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MECHANISMS OF INTER-ORGAN CROSSTALK MEDIATED BY TRYPTOPHAN METABOLISM

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Mechanisms of inter-organ crosstalk mediated by tryptophan metabolism
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To all the curious minds.

Keep asking questions.

"***Trust your feet***"

Climbers' common knowledge

POPULAR SCIENCE SUMMARY

Food provides us with many nutrients and energy needed for daily life. Many nutrients double up in their role; they work both as fuel and as building blocks for the cells. A good example of this is amino-acids, which are the building blocks of proteins. Amino-acids can also work as fuel or be metabolized into signaling molecules. Signaling molecules, as the name indicates, signal to the cells about changes that are happening in the body and how the cells should react to them. In the work of this thesis, we looked into the effects of the amino-acid tryptophan. Tryptophan is an essential amino-acid, which means the human body cannot produce it and must ingest it from diet. Ingested tryptophan can follow one of three paths: protein synthesis, metabolization into serotonin (a neurotransmitter with a central role in mood regulation, often referred to as the "happiness" neurotransmitter) or be degraded in what is known as the kynurenine pathway of tryptophan degradation (KP). Intermediate molecules of this pathway (which we can call metabolites) are signaling molecules and have been associated with energy regulation and immune tolerance but also inflammation, neuroinflammation, cancer, mental disease and more. Previous studies have shown that exercise helps to reduce the bad effects of over-activation of this pathway and improve the good ones.

The work of this thesis is comprised of 3 studies:

In **study I**, we investigated if tryptophan supplementation in the food, together with physical exercise, has an effect on the KP and its known effects. For this end we used mice, and separated them into 4 groups: control diet or tryptophan diet without access to a running wheel (that we call sedentary), and control diet or tryptophan diet with access to a running wheel. The mice on a tryptophan diet ran more in the wheels than controls. We investigated if this could be due to increased anxiety, but in the behavioral tests we performed we did not see a difference. We also looked into well-established markers of inflammation and dysregulation in the brain, without difference. Markers of energy metabolism in muscle or fat of these mice was also not changed. We measured the metabolites of the KP in circulation in the blood of these mice. All the mice with tryptophan diet had increased metabolites of the KP, but the ones that had access to the running wheel had less than the sedentary. We concluded that the tryptophan supplementation in the diet was enough to raise the KP metabolites in circulation, but not enough to cause the changes described in the literature. It is however possible that a higher one-time dose would trigger a different response.

In **study II**, we measure the KP metabolites in stroke patients in primary rehabilitation. Stroke is caused by the lack of blood supply to the brain. It can be classified as ischemic or hemorrhagic. 80 percent of strokes are of ischemic type, which is characterized by a blockage in a blood supplying artery, whereas in hemorrhagic stroke the decrease of blood supply is due to a rupture of artery and bleeding. KP metabolites have been described in the literature as elevated immediately after stroke, and we wanted to investigate if they are still elevated once patients reach primary rehabilitation. Indeed, in the ischemic stroke patients, KP metabolites were elevated and showed a tendency to decrease slowly, indicating that there was still ongoing

inflammation. In our small sample of hemorrhagic patients, the results were different than the ischemic. The metabolites of these patients increased over time, rather than decreased, indicating also different development of the inflammation. This could be useful when designing therapy strategies for these patients, as more insight provides an opportunity for more personalized care.

In **study III**, we characterized a protein called LMCD1 in skeletal muscle. Skeletal muscle is the type of muscle that is responsible for movement (think of your quads, or biceps). Using a mouse model, we increased the amount of LMCD1 in their muscles. The muscles with increased LMCD1 were bigger, and more resistant to fatigue. We further investigated how LMCD1 induced these changes, and we found that it interacts with a protein called calcineurin that regulates calcium signaling in the muscle. Calcium signaling is responsible for muscle contraction: when calcium increases inside the muscle cell, it contracts, and when calcium decreases, the cell relaxes. So, LMCD1 improves the efficiency of this event, improving muscle mass, force, and resistance to fatigue. Loss of muscle mass and impaired force is observed in aged people and in some diseases; understanding how it is regulated helps to develop therapeutic targets that improve this aspect of their conditions.

Elderly people naturally have muscle mass loss, which is aggravated when they become inactive (as when they are immobilized due to stroke). Increased inflammation (as reflected by the increased KP metabolites we observed in **study II**), and the common neurological effects of stroke only aggravate this problem. In **study I** we looked at how nutrition affects metabolism and potentially exercise volition, and in **study III** we looked at how to improve muscle mass and metabolism. Understanding how all these adaptations are regulated contributes to the development of therapy strategies for conditions like stroke.

ABSTRACT

Physical exercise and nutrition balance the "energy in-energy out" equilibrium that keeps the system at a dynamic homeostasis, energetically speaking. However, this is a simplistic vision of a dynamic system that is in a constant fine-tuning of all of its parts. Physical exercise benefits for metabolic health go beyond its "energy out" role: it modulates whole body metabolism, insulin sensitivity and induces favorable remodeling in other tissues through secreted factors. Old age and disease negatively impact muscle mass, which in turn has negative consequences on long-term health and quality of life. In the same fashion, nutrition goes beyond "energy in". Many nutrients' role goes beyond supplying energy or becoming building blocks for cells – they can also contribute as signaling molecules, and/or can be metabolized to bioactives. Such is the case for tryptophan (TRP). TRP is an essential amino-acid and is also the precursor for the neurotransmitters serotonin and melatonin. The majority of TRP is metabolized through the kynurenine pathway of TRP degradation (KP). Some KP intermediaries have been linked to inflammation, neuroinflammation, cancer, immune evasion, depression and other neurological diseases. Other intermediaries have been linked to improved energy metabolism, neuroprotection, immune-regulation, and fat depot browning. Interestingly, endurance physical exercise has been shown to tilt the metabolism of the KP towards the protective branch.

In **paper I**, we investigated if excess TRP supplementation in diet has an effect in physiology and behavior in a rodent model, and if access to a running wheel influenced the observations. All mice in TRP diet had increased KP metabolites in circulation, but access to a wheel ameliorated the load of most metabolites. Comparing the "+wheel" groups, mice with the TRP supplementation run more, but show no further muscle adaptations to exercise when compared to control-fed mice. Mice also displayed no difference in behavior tests nor brain gene expression markers when compared to the sedentary groups (without wheels). TRP metabolites had short half-life in circulation, returning to normal values 4 hours after the inactivity period started. Our results suggest that the increased TRP dosage supplemented in diet, without further inflammatory stimuli, is still within the body´s capacity of metabolization.

In **paper II**, we measured the KP metabolites in stroke patients in the early rehabilitation phase. KP is dysregulated immediately after stroke, but its long-term behavior and effects on outcome are unknown. We observed that in ischemic stroke, most metabolites of the pathway show a tendency to slowly decline over the primary rehabilitation phase. The pathway is thus still active and recovering, and this may influence response to therapy. In a small subgroup of patients suffering from hemorrhagic stroke, we observed the opposite behavior in the metabolites, i.e. they were slowly increasing. This indicates that ischemic and hemorrhagic stroke are not only different in entomology but also in recovery, which suggests different therapeutic approaches may be necessary for optimal recovery.

In **paper III**, we investigated the role of LMCD1 in skeletal muscle by ectopically expressing LMCD1 in mice with an intramuscular injection of adenovirus. LMCD1 improved force and resistance to fatigue with less calcium requirements, indicating improved calcium signaling. This event is dependent on calcineurin and that the LMCD1-calcineurin induces repression of

myoregulin, an inhibitor of SERCA. LMCD1 also increases markers of muscle mass like IGF-1 and PGC-1α4, and pathway activity related to muscle mass of mTOR and MAPK.

LIST OF SCIENTIFIC PAPERS

I. **Valente-Silva P**, Cervenka I, Ferreira DMS, Correia JC, Edman S, Horwath O, Heng B, Chow S, Jacobs KR, Guillemin GJ, Blomstrand E, Ruas JL. **Effects of Tryptophan Supplementation and Exercise on the Fate of Kynurenine Metabolites in Mice and Humans**. Metabolites. 2021 Aug 3;11(8):508. doi: 10.3390/metabo11080508. PMID: 34436450; PMCID: PMC8400416.

II. **Valente-Silva P**, Åkesson E, Von Euler M, Cronfalk BS, Ruas JL. **Comprehensive evaluation of circulating kynurenine pathway metabolites during stroke rehabilitation**. *Manuscript.*

III. Ferreira DMS, Cheng AJ, Agudelo LZ, Cervenka I, Chaillou T, Correia JC, Porsmyr-Palmertz M, Izadi M, Hansson A, Martínez-Redondo V, **Valente-Silva P**, Pettersson-Klein AT, Estall JL, Robinson MM, Nair KS, Lanner JT, Ruas JL. **LIM and cysteine-rich domains 1 (LMCD1) regulates skeletal muscle hypertrophy, calcium handling, and force**. Skelet Muscle. 2019 Oct 31;9(1):26. doi: 10.1186/s13395-019-0214-1. PMID: 31666122; PMCID: PMC6822430.

CONTENTS

LIST OF ABBREVIATIONS USED IN THE TEXT

3-HAA	3-Hydroxyanthranilic acid
3-HK	3-Hydroxykynurenine
AA	Anthranilic acid
AhR	Dioxin receptor
AMPK	AMP-activated protein kinase
ANGPTL4	Angiopoietin-like-4
ATF2	Activating transcription factor 2
BAIBA	Beta aminoisobutyric acid
BBB	Blood-brain-barrier
BCAAs	Branched chain amino acids
BDNF	Brain-derived neurotrophic factor
CamK	Calcium/calmodulin-dependent protein kinases
cAMP	cyclic AMP
CNS	Central nervous system
CREB	CAMP responsive element binding protein
CRP	C-reactive protein
CSF	Cerebrospinal fluid
DHPR	Dihydropyridine receptor
EDL	*Extensor digitorum longus*

ER	Endoplasmic reticulum
ERRα	Estrogen-related receptor alpha
ESR	Erythrocyte sedimentation rate
FA	Fatty acids
FDB	*Flexor digitoris brevis*
FFA	Free fatty acids
FGF21	Fibroblast growth factor
FNDC5	Fibronectin type III domain 5
GAS	*Gastrocnemius*
GLUT	Glucose transporter
GPR	G-protein-coupled receptors
HAAO	3-Hydroxyanthranilic acid dioxygenase
HDACs	Histone deacetylases
HFD	High fat diet
HIF-1α	Hypoxia-inducible factor 1 alpha
IDO	Indoleamine 2,3-dioygenase
IFN-γ	Interferon gamma
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IRS	Insulin receptor substrate

KATs	Kynurenine aminotransferases
KMO	Kynurenine 3-Monooxygenase
KO	Knockout
KP	Kynurenine pathway of tryptophan degradation
KYN	Kynurenine
KYNA	Kynurenic acid
KYNU	Kynureninase
LATs	Large neutral amino acid transporters
LMCD1	LIM and cysteine rich domain 1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MAS	Malate aspartate shuttle
MDA	Malondialdehyde
MEF2	Myocyte enhancer factor 2
Metrnl	Meteorin-like
MLN	Myoregulin
mTOR	Mammalian target of rapamycin
mtTFA	Mitochondrial transcriptional factor A
MURF1	Muscle RING-finger protein-1
MyHC	Myosin heavy chain

NAFLD	Non-alcoholic fatty liver disease
NF-KB	Nuclear factor KB
NFAT	Nuclear factor of activated T-cells
NIHSS	National Institutes of Health Stroke Scale
NLR	Neutrophil/Lymphocyte ratio
NMDAR	N-methyl D-aspartate receptor
NRF	Nuclear respiratory factors
PA	Picolinic acid
PDK-1	3-Phosphoinositide-dependent protein kinase 1
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPAR	Peroxisome proliferator-activated receptor
QA	Quinolinic acid
QPRT	Quinolinate phosphoribosyltransferase
ROS	Radical oxygen species
RyR	Ryanodine receptor
SERCA	Sarco/endoplasmic reticulum Ca^{2+} ATPase
SR	Sarcoplasmic reticulum
TA	*Tibialis anterior*
TDO	Tryptophan 2,3 dioxygenase
TFAM	Mitochondrial transcriptional factor A

TNFα	Tumor necrosis factor alpha
Treg	Regulatory T cells
TRP	Tryptophan
TSE	Metabolic cages
VEGF	Vascular endothelial growth factors
VGLUT	Vesicular glutamate transporter
WAT	White adipose tissue
XA	Xanthurenic acid
α7-nAChR - α7	Nicotinic acetylcholine receptor

1 INTRODUCTION

The idea of exercise and diet as both prophylaxis and cure for disease is not new. Since 6000BCE in India, when Susrata was the first recorded physician to prescribe exercise, to the Chinese East Han Dynasty (25 – 250 BCE), when Hua T'O affirmed *"The body needs exercise (…) for exercise expels the bad air in the system promotes free circulation of the blood and prevent sickness"* [1]. In ancient Greece, exercising was seen as a national duty and the gymnasium was a common place for training, socializing, and embarking on philosophical discussions under the protection and patronage of the Gods. In the words of Hippocrates "(…) *food and exercise, while possessing opposite qualities, yet work together to produce health*" [1].

Today, there is evidence that physical activity and physical exercise can help prevent and ameliorate a wide range of diseases from metabolic and cardiovascular conditions to neurodegenerative and mental health disorders. For example, in type 2 Diabetes, exercise helps to improve insulin sensitivity and reduce chronic inflammation by acting directly and indirectly on muscle, fat depots and the immune system [2,3]. Lifestyle interventions including regular exercise and dietary modifications are more successful than pharmacological intervention alone in the treatment of type 2 Diabetes and aging-related sarcopenia [4,5]. In addition, a relatively low threshold of physical activity (>3500 steps/day or >2.5h walking/week) [6,7] is enough to offer significant protection to glucose intolerant patients from transition into type 2 Diabetes. Regarding cardiovascular disease, physical exercise helps to reduce hypertension, improve cholesterol and fatty acid metabolism, and improve blood vessel health [8,9]. Physical exercise also reduces the risk for a plethora of other maladies like non-alcoholic fatty liver disease (NAFLD), cancer, dementia, Alzheimer´s disease, Parkinson´s disease and more [10–14], and helps to improve and stabilize mood in depression and major depressive disorder [15–18].

Endurance exercise (such as jogging or cycling) improves muscle energy metabolism, while resistance training increases muscle mass and strength. Endurance exercise is probably the most adopted type of exercise and has recognized effects on insulin-independent muscle glucose uptake and metabolism, as well as insulin sensitivity. At the cellular level, it increases muscle oxidative capacity by increasing mitochondrial biogenesis and respiration, and improves energy metabolism and metabolic flexibility [2,3,19–21].

Resistance exercise increases muscle glycolytic and oxidative capacity [22]. There is some evidence that resistance exercise training also helps improving insulin sensitivity in diabetic patients [23–25] and improves lean muscle mass and increases peak oxygen consumption rate in obese patients [10,26].

The second part of this centuries-old knowledge is diet and nutrition. Improving metabolism requires a balance between energy expenditure and proper nutrient intake. However, the importance of nutrition goes beyond supplying the correct fuel or building blocks necessary for cell function. Many nutrients and their metabolites can act as signaling molecules, adding a new level of significance to what we ingest. In the gut, nutrients can be metabolized by gut bacteria and generate important signaling molecules for gut-host communication. One such

example of importance to the work of this thesis is the essential amino acid tryptophan (TRP). TRP is historically known as the precursor for serotonin, a neurotransmitter with a role in mood regulation. Serotonin also plays an important role in gut motility and pain perception. TRP can be used for protein synthesis, or degraded through the kynurenine pathway of TRP degradation (KP). The end product of this pathway is NAD^+, but several intermediaries have been shown to have a role in mood, inflammation, disease and circadian regulation [27–30]. TRP can also be metabolized by gut microbiota into indole compounds that can then serve as interaction molecules with the host [31,32].

2 SKELETAL MUSCLE

There are more than 600 muscles in the human body, of which more than 400 are skeletal muscles. In this chapter, we will focus on skeletal muscle in the context of movement and its adaptations to physical exercise.

Skeletal muscle accounts for 40% of body mass in a healthy adult. It is composed of muscle fibers, polynucleated elongated cells with the equipment to contract upon stimuli (actin/myosin filaments), for calcium handling (as we will go over in detail in the next section) and different ways of generating energy for the contractile force.

Muscle fibers are often categorized based on their fuel preference, fatigability, and time to peak tension (twitch). They can be classified in a spectrum that goes from glycolytic fast-twitch fatigable fibers (type II) to oxidative slow-twitch fatigue resistant (type I). Fast twitch fibers preferably use glucose for ATP production, have a higher strength of contraction and have higher fatigability. On the other hand, slow twitch fibers have higher oxidative capacity from fatty acids (FA) and are fatigue resistant (**Table 1**) [2,33–35].

Table 1. Characteristics of Human Skeletal Muscle Fiber Types. Adapted from [2].

	Type I	**Type IIa**	**Type IIx**
Percent distribution in whole muscle *(vastus lateralis)*	54.0 ± 12.2	32.3 ± 9.1	13.0 ± 7.6
Force production (power output)	Weak	Intermediate	Strong
Time to peak tension	Slow	Fast	Fast
Fatigability	Fatigue resistant	Fatigue resistant	Fast fatigable
Metabolic characteristics	Oxidative	Oxidative-glycolytic	Glycolytic
Exercise-type dominance	Prolonged low intensity	Moderate duration, high intensity	Short duration, maximal effort

Fiber type composition of skeletal muscle (**Table 2**) has been shown to change in mouse models of exercise, including mouse transgenic models that recapitulate specific exercise components [34,36–39]. Electrical stimulation, which can be used as an *in vitro* mimic of exercise, also shows type II to type I phenotypical changes [37].

Table 2. Myosin heavy chain (MyHC) in commonly studied muscle beds from C57B/6 mice. Values are given as median ± semi amplitude for *extensor digitorum longus* (EDL), soleus, *tibialis anterior* (TA) and *gastrocnemius* (GAS) and fiber % for *flexor digitorum brevis* (FDB). Adapted from [33] and [35].

	EDL	**Soleus**	**TA**	**GAS**	**FDB (%)**
Type I	0 ± 0.688	41.5 ±12.21	0 ± 0	0.81 ± 2.34	4.4 +/- 2.9
Type IIa	10.82 ±6.6	57.56 ±13.32	25.52 ± 15.15	17.01 ± 12.64	43.9 +/- 1.9
Type IIx	0 ± 0	0.15 ± 2.82	0 ± 0	0 ± 0	51.6 +/- 4.8
Type IIb	88.3 ±6.6	0 ± 0	74.48 ± 15.15	84.5 ± 15.29	0

Improved energy metabolism is one of the tissue adaptations that occurs at the transcriptional, translational, and post-translational levels. It is brought about by several mechanism acting in parallel, resulting in increased glucose transporters (GLUTs), FA transporters, improved flux through glycolysis and mitochondrial biogenesis, with net increase and more efficient energy production (**Figure 1**). This demands an increase fuel and oxygen supply resulting in an increase in angiogenesis (to increase the amount of blood vessels to the muscle) and myoglobin (oxygen storing protein in muscle).

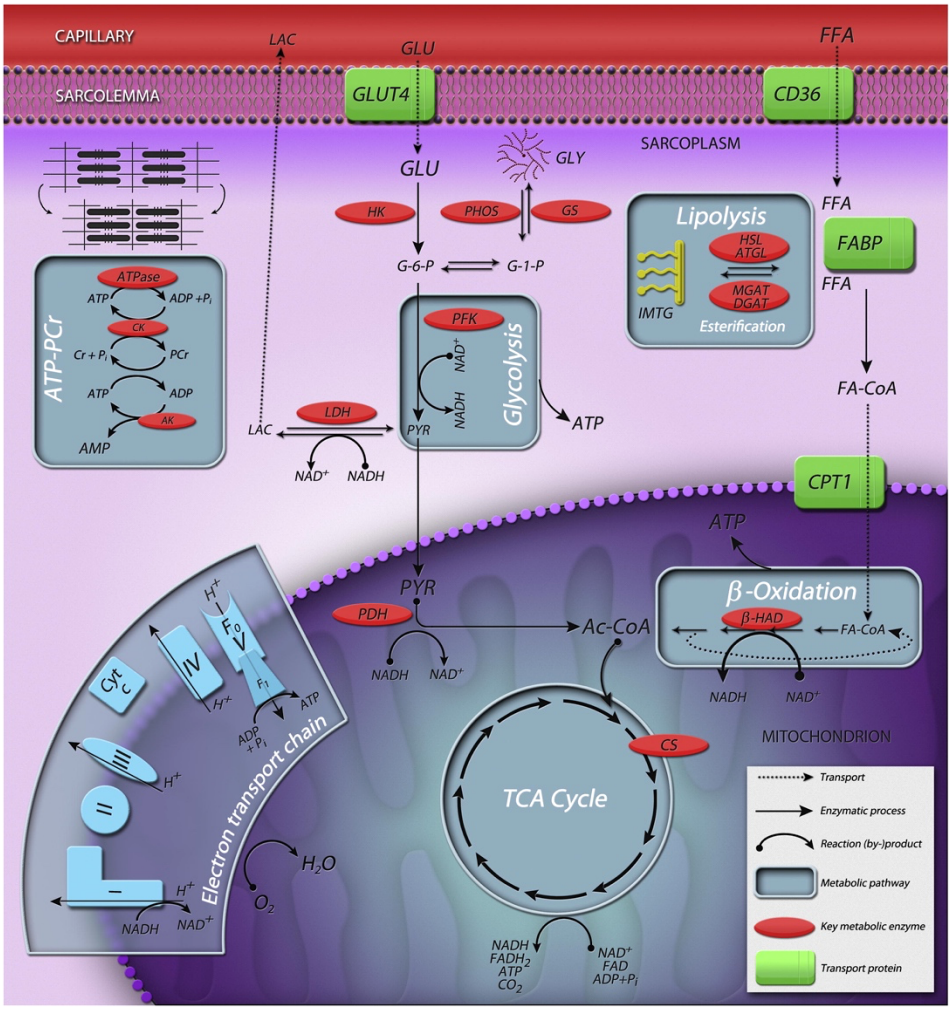

Figure 1. Energy metabolism adaptations in skeletal muscle in response to exercise stimuli. Abbreviations: Ac-coA - acetyl-CoA ; AK - adenylate kinase (myokinase); ATGL - adipose triglyceride lipase; CK – creatine kinase; CPT1 - carnitine palmitoyltransferase 1; CS - citrate synthase; Cyt c - cytochrome c; DGAT - diacylglycerol acyltransferase; FABP - fatty acid binding protein; FAT/CD36 - Fatty acyl translocase; FFA – free fatty acids; GLU – glucose; GLUT4 – glucose transporter type 4; GLY – glycogen; GS - glycogen synthase; HK -hexokinase, ; HSL – hormone sensitive lipase; IMTG – intramuscular triglycerides; LAC - lactate ; LDH - lactate dehydrogenase; MGAT - monoacylglycerol acyltransferase; PDH - pyruvate dehydrogenase; PFK – phosphofructokinase; PHOS - glycogen phosphorylase; PYR – pyruvate; TCA - tricarboxylic acid. Adapted from Egan and Zierath, 2013, Exercise metabolism and the molecular regulation of skeletal muscle adaptation, Cell Metabolism [2]. With permission from Elsevier.

The adaptations to physical exercise significantly impact systemic energy consumption and metabolic rate, since their ultimate aim is to increase muscle capacity to produce the necessary energy and contractile force [10,37]. Therefore, for muscle to function properly, it requires the integration and of various systems for adequate nutrient and oxygen supply. As we will see in **section 3**, muscle communicates to the rest of the body through secreted factors called myokines, coordinating the required changes in other tissues.

Intracellularly, there are many systems working in a concerted manner to orchestrate muscle contraction. Calcium signaling is one of the principal drivers of muscle contraction and is also an integrator of signaling with gene expression response, as we will discuss in the next chapters.

2.1 LOCOMOTION AND MUSCLE CONTRACTION: THE ROLE OF CALCIUM

Walking, running, dancing, or indeed just typing these words requires a concerted action of skeletal muscle contraction and relaxation, allowing the movement. Simplistically, muscle contraction occurs by binding of the myosin head to the actin filament, powered by ATP. Myosin binding sites in the actin filament are protected by troponin C and tropomyosin. Once calcium binds to troponin C this induces conformational changes that moves troponin C and tropomyosin exposing the myosin binding site in the actin filament, binding of myosin to actin and consequent contraction occurs [40].

At the intracellular level, muscle contraction is dependent on depolarization, G-protein-coupled receptors (GPRs), phosphorylation events and calcium concentration. While extracellular calcium has a concentration of 2-4 mM, in the myoplasma the calcium concentration is 100nM at rest and 0.4mM in the sarcoplasmic reticulum (SR) [41]. To initiate contraction, acetylcholine released by the motor neurons at the neuromuscular junction causes depolarization of the muscle cell membrane. This in turn activates L-type Ca^{2+} channels in the membrane (dihydropyridine receptor, DHPR), that are coupled to the ryanodine receptor (RyR) in the SR. Opening of the RyR causes an e-flux of calcium from the SR into the myoplasm, which binds to the troponin C complex inducing a conformational change that exposes the myosin binding sites on the actin filament, causing contraction. Ca^{2+} is pumped back into the SR by the Sarco/Endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump. Decreased myoplasmic Ca^{2+} levels cause Ca^{2+} to unbind from troponin C, with consequent return of tropomyosin to cover the actin binding site, causing relaxation (**Figure 2**) [41].

Regulation of SERCA is important for fiber contraction and muscle performance. In cardiac muscle, the endogenous proteins phospholamban and sarcolipin regulate SERCA Ca^{2+} transport activity. Phospholamban is also expressed in smooth muscle and slow twitch skeletal muscle, whereas sarcolipin, and the more recently discovered and myoregulin (MLN), are expressed in fast-twitch muscle [42,43]. Another important player in muscle function is the LIM and cysteine rich domain 1 (LMCD1), that was shown to regulate cardiac hypertrophy through calcineurin and nuclear factor of activated T-cells (NFAT) activation both *in vitro* and *in vivo* [44]. It was also shown to repress GATA6 (important for development) in an *in vitro* model [45]. More recently, and within the work of this thesis, we have shown it also has an important role in skeletal muscle hypertrophy (but interestingly not atrophy), force and calcium handling through repression of MLN [46].

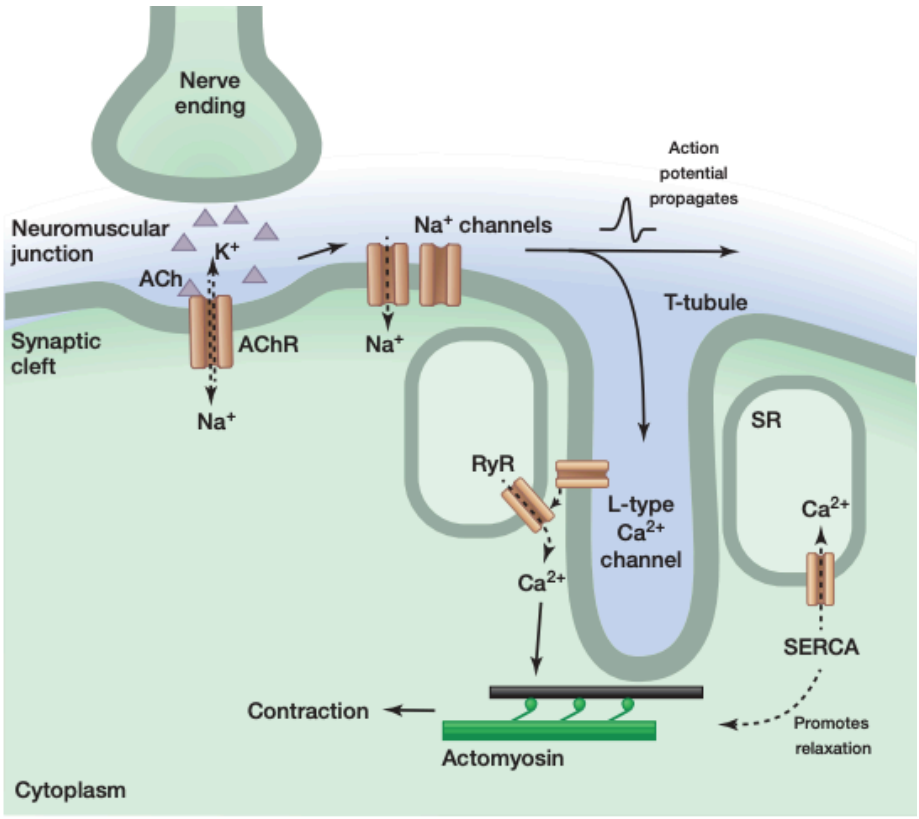

Figure 2. Calcium regulation of muscle contraction. Ach – acetylcholine; AChR – acetylcholine receptor; RyR - ryanodine receptor; SERCA - SR/ER calcium ATPase; SR – sarcoplasmic reticulum. Adapted from Kuo and Ehrlich, 2015, Signaling in muscle contraction, Cold Spring Harbor perspectives in biology [41]. Reprinted with permission. Copyright Cold Spring Harbor Laboratory Press.

Calcium also regulates cell metabolic enzymes involved in glycogen degradation and glycolysis and mitochondrial respiration through Ca^{2+}-Cam-dependent phosphatase calcineurin and calcium/calmodulin-dependent protein kinases (CamK). CamK and calcineurin also modulate gene transcription responses, driving the gene expression response to exercise stimuli [41,47–50].

2.2 TRANSCRIPTION REGULATION IN MUSCLE

Skeletal muscle is a plastic organ with the capacity to adapt to novel bioenergetic situations, as is the case of exercise. Many of these adaptations begin with extrinsic factors to muscle such as mechanical stretch, signaling peptides, steroid hormones and catecholamines, and nutrient availability [10]. Some of these adaptations include oxygen sensing and transport, nutrient uptake and metabolism, energy production, among others. During exercise, many of these signaling pathways ultimately lead to transcriptional changes in muscle. A network of transcription factors, nuclear receptors and co-regulators integrates exogenous stimuli with gene expression and adaptive responses. Calcium, in addition to its role of integrating external stimuli to muscle contraction, also coordinates the transcriptional responses to said stimuli (**Figure 3**).

2.2.1 The role of Calcium

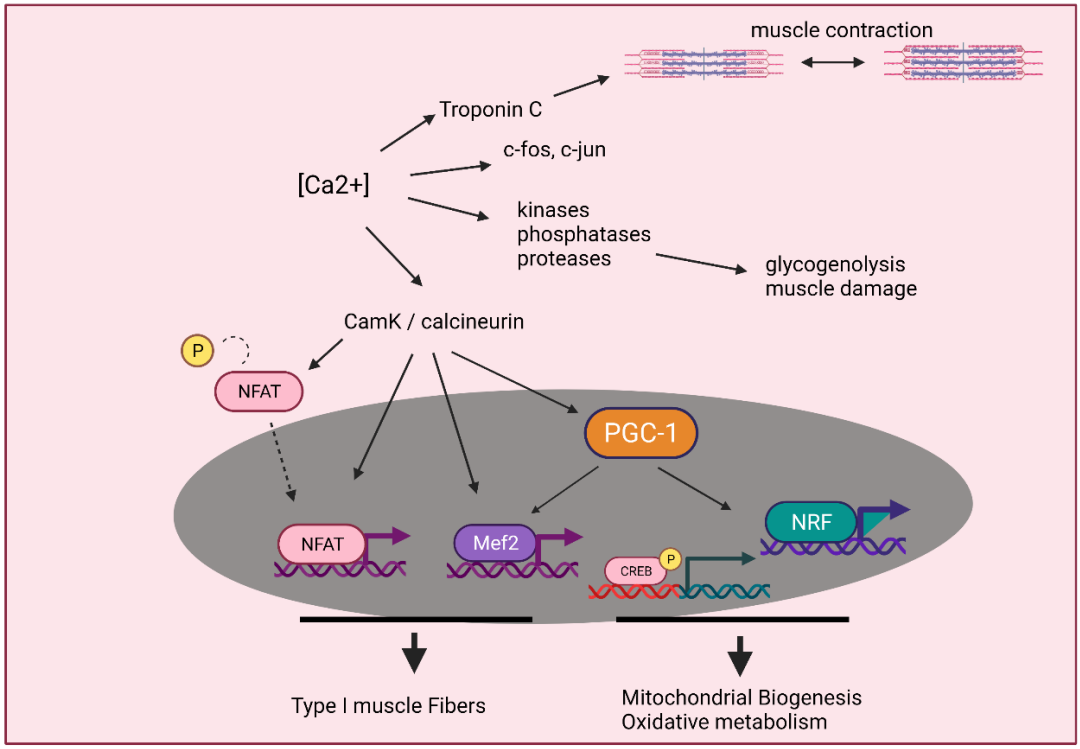

Figure 3. Schematic representation of intra-cellular events in skeletal muscle mediated by Calcium. PK – protein kinases; PP – protein phosphatases. See text for details. Adapted from [36] and [50]. Created with BioRender.com.

Upon signaling from the neuromotor neurons there is a rise in cytosolic calcium. As we saw earlier, this results in muscle contraction and consequently, movement. Many proteins that control transcription and cell activity are also sensitive to calcium signaling, such as calcineurin and CamKs. Calcineurin controls transcription through the dephosphorylation of NFAT. Dephosphorylated NFAT translocates to the nucleus, where it activates the transcription of slow-fiber gene expression [48]. Calcium-activated CamK II and IV phosphorylate histone deacetylases (HDACs) leading to their nuclear exclusion, removing its repression of myocyte enhancer factor 2A (MEF2) [2,10,48,50]. MEF2 target genes relate to glucose and lipid metabolism [51,52], and skeletal muscle plasticity [2]. CamK IV also activates cyclic AMP (cAMP) response element-binding protein (CREB) and transcription of its target genes [47,53] (**Figure 3**).

2.2.2 PGC-1α

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is a transcriptional co-activator that has a central role in transcriptional regulation of metabolic adaptations in various tissues. It was first discovered in brown adipose tissue in responsive thermogenesis [54]. Today, PGC-1α gene, and some of its isoforms, are known to be involved in the adaptation to diverse challenges such as exercise or fasting in several tissues [55] namely liver [56], skeletal muscle [36,39,57] and adipose tissue [54]. Interestingly, PGC-1α promoter is also regulated

by both CaMK IV and calcineurin activity [53]. There is also some evidence for a role of brain PGC-1α in whole body energy regulation [58,59].

Conversely, PGC-1α loss or decreased levels have been observed in diseases and disease models that have a component of muscle atrophy such as Duschenne's muscular distrophy or cancer cachexia. Further, over-expression of PGC-1α in an mdx model of Duschenne's muscular dystrophy [60] and muscle atrophy due to denervation or disuse [61] could partially rescue the phenotype. PGC-1α helps to prevent muscle wasting by partially inhibiting the FoxO3-induced E3 ubiquitin ligases muscle RING-finger protein-1 (Murf1) and Atrogin-1 [61].

PGC-1α regulates gene transcription by partnering with several other factors like nuclear receptors, transcription factors and other transcriptional co-activators forming transcriptional complexes that include chromatin remodeling, co-activators, the mediator complex, and splicing factors [62].

At the bioenergetics level, PGC-1α coordinates adaptations to more efficient fuel utilization (glycolysis, oxidative phosphorylation), fuel supply (angiogenesis, increase of GLUT and FA transporters) and improvement in force (muscle mass, fiber type) (**Figure 4**).

In cultured muscle cells, PGC-1α regulates mitochondrial biogenesis and energy metabolism through regulation of the nuclear respiratory factors (NRFs, NRF-1, NRF-2) and mitochondrial transcriptional factor A (mtTFA, TFAM) [63,47]. PGC-1α is highly expressed in muscle beds rich in the oxidative type I and type IIa muscle fiber, like soleus muscle [36].

PGC-1α also regulates angiogenesis, through the co-activation of the transcription factor estrogen-related receptor alpha (ERRα), that induces angiogenic factors including vascular endothelial growth factors (VEGF) [64] (**Figure 4**).. This induction is at least in part dependent on β-adrenergic signaling and independent of hypoxia-inducible factor 1 alpha (HIF-1α) signaling. Interestingly, adrenergic stimuli strongly induce PGC-1α expression from an alternative promoter, resulting in the expression of different PGC-1α isoforms [55,64,65].

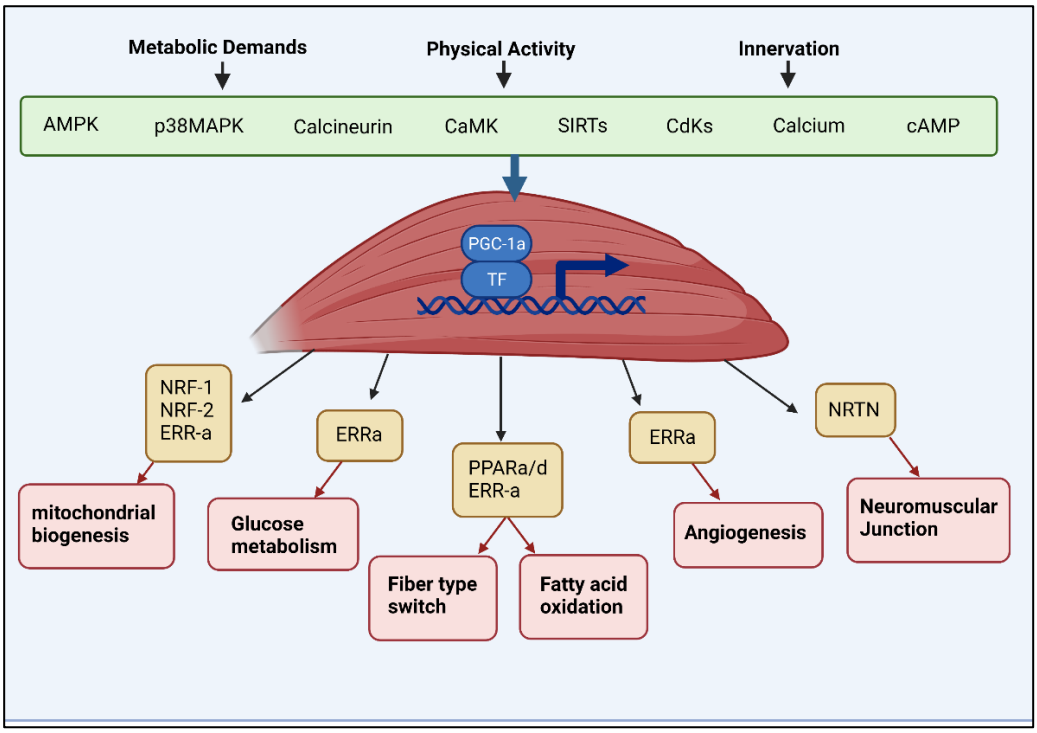

Figure 4. PGC-1α regulatory function in skeletal muscle. Different stimuli indices different skeletal muscle adaptations regulated by PGC-1α. CdKs – cyclin-dependent kinases; NRTN – neurturin. See text for details. Created with BioRender.com.

One isoform resulting from this alternative promotor is named PGC-1α4, which has been associated with muscle mass regulation by induction of insulin-like growth factor 1 (IGF-1) and repression of myostatin. Further, PGC-1α4 stimulates mTOR activity, promoting a hypertrophic phenotype in a mouse model of skeletal muscle over-expression [39,62].

PGC-1α1 also interacts with peroxisome proliferator-activated receptor (PPAR) α/δ to drive the expression of kynurenine aminotransferases (KATs) both in mouse [66] and human [67] skeletal muscle, with consequences for muscle energy utilization through improved malate-aspartate shuttle (MAS) capacity [68] and indirectly impacting also on adipose tissue browning and brain [69].

2.2.3 At the intersection between signaling and gene transcription response

Many proteins and cascade responses are directly or indirectly involved in linking signaling to cellular responses and adaptations. In addition to calcium-regulated proteins, mitogen-activated protein kinases (MAPKs) and AMP-activated protein kinases (AMPKs) are two important families of integrator proteins.

2.2.3.1 Mitogen-activated protein kinase (MAPK) family

The MAPK family integrates extrinsic signals from mechanical stress, cytokines, and growth factors to cell/tissue adaptations. They regulate differentiation, hypertrophy, inflammation, and gene expression, amongst other physiological processes [2,10,70]. This family includes, for example, ERK1/2, JNK and p38. MAPKs phosphorylate various substrates in the cytoplasm or nucleus, including transcriptional factors and co-activators, linking external stimuli to the gene transcription response. For example, p38 MAPK increases the activity of transcription factors such as MEF2 and activating transcription factor 2 (ATF2) which regulate PGC-1α transcription upon calcium stimuli [38,49,71].

During exercise, there is an increase in radical oxygen species (ROS) production by the mitochondria and other cellular processes. ROS also signals through MAPK and the transcription factor nuclear factor-KB (NF-KB) [72].

2.2.3.2 AMP-activated protein kinase (AMPK)

The AMPK, together with sirtuins, integrate intrinsic cellular signals such as changes in NAD/NADH and AMP/ATP, respectively. They also regulate transcription of genes involved in energy metabolism through PGC-1α activation, such as increased glucose transport and lipid metabolism, and decrease anabolic activity. AMPK modulates induction of the transcription factors NRF1, MEF2 and HDACs. These adaptations ultimately lead to the adaptations to exercise such as mitochondrial biogenesis, substrate utilization, and fiber type switching [10].

All these transcriptional and, ultimately, phenotypical adaptations aim to equip skeletal muscle with the means and capacity to produce the necessary energy and contractile force (**Figure 5**). However, they require the coordination of several systemic processes – for example, increased mitochondrial capacity requires increased fatty acid supply, which requires changes also in the adipose tissue. So, the muscle communicates to the other tissues through a myriad of secreted molecules and factors – the myokines.

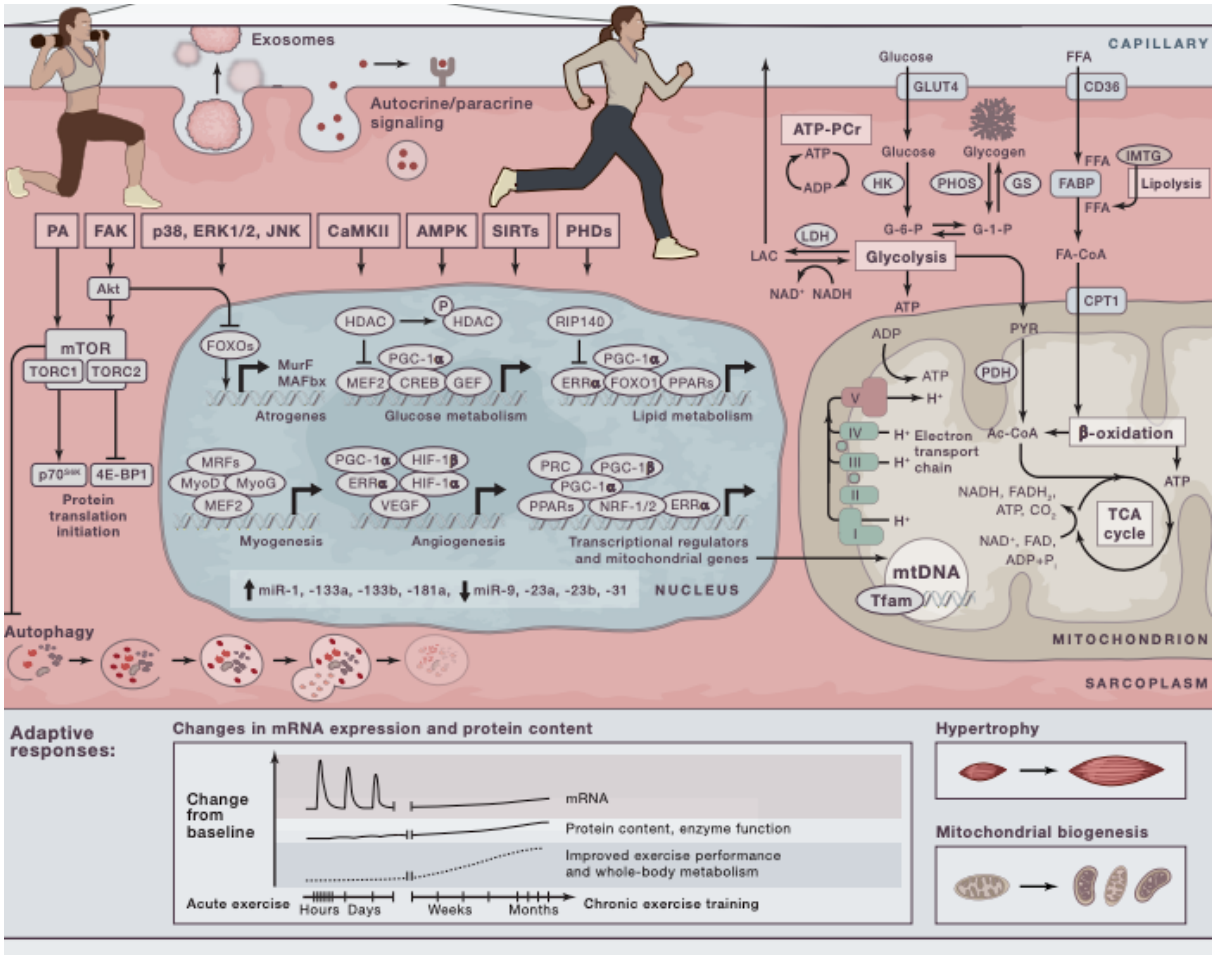

Figure 5. Overview of transcriptional adaptations to exercise in skeletal muscle. Abbreviations: 4E-BP1 - eukaryotic translation initiation factor 4E-binding protein 1; Ac-CoA - acetyl-CoA; ADP - adenosine diphosphate; AMP - adenosine monophosphate; AMPK - AMP-activated protein kinase; CaMK - Ca2+ /calmodulin-dependent protein kinase; CD36 - fatty acid translocase; CPT1 - carnitine palmitoyltransferase 1; CREB - cyclic AMP response element-binding protein; ERK - extracellular signal-regulated kinase; ERR - estrogen-related receptor; FA-CoA - fatty acyl CoA; FABP - fatty acid-binding protein; FAD - oxidized form of flavin adenine dinucleotide; FADH2 - reduced form of FAD; FOXO - forkhead transcription factor O-box subfamily; G-1-P - glucose 1-phosphate; G-6-P - glucose 6-phosphate; GEF - GLUT4 enhancer factor; GLUT4 - glucose transporter type 4; GS - glycogen synthase; HDAC - histone deacetylase; HIF - hypoxia-inducible factor; HK - hexokinase; IMTG - intramuscular triglyceride; JNK - c-Jun N-terminal kinase; LAC - lactate; LDH - lactate dehydrogenase; MAPK - mitogen-activated protein kinase; MEF2 - myocyte enhancer factor 2; miR - microRNA; MRF - myogenic regulatory factor; mtDNA - mitochondrial DNA; mTOR - mechanistic target of rapamycin; MyoD - myogenic differentiation 1; MyoG - myogenin; NRF - nuclear respiratory factor; PDH - pyruvate dehydrogenase; PHOS - glycogen phosphorylase; PGC-1 - PPARg co-activator 1; PPAR - peroxisome proliferator-activated receptor; PRC - PGC-1-related coactivator; PYR - pyruvate; RIP140 - nuclear receptor-interacting protein 1; SIRT - sirtuin; SRF - serum response factor; Tfam - mitochondrial transcription factor A; TORC - target of rapamycin complex; VEGF - vascular endothelial growth factor. From Egan, Hawley and Zierath, 2016, Snapshot: Exercise Metabolism, Cell Metabolism [73]. With permission from Elsevier.

3 MUSCLE-CENTRIC INTER-ORGAN COMMUNICATION: MUSCLE AS AN ENDOCRINE, PARACRINE AND AUTOCRINE ORGAN

The existence of secreted factors produced by the muscle with a distal effect has long been hypothesized. Cytokines, peptides, and other molecules secreted by muscle that have an endocrine or paracrine effects are commonly referred to as "myokines" [74,75] (**Figure 6**).

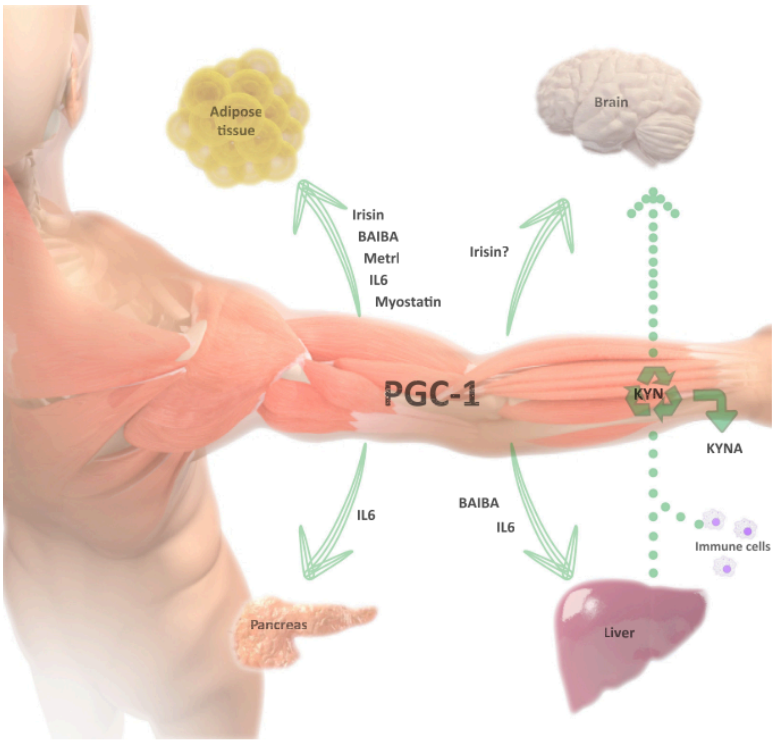

Figure 6. Exercise factors with systemic effects. From Correia, Ferreira and Ruas, 2015, Intercellular: local and systemic actions of skeletal muscle PGCs, Trends in Endocrinology and Metabolism, [62]. With permission from Elsevier.

3.1 MUSCLE-SECRETED FACTORS WITH AN ENDOCRINE EFFECT

Interleukin-6 (IL-6) was the first myokine to be discovered as a secreted factor from the exercising muscle into circulation [74]. In skeletal muscle, its expression is dependent on p38 MAPK and NF-KB [76]. Muscle-secreted IL-6 increases hepatic fat oxidation and gluconeogenesis, lipolysis in muscle and stimulate the release of glucagon-like peptide 1 (a hormone that stimulates insulin secretion), contributing also in this manner to whole body glucose homeostasis [74,77].

Interestingly, IL-6 (and also tumor necrosis factor alpha – TNF-α) are increased in obese patients. Mouse knock-out (KO) studies and specific cytokine studies show that depletion of IL-6 and TNF-α increases circulating cholesterol and body weight, and aggravates other

metabolic parameters, suggesting that in this case increased IL-6 and TNF-α are part of a compensatory mechanism in obesity [78].

Hypoxia, like the one observed in tissues of obese patients, leads to cell death, endoplasmic reticulum (ER) stress, lipolysis and inhibited lipogenesis in adipose tissue. Pro-inflammatory cytokines are generally believed to contribute to insulin resistance. However, they can also be useful in increasing energy expenditure by stimulating adipose tissue remodeling, and improving tissue blood supply [78,79].

3.1.1 Specific effects on adipose tissue

Adipose tissue "browning" agents are also secreted by the muscle, such as irisin [80], meteorin-like (Metrnl) [81] kynurenic acid (KYNA) [66], and β-aminoisobutyric acid (BAIBA) [82]. The latter also affects β-oxidation in hepatocytes by activating PPARα. Interestingly, BAIBA levels also inversely correlated with cardiometabolic risk factors [82].

Irisin is a controversial myokine. It is transcribed from the fibronectin type III domain 5 (FNDC5) gene upon induction by exercise. It induces browning in white adipose tissue (WAT) both *in vitro* and *in vivo* [80]. In mice, it also mediates the transcription of exercise-induced increase of hippocampal brain-derived neurotrophic factor (BDNF), induces a neuroprotective gene program, and improves synaptic plasticity and memory [83,84]. However, these effects seem to be mediated by local FNDC5 expression, and if muscle-secreted irisin can reach the brain and have similar effects is yet to be determined. The human FNDC5 gene has a mutated start codon, and reports on FNDC5/irisin expression, secretion and effects in humans are contradictory [85–88]. Irisin detection itself also proved a methodological challenge, and detection my mass spectrometry seems to be the most reliable method yet. Using this methodology, an increase in plasma irisin was observed in humans with exercise [89].

Metrnl expression is stimulated by PGC-1α4. In a mouse model, this myokine promotes browning of WAT and an increase in energy expenditure with improved glucose utilization. Interestingly, Metrnl does not appear to act directly on the adipocytes; but through macrophage alternative IL-4/IL-13-dependent activation to enter the adipose tissue and activate the thermogenic gene program [81].

3.2 NON-EXCLUSIVE MUSCLE FACTORS

Metrnl, fibroblast growth factor 21 (FGF21) and angiopoietin-like-4 (ANGPTL4) are examples of factors that are secreted by other organs than skeletal muscle in response to exercise. FGF21 and ANGPTL4 are also released by the liver during exercise and regulate circulating triglycerides concentration, metabolism and skeletal muscle mass and strength [14] (**Figure 6**).

ANGPTL4 is expressed in adipose tissue and in the liver during exercise and fasting [90]. It regulates lipid metabolism by promoting adipose tissue lipolysis and inhibiting lipoprotein lipase activity [91,92].

In the liver, FGF21 stimulates fatty acid oxidation, ketogenesis and gluconeogenesis [93,94]. In brown adipose tissue, it promotes glucose uptake and thermogenesis and in WAT it promotes adipogenesis [94,95]. Systemically, FGF21 potentiates insulin induced glucose uptake [95–97].

3.3 MUSCLE FACTORS WITH AN AUTOCRINE AND PARACRINE EFFECT

Some myokines have an autocrine effect, like myostatin, IL-6 and BDNF [75]. Myostatin, for example, has a role in regulation of muscle mass, as shown by the increased muscle mass of the myostatin-null mice [98]. *In vitro*, myostatin inhibits satellite cell activation and myoblast differentiation [99]. Myostatin-null mice also display browning of WAT adipose tissue, adipose tissue lipolysis and increased fatty acid oxidation [98].

BDNF is expressed in muscle after exercise and *in vitro* upon electrical stimulation [100]. In skeletal muscle, BDNF is involved in satellite cell differentiation, muscle regeneration and AMPK signaling, which controls fatty acid oxidation [11,100,101]. BDNF also increases in circulation immediately after exercise but *in vitro* and *in vivo* experiments suggest it is not released from skeletal muscle [100]. It has been hypothesized that circulating BDNF is of central nervous system (CNS) origin [11,100].

Taking together all the aforementioned muscle-centric changes, it is not surprising that lack of exercise leads to many morbidities and increased risk factors. Indeed, exercise affects not only skeletal and cardiac muscles, but the body as a whole.

4 TRYPTOPHAN METABOLISM

One of the above-mentioned muscle-secreted molecules is KYNA, that is a product created by conversion of TRP. TRP is an essential amino acid, which means the human body does not have the necessary machinery to produce it and needs to ingest it from diet. Amino acids are classically known as the building blocks for proteins, but many have bioactive roles or are metabolized into bioactive molecules.

Only about 1% of the ingested TRP contributes to protein synthesis. 4-5% is converted to serotonin, a neurotransmitter tightly linked to mood regulation. Serotonin can also be further converted to melatonin, which is involved in sleep and circadian regulation. The majority of ingested TRP (about 95%) is catabolized by what is known as the kynurenine pathway of tryptophan degradation (KP) [102] (**Figure 7**). KP metabolites have systemic effects in inflammation and mental health that are strongly regulated by exercise.

Serotonin and melatonin, despite being mostly known as neurotransmitters, have the highest concentration in the gut. Gut serotonin has a role in gut motility and accounts for about 90% of total serotonin in humans. Melatonin is 400 higher in gut than in pineal gland. There, it has a role in moderating gut permeability and immune activation [29,32].

Upon ingestion, TRP is absorbed and transported through the large neutral amino acid transporters (LATs) and competes with several other branched chain amino acids (BCAAs) like phenylalanine, leucine, isoleucine and valine for the transport site. TRP availability for cellular uptake and usage is therefore not only dependent on TRP concentration, but also on the concentration of the competing amino acids. Furthermore, only free TRP is taken up by cells and under normal conditions about 80-90% of TRP circulates in plasma bound to albumin. TRP is displaced from its albumin binding site by FA; a rise in circulating free fatty acids (FFA) levels, as it is observed for example in moderate physical exercise, will increase free TRP levels in plasma and thus, the fraction available for cell uptake [103]. Indeed, TRP and some of its downstream metabolites in the serotonin pathway have been observed to increase in plasma and several brain regions in an exercise protocol in rats [104].

Gut microbiota also impacts on host's TRP metabolism, as suggested by a study that showed germ free mice have increased levels of total TRP in circulation. Microbes can produce other derivatives of TRP that interact with host such as tryptamine and indol compounds. Reversely, host TRP metabolism has an effect in microbial proliferation and microbiota diversity [32].

4.1 THE KYNURENINE PATHWAY OF TRP DEGRADATION

The KP (**Figure 7**) is the main pathway of degradation of ingested TRP. When left to run its course, the KP results in NAD^+ production, but many intermediaries of this pathway have a biological action, namely in cancer cell survival (Kynurenine (KYN), 3-Hydroxykynurenine (3-HK)), inflammation (3-HK, KYN, Quinolinic acid (QA)), neuroinflammation (3-HK, QA),

immune system (KYN, KYNA) cardiovascular disease (KYN, 3-HK, 3-HAA) and mental health (KYN, KYNA) [105–111].

Under basal conditions, the KP in mainly active in the liver, where tryptophan 2,3 dioxygenase (TDO) is the rate limiting enzyme catalyzing the conversion of TRP to KYN. TDO is regulated by TRP levels, oestrogens and glucocorticoids [29]. The latter are of particular interest in several pathophysiological situations, as many risk factors for disease such as lack of physical activity and stress elevate glucocorticoids in circulation.

Indoleamine 2,3-dioygenase (IDO) is another enzyme capable of catalyzing the first step of the KP. It plays a major role in maternal immune tolerance of the fetus, and pharmacological inhibition of IDO leads to rejection of the fetus by maternal T cells and consequent abortion [112]. This discovery revealed the importance of this pathway in immune regulation, and current research highlights its importance in immune and inflammation. In addition to the placenta, IDO is expressed in monocytes, macrophages, dendritic cells and microglia. is highly activated upon inflammatory stimuli, becoming of importance in pathological settings especially in the brain, immune compartment and gut [107,113–115].

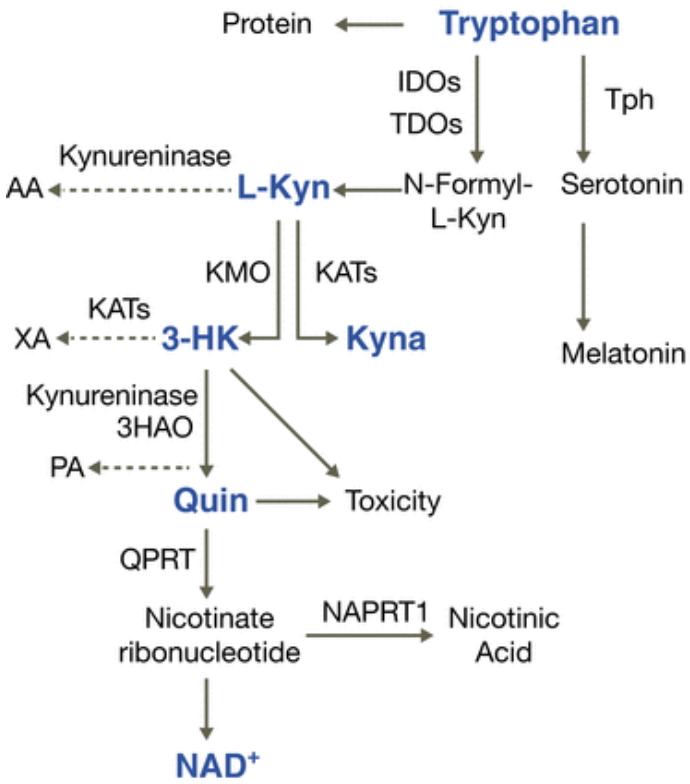

Figure 7. The Kynurenines pathway of Tryptophan degradation. From Valente-Silva, Ruas, 2018, Tryptophan-Kynurenine Metabolites in Exercise and Mental health, Hormones Metabolism and the benefits of exercise, Springer, [30]. With permission under the Creative Commons Attribution 4.0 International License.

4.1.1 Bioactive roles of KP intermediaries

KYN is the metabolite produced by TDO/IDO activation. It has a role in immunotolerance, inflammation, and immune maturation, and it was found to also contribute to the regulation of arterial vessel relaxation [116].

KYN is metabolized to KYNA by KATs. KYNA has a neuroprotective role as an antagonist of N-methyl D-aspartate receptor (NMDAR) and α7-nicotinic acetylcholine receptor (α7-nAChR) [27,28,115]. KYNA concentrations were found to be decreased in the cerebrospinal fluid (CSF) of Alzheimer's and Parkinson's disease patients [117]. Curiously, high levels of KYNA have also been reported in the brain of schizophrenic patients [118], but if this elevation is a contributor to the disease or a consequence of unbalance neurochemistry is debated in the field.

In the periphery, KYNA was able to ameliorate adiposity and weight gain through increased energy expenditure by promoting WAT browning through activation of GPR35. It further contributed to ameliorate the inflammatory environment in adipose tissue, rendering some protection against high fat diet (HFD)-induced ailments [69].

KYNA was also shown to act as a nutritional cue to induce satiety in a *Caenorhabditis elegans* (*C. elegans*) model [119], suggesting yet another mechanism in which KYNA has an effect in body metabolism. Rats exposed to KYNA supplementation during the breastfeeding period showed significant reduction of body weight gain [120]. In addition, in a study comparing maternal milk vs baby formula, KYNA was significantly reduced in the later, in which the authors speculate this may be a possible mechanism for the observed correlation of baby formula feeding and adulthood obesity and metabolic impairment [120].

KATs also convert 3-HK into xanthurenic acid (XA). In the brain, XA is stored in neuronal vessels and released in an activity dependent manner [121,122]. It has a modulating effect in hippocampal activity as an endogenous agonist of metabotropic glutamate receptors and an inhibitor of vesicular glutamate transporter (VGLUT) [122,123], although its direct binding to the receptors is questioned [122].

In the periphery, XA has been proposed as a player in diabetes. XA complexes with insulin, reducing its activity [124,125] and with Zn^{2+} causing toxic complexes in the β cells [126]. Urinary XA levels, as well as KYN and KYNA levels, are significantly elevated in patients with type 2 Diabetes [127]. In addition, in obese patients 1 year after bariatric surgery, XA decrease was associated with improved insulin sensitivity index [128].

Reduction of the level of circulating vitamin B6 is associated with a variety of conditions, including rheumatoid arthritis, cancer and stroke, as well as chronic inflammation (as seen in obese and diabetic patients) [129–131]. Vitamin B6 is a cofactor for kynureninase (KYNU), an enzyme that converts 3-HK into QA. Reduction of flux though the NAD production branch could potentially increase the conversion of 3-HK into XA.

In isolated rat pancreatic islets, both XA and KYNA inhibited glucose-stimulated proinsulin synthesis [132], although at millimolar level, raising the question of biological importance. The

authors speculate that local overproduction of these metabolites could induce the observed effects in a pathological model.

Many of the intermediaries of the KP have been studied in the context of inflammation and disease.

5 KYNURENINE PATHWAY IN INFLAMMATION AND DISEASE

KP activity is altered during inflammation, as many of the enzymes are sensitive to induction by pro-inflammatory stimuli, and many metabolites are involved in inflammatory events.

5.1 REGULATION OF KP ENZYMES BY INFLAMMATORY FACTORS

Inflammatory stimuli are potent inducers of IDO in the immune compartment [107,113–115]. Many different cytokines can induce IDO both at gene and protein expression level (TNF-α, IL-1β, IL-6, etc), amongst which interferon gamma (IFN-γ) is the most potent one [111,113,133,134].

Other steps of the pathway are also sensitive to inflammatory stimuli by INF-γ, such as kynurenine 3-monooxygenase (KMO), KYNU, 3-hydroxyanthranilic acid dioxygenase (HAAO), quinolinate phosphoribosyltransferase (QPRT) and KATs 1 and 2 [113,135,136] (reviewed in [107]). As a result, several pro-inflammatory intermediates, such as KYN, 3-HK, and QA accumulate.

Inhibition, knock-down or knock-out (KO) of KMO, KYNU or IDO in various animal models of disease resulted in improvement of disease phenotype and amelioration of cellular and molecular markers [137–141]. IDO-induced inflammation can be a factor for metabolic dysfunction. Mice fed a HFD show increased KYN/TRP ratio (seen as proxy for IDO activity) in plasma, adipose tissue and muscle, while IDO KO mice were protected from weight gain, inflammation and insulin resistance [142].

5.2 KP METABOLITES IN INFLAMMATION

In human healthy volunteers, injection with pro-inflammatory stimuli show alterations in some of the KP metabolites up to 48 hours post challenge [111,143]. One study using endotoxin saw changes in KYN, KYNA and TRP levels that correlated with the levels of the cytokines IL-6 and TNF-α [143]. In contrast, another study using lipopolysaccharide (LPS) infusion saw no difference in KYNA, picolinic acid (PA), and 3-HK at any time-point, but a decrease in TRP and KYN after infusion with a later increase at 24 and 48 hours. This study further found a relation between KYN/TRP with C-reactive protein (CRP), and QA/KYN with IL-6 and CRP, markers of peripheral immune activity involving TNF and IL-1β [111].

KYN, KYNA, XA are all ligands for the dioxin receptor (AhR), a transcription factor that mediates xenobiotic metabolism [32,107,144]. KP metabolites induce AhR-mediated decrease in the activity of natural killer cells, dendritic cells, and T-cells through proliferation of regulatory T cells (Treg), promoting an immune tolerogenic environment [133,144–147] (**Figure 8).**

Several gut bacteria derived TRP metabolites are AhR ligands as well. AhR and IDO1 play a crucial role in immune homeostasis at the mucosa [32].

KYNA action as an agonist for GPR35 can also reduce neuroinflammation. GPR35 modulates cAMP signaling and this can lead to inhibition of the several neuroinflammatory pathways [148]. In agreement with this hypothesis, increased brain KYNA correlated with amelioration of phenotypical and cellular and molecular markers in animals models of neurodegeneration [138,139].

Some tumor types also have high IDO activity with increased local KYN production, resulting in immune evasion [149,150]. Increased tumor IDO expression also correlates with poor clinical prognosis [151].

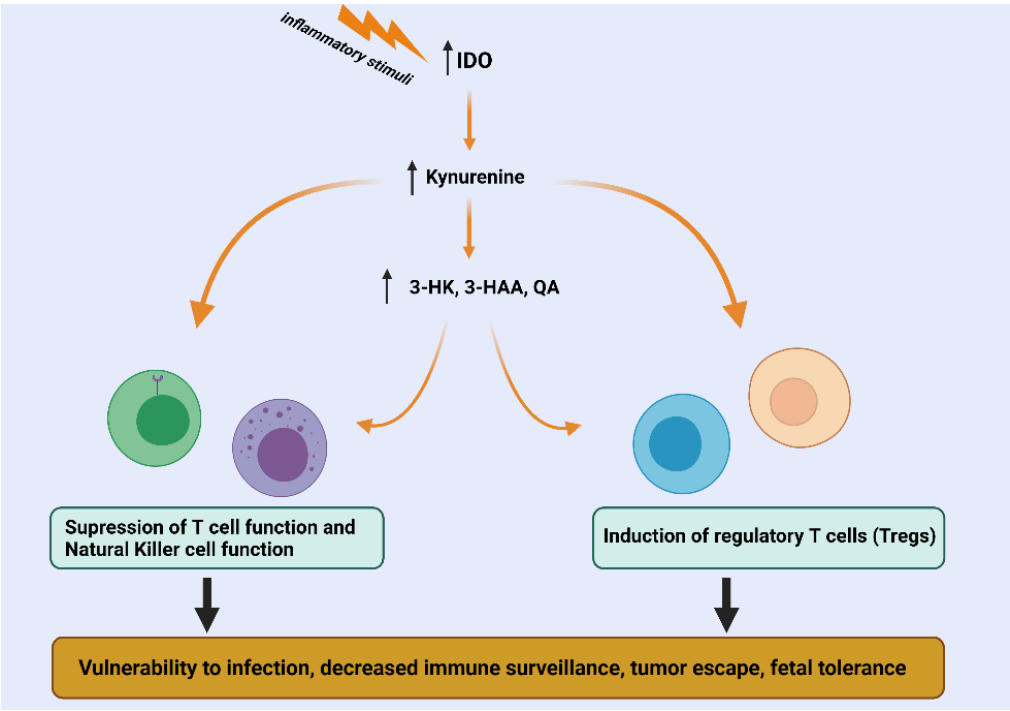

Figure 8. Immune tolerance and immune-surveillance evasion strategies mediated by IDO. Adapted from [115]. Created with BioRender.com.

3-hydroxyanthranilic acid (3-HAA) suppresses cytokine and chemokine production and has antioxidant properties [136,152,153]. In models of cardiovascular disease, 3-HAA reduces the uptake of oxidized LDL by macrophages, an initiating event for the formation of foam cells and

positively modulates inflammatory response. It is further able to lower plasma lipids by modulating liver cholesterol metabolism [154,155].

QA, 3-HK and 3-HAA are known ROS generators and contribute to glutamate excitotoxicity [114,156,157]. Sustained activation of NMDAR in β-cells has been linked to β-cells exhaustion [158]. Activation of these receptors by KP metabolites that are agonists for the NMDAR could potentially lead to β-cell failure and contribute to metabolic disease onset. Interestingly, 3-HK is a redox agent, that can either be anti or pro-oxidant. On one hand, it is a powerful ROS generator, oxidizing in the presence of oxygen and trace metals. On the other hand, 3-HK has scavenging properties and at higher concentrations has been shown to have anti-oxidant properties *in vitro* [152,153,159].

5.3 KYNURENINE METABOLITES IN THE BRAIN AND MENTAL HEALTH

There is an increasing body of evidence that systemic inflammation plays a role in mental disease. Depressed patients show elevations in pro-inflammatory cytokines [160,161]. Patients receiving immune stimulating treatments, suffering from autoimmune diseases or diseases with a big inflammatory component, have a higher risk of developing depression [143,162,163]. Finally, low dose endotoxin also induces depressive symptoms in healthy volunteers [143,164].

As we have discussed in the previous chapter, KP metabolites are tightly connected to inflammation in the periphery and in the brain (**Figure 9**). Inhibition of some branches of the pathway alleviates disease symptomatology and also attenuates the depressogenic effects of inflammation. For example, in mice, KO or inhibition of IDO [165], KMO-KO or HAAO-KO [166] are resistant to LPS induced depressive symptoms, suggesting the KP is a necessary mediator for inflammation induced depression. In humans, abnormal KP metabolite levels are observed in mental and psychotic disease. Positive response to therapies correlate with changes in the metabolite concentrations towards normalization [109,167–170].

Peripheral KYN is able to cross the BBB. Indeed, about 60% of brain KYN comes from the periphery, while the rest is produced locally by astrocytes and microglia. When peripheral KYN concentrations increase due to, for example, inflammation or chronic stress, the increased KYN load in the brain causes depressive-like symptoms by a mechanism not clearly elucidated as well as neuroinflammation by further catalysis to 3-HK and QA [161]. Microglia preferentially produce QA, whereas astrocytes produce KYNA. Astrocytes lack KMO and thus cannot produce 3-HK. KMO is expressed in macrophages/microglia and is upregulated by inflammatory cytokines [113,157]. Upon inflammation-induced elevation of extracellular levels of 3-HK, astrocytes can uptake it and further metabolize it to generate QA [113] .

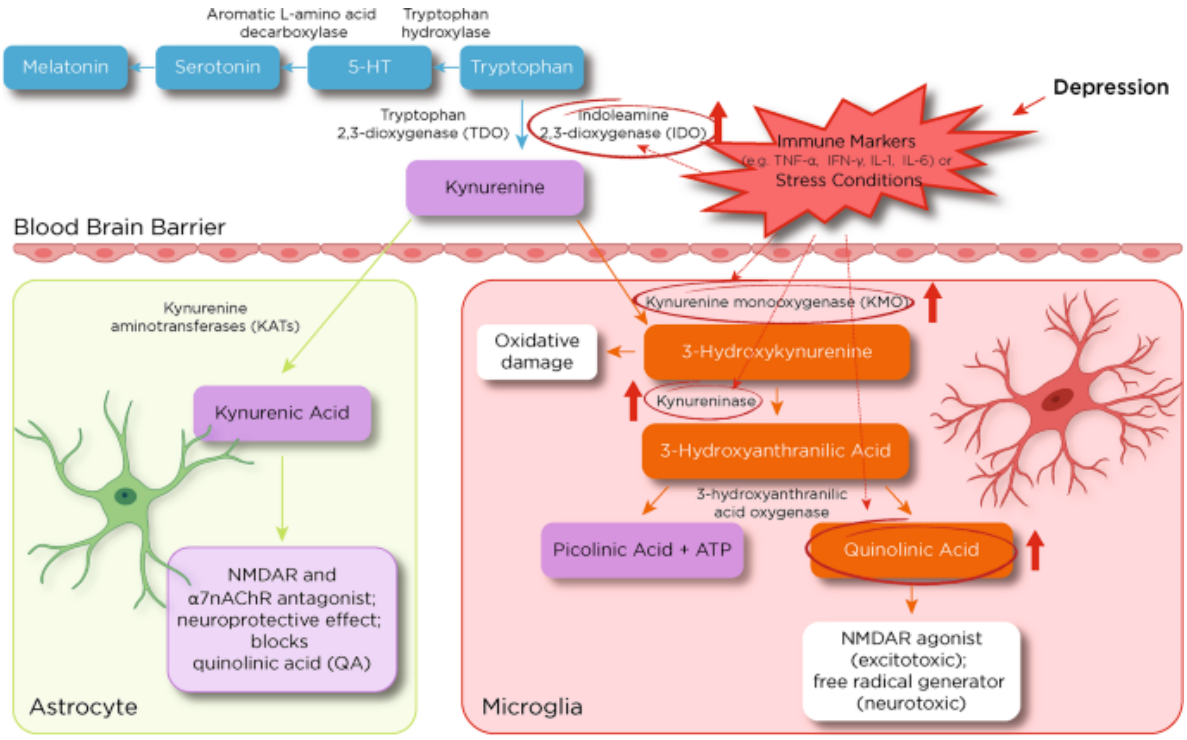

Figure 9. Actions of KP metabolites in the brain. Adapted from Kadriu et al, 2019, The kynurenine pathway and bipolar disorder: intersection of the monoaminergic and glutamatergic systems and immune response, Molecular Psychiatry [161]. With permission from Springer Nature.

QA was the first KP metabolite to be experimentally reported to have a neurobiological role. In the early 80s, Stone and Perkin showed that QA can act as an NMDAR agonist and induce neurotoxicity [171]. Many of the QA effects seem to be directly or indirectly through its role as an NMDAR agonist, namely glutamate toxicity and excitotoxicity, glutamate release and inhibition of glutamate re-uptake by astrocytes. Excess QA also causes destabilization of the cellular cytoskeleton by increasing phosphorylation of structural proteins. As a ROS generator, QA also contributes to lipid peroxidation and toxicity. It further induces disruption of autophagy and induces astrocyte apoptosis and may have a role in phosphorylated Tau accumulation in Alzheimer's disease [172,173].

The effect of the KP in glutamatergic transmission and neurotoxicity may account at least partially for the link between inflammation and depression/mental illness (**Figure 9**). The hippocampus has a particular high density of NMDAR and as such is of particular interest in KP induced neuro-disease. Hippocampal volume (that reflect dendritic atrophy) is negatively correlated with the putative neuroprotective ratios KYNA/3-HK and KYNA/QA in patients with major depressive disorder and bipolar disorder [174,175]. Finally, mice deficient in IDO or KMO are protected from endotoxin-induced cognitive deficits when tested for recognition memory, a task highly dependent on the hippocampus [176].

KYNA, QA and 3-HAA cross the blood-brain-barrier (BBB) poorly [177], although in some circumstances peripheral KYNA and QA have been observed to cross and affect the brain. In

gerbils, peripheral administrated radiolabeled QA is detectable in the brain and CSF [178]. Peripheral KYNA administration following brain injury has been shown to be neuroprotective in different animal models (reviewed in [179]), although this may be partially due to loss of BBB integrity. BBB integrity is altered in many diseases including brain trauma and edema, stroke, epilepsy, multiple sclerosis, Alzheimer´s disease and Parkinson's disease, and possibly in several psychiatric disorders [180]. Thus, impermeability of the BBB may depend on the degree of the underlying inflammation, amongst other unknown factors.

5.4 KP IN ACUTE INFLAMMATION

KP metabolism is also altered in acute inflammation events, such as traumatic brain injury, or stroke [134,136].

Risk factors of stroke, such as cardiovascular disease, atherosclerosis, and partial occlusion by carotid artery stenosis have been correlated with dysregulations of the KP [136,181,182]. This suggests that the KP is already primed towards the pro-inflammatory branch before the acute event, when it becomes further exacerbated.

5.4.1 Stroke

Stroke is the 2nd leading cause of death worldwide after ischemic heart disease and is the 3rd leading cause of long-term disability. Three months after stroke, a third of the patients have either died or have lost independence for daily activities [183,184].

Stroke is characterized by an interruption of the blood flow into the brain, with the source of this interruption usually broadly classified as ischemic or hemorrhagic. Ischemia is the most common primary cause accounting for about 80% of cases, and is characterized by an occlusion of the blood supply. A hemorrhagic stroke is caused by a rupture of blood supply arteries [185].

During ischemia, a rapid chain of inflammatory events ensues involving hypoxia, glutamate excitotoxicity, calcium overload and ROS damage. This ischemic stress leads to activation of microglia, immune cell infiltration and programmed cell death at the injured site and penumbra [186,187]. Immune activation triggers the release of various inflammatory cytokines, amplifying the inflammatory response, and leading to IDO and consequently KP activation. Animal models of stroke support the hypothesis of KP dysregulation, with increased IDO, KYNU, KMO and HAAO activity and QA and KYNA levels in several brain regions (reviewed in [179]).

In human, there is a depletion of circulating TRP during the acute phase of stroke. This is accompanied by a change in other metabolites and inflammatory markers, such as KYN, KYNA, KYN/TRP ratio, 3-HAA, melatonin, tryptamine, higher malondialdehyde (MDA, marker of oxidative stress) and neopterin [136,156,188,189]. MDA further correlated negatively with KYNA and KAT activity [136]. One study shows a rapid spike increase in KYN levels that

normalized after 24 hours [156], whereas others found no change in KYN but rather in its downstream metabolites [189]. These discrepancies can perhaps be explained by the rapid kinetics, different methodology and time of collection. On the other hand, they also highlight the high individual and pathological variability of stroke, and how patients might benefit from better understanding of the variations of outcomes for better personalized care.

The KYN/TRP ratio is often used as an "inflammatory ratio", as it is a proxy for IDO activation that is dependent on inflammatory stimuli. There is some debate regarding the validity and usefulness of this ratio as a specific stroke biomarker [136]. KYN/TRP is high after stroke and correlates with National Institutes of Health Stroke Scale (NIHSS) score and infarct volume in acute ischemic stroke [156]. However, it also correlates with other general inflammatory parameters such as levels of CRP, erythrocyte sedimentation rate (ESR), neutrophil lymphocyte ratio (NLR) and INF-γ activity [114,136]. Therefore, KYN/TRP may be a good proxy of the general inflammatory state but may lack the specificity to be useful as a specific stroke biomarker [136].

Lipid peroxidation and the 3-HAA/anthranilic acid (AA) ratio also correlated with stroke volume, in a study involving human patients. This was due to both a decrease in 3-HAA and an increase in AA [156]. Not much is known about the bioactive role of AA. 3-HAA has been shown to have anti-inflammatory properties by upregulating Tregs, inhibiting T cell response and inducing T cell apoptosis, and inhibiting dendritic cell activation. The effects of 3-HAA seem to be at least partially mediated by 3-phosphoinositide-dependent protein kinase 1 (PDK-1), NF-KB, and the p38/c-Jun pathways [105,190,191].

The degree of activation of the KP further correlates with stroke severity and long-term outcome [114,179]. Neuroimmune activation, ROS damage and inflammation during stroke and are major contributors to stroke-related long-term injury [187]. Over-stimulation of NMDAR could be involved in necrotic brain damage after ischemic stroke [186–188].

This is also seen in other acute inflammatory events with long resolution time such as traumatic brain injury, during which elevated QA in CSF and blood are a negative prognostic indicator and are associated with increased depressive and anxiety symptoms [134].

In stroke, about 30% of surviving patients develop depression [179,192] and 35 to 92% develop post-stroke fatigue [193]. Differences in numbers likely reflect difference in populations and methodology used. Post-stroke depression is a complex phenomenon that depends on multiple biological factors such as location and extent of injury, persistent inflammation, severity of stroke, sex (females are more at risk), clinical history, and environmental factors such as socioeconomical level and degree of impairment [192,194]. Pro-inflammatory cytokines and sustained prolonged inflammation activate IDO and increase KP activity, offering a possible link. Indeed, in these patients increased 3-HK and KYNA associated with depressive symptoms [194]. The increase in KYNA also observed in this context may be due to general activation of the pathway.

Pro-inflammatory cytokines likely also contribute to the onset of post-stroke fatigue [192,193]. Activation of the KP through increased IDO causes TRP depletion. Decreased TRP index (TRP/other BCAAs) is associated with increased fatigue score [193]. This is thought to be due to a consequent reduction in serotonergic and orexin neurotransmission, which contribute to fatigue and depression onset [192,193].

Some animal studies have also investigated the therapeutic value of the pathway. Prophylactic treatments with KYN or KMO inhibitors seem to yield promising positive results [195–197], and possibly act through either increase of brain KYNA or increase of corticocerebral blood flow. In contrast, when KYN is given after the induced stroke, it worsened the symptoms and outcomes [198]. These results offer promise in modulators of the KP as a preventative therapy, and as a potential therapeutical target.

5.5 EFFECTS OF EXERCISE IN KP AND INFLAMMATION

The KP ultimately results in NAD production, and it has been speculated that during exercise, KP upregulation functions in part to meet energy demands [199].

Exercise increases KATs expression in skeletal muscle of both mice [66] and humans [67], including healthy older man over 65 years of age [200]. This upregulation, as we have seen before, has a positive effect in muscle energy utilization, while converting KYN into KYNA as a secondary reaction of the MAS. In mice, this upregulation of KATs provided them with protection against stress-induced depression by preventing the accumulation of peripheral KYN, adding a new layer to muscle-brain communication in the context of mental health.

In depressed patients, results of exercise on the KP activity are mixed. It is important to note that in these studies, exercise protocols and patient engagement can vary (chronic vs acute, supervised vs unsupervised) as well as timing of samples (immediately after exercise vs hours, days or weeks after). Interestingly, despite no changes in the KP metabolites measured, patients still had a decrease in the self-reported levels of depression [201,202].

Increased levels of KP metabolites have been reported in patients with breast and gastroesophageal cancer, and an exercise protocol was able to alleviate the KP metabolite load [203,204].

Acute endurance and resistance exercise also acutely affect the KP [205]. Exercise causes a spike in cortisol and inflammatory cytokines, such as IL-6, that can transiently activate IDO and cause a spike in KYN. This could potentially contribute to exercise-induced alleviation of disease symptoms through KYN immune modulation properties.

TRP metabolism and the serotonergic system are implicated in central fatigue, which can be characterized as failure to perform not due to muscle fatigue but due to inadequate input from the CNS. It has been suggested that during exercise, the concomitant decrease of BCAAs in circulation and increase of FFA contribute to TRP availability to be uptaken by the brain, where

it is metabolized to serotonin [103]. Serotonin is increases in some brain regions of rats after exercise [104]. This increase in serotonin would then contribute to sleep and fatigue perception. However, in humans, TRP supplementation has been shown to decrease fatigue perception and improve performance without differences in heart rate or hormonal responses [206]. Further, decrease of muscle serotonin has been related to decreased exercise performance [207]. Whereas no conclusion can be made, we can speculate that in CNS serotonin contributes to fatigue onset, while in the periphery TRP helps to support movement and serotonin improves muscle-CNS communication.

6 AIMS

The overarching aim of this thesis was to investigate the regulatory mechanisms between nutrition and exercise, and how they can provide a new tool for biomarkers and personalized therapies.

With **Paper I** we aimed to understand if chronic supplementation of tryptophan had an effect in physical activity and mood in a rodent model.

In **Paper II** we investigated the biomarker potential of the KP metabolites in the early rehabilitation phase of stroke patients.

Finally, in **Paper III** we investigated the role of LMCD1 in muscle force and its mechanism of action.

7 DISCUSSION

7.1 PAPER I

KP metabolites are biologically active and can influence biological functions, both in health and in disease. These metabolites originate from TRP, an essential amino acid. Protein intake (either by increasing protein-rich food or protein shakes) is trending, especially amongst fitness communities. However, specific amino acid composition is not standardized, and potential effects of bioactive amino acids (such as TRP) are unknown.

Our laboratory has previously shown in a mouse model that overexpresses PGC-1α1 specifically in skeletal muscle (as a model for endurance exercise) has increased expression of KATs in skeletal muscle [66], resulting in increased KYN to KYNA conversion. This was further validated in humans, in skeletal muscle of endurance-trained individuals [67]. The increase of KAT expression increases MAS capacity in skeletal muscle, since these enzymes also participate in the MAS reactions. The MAS allows for electrons resulting from cytosolic glycolysis to cross the mitochondria inner layer (that is impermeable to NADH itself) and delivers them to the respiratory complex I. This contributes to improved energy utilization. Systemically, this has the effect of lowering circulating KYN by converting it to KYNA. KYN is increased in stress and inflammation. It crosses the BBB where it has a neurotoxic effect and has been related to depressive-like behavior in mice and in some human studies. KYNA does not cross the BBB and is therefore restricted to the periphery. A recent study from our lab has shown that KYNA in the periphery has a browning effect in fat [69].

In **paper I** we wanted to understand if chronic supplementation of TRP, the precursor of KYN, with and without exercise, would result in increased TRP uptake and further influence the circulating KP metabolites and their physiological effects. To this end, we fed C57B6/J mice with a control diet or with a diet supplemented with five times the TRP amount, with and without access to a wheel. We performed extensive gene expression analysis of energy-related pathways and inflammation in several metabolically relevant tissues, such as *gastrocnemius* muscle, fat depots, and brain. We also performed behavioral tests for anxious-depressive phenotype and a complete KP metabolite analysis in plasma. We further fed another group of mice with control or TRP enriched diet for 5 weeks and evaluated metabolic parameters with metabolic cages (TSE) and intraperitoneal glucose tolerance test.

Mice fed with TRP supplementation run more, but without changes in energy metabolism transcriptional signature in skeletal muscle compared to control-fed running mice

As expected, exercise increased markers of mitochondrial energy metabolism and angiogenesis in muscle. Interestingly, mice with TRP supplementation have more bouts of running in the wheel and cover a higher distance. We hypothesized this could be due to improved fuel utilization, since the KP ultimately results in NAD production, decreased fatigability, or due to changes in neurochemistry that would cause them to stay in the wheel longer and/or access to

the wheel more often. However, they did not show differences in body weight, body composition nor further skeletal muscle adaptations than the exercise-control group. Potentially, the extra exercise might not have been enough to trigger further adaptations that the ones already triggered by the exercise. Further, diet alone was not able to elicit any changes. Possibly, the amount of TRP in the chronic supplementation was still within the capacity of normal enzymatic expression.

Mice running behavior cannot be explained by behavioral changes nor changes in gene expression in the brain

The KP is altered in many disorders of mental illness, including anxiety and depression [167,168,169,170,161,109,134]. To understand if the running phenotype could be explained by an anxious-depressive phenotype, we performed open field and elevated plus maze tests, which are standard tests for anxiety and locomotion. We saw no indication in these tests of such phenotype. These are well established tests that are widely used as a screening tool, but they may lack the finesse to pinpoint smaller, more subtle phenotypes. However, we saw no changes in gene expression in hippocampus and hypothalamus of genes associated with mood, inflammation, neuronal integrity or TRP metabolism, which renders this hypothesis unlikely.

Interestingly, in a study of TRP supplementation in humans, TRP supplementation also increased performance due to reduced fatigue perception [206]. Muscle serotonin is also hypothesized to play a role in glucose metabolism and neuro-muscular communication in exercise and fatigue and would explain the phenotype observed by us in mice and others in humans [207,208].

KP metabolites have a short half-life in circulation

Mice are nocturnal animals, meaning they have their most active period during the dark phase, including feeding. To evaluate if the TRP plasma levels change upon stopping food ingestion, we collected samples from mice fed with high TRP at the start of the mice inactivity cycle (6AM in our facility) and 4 hours into the inactivity cycle (10AM). After 4 hours into the inactivity period, the KP metabolites in circulation had mostly returned to control levels.

Our results showed a rapid metabolism of TRP, which highlights the importance of the experiment timing. Further, much of the literature regarding the link of TRP metabolites to inflammation and negative effects refer to a chronic, sustained elevation of the pathway, often in an already pro-inflammatory setting. In contrast, our mice had a chronic supplementation in diet but no further disease stimuli.

Of note, the behavioral tests were also performed in the time frame that the KP metabolites would already have returned to baseline. This also supports the idea that without further inflammatory stimuli, returning of the KP to baseline is enough to return other physiological functions to normal as well. Possibly, the chronic supplementation in our protocol still fell below the capacity of normal KP capacity.

Diet supplementation was enough to raise KP metabolites in plasma, and access to the wheel reduced the burden in the neurotoxic branch

We collected blood from the mice that had access to a wheel between 6AM and 8AM. As expected, most of the metabolites of the KP were elevated in the sedentary group with TRP supplementation. They were also elevated in the mice fed TRP with access to a wheel (in comparison to mice fed control diet), but access to a wheel alleviated the burden of almost all pathway metabolites. This indicates that exercise increased flux through the pathway and prevented accumulation of intermediaries. End products, such as KYNA, would be rapidly excreted in urine.

As discussed before, this increase did not have an effect in the molecular or behavioral markers tested for. This could be due to the dosage of TRP in diet, timing of the experiments or both. It would be interesting to test whether a bolus of TRP or performing the experiments at the mice peak activity period would yield different results.

TRP supplementation while exercising also increases KP metabolites in humans. Further, trained subjects showed a higher increase in TRP and KYN than untrained subjects

We recruited five untrained (4 males and 1 female) and four endurance-trained subjects (2 males and 2 females) healthy volunteers to investigate if TRP supplementation and exercise also showed differences in KP metabolites in humans. They exercised on a mechanically braked cycle ergometer at 60% VO_2 max taking either placebo (flavored water) or water containing a total of 15mg TRP/kg body weight, in boluses throughout the experimental time.

KP metabolites were not changed when the volunteers took placebo. With TRP supplementation, several metabolites of the pathway were elevated, up to 200 minutes after the exercise had ended. Interestingly, the trained group had elevated TRP and KYN, indicating a potential higher TRP displacement from albumin by FFA and increased availability for KYN production. Increased TRP in circulation can also be uptaken into the brain and potentially contribute to serotonin-induced central fatigue [104] or contribute to muscle serotonin and potentially improve fatigue [206]. As a future perspective, it would be interesting to investigate free TRP vs albumin bound TRP in circulation, as well as muscle serotonin content and fatigue perception. It would also be interesting to evaluate for how long the KP remain elevated after the exercise and supplementation stop, and document changes in mental state or mood changes with follow up interviews.

7.2 PAPER II

KP is dysregulated with many metabolites elevated in the acute phase of stroke [114,136,156,179]. Further, there has been some attempts at correlating metabolite levels in the acute phase with characteristics of stroke and outcomes. For example, 3-HAA/AA was reported to correlate with infarct volume [156] and KYN/TRP value with NIHSS score [114].

In **paper II**, we tried to understand if KP metabolites also influenced the post-acute phase of stroke, in early rehabilitation phase. To this end we recruited 25 patients after discharge from acute hospital care and upon admission to the primary rehabilitation center. Blood was collected at arrival and discharge from the clinic and analyzed for KP metabolites. Patient´s medical data was retrieved from the medical records.

KP metabolites are still elevated in early rehabilitation phase

Patients are admitted to the rehabilitation care in different days after their acute stroke event, depending on their improvement speed in the acute care. We analyzed the metabolites in a time scale (days after stroke), and found the metabolites were still elevated with a slow tendency to decrease. Long sustained elevation of the pathway is seen in chronic inflammation and contributes to depression and potentially to chronic fatigue. Indeed, in stroke patients KP dysregulation is correlated to depression and chronic fatigue up to a year after the ischemic event. This work is relevant in showing that strategies to help normalize the KP early on may have consequences for long-term rehabilitation and mental health.

Stroke origin-type may affect KP pattern

A small number of patients in our sample (n=5) presented hemorrhagic stroke, and the remaining (n=20) were ischemic, in line with published statistics (20% vs 80%, respectively) [185]. Despite a small sample, the KP metabolites seem to follow a different pattern, with many increasing over time (vs decreasing in ischemic patients).

In hemorrhagic stroke, the lack of blood flow to the brain is caused by a bleeding or blood vessel burst. In this condition, not only there is a suspension or drastic reduction of blood flow to the brain, but the vasculature is compromised. So, re-perfusion is potentially less efficient and increases damage to a greater extent than in ischemic stroke. These patients typically present a worse prognosis, and this could also account, at least in part, for the different KP pattern we observed in our population sample. Hemorrhagic patients also typically present a longer hospital stay. This is of significance as muscle mass loss is increased in elderly, and periods of immobility drastically affect muscle mass, and by proxy, independence and quality of life.

Specific biomarkers, such as the KP metabolites that are different between ischemic and hemorrhagic stroke, would therefore be useful to monitor or try to predict long term progression.

KP metabolites also link inflammation to neuroinflammation, and mental health. They offer a promising prognosis marker, and possible therapies targeting the modulation of the KP may have long term consequences for mental health outcome of these patients. This is relevant in the context of post-stroke depression and post-stroke fatigue, that affect a significant part of the afflicted patients.

7.3 PAPER III

LMCD1 is present in various tissues, with the highest expression in skeletal muscle [209]. LMCD1 was shown to repress GATA6 expressed from both lung and cardiac tissue-specific promoters in a 293 cells in vitro model [45] and contribute to cardiac hypertrophy *in vivo* and *in vitro* through calcineurin and NFAT activation [44].

In **paper III**, we observed that LMCD1 expression is decreased in muscle atrophy diseases and aged individuals. In mice, LMCD1 was highly expressed in heart and skeletal muscle, in accordance with the literature. In a combined exercise protocol, LMCD1 expression increased in the skeletal muscle of young but not of old individuals. Taken together, this data led us to investigate if LMCD1 was a driver or a consequence of changes in muscle mass.

To understand the mechanism of action of LMCD1, we transiently overexpressed or silenced LMCD1 *in vivo* and *in vitro* and analyzed gene and protein expression, force, and calcium levels.

LMCD1 regulates muscle mass, protein synthesis and fiber size

In vivo transient overexpression of LMCD1 in gastrocnemius muscle with an adenoviral vector resulted in increased PGC-1α4 and IGF-1 expression and decreased MURF1 and myostatin expression, which are correlated with increased muscle mass and protein synthesis. We also observed a slight shift towards oxidative fibers, with increase expression of MyHC I, IIa and IIx and decrease in IIb. Silencing of LMCD1 reduced protein synthesis without an atrophy phenotype.

LMCD1 acts mostly through calcium signaling, rather than direct regulation of gene expression

In primary mouse myotubes transduced with an LMCD1 overexpressing adenovirus, kinase array showed protein synthesis and calcium signaling. In line with these findings, MAPKs and mTOR were some of the top ranked pathways. These pathways are involved in muscle mass regulation. The array also highlighted kinases that are involved in cytoskeleton remodeling and calmodulin binding. CREB is activated after calcium and ß-adrenergic stimulation and is known to control the anabolic changes observed, including expression of PGC-1α4. These results indicate that LMCD1 acts primarily through signaling rather than direct regulation of gene expression.

LMCD1 increases fiber force and reduces fatigue by decreasing the requirement for calcium handling through calcineurin activation

Isolated mouse muscle fibers overexpressing LMCD1 showed increased specific force with less SR Ca^{2+} release and are fatigue resistant. LMCD1 seems to protect against fatigue by increasing the re-uptake of Ca^{2+} into the SR. This re-uptake is mostly through the SERCA pump system that re-uptakes cytoplasmic Ca^{2+} after release. LMCD1 overexpression decreases MLN

expression, which is a repressor of SERCA. The opposite was observed when silencing LMCD1 with an shLMCD1 adenovirus.

Overexpression of LMCD1 also increased the protein levels of calcineurin A and B. Calcineurin is a Ca^{2+}-dependent phosphatase that regulate transcriptional responses of muscle to exercise. Inhibition of calcineurin ablated the effects of LMCD1. In fact, inhibition of calcineurin even with overexpression of LMCD1 had the same results as silencing LMCD1. Therefore calcineurin is necessary for LMCD1 action in improving SERCA re-uptake into the SR and improving calcium requirements and signaling.

Calcineurin also regulates muscle mass and gene expression control of muscle mass [53]. When LMCD1 was overexpressed, we observed an increase in mTOR signaling pathway and phosphorylation of AKT and Insulin Receptor Substrate (IRS), all involved in protein synthesis. Silencing of LMCD1 ablated the signaling activation.

We therefore conclude that LMCD1-calcineurin axis is not necessary but is sufficient for muscle hypertrophy and increased force, calcium signaling and fatigue resistance.

8 CONCLUSION AND FUTURE PERSPECTIVES

With **paper I**, we showed that moderate chronic supplementation of tryptophan is enough to elevate KP metabolites in circulation, and access to a wheel decreases the metabolite load. Upon entering the inactivity phase, the metabolites quickly return to baseline within 4 hours, revealing their short half-life in circulation. This increase, however, did not seem to affect physiology, adaptation and brain functioning in a mouse model. It is to be determined if in an inflammatory/stress context, a higher dose, or bolus dose (as one would take in a supplement) would result in observable phenotypical changes.

With **paper II**, we evaluated the KP metabolites in stroke patients, during the rehabilitation phase. We observed the metabolites still exhibit a decreasing pattern at this stage, with a slow kinetics. Further, there is a suggestion that type of stroke (ischemic vs hemorrhagic) also affects the KP differently. These results encourage future research into the KP metabolites as biomarkers for stroke recovery, which may provide insight into patient state and facilitate better personalized therapy and the use of KP modulators as therapies, which may facilitate recovery.

In **paper III**, we identified and characterized a new regulator of muscle mass and force. LMCD1 acts through calcineurin and calcium signaling. Understanding the players in muscle mass regulation opens ways to therapeutic approaches in situations that do not allow for a normal exercise protocol, as in muscle dystrophies and stroke. Further, lack of physical exercise exacerbates inflammation and mental health deterioration, which may also benefit from muscle-sparing approaches.

In **paper I** and **paper III**, we looked at effects of nutrition in muscle, and how changes in muscle could impact on whole body metabolism and function. These changes are often mediated by muscle-secreted myokines, which are responsible for many whole-body positive adaptations to exercise and understanding their regulation can help develop therapy strategies for conditions like stroke.

Elderly people already have increased loss of muscle mass and impaired response to training as we saw in **paper III**. Stroke is more prevalent in elderly, and in addition to the inflammatory and neurological effects, there is also substantial loss of muscle mass due to immobility and inactivity. Our discoveries in LMCD1 provide new insights on muscle mass and force increase and maintenance. They add to the body of work necessary for the development of therapies to increase muscle mass and force in diseases that do not allow for training at a capacity that would elicit these changes.

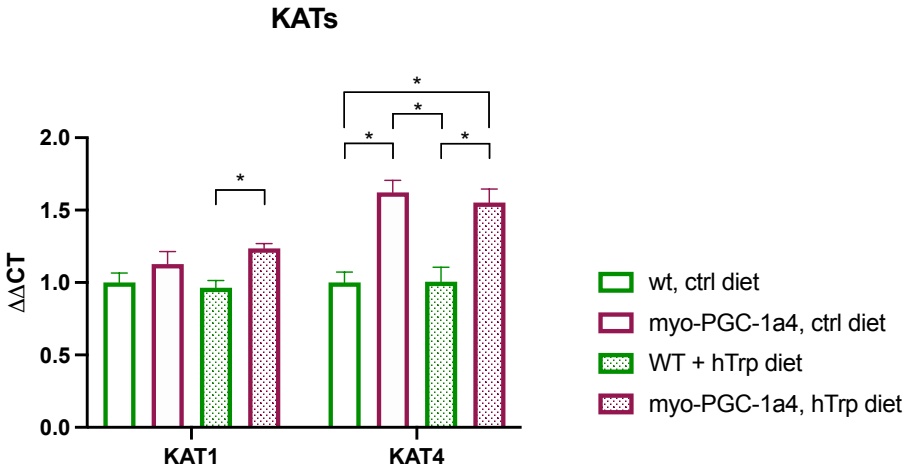

Figure 10. gene expression analysis of gastrocnemius muscle by qRT-PCR analysis. Gene expression data is normalized to the expression of a housekeeping gene (hypoxanthine phosphoribosyltransferase, HPRT) and presented as relative to WT control diet.

In a mouse model of resistance exercise over-expressing PGC-1α4 isoform in skeletal muscle, KAT expression is slightly increased (Valente-Silva P, Martinez-Redondo V, Ruas JL, unpublished data, **Figure 10**), although not to the level of the endurance model over expressing PGC-1α1 used in [66]. Manipulating the resistance-arm of skeletal muscle adaptations could still provide some protection against increase of the pro-inflammatory KP metabolites, while providing the protection against muscle mass loss. The work of this thesis opens new avenues for improved personalized therapies based on analysis of KP metabolites as biomarkers and manipulation of muscle mass.

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