smaller in dKO muscle at 7 dpi compared to WT mice (**Fig. 5c–e**).

Overall, these data demonstrate that loss of NRK1 and 2 affects muscle regeneration independent of MuSC activation by delaying maturation of newly formed myofibers at the time when NAD^+ levels fail to recover.

Dietary NR supplementation improves NAD^+ regeneration after muscle injury in WT mice

Our results presented above showed that the NRK-mediated conversion of endogenous NR to NAD^+ becomes rate-limiting for myofiber maturation during muscle regeneration. This prompted us to ask whether exogenous NAD^+ boosting through dietary

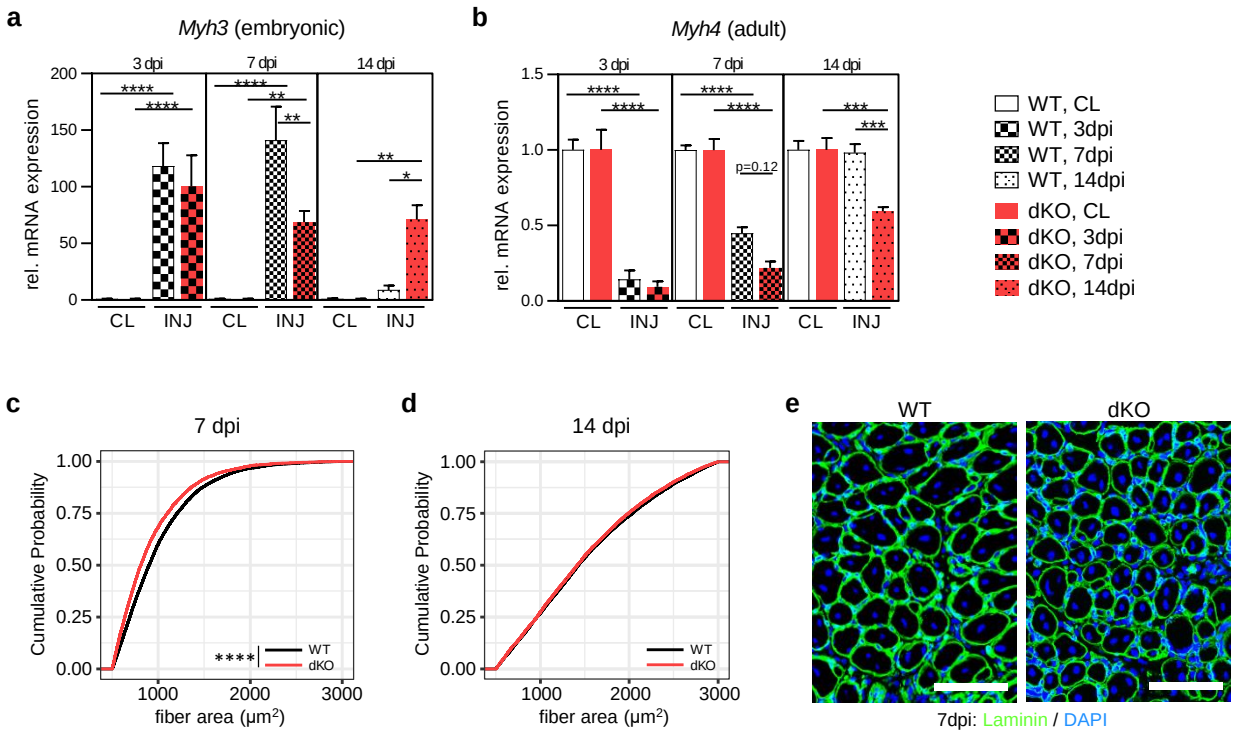

Figure 5: Delayed maturation of newly formed fibers retards muscle regeneration in dKO muscle. Mice were subject to unilateral CTX injection into hindlimb muscle, tissues were collected 3, 7, and 14 days post injection (dpi). **(a)** *Myh3* and **(b)** *Myh4* mRNA expression in total TA muscle. **(c–d)** Cumulative distribution of cross sectional area of regenerating fibers with centralized nuclei at **(c)** 7 dpi and **(d)** 14 dpi. **(e)** Representative images of TA muscle sections stained with Laminin (green) and DAPI for nuclei (blue) at 7 dpi. Scale bar: 100 μm. mRNA measured by qPCR relative to *Atp5b*, *Eif2a*, and *Psmb4* as housekeeping genes. Results shown are mean +/-s.e.m., *p<0.05, **p>0.01, ***p<0.001, ****p<0.0001 versus the respective CL leg of each genotype by **(a,b)** one-way ANOVA followed by Sidak's post-hoc test or **(c,d)** Kolmogorov-Smirnov test, n=8.

NR could be beneficial for muscle regeneration of WT mice.

In line with previous reports (Frederick et al., 2016), dietary treatment with NR in young WT mice increased NAD^+ levels in liver, but not in uninjured muscle where NAM salvage is dominant (**Fig. 6a** and data not shown). However, NR supplementation resulted in faster recovery of NAD^+ levels during muscle regeneration, with NAD^+ content being elevated by 35% in NR-treated mice at 7 dpi (**Fig. 6b**), when NAM salvage is low and NRKs are rate-limiting. Additionally, similar to previous reports (Zhang et al., 2016), NR increased *Pax7* expression in uninjured muscle (**Fig. 6c**) and further activated expression of *Pax7*, *MyoD1* and *Myogenin* after injury, the prototypical markers of muscle stem cell amplification and commitment (**Fig. 6d–f**). In line with our observations in NRK1/2 dKO mice, dietary NR treatment further promoted expression of embryonic *Myh3* and efficient recovery of adult *Myh4* (**Fig. 6g,h**).

These results demonstrate that NR promotes NAD^+ recovery and suggest that NR supports myogenic activation and myofiber maturation during skeletal muscle regeneration of young WT mice. Thus, the flux of NAD^+ biosynthesis predominantly driven by NRKs during muscle regeneration is not saturated by endogenous substrates and can be enhanced nutritionally by NR to promote faster regeneration after muscle injury.

DISCUSSION

NAM salvage is commonly accepted to be the critical NAD^+ producing path in skeletal muscle (Frederick et al., 2016; Fletcher et al., 2017). Nevertheless, the tissue-specific expression of a second NRK, NRK2, strongly suggests that NR plays a specific role as an NAD^+ precursor in the muscle (Fletcher and Lavery, 2018). Despite the overlapping function of the NRK isozymes in hydrolizing NR and possible compensatory mechanisms, available literature mainly focuses on NRK1 or NRK2 single KO (sKO) mice and

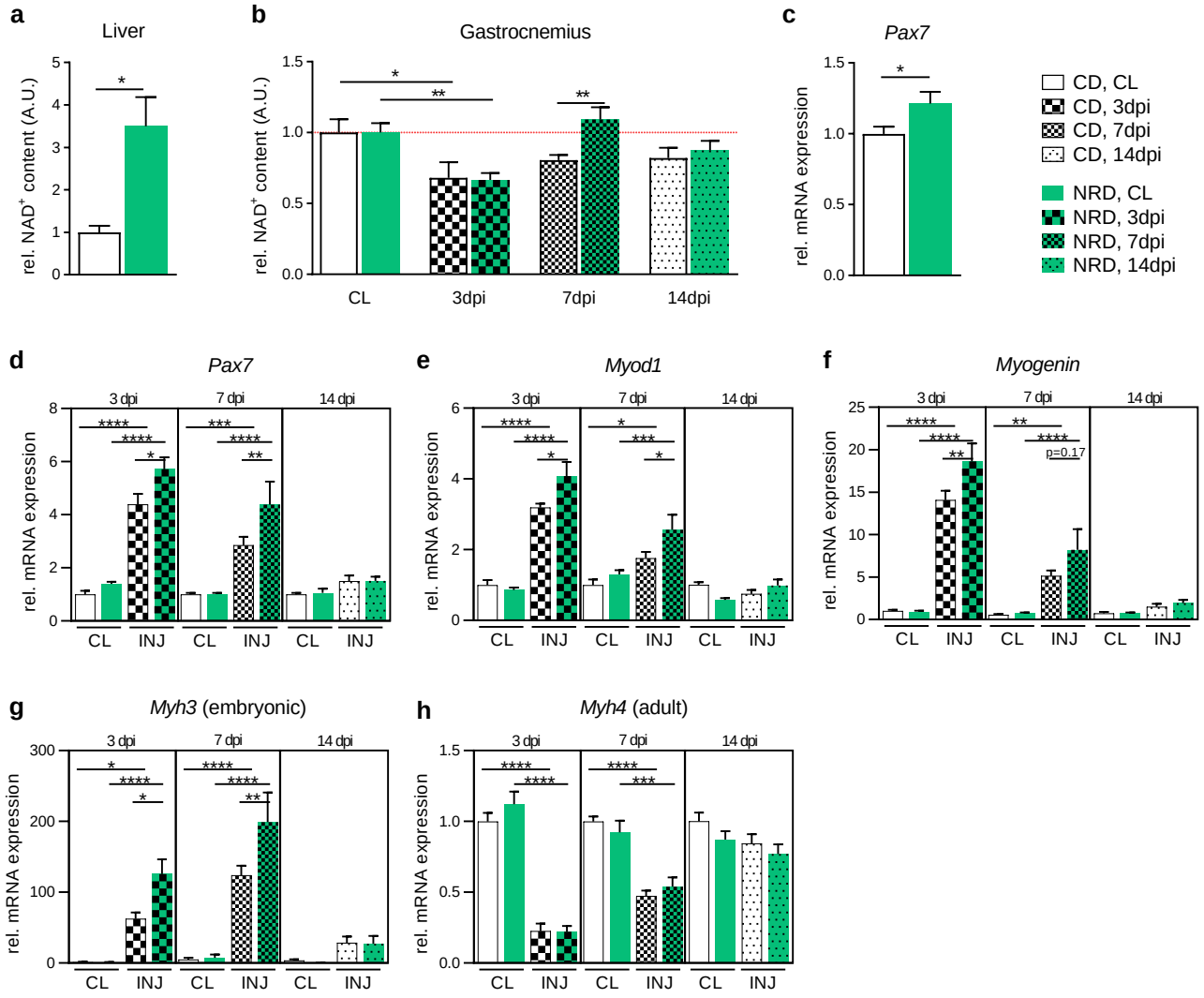

Figure 6: Dietary NR supplementation activates MuSCs and improves NAD$^+$ regeneration after muscle injury. WT mice received control diet (CD) or diet supplemented with 400 mg NR/kg/day (NRD) starting 1 week before unilateral CTX injection into hindlimb muscle. Tissues were collected 3, 7, and 14 days post injury (dpi). **(a,b)** Relative NAD$^+$ levels measured by enzymatic cycling assay in **(a)** liver after 10 days of dietary NR, and **(b)** gastrocnemius muscle. **(c)** *Pax7* mRNA expression in CL muscle after 10 days of dietary NR. **(d–h)** mRNA expression of MuSC marker *Pax7*, myogenic transcription factors *MyoD1* and *Myogenin*, and myosin heavy chain isoforms *Myh3* and *Myh4*. mRNA extracted from TA muscle and measured by qPCR relative to *Atp5b*, *Eif2a*, and *Psmb4* as housekeeping genes. Results shown are mean +/-s.e.m., *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus the respective CL leg of each genotype by **(a–c)** Student's t-test or **(d–h)** one-way ANOVA followed by Sidak's post-hoc test, n=8.

only a limited amount of data describing the phenotype of NRK1/2 dKO muscle has been presented up to date.

It was reported that NRK1/2 sKO and dKO mice do not exhibit significant differences in their muscle tissue NAD$^+$ levels neither at baseline nor during aging (Ratajczak et al., 2016; Fletcher et al., 2017; Deloux et al., 2018). Our results corroborate these findings, and bring fundamental new insights by demonstrating that an NRK-mediated flux is active in healthy muscle since the absence of NRKs leads to a significant accumulation of NR in tissue. This important result highlights the rate-limiting nature of NRKs for the efficient utilization of NR as a muscle NAD$^+$ precursor, as previously shown in primary liver and muscle cells isolated from NRK1/2 dKO mice (Ratajczak et al., 2016; Fletcher et al., 2017).

Muscle NAD$^+$ biosynthesis relies mainly on NAM salvage by NAMPT, which contributes to up to 85% of muscle NAD$^+$ synthesis in healthy muscle (Frederick et al., 2016; Fletcher et al., 2017; Liu et al., 2018). In our study, NAM levels did not differ be-

tween the genotypes as it is under high metabolic flux, but we observed significantly lower levels of the NAM excretion product MeNAM. This observation may be linked to a generally slower NAD^+ turnover in NRK1/2 dKO muscle. However, increased expression of *Nampt* and *Nmnat1* suggests that in dKO muscle, the flux through NAM recycling is favored over NAM excretion in the absence of the NRK-mediated flux into NAD^+ biosynthesis.

Even though the loss of NRKs can be compensated in the homeostatic muscle, our data demonstrate a distinct role of NRKs in certain pathological situations such as during the initial phase of muscle regeneration, where the salvage of NAD^+ from NAM is no longer dominant. Our novel findings therefore broaden the current knowledge of the biological function of NRKs in skeletal muscle. Due to its muscle-specific expression, the role of NRK2 has been fairly studied (Li et al., 1999, 2003; Goody et al., 2010; Fletcher et al., 2017; Deloux et al., 2018). In C2C12 mouse myoblasts, NRK2 was first described as the muscle integrin binding protein (MIBP) and reported to localize to the plasma membrane, binding the cytosolic tail of the laminin receptor integrin $\alpha7\beta1$ (Li et al., 2003). Zebrafish muscle development strongly depends on NRK2b (Goody et al., 2010), and even though mice deficient of NRK1 and/or NRK2 develop grossly normal, first data on aged NRK2 sKO mice demonstrated a maladaptive response to exercise (Deloux et al., 2018). Additionally, *Nmrk2* has been observed to be upregulated in response to energy stress in the failing heart of a mouse model of dilated cardiomyopathy as well as in human failing heart (Diguet et al., 2018).
Interestingly, *Nmrk2* appears to be a stress-responsive transcript also in non-muscle tissues, as observed in dorsal root ganglions after sciatic nerve resection (Sasaki et al., 2006). In the present study, we used sciatic nerve resection to model the absence of contraction stimuli on muscle as happens for instance after spinal cord injury. Loss of innervation activates the expression of atrogenes that drive ubiquitin-mediated proteolysis through the proteasome pathway (Beehler et al., 2006; Moresi et al., 2010). In contrast to the observations in resected nerves (Sasaki et al., 2006), *Nmrk2* was downregulated in denervated muscle, while expression of *Nmrk1*, *Nampt* and *Nmnat1* was unaffected (Fig. 2a,b and data not shown). The modest defect of dKO mice in regulating NAD^+ indicates a possible role for NRK1 during neurogenic muscle atrophy. Nevertheless, the activation of the transcriptional network controlling muscle atrophy was similar in dKO and WT muscle, suggesting a minor involvement of NAD^+ alterations during these processes.
In contrast, the severe damage of muscle fibers after CTX-induced muscle injury leads to a transient decline in NAD^+ levels early after injury. dKO muscle showed a clear delay in recovery of tissue NAD^+, demonstrating that NRKs are rate-limiting in this process. In WT muscle, *Nampt* and *Nmnat1* were strongly downregulated during the early steps of regeneration, but *Nmrk1* and *Nmrk2* were stably expressed throughout regeneration, with *Nmrk2* even showing a tendency to increased expression at the time of myogenic differentiation. A similar transient up-regulation of *Nmrk2* has been reported after freeze injury in mouse muscle (Aguilar et al., 2015). *In vitro*, *Nmrk2* is up-regulated during differentiation of C2C12 mouse myoblasts as well as during primary mouse myoblast differentiation (Fletcher et al., 2017), indicating an involvement of NRK2 in the orchestration of muscle maturation. In line with this, we observed that newly regenerated fibers were significantly smaller in dKO muscle, and exhibited delayed maturation with extended expression of embryonic *Myh3*. The efficient re-organization of the extracellular matrix composing the basal lamina facilitates recovery of muscle architecture as well as innervation and formation of new neuromuscular junctions. This process is imperative for fiber maturation, as the nature of neurogenic input determines the contractile characteristics of each newly-formed myofiber (Pette and Vrbová, 1985; Pette and Staron, 2001; Schiaffino and Serrano, 2002).
Based on their findings during zebrafish muscle development, Goody and Henry (2018) proposed a model in which membrane-located NRK2 constantly generates a membrane-proximal pool of NAD^+. In this model, muscle damage leads to NAD^+ leakage into the extracellular space and might also stimulate the active transport of NAD^+ across the sarcolemma of intact fibers (Zolkiewska, 2005). Extracellular ADP-ribosyltransferases (i.e. ART1) are then able to utilize the NAD^+ for ADP-ribosylation of integrin $\alpha7\beta1$, which enhances the affinity for integrin-laminin binding (Zhao et al., 2005). This contributes to increased basal lamina laminin organisation and further enables the intracellular localization of paxillin to cell-matrix adhesion complexes, finally re-

sulting in efficient adhesion of regenerated muscle fibers (Goody et al., 2010).

Testing this concept in rodents is rather challenging, as murine models of NRK2 sKO display functional compensation by NRK1 (Fletcher et al., 2017). However, our data from NRK1/2 dKO mice provide the first evidence that NRK1 and 2 become the critical NAD^+ producers after muscle injury. Endorsing the presented model, we propose that deficient extracellular matrix adhesion of newly formed fibers results in delayed fiber maturation. Furthermore, based on their redundant function (Fletcher et al., 2017), we suggest that both NRKs on their own have the potential to facilitate efficient tissue repair after muscle damage.

As NAD^+ plays a major role in the regulation of the myogenic program during regeneration (Ryall et al., 2015), we asked whether the slower recovery of tissue NAD^+ levels affects the regenerative capacity of MuSCs. The efficient activation of MuSCs in dKO muscle shows that NRKs are not rate-limiting for the intrinsic regulation of NAD^+ in MuSCs which appears to be independent from muscle fiber NAD^+ metabolism. Nevertheless, it was shown that MuSCs benefit from dietary NR supplementation (Zhang et al., 2016). Therefore, we tested whether exogenous NR can be beneficial during regeneration of healthy WT muscle. Recently, dietary NR has been shown to alleviate MuSC senescence and thereby improve MuSC number and function (Zhang et al., 2016). Furthermore, in aged mice as well as *mdx* mice, NR supplemented diet was shown to improve muscle regeneration after CTX (Zhang et al., 2016).

Albeit NR diet did not lead to changes in muscle NAD^+ content (similar to previously reported by Frederick et al. (2016)), it still had a positive effect on the regenerative capacity of muscle as regeneration of NAD^+ was more efficient in injured muscle of supplemented mice. Furthermore, we measured higher levels of the MuSC marker *Pax7* in supplemented mice, which translated into a higher level of transcriptional MuSC activation after injury. These data propose once more that NR serves as an important NAD^+ precursor in the challenged muscle and confirm the relevance of NRK-driven NAD^+ synthesis during regeneration. However, further work will be required to determine the effects of exogenous NR on the efficacy of muscle regeneration in terms of size and number of regenerating fibers.

In conclusion, in this work we showed that NRK1/2 dKO mice exhibit altered NAM salvage. Transcriptional regulation of atrophy related signaling is not perturbed in dKO muscle. However, in the context of muscle damage, our results demonstrate for the first time that NRK1/2 become the limiting NAD^+ producing enzymes and are required for efficient fiber maturation during regeneration. Our data further suggest that dietary supplementation of NR enhances the flux through NRKs to stimulate myogenic activation and repair, and to improve skeletal muscle regeneration.

METHODS

NRK1/2 dKO mice. Full-body NRK1/2 dKO mice were generated on a pure C57BL/6NTac background (Taconic Biosciences). Briefly, exons 3 to 7 of the *Nmrk1* gene or exons 2 to 7 of the *Nmrk2* gene were flanked with loxP sites. Floxed mice were crossed with mice expressing Cre recombinase under the general promoter of the Gt(ROSA)26Sor gene (Cre deleter). The deletion of these exons was validated by PCR, and resulted in the complete loss of the NRK1 or NRK2 protein. Mice carrying the whole-body, including germ line, *Nmrk1* or *Nmrk2* deletion were further bred to eliminate expression of the Cre recombinase. NRK1/NRK2 dKO mice were obtained by crossing sKO mice (J. Ratajczak).

Animal work. All animal experiments were carried out according to Swiss and EU ethical guidelines and approved by the local animal experimentation committee of the Canton de Vaud under license number VD 2764. Mice kept under a 12/12 h dark/light cycle and housed by 3–5 in ventilated cages with *ad libitum* access to water and food. For the dietary supplementation study, pellets were prepared by mixing powder diet (ResearchDiets, D12450JMi) with water alone (control diet, CD) or water containing NR (NR diet, NRD, Biosynth) as previously described (Feige et al., 2008). Mice received 400 mg NR per kg bodyweight per day, diet was changed every 2–3 days. Age- and body weight-matched animals were randomly assigned into different treatments.

Cardiotoxin (CTX)-induced muscle regeneration. Skeletal muscle injuries were performed as previously described (Lukjanenko et al., 2019) (n=8, 11-14 weeks old mice, 3/14 dpi group male mice, 7 dpi group female mice. NR diet study: n=8, 10 weeks old male mice for all groups). Mice were anesthetized with isofluran inhalation prior to shaving the one leg and intramuscular injection of 10 µM cardiotoxin (CTX, losartan, # L8102). Using a 22 gauge needle (Hamilton), tibialis anterior (TA) and gastrocnemius muscles were injected through one and two injections of 50 µL CTX, respectively. For muscle collection, mice were euthanized 3, 7, and 14 days post injection (dpi) with CO_2 (air mixture 85:15) in an inhalation chamber followed by cervical dislocation. Both contra-lateral (CL) and injured muscles were dissected free of fat, weighted and snap frozen in liquid nitrogen. TA muscles were cut in two parts, one part was snap frozen in liquid nitrogen for total RNA and NAD^+ extraction, the other part was embedded into optimal cutting temperature (OCT) compound and then frozen for histological analyses in isopentane cooled with liquid nitrogen.

Sciatic nerve resection. Mice were anesthetized with isofluran inhalation prior to shaving one leg and performing an incision in the mid-thigh region. The sciatic nerve was isolated and a piece of approximately 5 mm was resected. The incision was closed with suture thread. Tissue collection was performed 7 days after surgery as described above.

Immunohistochemistry. Frozen embedded TA muscle was sectioned to 10 µm on a cryostat and histology stainings were performed as previously described (Dammone et al., 2018). For Pax7-Ki67 and Pax7-MyoD staining, respectively, sections were dried for 10 min, then fixed for 10 min in 4% PFA, and permeabilized in cold methanol (-20°C) for 6 min. For antigen retrieval, slides were immersed twice in hot acid citric (0.01 M, ph 6) and then in a boiling water bath for 5 min each. Sections were subsequently blocked in 4% BSA (IgG-free Bovine Serum Albumin (BSA), Jackson #001-000-162) in PBS for 3h, followed by 30 minutes blocking with a goat-anti-mouse FAB (Jackson #115-007-003) at 1/50 in PBS. Chicken anti-laminin (Lifespan Bioscience #LC-C96142-100) and rabbit anti-Ki67 (Abcam #ab15580) or rabbit anti-MyoD antibody (Santa-Cruz #sc-304 or Abcam # ab198251) were used at 1/200, 1/100, and 1/100 in blocking solution, respectively, and incubated for 3 h. Mouse anti-Pax7 (DHSB, purified) at 2.5 µg/mL was then incubated overnight at 4°C. Secondary antibodies Alexa488-goat anti-rabbit (1/1000) and Alexa647-goat anti-chicken (1/1000) were incubated for 1 h in blocking solution. Pax7 signal was amplified using goat-anti mouse IgG1-biotin at 1/1000 (Jackson ImmunoResearch #115-065-205) followed by Streptavidin Alexa555 at 1/2000 (Life Tech. S-21381), together with Hoechst. Stained tissue sections were imaged using an Olympus VS120 slide scanner and analyzed with the VS-ASW FL software measurement tools or the MetaXpress software using an automated image processing algorithm developed internally (Guillaume Jacot), followed by manual quality control. The number of Pax7, MyoD and Ki67 positive cells was determined manually by counting at multiple randomly chosen areas of the injured region. Cross sectional area (CSA) of was detected by laminin/DAPI stainings and measured for all regenerating myofibers with centralized nuclei of the section.

Gene expression. Total mRNA from tissues was extracted with TRIzol (Life Technologies) and the microRNA extraction kit (Qiagen) according to manufacturer's instructions. mRNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's protocol. Expression of mRNA was then measured by quantitative polymerase chain reaction (qPCR) using SYBR Green real time PCR technology and the Light Cycler 480 (Roche). Gene expression was normalized to housekeeping genes *Atp5b, Eif2a* and *Psmb4* for CTX experiments and to *Actinb* in the denervation study. Relative gene expression between groups or genotypes was assessed using the ΔΔCt method. Primers used: *Actinb* (fw CTTCTTGGGTATGGAATCCTG; rev ACTGTGTTGGCATAGAGGT), *Aox1* (fw CGATTGAGGAAGCTATACAACACAA; rev CCTGGCCGCCTATGTGTATT), *Atp5b* (fw ACCTCGGTGCAGGCTATCTA; rev AATAGCCCGGGACAACACAG), *Atrogin1* (fw TGCTCCGTCTCACTTTCCCC; rev AGTGTTGTCGTGTGCTGGGA), *CD11b* (fw GCCTGTGAAGTACGCCATCT; rev GCCCAGGTTGTTGAACTGGT), *Eif1a* (fw CACGGTGCTTCCCAGAGAAT; rev TGCAGTAGTCCCTTGTTAGCG), *F4/80* (fw CTCTTCTGGGGCTTCAGTGG; rev TGTCAGTGCAGGTGGCATAA), *Hdac4* (fw GCTGACCTCAGTGTTCGTCA; rev CTATCCACCCCAACACCACC), *IL-1beta* (fw TGCCACCTTTTGACAGTGATGA; rev TGCCTGCCTGAAGCTCTTGT), *IL-6* (fw GGTGACAACCACGGCCTTCCC; rev AAGCCTCCGACTTGTGAAGTGGT), *Msr1* (fw ATTGGCTTCCCTGGAGGTCG; rev GGAGTTATACTGATCTTGATCCGCC), *Murf1* (fw GCTACCTTCCTCTCAAGTGCCA; rev CAGCCCTTGGAGGCTTCTACA), *Myh3* (fw ACAGTCAGAGGTGTGACTCAGC; rev TCCGACTTGCGGAGGAAAG), *Myh4* (fw CATCTGGTAACACAAGAGGTGC; rev ACTTCCGGAGGTAAGGAGCA), *Myod1* (fw GCAGATGCACCACCAGAGTC; rev GCACCTGATAAATCGCATTGG), *Myogenin* (fw GTGCCCAGTGAATGCAACTC; rev CGCGAGCAAATGATCTCCTG), *Nampt* (fw TCGCAAGAGACTGCTGGCATA; rev AGAGCAATTCCCGCCACAGT), *Nmnat1* (fw TGGCTCTTTTAACCCCATCAC; rev TCTTCTTGTACGCATCACCGA), *Nmrk1* (fw CCCAACTGCAGCGTCATATC; rev CCTTGAGCACTTTCCAAGGC), *Nmrk2* (fw GACCAGTCACCTCCAGTCCC; rev TTGGTCACCCCTCCAATGCC), *Nnmt1* (fw TGCCTGGGTGCTGTAAAAGG; rev CCCAGAGGTTCTGGTCTGTG), *Pax7* (fw CTGGAAGTGTCCACCCCTCT; rev TTGTGACGGATGTGGTTCGG), *Pnp* (fw GCATTCCACTCCCGGATCG; rev TAGAGAGCTACTTGGACCCCAC), *Ppargc1a* (fw AAGTGTGGAACTCTCTGGAACTG; rev GGGTTATCTTGGTTGGCTTTATG), *Psmb4* (fw GCGAGTCAACGACAGCACTA; rev TCATCAATCACCATCTGGCCG), *Tnfa* (fw AGCCGATGGGTTGTACCTTG; rev ATAGCAAATCGGCTGACGGT).

NAD^+ measurements and LC-MS metabolomics. NAD^+ levels were measured with an enzymatic cycling assay as previously described (Dall et al., 2018). Briefly, ca. 15 mg muscle tissue was lysed in 200 µL 0.6 M perchloric acid using a TissueLyser II (Qiagen). After centrifugation, the supernatant was diluted 500-fold in 100 mM Na_2HPO_4 pH 8.0. 100 µL of diluted sample was combined with 100 µL reaction mix (100 mM Na_2HPO_4 pH 8, 2% ethanol, 90 U/mL alcohol dehydrogenase, 130 mU/mL diaphorase, 10 µM resazurin, 10 µM flavin mononucleotide, 10 mM nicotinamide), and the fluorescence increase at Ex/Em 540/580 nm was measured over 10 min. NAD^+ content was calculated from a standard curve and normalized to tissue weight. For the dietary NR study, NAD^+ was extracted from ca. 20 mg of frozen tissue and measured using the colorimetric EnzyChrom NAD^+/NADH assay kit according to manufacturer's instructions (BioAssay Systems).

Sample preparation for LC-MS metabolomics analysis was based on Ratajczak et al. (2016). Samples (2-10 mg liver; 9-18 mg muscle; 60 µL plasma) were reconstituted in 200 µL of 100 mM ammonium acetate acetonitrile:water 85:15 (v/v). Liver and muscle extracts were homogenized using a TissueLyser II (Qiagen) for 1.5 min at 20 Hz, followed by protein precipitation on ice for 1 h. Freshly thawed plasma was vortexed for 20 s. All samples followed 10 min centrifugation at 15.000 rpm at 4°C. The supernatants were either diluted 1:10 in 100 mM ammonium acetate acetonitrile:water 85:15 (v/v) (plasma), or directly transferred (liver, muscle) to glass vials for hydrophilic interaction chromatography (HILIC) ultra-high performance liquid chromatography mass spectrometry (UHPLC-MS) analysis. The UHPLC consisted of a binary pump, a cooled autosampler, and a column oven (Vanquish UHPLC+ Focused, Thermo Scientific), connected to an orbitrap mass spectrometer (Orbitrap Fusion Lumos Tribrid, Thermo Scientific) equipped with a heated electrospray (H-ESI) source. Samples were injected into an analytical column (2.1 mmx150 mm, 5 µm pore size, 200Å HILICON

iHILIC®-Fusion(P)), guarded by a pre-column (2.1 mm x 20 mm, 200Å HILICON iHILIC®-Fusion(P) Guard Kit) operating at 35°C. The mobile phase (10 mM ammonium acetate at pH 9, A, and acetonitrile, B) was pumped at 0.25 mL/min flow rate over a linear gradient of decreasing organic solvent (0.5-16 min, 90-25% B), followed by re-equilibration for a total running time of 30 min. On-the-fly alternating negative (3 kV) and positive (3.5 kV) ion modes was used for ionization and the following source parameters were applied: 20 sheath gas, 15 aux gas, ion transfer tube temperature 310°C, vaporizer temperature 280°C. MS acquisition was performed at 60k orbitrap resolution, in centroid mode, by scanning between the mass range 85 to 850 Da. The software Xcalibur v4.1.31.9 (Thermo Scientific) was used for instrument control, data acquisition and processing. Positive ion mode extracted chromatograms using the corresponding accurate mass of NR, Nam, NA, and NAD^+ were integrated and used for relative comparison. Retention time and mass detection was confirmed by authentic standards.

Statistical analysis. GraphPad Prism Software version 7.02 for Windows was used for preparation of graphs and statistical analysis. Statistical methods were chosen as stated in the figure legends. Student's t-test was used for comparison of 2 groups (WT vs. dKO). Comparison of multiple groups with single end point (denervation study) was done by one-way ANOVA and Bonferroni's multiple comparisons post-hoc test. Comparison of multiple groups and time points (CTX experiments) was performed by one-way ANOVA and Sidak's multiple comparisons post-hoc test. All data are expressed as mean value +/- s.e.m. Kolmogorov-Smirnov test was used for comparison of fiber area distribution.

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AUTHOR CONTRIBUTIONS

T.S., A.P. and J.F. designed the study. C.C. generated NRK1/2 dKO mice. T.S., P.C. and A.P. performed animal work and experiments. M.P.G. and S.M. performed LC-MS experiments. T.S. and G.J. performed histology analysis. T.S. and J.F. analyzed data and wrote the manuscript. All authors approved the final manuscript.

SUPPLEMENTARY DATA

Figure S1 — Expression of NAM excretion enzymes is not changed in dKO muscle.

Figure S2 — CTX-induced immune response is not altered in dKO muscle.

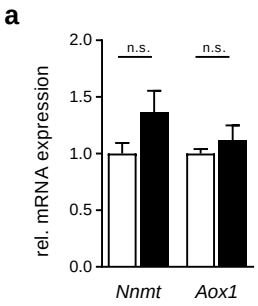

Figure S1: **Expression of NAM excretion enzymes is not changed in dKO muscle.** **(a)** mRNA expression levels of *Nnmt* and *Aox1* in TA muscle, n=16. Results shown are mean +/-s.e.m. with *n.s.* p>0.05 versus WT determined by Student's t-test. mRNA expression measured by qPCR relative to *Atp5b*, *Eif2a*, and *Psmb4* as housekeeping genes.

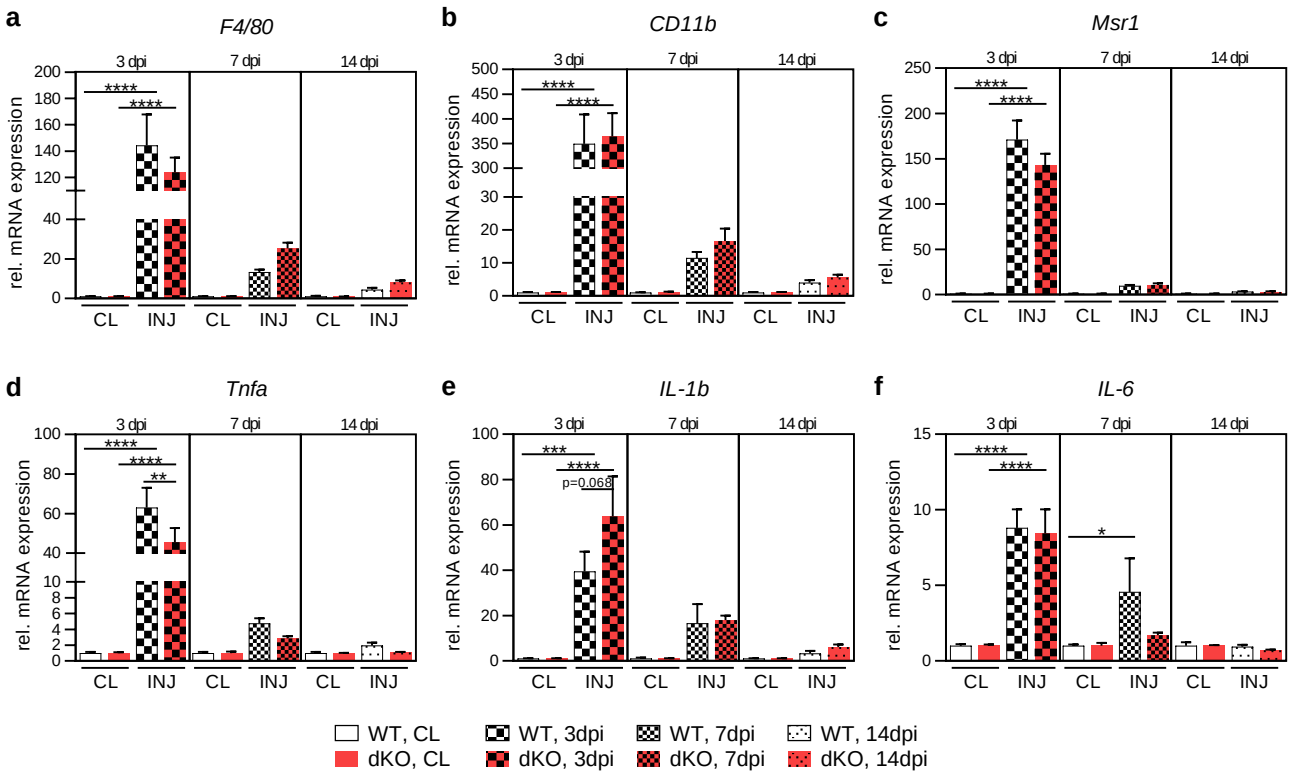

Figure S2: **CTX-induced immune response is not altered in dKO muscle.** mRNA expression of cellular surface markers of pro-inflammatory macrophages **(a)** *F4/80* and **(b)** *CD11b*, and anti-inflammatory macrophages **(c)** *Msr1*. mRNA expression of cytokines **(d)** *TNFa*, **(e)** *IL-1b*, and **(f)** *IL-6*. Gene expression measured by qPCR extracted from total TA muscle, n=6-8, results shown are mean +/-s.e.m., *p<0.05, **p>0.01, ***p<0.001, ****p<0.0001 versus the respective CL leg of each genotype by one-way ANOVA followed by Sidak's post-hoc test.

5 Discussion and perspectives

Given our current demographic development and the high prevalence of sarcopenia in the aging population, we can expect a dramatic increase of sarcopenic patients, which will pose a considerable health care burden in the near future (Goates et al., 2019; Pinedo-Villanueva et al., 2019). The specification of a consensus definition of sarcopenia based on measurable parameters (Cruz-Jentoft et al., 2010, 2019; Chen et al., 2014), and its recent recognition as a disease (Anker et al., 2016) paved the way for an emerging field of high-level research elucidating molecular mechanisms of sarcopenia and investigating therapeutic strategies for its treatment and prevention. However, so far, muscle aging has only been studied as a function of age, comparing old sarcopenic patients with young healthy controls (i.e. Giresi et al. (2005); Short et al. (2005)). While this helps to elucidate the physiological and metabolic changes skeletal muscle experiences during aging, studies comparing pathological to "normal" reduction of muscle function are scarce.

In the MEMOSA project presented in **Chapter 2**, we were the first to perform a study that characterized the molecular signature of human sarcopenia uncoupled from natural aging as we compared the muscle transcriptome of sarcopenic patients to age-matched healthy controls. Moreover, we demonstrated that sarcopenia has a common transcriptional profile across Asian, Caucasian, and Afro-Caribbean ethnicities.

The decrease of skeletal muscle mitochondrial function is a well-accepted feature of aging (Johnson et al., 2013; Cartee et al., 2016). For the first time, our data show that mitochondrial bioenergetics is also the major discriminating factor between sarcopenic patients and elderly with normal muscle function. We demonstrate that sarcopenic muscle exhibits accelerated loss of mitochondrial function that is correlated to clinical parameters of sarcopenia. Our data identify defects at multiple levels of mitochondrial regulation such as upstream transcriptional networks, mitochondrial dynamics, mitochondrial protein synthesis and quality control. All these alterations translate into a lower amount of mitochondria, downregulated expression of mitochondrial proteins as well as functional deficiency of the respiratory chain complexes I–IV in sarcopenic compared to healthy muscle.

Current recommendations to promote healthy muscle aging and manage sarcopenia focus on physical

activity and nutritional interventions (Denison et al., 2015; Lozano-Montoya et al., 2017). Indeed, exercise training is not only very effective to combat the decline in muscle mass and strength, it also improves mitochondrial respiratory capacity (Cartee et al., 2016). On the other hand, therapeutic interventions have been investigated, but most studies focused on aged individuals in general rather than on diagnosed sarcopenic patients (De Spiegeleer et al., 2018). For instance, vitamin D and hormone replacement therapy have shown promising results to improve functional muscle parameters in aged subjects, however to date no drugs have been approved for the treatment of sarcopenia (Cruz-Jentoft and Sayer, 2019). Our results highlight regulatory nodes and signaling pathways that could be therapeutically targeted to improve mitochondrial biogenesis, quality control, and redox homeostasis as a valid approach to treat sarcopenia.

Preclinical studies have linked age-related declines in intracellular NAD^+ levels to mitochondrial dysfunction in aged skeletal muscle (Gomes et al., 2013; Cantó et al., 2015). Albeit reports on reduced NAMPT levels in aged muscle suggested the same to be true in humans (Costford et al., 2010; de Guia et al., 2019), a recently published clinical trial challenged these findings and showed that human skeletal muscle NAD^+ levels are not compromised with chronological aging *per se* (Elhassan et al., 2019a). Our results obtained from the MEMOSA study are the first to show that human sarcopenic muscle (compared to aged control muscle) exhibits lower NAD^+ content, which is likely a consequence of reduced NAD^+ production. We further demonstrate that NAD^+ levels are correlated to muscle strength and function in our study subjects.
Declining NAD^+ was shown to affect the activity of NAD^+-dependent enzymes such as SIRT1, an energy sensing regulator of mitochondrial metabolism upstream of PGC1α (Mouchiroud et al., 2013). Therefore, different therapeutic strategies are being explored in an approach to increase cellular NAD^+ and SIRT1 activity. Even though the pure age-associated reduction in human skeletal muscle NAD^+ levels is still a matter of debate, recent research shows that exercise training is an effective means to increase NAD^+ salvage capacity (expression of NAD^+ salvage enzymes) in aged human muscle (Costford et al., 2010; de Guia et al., 2019). This is in line with preclinical data additionally reporting increases of NAD^+ and mitochondrial function in response to exercise (i.e. Cantó et al. (2010)). Certainly, the applicability of such intervention may be limited in sarcopenic patients either by the severity of the condition or secondary factors.
An emerging strategy to increase NAD^+ levels is through supplementation of dietary vitamin B_3 precursors of NAD^+ that were shown to improve mitochondrial function in preclinical models (Cantó et al., 2015; Connell et al., 2019). Particularly nicotinamide riboside (NR) has emerged as a promising candidate to ameliorate pathological muscle conditions and revert age-related mitochondrial dysfunction and stem cell senescence in mouse skeletal muscle (Cerutti et al., 2014; Khan et al., 2014; Ryu et al., 2016; Zhang et al., 2016). However, the investigative dimensions and proposed mechanisms of these studies were mostly restricted to readouts of mitochondrial function with insufficient focus on metabolization of NR and NAD^+, especially in aged animals.

In our work presented in **Chapter 3** we are the first to describe the metabolization of NR in aged tissue and further present a characterization of the global transcriptional response elicited by NR in rats. Specifically, we identified pathways that are altered in sarcopenic muscle, i.e. fibrosis, and showed that NR/NAD^+ has the potential to modulate and even normalize transcriptional patterns related to these pathways. Interestingly, our transcriptional profiling also revealed that the molecular response to NR is partially blunted in old animals and suggests that aged muscle could be in a state of partial NAD^+ resistance. We show that this is not due to a defect in the conversion of NR to NAD^+, and suggest that differential utilization of NAD^+ as well as altered excretion of NAM in sarcopenic muscle might contribute to this observation. With these results we are the first to show that age affects the metabolization, the transcriptional efficacy, and thus the therapeutic potential of NR and has to be taken into consideration when designing therapeutic strategies using this compound.
Two first clinical studies are now reporting on the effect of NR supplementation in aged, but not sarcopenic, individuals (Dolopikou et al., 2019; Elhassan et al., 2019b). The first study reports that a single oral dose of NR acutely improved redox homeostasis and exercise performance in old (but not in young) subjects (Dolopikou et al., 2019). A second, longer-term study showed that three weeks of oral NR supplementation did not change hand-grip strength of aged participants and also reported that the transcriptional signature of mitochondrial function was decreased with NR, in contrast to base-line and placebo (Elhassan et al., 2019b). While the findings of the latter study are in contrast to reports from mouse studies (i.e. (Zhang et al., 2016)), these results are similar to our findings after acute NR treatment in rats. This highlights that our transcriptomics data may provide a comprehensive basis for future investigations of the action spectrum of NR in aged humans and it will be interesting to further explore the new human datasets for overlaps with the specific pathways we identified in rat muscle. Furthermore, our data suggests that the design of future studies aiming to elucidate the effect of NR in sarcopenic subjects should amend "classical" primary readouts related to muscle strength and function with secondary readouts, i.e. for markers of fibrosis, which could influence muscle quality on a more long-term basis beyond the investigative period.

Despite the ambiguous results of first human trials (Dollerup et al., 2018; Martens et al., 2018; Dolopikou et al., 2019; Elhassan et al., 2019b), interest in therapeutically targeting NAD^+ synthesis is gaining momentum as other clinical trials are underway, i.e. aimed at understanding the effect of NR in mitochondrial diseases or obesity/insulin resistance (Connell et al., 2019). As the potential of NR as an NAD^+ precursor is largely dependent on the enzymatic activity of NR kinases (NRKs) (Ratajczak et al., 2016; Fletcher et al., 2017), comprehensive knowledge on their physiological function(s) will be of major importance. Recent muscle research has mainly focused on deciphering the role of muscle-specific NRK2 in genetic loss-of-function models, with the limitation of a functional compensation through NRK1 activity (Fletcher et al., 2017; Deloux

et al., 2018). Our results from NRK1/2 dKO mice presented in **Chapter 4** are the first to demonstrate that an endogenous NRK-dependent flux actively converts NR into NAD^+ in healthy skeletal muscle. We further show that NRKs are required for efficient NAD^+ salvage and myofiber maturation during muscle regeneration. This sheds new light on intrinsic functions of NRKs in the muscle and unveils the importance of endogenous NR for NAD^+ homeostasis in the challenged muscle. Our findings align well with previously published data on the role of NRK2 (aka muscle integrin binding protein, MIBP) in C2C12 muscle cell differentiation and adhesion as well as during zebrafish muscle development (Li et al., 1999, 2003; Goody et al., 2010). With this data we provide a missing link for a damage response model that places NRK-mediated NAD^+ synthesis as the key driver of myofiber embedding into the extracellular matrix during muscle regeneration (Goody and Henry, 2018). However, deciphering the mechanistic aspects that connect NRK activity and NAD^+ availability with myofiber maturation will be important to fully endorse the proposed model.

Finally, in line with our findings as well as studies in aged and dystrophic mice (Zhang et al., 2016), we showed that augmenting NRK activity by exogenous NR improves the regenerative capacity in wild-type mice and could therefore be useful to support muscle recovery after damage induced by accidents, surgery or strenuous exercise. Our finding that specific conditions induce preferential utilization of unique types of NAD^+ precursors shows that the therapeutic potential of NAD^+ boosting may strongly depend on the choice of precursor. Therefore, further studies are needed to fully elucidate the role of NRKs and endogenous NR dynamics in the maintenance of tissue function in muscle and other tissues.

5.1 Conclusion

In this thesis, I demonstrated that human sarcopenia is tightly linked to mitochondrial bioenergetics and NAD^+ metabolism. I discovered that metabolization and efficacy of NR is changed during aging, and showed that NR supplementation is nevertheless a promising approach to normalize specific transcriptional dysregulations associated to sarcopenia in rats. Finally, I described NR as a unique NAD^+ precursor during muscle regeneration in mice and I am confident that my findings on the specific role of NRKs in the muscle will inspire future research and set the stage for the development of patient-specific NAD^+ boosting therapies.

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Work & research experience

Apr. 2015-Sep.2019 — Doctoral assistant, Nestlé Institute of Health Sciences (NIHS), Lausanne, Switzerland

Apr. 2015-Sep.2019 — Teaching assistant, Ecole Polytechnique Fédérale de Lausanne (EPFL), Switzerland
M.Sc. courses "Biological Chemistry 2" & "Biological Chemistry 3" (300 hours teaching)

Oct. 2014-Feb.2015 — Teaching assistant, University of Konstanz, Germany
B.Sc. courses "Plant physiology" & "Animal physiology"

Nov. 2012-May 2013 — Internship, Pharmacology Group (Prof. Dr. G. Muench), School of Medicine, University of Western Sydney, Campbelltown, NSW, Australia
Projects: "Establishment of a high-performance liquid chromatographic method for the quantification of α-dicarbonyls in blood" & "Determination of methylglyoxal as a biomarker for cognitive decline in diabetic patients"

May 2011-May 2012 — Student assistant, GATC Biotech AG dept. Custom Sequencing, Konstanz, Germany

Jan.-Jun. 2010 — Internship, StudEx scholarship, Space Biology Group (Prof. M. Egli), Swiss Federal Institute of Technology (ETH) Zurich, Switzerland
Project: "Electrophysiological study investigating cellular effects of weightlessness induced muscle atrophy" Participation in 52nd ESA Parabolic Flight Campaign, Bordeaux, France

2009-2013 — Student assistant in different research groups, University of Konstanz, Germany: Summerer Group, chemical biology; Kroth Group, plant ecophysiology; Meyer Group, evolutionary biology; Botanical garden, Dr. G. Schmitz

Educational background

Apr. 2015-Sep. 2019 — PhD candidate in molecular metabolism, Nestlé Institute of Health Sciences (NIHS) & Ecole Polytechnique Fédérale de Lausanne (EPFL), Switzerland
PhD thesis: "Targeting mitochondrial bioenergetics and NAD^+ metabolism during sarcopenia and muscle regeneration" Dr. J. Feige (NIHS) & Prof. E. Meylan (EPFL), EDMS Doctoral School of Molecular Sciences

2011-2014 — M.Sc. of Biological Sciences, University of Konstanz, Germany
M.Sc. thesis: "Evaluation of coenzyme Q_{10} for the prevention of UV-induced damages in skin cells" Prof. A. Bürkle (Univ. Konstanz) & Prof. J. Bergemann (Sigmaringen)

2009 — Exchange semester, ERASMUS scholarship, University of Tromsø, Norway

2007-2011 — B.Sc. of Biological Sciences, University of Konstanz, Germany
B.Sc. thesis: "Personality and urbanization in European blackbirds *Turdus merula* – a neophobia experiment" Prof. M. Wikelski, Max Planck Institute for Ornithology, Radolfzell, Germany

Scholarships

Jan.-Jun. 2010	Leonardo da Vinci scholarship funded by StudEx, ETH Zurich, Switzerland
Aug.-Dec. 2009	ERASMUS scholarship, University of Tromsø, Norway

Conferences & Presentations

September 2019	*CatCave9*, Lausanne, Switzerland (Invited guest scientist)
June 2017	FASEB *NAD+ Metabolism and Signaling,* New Orleans, USA (Poster)
September 2016	*Muscle stem cells, metabolism and ageing* LIMNA Symposium, Lausanne, Switzerland (Selected short talk & poster)
June 2016	*Aging and Metabolism* Cell Symposium, Sitges, Spain (Poster)

Extracurricular activities

Leadership experience	Co-Organizer of EPFL PhD summer school "*Micro and Optical Technologies in Biomedical Science*" (Sep. 2018); Guest organizer of *7th LIMNA Symposium*, Lausanne, Switzerland (Apr. 2017); Organizer of EPFL PhD winter school "*Gut matters: Human health, gut microbiome and nutrition*" (Mar. 2017)
Voluntary work	Election worker for governmental, local and European elections (City of Konstanz), youth work with girl scouts 6-13 years of age (PSG Wernau)

Skills & competences

Languages	German (native), English (excellent), French (good), Norwegian (basic)
IT	Windows, LaTeX, MS Office, GraphPad Prism, ImageJ, MetaXpress, Adobe Illustrator
Driving licence	Car (B), motorcycle (A)
Interests	Creative work, sewing, diving (CMAS**), mountain sports, travelling

Laboratory techniques

Preclinical studies	Mouse breeding, intraperitoneal & subcutaneous injections, muscle injury by intramuscular injections, sciatic denervation, muscle dissection & organ collection, tissue embedding for histological analysis
Cell culture	Cell line culture, primary cell isolation & culture, gene reporter assays, adenoviral knockdown & overexpression, transfection
Molecular biology	DNA & mRNA extraction, qPCR, basic micro array analysis, protein purification, SDS-page, western blotting, NAD^+ metabolite extraction, dual luciferase assay, immuno-histochemistry, ELISA, HPLC

Publications

Sonntag, T., Cichosz, P., Jacot, G., Giner, M.P., Pannérec, A., Moco, S., Cantó, C., Feige, J.N. (2019). "Nicotinamide riboside kinases are rate-limiting for skeletal muscle regeneration" in preparation.

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