EXTRACELLULAR VESICLE REGULATION BY EXERCISE IN HEALTH AND DISEASE

Geoffrey Warnier – 2024

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LIST OF ABBREVIATIONS

ADSC	Adipose-derived stem cells
Akt	Protein kinase b, PKB
ALIX	X-interacting protein ALG2
APO A1	Apolipoprotein A1
APO B	Apolipoprotein B
aSMase	Acidic phingomyelinase
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
CD9	Cluster of differentiation 9
CD63	Cluster of differentiation 63
CD81	Cluster of differentiation 81
CNS	Central nervous system
CRP	C-reactive protein
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
ELV	Exosome-like vesicle
ESCRT	Endosomal sorting complex required for transport
EV	Extracellular vesicle
Flot1	Flotillin 1
HbA1c	Glycated hemoglobin
HDL	High-density lipoprotein
HIF-1α	Hypoxia-inducible factor 1α
HIIT	High intensity interval training

HOMA	Homeostatic model assessment
HOMA-IR	Homeostatic model assessment of insulin resistance
HOMA-β	Homeostatic model assessment of β-cell function
HSP60	Heat shock protein 60
HSP70	Heat shock protein 70
ILVs	Intraluminal vesicles
IL-1β	Interleukin 1β
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-13	Interleukin 13
IL-15	Interleukin 15
IRS1	Insulin receptor substrate 1
ISEV	International Society for Extracellular Vesicles
JNK	c-Jun N-terminal kinase
LDL	Low-density lipoprotein
LPS	Lipopolysaccharides
MCP1	Monocyte chemoattractant protein-1
miRNAs	Micro-ribonucleic acids
MLSS	Maximal lactate steady state
mRNAs	Messenger ribonucleic acids
MVB	Multivesicular body

MV	Microvesicle
muRF1	Muscle RING-finger protein-1
NAFLD	Non-alcoholic fatty liver disease
NF-κB	Nuclear factor-kappa B
NLR	Nucleotide-binding oligomerization domain-like receptor
nSMase	Neutral sphingomyelinase
NTA	Nanoparticle tracking analysis
OGTT	Oral glucose tolerance test
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PRDX	Peroxiredoxin
PRR	Pattern recognition receptor
QUICKI	Quantitative insulin sensitivity check index
RER	Respiratory exchange ratio
RNA	Ribonucleic acid
RPE	Rate of perceived exertion
SEC	Size exclusion chromatography
SGCA	α-sarcoglycan
SkMEV	Skeletal muscle derived-EV
SOD	Superoxide dismutase
SMase	Sphingomyelinase
SNAP	Synaptosomal-associated protein
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor

TEM	Transmission electron microscopy
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha
tRNAs	Transfer ribonucleic acids
TSG101	Tumor susceptibility gene 101
T2D	Type 2 diabetes
VAMP7	Vesicle-associated membrane protein 7
VO_2max	Maximal oxygen uptake
WHO	World health organization
1-RM	One-repetition maximum

ABSTRACT

Regular exercise confers remarkable health benefits, decelerates biological aging, and prolongs lifespan. Although the exact underlying mechanisms are still under study, these positive effects could be partly attributed to the release of bioactive molecules called exerkines. While initially thought to be released by the classical secretory pathway, recent evidence suggests that these molecules could also be released and transported to their target by exosome-like vesicles (ELVs). These vesicles could thus participate in the adaptive process to exercise by allowing communication between cells at both local and remote levels. Despite a growing interest in the scientific community in recent years, the regulation of these vesicles during exercise remains poorly understood. Additionally, studies following the international guidelines from the International Society for Extracellular Vesicles (ISEV) are rare, which prevents strong conclusions.

To fill this gap in the literature, three studies have been conducted as part of this PhD thesis. The first study investigated ELV release during submaximal endurance exercise in normoxic and hypoxic conditions in healthy and prediabetic subjects. Blood samples were taken before, during and directly after exercise to evaluate the kinetics of release. In the second study, we evaluated the effect of chronic exercise on basal circulating ELV regulation as well as their miRNA content. To this end, endurance trained athletes were recruited to participate in a 6-week training protocol at different simulated altitudes (sea-level, 2000 m, 3000 m or 4000 m). ELV amount and content were analyzed from blood collected before and after the training intervention. Finally, the third and last study addressed the impact of both acute and chronic

exercise on ELV regulation in the context of obesity and low-grade inflammation. ELV amount and proteome were studied to investigate the link between ELV content and exercise-related inflammation reduction.

These three parts of the project allowed a better understanding of exercise effects on ELV regulation. We showed that low intensity endurance exercise regulates ELV release in a duration/intensity-dependent manner, and that the typical ELV increase by exercise was blunted in prediabetes and in hypoxic conditions. Contrasting with the effect of a single session, the impact of long-term training might be limited to smaller magnitude of changes, especially in already trained populations. Here as well, we found that hypoxia and metabolic pathologies could interact with regular exercise, possibly inhibiting the effects of the latter on basal ELV levels. Beyond the quantitative changes of circulating ELVs, we found that both acute and chronic low intensity endurance exercise regulated ELV proteome diversity and abundance. In particular, it seems that the potential anti-inflammatory effects of ELVs were related to changes in antioxidant enzymes abundance rather than a modulation of pro- or anti-inflammatory proteins in those vesicles.

Overall, while much work remains to be done, our three studies have contributed to a better understanding of both acute and chronic effects of exercise on ELV regulation.

Chapter I: Introduction

1. Extracellular vesicles

1.1 Brief history of the discovery of extracellular vesicles

Although the first experiments in which extracellular vesicles (EVs) were specifically identified as such began during the 80s and 90s, the earliest descriptions of structures that can be retrospectively related to EVs actually date back to the 40s [1]. EV research started with the studies of Chargaff and West and their work on coagulation. These two researchers were the first to observe the sedimentation of tiny particles by high-speed centrifugation [2]. In 1967, Peter Wolf further characterized phospholipid-rich microparticles released from platelets during clotting as "platelet dust" [3]. From the mid-60s and the early 80s, several electron microscopy studies described vesicular structures [4-6]. Interestingly, the paper of Nunez and his colleagues was one of the first to report the presence of multivesicular bodies close the apical membrane [7]. Finally, in 1983, two seminal papers studying reticulocyte maturation and transferrin receptor highlighted the fusion of the multivesicular body with the plasma membrane resulting in the release of intraluminal vesicles outside the cell [8, 9]. Those vesicles were then defined as exosomes [10]. At this stage, it was suggested that exosomes were "a major route of externalization of obsolete membrane proteins" [11] and the idea that these vesicles were just a way for the cell to shed waste remained for some years.

Although exosomes had been shown to be enzymatically active as early as 1989 [12], it was not until a few years later that research revealed the potential functional role of those vesicles. One of the turning points was the work

conducted in 1996 by Raposo and colleagues showing that EVs from immune cells were capable of presenting antigens and, thus, to induce specific responses in target cells [13]. This discovery, followed by others in the subsequent years [14, 15] brought to light the involvement of EVs in intercellular communication and their functional roles in biological processes. In the early 2000s, a growing interest emerged for these vesicles, resulting in a deep exploration of their nature and functions in different contexts and cell types [1]. Several studies, for instance, showed that EVs could functionally transfer RNA cargo [16-18]. From 2010 onwards, EV research took a different turn, with an exponential increase in the number of articles published. This growing number of articles, along with a wide variety of nomenclatures and methods, led to the creation of the International Society for Extracellular Vesicles (ISEV) in 2011 and the establishment of international guidelines in 2014 [19], which were updated in 2018 [20] and 2024 [21].

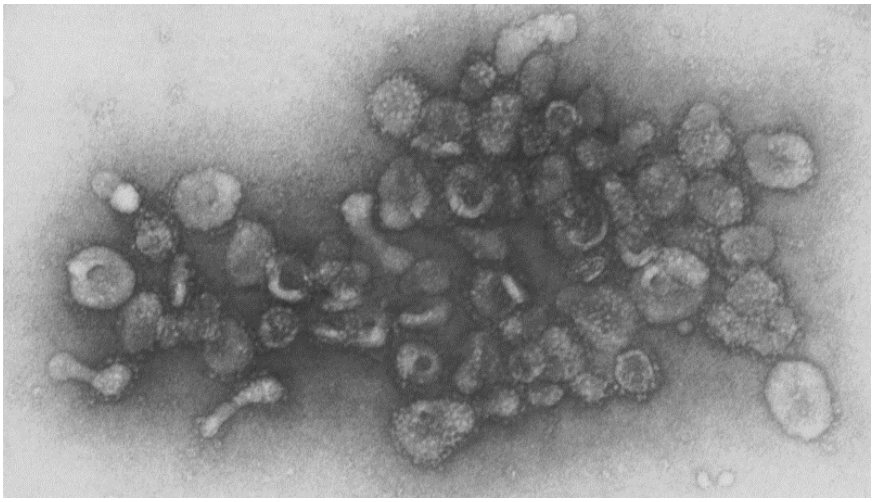

Figure 1. Extracellular vesicles released by reticulocytes. Image from Pan et al. (1983) [9].

1.2 Exosomes, microvesicles and apoptotic bodies

Over the years, numerous terms were used when describing vesicular structures. Terms like “microparticles”, “virus-like particles”, “ectosomes”, “oncosomes” or “exosomes” were applied interchangeably, creating some confusion. A precise nomenclature was therefore needed to ensure a better understanding, reproducibility, and standardization in the field. Currently, EVs are classified in three subtypes based on their biogenesis and release mechanisms: apoptotic bodies, microvesicles and exosomes (Fig. 2).

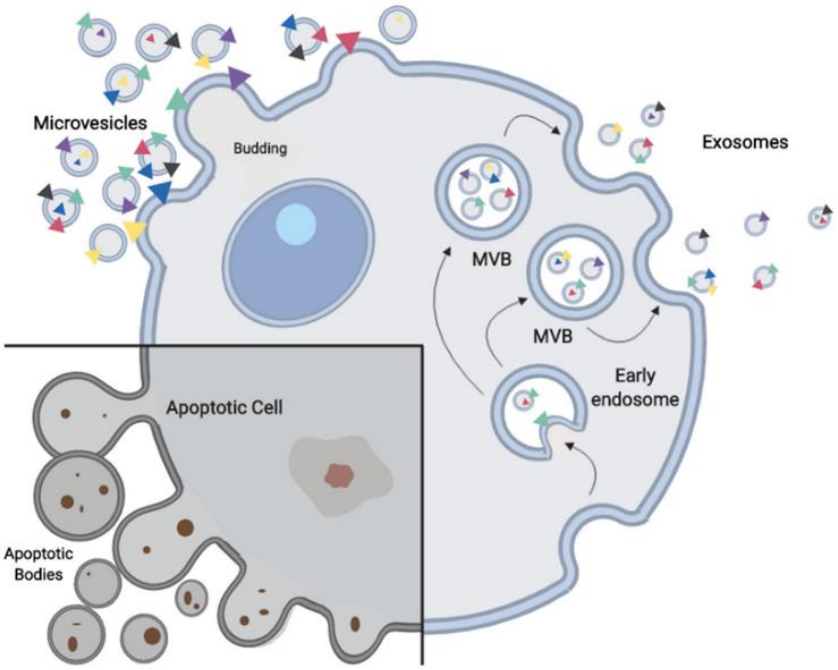

Figure 2. Extracellular vesicle formation. Apoptotic bodies and microvesicles directly bud from the plasma membrane whereas exosomes are formed inside the cell. In the case of exosomes, intraluminal vesicles bud within early endosomes, forming multivesicular bodies (MVB). MVB can then fuse with the plasma membrane to release exosomes into the extracellular space. Figure from Angulo et al. (2019) [22].

Apoptotic bodies are the largest EVs (~500-4000nm) and are released as a consequence of programmed cell death [23]. During apoptosis, the cell undergoes different stages ultimately leading to a split of the cellular content in distinct membrane-enclosed vesicles termed apoptotic bodies. These vesicles are thus often characterized by the presence of nuclear content and/or organelles in their lumen. Microvesicles (MVs) are formed directly from the outward budding of the plasma membrane with diameters ranging from ~100 nm to 1000 nm [24]. MVs can be differentiated from apoptotic bodies by both size and formation mode, and also by their content and membrane-specific antigens. Finally, exosomes, the smallest subtype of vesicles (~30-150 nm), are formed inside the cell via the endosomal pathway and are released through the fusion of the multivesicular body with the plasma membrane [25].

However, when isolating EVs, it is not always possible to identify their biogenesis pathway. Moreover, no consensus has yet been reached on specific markers for EV subtypes, and their size does not allow them to be accurately discriminated given the existing overlap. Taken together, these elements led the ISEV to encourage utilization of "EVs" as a generic term for "particles naturally released from the cell that are delimitated by a lipid bilayer and cannot replicate". Unless the subcellular origin can be established, terms as MVs or exosomes should be avoided. Furthermore, a clear description of EV physical characteristics, biochemical composition and origin tissue/condition is highly recommended [20].

1.3 Biogenesis and release of extracellular vesicles

As mentioned above, exosomes and MVs exhibit distinct modes of formation. Consequently, it is reasonable to assume that their cellular machineries would

differ as well. However, it is interesting to note that some mechanisms may be shared between those vesicles. In addition, the implication of different biogenesis pathways seems to vary according to the producing cell type [26]. As this thesis does not intend to describe extensively those mechanisms, only the main biogenesis and secretion pathways of exosomes and MVs will be presented in the next sections.

1.3.1 Exosomes

Exosomes being related to the multivesicular body, their formation starts with the endocytic pathway and the internalization of plasma membrane giving rise to an early endosome. These early endosomes then mature into late endosomes while accumulating intraluminal vesicles (ILVs) in their lumen. From that point, endosomes are defined as "multivesicular endosomes" or more frequently "multivesicular bodies" (MVB). The best-described mechanism for ILV formation in MVBs is driven by the endosomal sorting complex required for transport (ESCRT). ESCRT is composed of ~30 proteins that assemble into four complexes (ESCRT-0, -I, -II and -III) with associated proteins [ALG-2-interacting protein X (ALIX) and vacuolar protein sorting-associated protein 4 (VPS4)] [27]. ECSRT-0 is responsible for the recognition and the sequestration of ubiquitinated proteins at the endosomal membrane. ESCRT-I and -II induce a membrane deformation into buds inwards the endosome. Finally, ESCRT-III allows membrane fusion and drives vesicle scission, whereas the associated proteins seem to participate in the dissociation and recycling of the ESCRT complexes [27]. In addition to the ESCRT-dependent pathway, it seems that other independent mechanisms operate in ILV formation (Fig. 3).

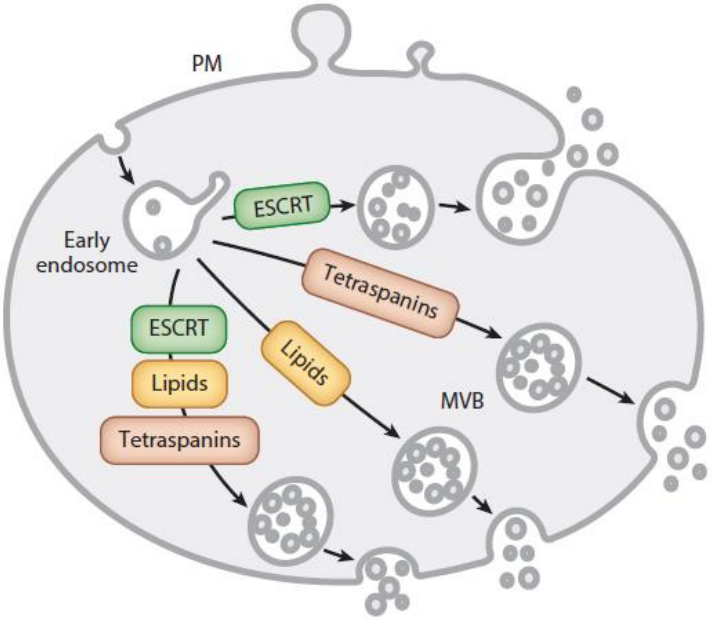

Figure 3. Molecular machineries of exosome biogenesis. Multiple mechanisms are involved in biogenesis of multivesicular body (MVB), ultimately releasing exosomes. Endosomal sorting complex required for transport (ESCRT) components, tetraspanins and lipids have been described to participate in MVB formation. Whether these pathways act simultaneously on the same MVB or on different MVBs remains to be determined. PM: plasma membrane. Figure from Colombo et al. (2014) [25].

The first pathway involves transmembrane proteins of the tetraspanin family (CD63, CD9 and CD81) [28], while the second is driven by specific lipids (ceramide and phosphatidic acid) and enzymes (neutral sphingomyelinase and phospholipase D2) [29-31]. It is important to mention that, apart from operating independently, these three main mechanisms may also act in synergy (Fig. 3) [25]. The current literature suggests that the different pathways could either take place concomitantly or sequentially to form MVBs [26]. This variety of mechanisms could be at the root of the heterogeneity of MVBs within the cell, as well as of the different vesicle subpopulations. In sum, exosome biogenesis remains a complex process that has yet to reveal

some secrets. Once formed, MVBs can either fuse with the lysosome to be degraded or fuse with the plasma membrane to allow exosome secretion to the extracellular space. While the regulation of the balance between degradation and secretion is largely unexplored, both situations would certainly require transport and fusion processes. As for other intracellular transports, MVB mobility involves elements of the cytoskeleton, associated molecular motors and molecular switches. MVB are thus transported along the microtubule network to the plasma membrane or lysosomes via kinesins and dyneins, respectively [32]. These actions are allowed by multiple proteins from the Rab GTase family such as Rab5, 7, 11, 27a, 27b or 35 [27]. When reaching the plasma membrane, the MVB docks to it to enable the fusion of lipid bilayers. This final step of exosome secretion seems to be mediated by a calcium-regulated exocytosis involving SNARE proteins and synaptotagmin family members. Several proteins like SNAP-23, VAMP-7, and Ykt6 have been reported to participate in the release of exosomes outside of the cell [33-35]. Similarly to the variety of mechanisms involved in MVB biogenesis, it has been suggested that different Rab proteins and SNARE complexes could mediate the transport and secretion of distinct MVB subpopulations [25].

1.3.2 Microvesicles

In contrast with exosome biogenesis, MV formation modes remain less well characterized. Nevertheless, several mechanisms have been highlighted in various models. To enable bud formation through physical bending of the membrane and restructuration of the cytoskeleton, molecular rearrangements within the plasma membrane are required. A first path leading to MV formation involves phopspholipid reorganization via several calcium-

dependent enzymes, including members of the protease calpain, flippase, floppase and scramblase families [36]. A second one involves cytoskeleton elements and their regulators. More specifically, actin dynamics regulated by Rho GTPases and the Rho-associated protein kinase allow vesicle budding as well [37]. Finally, shared mechanisms with exosome biogenesis occur in MV formation, as mentioned in section 1.3. In addition to its role in ILV formation, the sphingomyelinase (SMase) family seems to also induce ceramide-dependent MV production. While nSMases are involved in ILV formation, aSMases participate in MV biogenesis. The latter suggests that different members of the SMase family specifically control the release of distinct EV populations, at least in the central nervous system (CNS) [31, 38].

Unlike exosomes, for which MVBs require transport, freshly formed MVs only need a fission process to be released into the extracellular space. This mechanism depends on the interaction of cytoskeleton actin and myosin at the plasma membrane together with a subsequent ATP-dependent contraction. As such, the activation of small GTP-binding proteins including ARF6 and ARF1, leads to actomyosin contraction, which allows the vesicles to bud off from the membrane [39-41]. ESCRT component TSG101 in concert with VPS4 and ARRDC1 are also reported to participate in the scission and release of MVs [42].

1.4 Extracellular vesicles composition and cargo selection mechanisms

The nature and abundance of EV cargo seems to depend on the cell type and is often influenced by physiological or pathological stimuli [25]. To date, the actual composition of EV subtypes remains unknown, but it is well

established that EVs contain proteins, lipids, and various nucleic acids (Fig. 4).

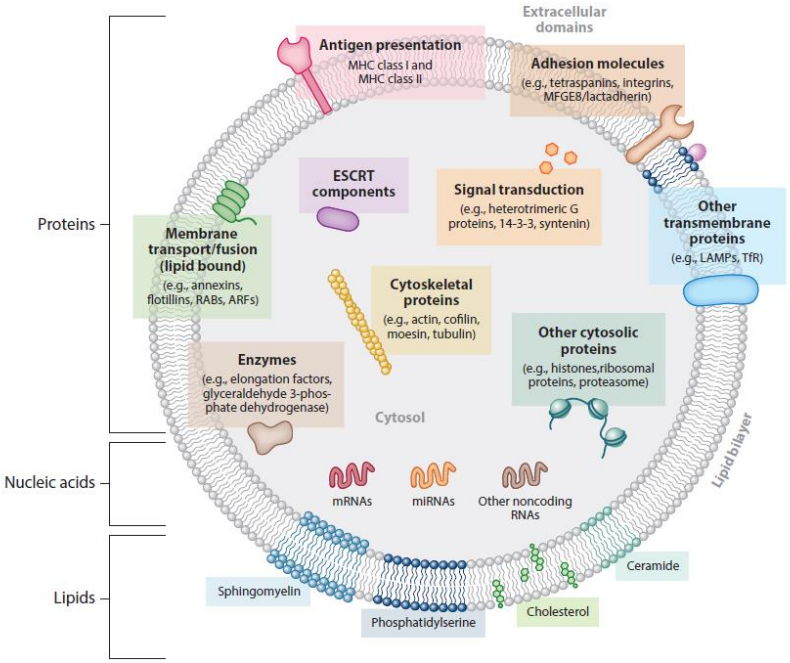

Fig. 4. Overall composition of extracellular vesicles (EVs). Schematic representation of the composition (families of proteins, lipids, and nucleic acids) and membrane orientation of EVs. Examples of tetraspanins commonly found in EVs include CD63, CD81, and CD9. Note that each listed component may in fact be present in some subtypes of EVs and not in others. For instance, proteasome and ribosome components, as well as histones, are probably secreted in large plasma membrane–derived EVs and/or apoptotic vesicles rather than exosomes. ARF: ADP ribosylation factor; ESCRT: endosomal sorting complex required for transport; LAMP: lysosome-associated membrane protein; MHC: major histocompatibility complex; MFGE8: milk fat globule–epidermal growth factor-factor VIII; RAB: Ras-related proteins in brain; TfR: transferrin receptor. Figure and caption from Colombo et al. (2014) [25].

Proteomic analyses highlighted that EVs contain a specific subset of cellular proteins. Among these proteins, some depend on the secreting cell type, others seem to be enriched in specific EV subpopulations, while several are found in most vesicles. The latter include proteins from endosomes, plasma

membrane and cytosol, but rarely from intracellular organelles (nucleus, mitochondria or Golgi) [25]. These observations confirm the specificity of EV formation and show that they represent a specific subcellular compartment instead of transporting cargo randomly packed in. Of note, although exosomes should be enriched in endosomal components, as compared to MVs, the two types of EVs display a large overlap in composition, which prevents the application of universal protein markers specific for each of them [27]. Among the most enriched proteins, one often finds those involved in EV formation and release, such as tetraspanins (CD9, CD63, CD81), TSG101 or ALIX, to name a few. These proteins are usually considered as EV markers and used to confirm the presence of EVs in collected samples [20].

While proteomic analyses of EVs have been conducted intensively, studies investigating vesicular lipid composition are less frequent. Interestingly, similarly to proteins, EV lipid composition is different from the whole cell membranes, reinforcing the idea of a specific subcellular compartment [43-45]. Indeed, EVs have been shown to be rich in cholesterol, sphingomyelin, ceramides, and phosphatidylserine organized in microdomains called lipid rafts. These rafts are capable of membrane curvature induction and, thus, membrane buds during EV formation, as mentioned above. While lipid composition seems to vary depending on the vesicle subtype [43, 46], the differences in lipid enrichment between exosomes and MVs remain unclear.

After the first description of nucleic acids in both MVs and exosomes in the late 2000s [16-18], numerous studies have been conducted to investigate the genetic material contained in EVs. This cargo includes mRNAs but is particularly enriched in a large variety of small non-coding RNAs such as

transfer RNAs (tRNAs) or micro-RNAs (miRNAs), while ribosomal RNAs (18S and 28S) appear to be absent [25]. Among the RNAs carried in EVs, miRNAs are undoubtedly the most studied. As for proteins and lipids, EV RNA composition differs from the parent cell, and the different EV subtypes seem to transport different subsets of RNA sequences [47, 48].

Like biogenesis and release, the selection of cargo into the vesicles is thought to be highly regulated and not a random event. For instance, although the mechanism is still not very well understood, the inclusion of soluble cytosolic proteins into ILVs could involve chaperones as HSC70 [49, 50]. In a TSG101- and VPS4-dependent process, chaperones would bind to the target protein and to phosphatidylserine from the outer membrane of the MVB, and thus enter the ILVs [51]. Modification by ubiquitylation [52] or farnesylation [53] has also been proposed to participate in protein sorting in ILVs. For MV loading, the mechanisms remain largely undefined, but cytoplasmic protein oligomerization at plasma membrane anchor seem to drive the proteins into the vesicle [54]. Apart from mechanisms incorporating proteins into EVs, RNA loading is likely to be regulated as well. Some proteins (ALIX, AGO2) and protein complexes (ESCRT-II, miRNA-induced silencing complex) could interact and bind to miRNA to further load it in EVs [55, 56]. Interestingly, miRNAs have been shown to be sorted in EVs based on specific sequences [57, 58]. miRNAs would possess either sequences allowing their secretion in EVs (EXOmotifs) or their retention in the cell (CELLmotifs). Additionally, different cell types, including brown and white adipocytes, endothelial cells, hepatocytes, and myocytes, make preferential use of specific sorting sequences, thus determining cell-specific miRNA profile [57]. Finally, the EV lipid composition seems to be mainly determined by the

lipid rafts composition. Indeed, the lipids present in EVs compose the membrane of those vesicles. Lipid rafts being the preferential vesiculation sites at the plasma membrane, their formation and composition greatly influence the lipid enrichment of the released vesicles [25, 59].

1.5 Extracellular vesicles interaction with recipient cells

To act as messengers in intercellular communication, EVs secreted by one cell must be able to interact with a recipient cell to induce changes in its physiology. The potential functions of EVs in physiological or pathological processes, therefore, depend on their ability to interact with target cells and/or to deliver their content to them. In some case, simple binding of the vesicle to the cell membrane through ligand-receptor interaction enables signal transduction without cargo delivery. It is, for instance, the case with the presentation of MHC-peptide complexes on the EV surface to antigen-specific T cells [60, 61]. In other cases, EV cargo must be transferred inside the cell via several pathways (Fig. 5) [62].

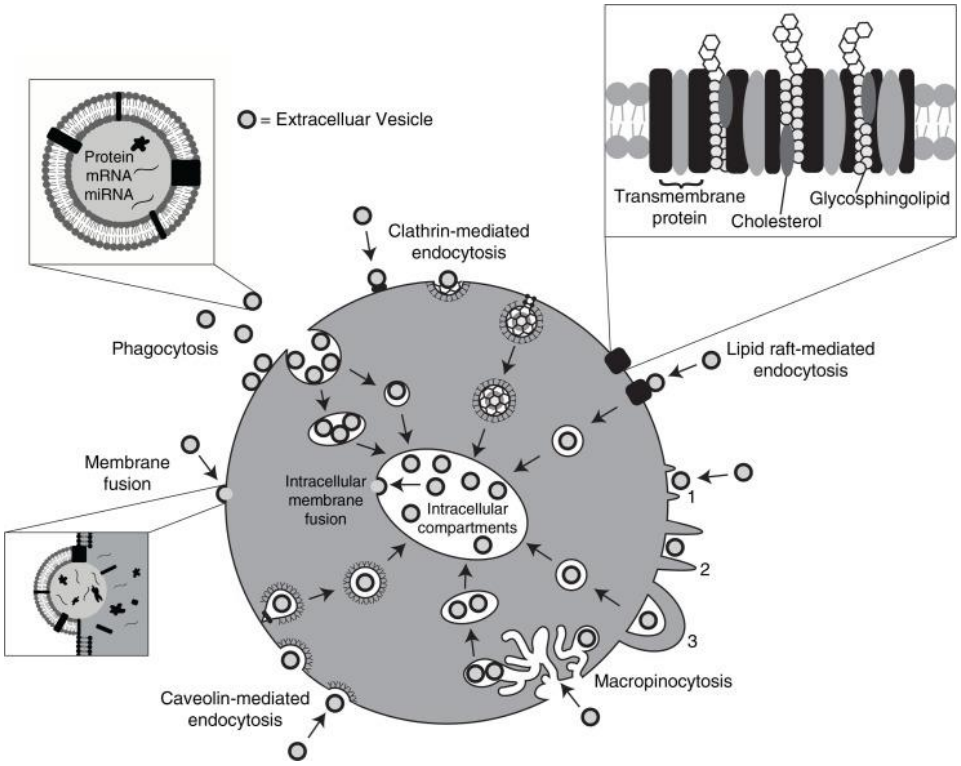

Fig. 5. Pathways involved in extracellular vesicle uptake by target cells. Various mechanisms have been identified for the uptake of extracellular vesicles (EVs). EVs can be internalized by clathrin-dependent endocytosis or clathrin-independent endocytosis such as caveolin-mediated endocytosis, macropinocytosis, phagocytosis or lipid rafts-induced endocytosis. EVs may also deliver their cargo (proteins, mRNA, and miRNA) by fusion with the plasma membrane. Alternatively, internalized EVs may fuse with the endosomal membrane following endocytosis to enable the release of their content and thus elicit cellular responses. Figure from Mulcahy et al. (2014) [62].

EVs can either release their content inside the recipient cell by direct fusion with the plasma membrane or be internalized. Although direct fusion might occur during EV internalization, it is unlikely that this path is the main route of entry. Instead, most experimental data suggest that EVs are generally taken up into endosomal components via endocytosis. Once at the plasma membrane, EVs can be internalized by clathrin-dependent endocytosis or clathrin-independent endocytosis such as caveolin-mediated endocytosis,

macropinocytosis, phagocytosis or lipid rafts-induced endocytosis [62]. The exact contribution of these different mechanisms remains to be elucidated but is probably influenced by both EV and recipient cell characteristics. Proteins and glycoproteins (tetraspanins, integrins) found on the surface of both the vesicle and the recipient cell seem to influence the uptake route used by a given EV, suggesting some tissue specificity in this process [62]. Finally, once inside, the vesicle still needs to unpack its content to elicit a phenotypic response. EVs may fuse with endosomal limiting membrane to release their cargo, but this final step is currently very poorly understood.

1.6 Extracellular vesicles functions

EV secretion appears to occur in nearly all cell populations. In addition, EVs have been isolated from most of body fluids (blood, saliva, cerebrospinal fluid, semen, breast milk, ...) [63-68]. It was therefore suggested that these vesicles could be involved in certain biological processes. Due to their recent notoriety, EVs have been studied in both physiological and pathological models, leading to the attribution of numerous functions. Back in the 60s, platelet-derived vesicles were first shown to promote the coagulation cascade [3], while the work conducted by Johnstone and Stahl's groups in the 80s highlighted the role of EVs in reticulocyte maturation via transferrin receptor externalization [8, 9]. Later, vesicles aroused the curiosity of immunologists because of their ability to present antigens and stimulate immune responses [13]. Thanks to their capacity to transfer proteins and RNAs, vesicles participate as well in both local and distant intercellular communication. Finally, EVs are involved in several other biological processes in different systems including, but not limited to, cardiovascular, renal, reproductive, central nervous, musculoskeletal, and gut microbiome systems [69].

As for a large variety of cellular processes, EVs can be affected by diseases [70, 71]. In most of the pathological conditions studied, their release and/or cargo seem to be deregulated. Patients with cancer, diabetes, obesity, neurodegenerative diseases, or atherosclerosis exhibit altered circulating EVs (number and/or content) [70]. In addition to reflect changes in the cellular status (which makes EVs interesting candidates as biomarkers), EVs can actively participate in the development of the disease. For instance, tumor cells secrete EVs contributing to tumor progression by promoting angiogenesis and tumor cell migration in metastases [72, 73]. In neurodegenerative diseases, EVs seem to be involved in the transport and dissemination of pathogenic proteins such as prions [74], β-amyloid peptide [75], superoxide dismutase [76] and α-synuclein [77]. Obese individuals exhibit higher levels of circulating EVs with altered cargo, which could contribute to inflammation and insulin resistance development [78]. By the secretion of miR-155, adipocyte-derived exosomes from obese mice have been shown to induce pro-inflammatory macrophage phenotype [79].

Taken together, the above elements highlight that EVs are important vehicles of intercellular communication and are implicated in various biological processes. These vesicles can be considered neither good nor bad, as their effects are dependent on the physiological state of the parent cell. EVs should rather be considered as “innocent bystanders that are manipulated” [71]. While EV release in pathological state seems to have detrimental effects in most cases, EV release during physical exercise has been proposed to be beneficial. In which extend EVs are involved in exercise-mediated health benefits? How are EVs regulated during exercise? The next sections will explore the links between exercise, health and EVs.

2. Extracellular vesicles and exercise

The following section is instigated from the review entitled "Extracellular Vesicles and Exosomes: Insights From Exercise Science" published in "Frontiers in Physiology" in 2021 that we co-wrote with Joshua Nederveen, Alessia Di Carlo, Mats Nilsson and Mark Tarnopolsky. The original text has been modified, updated, or removed to fit to the thesis format. The term exosome-like vesicle (ELV) will be used to cover all subpopulations of small vesicles (<200nm) when no difference can be made between exosomes and MVs.

2.1 Extracellular vesicles as mediators of multi-systemic exercise response?

Over the last 60 years, the study of exercise science has yielded the immutable fact that habitual exercise confers remarkable health benefits, decelerates biological aging, and prolongs lifespan. While low-intensity exercise is sufficient to improve overall health, training benefits are usually dose-dependent. For instance, higher intensities are linked with greater reductions in mortality risk [80]. There are a wealth of original studies, reviews and meta-analyses demonstrating that physical activity and exercise have beneficial effects across all organ systems in humans, thus protecting against a diverse spectrum of disease states. Although these benefits are most evident in organs directly involved in movement, respiration, and blood-flow (e.g., musculoskeletal, cardiorespiratory, and nervous systems), positive effects may also be seen in less obvious systems (e.g., integumentary, reproductive, and digestive).

The combined weight of the evidence suggests that exercise exerts its multi-systemic effects by facilitating juxtracrine, autocrine, and paracrine communication between cells. Exercise also appears to promote crosstalk between tissues/organs that are not located in a close spatial proximity (i.e., endocrine signaling). The provision of those remarkable health benefits is obviously complex and multi-factorial [81, 82], but likely partly attributed to the myriad of bioactive molecules released into circulation during exercise, collectively termed exercise factors or 'exerkines'. The pioneering work of Pedersen et al. [83, 84] identified interleukin 6 (IL-6) as the first muscle-derived exerkine released by skeletal muscle, and thus was classified as a myokine. The fact that skeletal muscle accounts for ~40% of the human bodyweight and changes its metabolic profile dramatically during exercise, underscores it as a likely origin of therapeutic factors. However, any tissue/cell type capable of secretion may theoretically add to the global 'exercise secretome,' including adipose tissue, liver, lymphocytes, endothelial cells, and platelets. Currently, over ~300 exercise factors have been identified, many of which appear to be contractile activity-regulated [85-87].

Myokines have the capability to act in a paracrine, autocrine, and/or endocrine fashion [88-90]. Studies have demonstrated that an acute bout of exercise can induce an increase in the production and the secretion of IL-6 by skeletal muscle [91, 92]. Once released, IL-6 can mediate local tissues, increasing glucose uptake and fatty acid oxidation in the muscle [93, 94]. It may also function as an endocrine agent, capable of increasing lipolysis of adipocyte tissues [95-97]. In addition to IL-6, several other muscle-derived factors have been discovered, including a vast number of pro- and anti-

inflammatory cytokines (e.g., IL-1α/β, IL-8, IL-10, and IL-15) [85-88, 98]. Among those, exerkines, osteocrin [99], brain-derived neurotrophic factor (BDNF) [100], follistatin-like 1 (FSTL1) [101], irisin [102] and fibroblast growth factor-21 (FGF-21) [103], vascular endothelial growth factor (VEGF) [104] and myostatin [105] have also been investigated for their therapeutic potential.

Until recently, the benefits that have been ascribed to systemic growth factors secreted during exercise have not been examined outside the classical secretory pathway. However, an additional pathway for secreting peptides and other signaling molecules out of the cell exists in the form of EVs [106]. The myogenic origin of some subpopulations of EVs have been probed in vitro, with results suggesting that myoblasts and myotubes are both capable of releasing EVs in culture [107-110], possibly originating from different processes [110]. Proteomic analyses performed on EVs released in the conditioned medium from human myotubes revealed an increased expression of proteins related to intercellular trafficking, plasma membrane development, endocytosis, protein synthesis, and free-radical scavenging [86]. In line with the concept that EVs are, in part, responsible for cell-to-cell communication, evidence from Forterre et al. [108] suggested that EVs released from myotubes could be taken up by myoblasts, subsequently upregulating myogenic differentiation [108]. Work from the same group established that EVs released from myoblasts and myotubes in vitro contain proteins and miRNAs that are specific to the vesicles and not the cells themselves [109]. This process of selective loading of EVs has also been examined in an in vitro model of muscle atrophy (i.e., dexamethasone application to in vitro myotubes). During myotube atrophy, there was a

marked decrease in miR-23a abundance in the myotubes concomitant with an increase in miR-23a expression in the isolated EVs, ultimately leading to an altered expression of downstream target genes [111]. Taken together, these data suggest that skeletal muscle can produce bioactive EVs. Our understanding of how skeletal muscle acts as an endocrine organ has evolved and now includes the release of EVs. However, the mechanisms by which signaling molecules, peptides, miRNAs can be secreted into EVs and subsequently shuttled to another organ/tissue in response to exercise remain to be elucidated.

2.2 Contribution of skeletal muscle to the extracellular vesicle pool

Extensive work has established skeletal muscle as an endocrine organ, and growing evidence supports the notion that muscles can release EVs into the circulatory blood. For instance, an intramuscular injection of fluorescently labeled EVs resulted in the appearance of fluorescence in proximal and contralateral muscles – reinforcing the notion of paracrine and endocrine action of muscle released EVs [112]. Nevertheless, challenges remain for the detection of skeletal-muscle derived-EVs (SkMEVs) in response to exercise, as there are limited methods to label and track SkMEVs within the systemic circulation. Instead, skeletal muscle specific markers have been used as a surrogate for specific SkMEV labels. Initially described by Guescini et al. [113], population of SkMEVs were identified using α-sarcoglycan (SGCA), a protein that is highly abundant in skeletal muscle. Subsequently they were verified from the EV cargo, which was enriched with skeletal muscle-specific microRNA (myomir) mir-206 [113]. In this study, while high-purity SkMEVs were positive for canonical EV markers TSG-101 and CD81, they only represented ~1 to 5% of the total plasma-isolated EV population, putting into

question the relative importance of skeletal muscle-derived EVs to the total pool of circulating EVs [113]. Another study provided interesting insights regarding SkMEV release and muscle typology. Nie and collaborators (2019) incubated mouse muscles in serum-free culture media for 24 h. Vesicular markers ALIX and TSG101 were then quantified in ELVs isolated from the media. Results showed that EVs were indeed released by the muscles, and that a higher amount (~5.5 fold) originated from oxidative vs glycolytic muscles (Fig. 6B). Despite not being conducted in an exercise configuration, this observation suggests that SkMEV release might be fiber-type specific [114]. More recently, those results were confirmed using other quantification techniques, such as EV protein measurement [115] or NTA [115, 116].

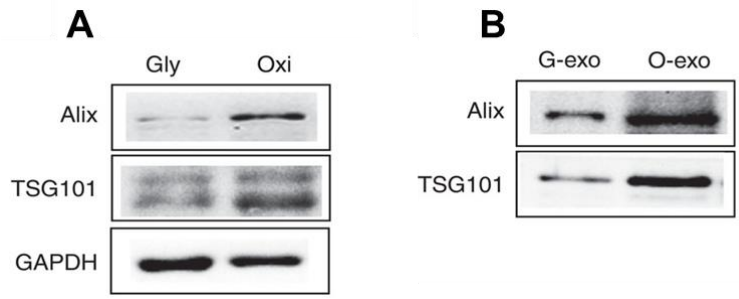

Figure 6. Muscle typology and ELV production. A) Representative images of ELV markers Alix and TSG101 in glycolytic (Gly) and oxidative (Oxi) muscle. B) Representative images of ELV markers Alix and TSG101 in ELVs derived from equal amounts of glycolytic (G-exo) and oxidative muscle (O-exo). Images from Nie et al. (2019) [114].

Given the challenge presented in tracking circulating ELVs derived from skeletal muscle, the real contribution of these vesicles to metabolism regulation, especially during exercise, remains to be elucidated clearly. The next sections will present the current comprehension of the effects of exercise (acute) and training (chronic) on total circulating ELV amount and content.

2.3 Effect of acute exercise on exosomes-like vesicle release

Although the field is still in its infancy, a few well-controlled studies have attempted to examine the effects of acute exercise on ELVs with complete particle characterization and expression analyses.

In 2015, Frühbeis et al. were the first to examine the dynamics of ELVs in response to short-duration, incremental cycle ergometry and treadmill running in healthy male volunteers [117]. In the first exercise setting (n = 8, ∼41 years), participants completed a graded cycling protocol starting at 50 W, which was increased incrementally by 50 W every 3 min until volitional exhaustion. On the second testing occasion (n = 4, age ∼27 years), participants completed a progressive running protocol starting at 6 km/h at 1.5% grade, which was increased by 2 km/h every 3 min until exhaustion. For both tests, ELVs were isolated from plasma by ultracentrifugation (2 h at 100,000 g). As per ISEV recommendations, nanoparticle tracking analysis (NTA) and immunoblotting were conducted to examine changes in ELV size and concentration [19, 20]. Compared to pre-exercise, plasma taken immediately following a maximal incremental cycling test showed a ∼2.7-fold and ∼1.8-fold increase in ELV concentrations as assessed by NTA, with a return to baseline levels within 90 min of rest (n = 2, descriptive data, see Fig. 7).

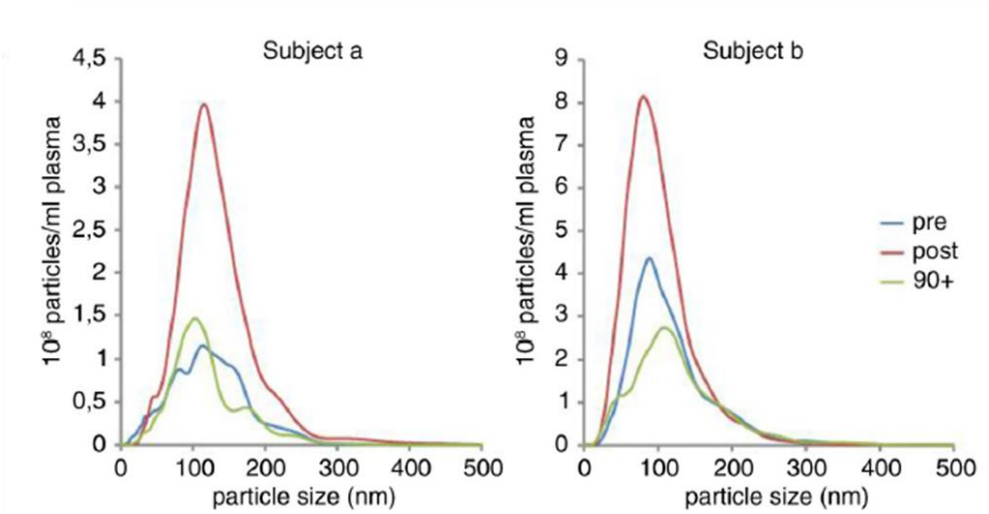

Fig. 7. Effect of cycling exercise on plasma ELVs. Particle concentration measured by nanoparticle tracking analysis in two subjects. Plasma samples were collected prior, directly after and 90 min after the end of an incremental cycling exercise test. Graphs from Frühbeis et al. (2015) [117].

Furthermore, markers such as HSP/HSC70 and flotillin 1 (Flot1) were elevated immediately after exercise (mean increase ~5.2-fold), before returning to baseline 90 min post-exercise, while the canonical ELV marker TSG101 seemed to be increased but statistically unchanged. Interestingly, the incremental treadmill test resulted in a different release dynamic than what was seen with cycling. A moderate increase in NTA-quantified ELVs (~1.5-fold) was observed immediately post-exercise, but remained sustained at this level for the following 90 min. Despite a sustained elevation in NTA-detected particle concentration following treadmill exercise, protein levels of HSP/HSC70 were not increased, while Flot1 was only elevated immediately post exercise (TSG101 was undetectable). Finally, both NTA and immunoblotting results confirmed that circulating ELVs returned to baseline levels 6 h post exercise and remained stable for the next 24 h.

In the latter study, mean vesicle diameters were ~120 and ~165 nm in the cycle vs. treadmill tests, respectively, suggesting that in addition to exosomes, the isolates also contained small MVs (~50–200 nm) and/or chylomicrons (75–600 nm). Notably, it is challenging to assess the true proportion of ELVs when using ultracentrifugation isolation. This method co-isolates significant amounts of lipoprotein particles, including chylomicrons, LDL and HDL (known to be significantly increased with acute exercise [118]), which may interfere with NTA data [119]. Furthermore, these results indicate that exercise modality may play a role in ELV release. Treadmill incremental tests typically activate more muscle groups as compared to standard cycle ergometry [120, 121], thereby increasing total blood flow [122, 123] and ultimately a higher maximal oxygen consumption [124, 125]. Although speculative, it is possible that those factors contributed to the observed differences in ELV kinetics and sizes between both modalities. Of note, these results should be considered with caution as the two modalities were not matched for age (which is known to impact circulating EVs), a very low number of participants (n=4) were included in the treadmill experiment, and the differences were mainly observed in NTA data.

To further elucidate the kinetics of ELVs during exercise, Frühbeis et al. (2015) also collected samples at each incremental step during the graded cycling test. The authors reported that ELV-related proteins, such as Flot1 and HSP/HSC70, increased at ~9–12 min of cycling (corresponding to a workload of 150–200 W), before plateauing between ~15 and 20 min (250–300 W), and rapidly returning to baseline levels 10 min post exercise. Importantly, these data provided the first insight into EV kinetics during exercise and indicated that circulating ELVs begin to accumulate prior to the

anaerobic/lactate thresholds and the exponential increase in plasma norepinephrine/epinephrine in healthy, male volunteers [126, 127].

In a follow-up study by the same group [128], the ELV response to incremental cycling ergometry was assessed by more sophisticated EV isolation methods in aerobically trained male athletes (~50 $mL \cdot kg^{-1} \cdot min^{-1}$ VO_2max). Following overnight fasting, participants completed an incremental cycling test starting at 40 W intensity, which was then increased by 40 W every 3 min until volitional exhaustion. Venous blood was drawn at rest, during exercise (at respiratory quotient of 0.9), and immediately following exercise. ELVs were then isolated using size exclusion chromatography (SEC) columns and immunobead capture (e.g., CD9+, CD63+, CD81+ isolation kits), followed by characterization of the exercise response by NTA, immunoblotting and transmission electron microscopy (TEM). In line with their previous work on ultracentrifugation-derived isolates [117], immunoblot analyses of SEC-ELVs revealed an increase in abundance of canonical exosome markers during and immediately post-exercise (e.g., CD9, CD63, and CD81) (Fig. 7B). Notably, circulating ELVs, as assessed by immunoblotting, began to rise at submaximal exercise intensities (RQ of 0.9), confirming previous findings on ultracentrifugation-derived isolates [117], and appeared to reach peak levels immediately post-exercise. Importantly, NTA analyses were unable to demonstrate this exercise-induced increase in SEC-ELVs (Fig. 8). Therefore, the authors concluded that semi-quantitative methods are more appropriate because they are less affected by interference by contaminating factors.

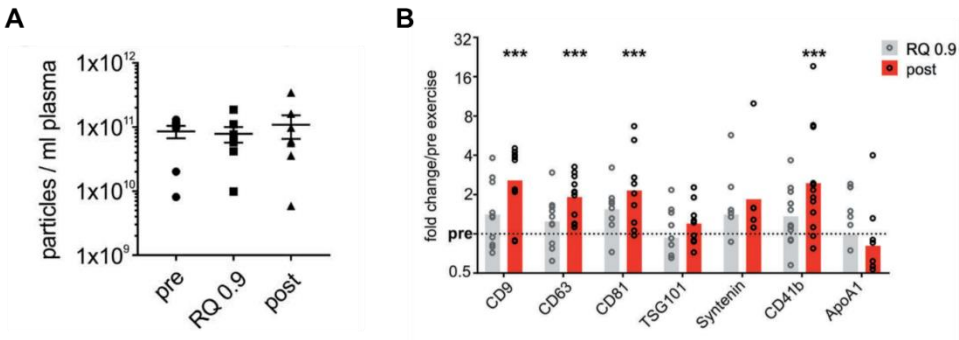

Figure 8. ELV quantification during incremental exercise. A) Particle counts in ELV samples collected pre-, at RQ 0.9 and post-exercise. B) Densitometric quantification of western blot signals. Individual (circle) and mean (bar) fold changes of western blot markers. Graphs from Brahmer et al. (2019) [128].

In a separate analysis, Brahmer et al. (2019) demonstrated that exercise-induced ELVs constituted a heterogenous population, mainly originating from lymphocytes (CD4+ and CD8+), monocytes (CD14+) endothelial cells (CD105+ and CD146+), and platelets (CD41+, CD42+, and CD62+) with less contribution from skeletal muscle (SCGA+ EVs) [128] confirming the results of Guescini et al. (2015) [113]. Together, these findings suggested that exercise stimulates ELV release at sub-maximal workloads (i.e., prior to the increase in systemic lactate and spike in catecholamines) and may be potentiated as exercise duration and/or intensity increases. This rapid rate of appearance may suggest that ELVs are primed by early exercise signals, such as shear-stress, mechano-transduction, excitation-contraction coupling, or other Ca^{2+}-mediated events, while later signals, such as hormone release (e.g., cortisol), HPA axis activation (e.g., catecholamines), metabolic stress, and/or reactive oxygen species, may potentiate the EV response [129-131]. Different biomechanical stresses including shear, tension, and compression, have been shown to modulate ELV biogenesis and release in various tissues [132]. Although the response is heavily dependent on both cell type and the nature

of applied stress, ELV release is increased by mechanical stress exposure in most cases. Of note, various mechanical stresses induce different responses among EV subpopulations. For instance, exosome release seems to be increased by tensile stress while it decreases MV release. Conversely, MV budding increases under compression [132]. Importantly, ELV release has also been shown to be Ca^{2+}-mediated [131], and genetic knockdown of synaptotagmin-7 (a Ca^{2+} sensor) has been shown to reduce ELV secretion [133]. Following a muscle action potential via motor neurons, Ca^{2+} is released from the sarcoplasmic reticulum into the local milieu [134], ultimately binding to troponin C to eventually allow for crossbridge cycling. The relatively large flux in Ca^{2+} during skeletal muscle repeated contractions (i.e., exercise) may result in the accelerated release of ELVs at a faster rate than in other tissues, but this remains to be elucidated. Finally, temperature rise observed during exercise could also favor an increase in ELV release. Indeed, temperature-dependent ELV secretion has been highlighted in vitro with higher amount at superior temperatures (39°C > 37°C > 35°C) [135]. Together, the rapid appearance of ELVs primarily from cells that are not separated by the blood-tissue barrier puts into question whether skeletal muscle, previously shown to release hundreds of exercise factors, significantly contributes to the exercise-derived ELV pool during acute exercise. Instead, the circulatory pool of ELVs observed following exercise may be contributed by several cell types (Fig. 9).

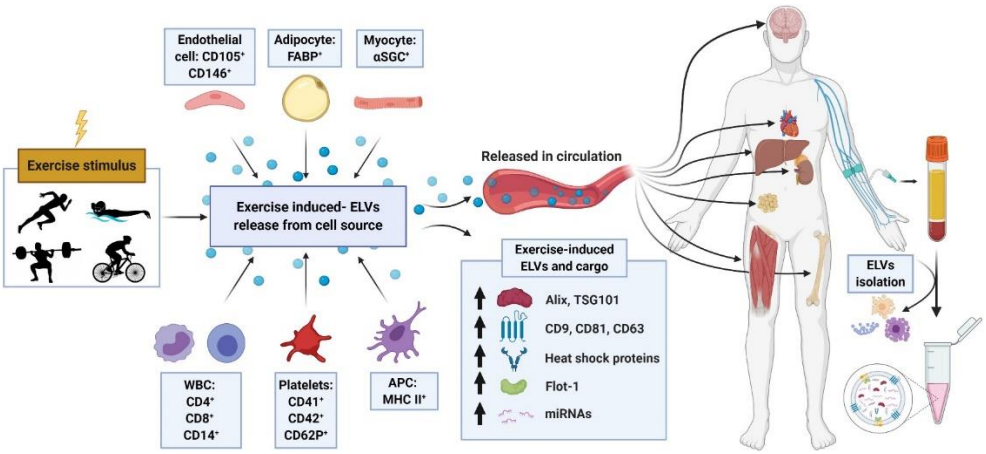

Figure 9. Schematic representation of the proposed origin, release, composition, and cargo of exosome-like vesicles (ELVs) following an exercise stimulus. Following either chronic training or a single bout of exercise, ELVs may be released from muscle or other cell populations and enter the systemic circulation. Exercise-induced alterations in plasma concentration, exosome markers (e.g., ALIX, TSG101, tetraspanins, flotillin, and heat shock proteins) and cargo (e.g., miRNA, 'myomiR' abundance) have been observed. APC, antigen presenting cell; α-SGC, α-sarcoglycan; FABP, fatty-acid-binding proteins; WBC, white blood cells; including monocytes and lymphocytes. Figure and caption from Nederveen et al. (2020) [136].

2.3.1 Effect of exercise intensity

Considering that total blood flow and/or number of muscle groups/fibers recruited during exercise may affect the magnitude and/or kinetics of EV release [117], the intensity of exercise may also dictate this response. However, there is currently limited research to support this notion in both animals and humans. For example, Oliveira et al. (2018) exercised rats for 40 min at low, moderate, or high exercise intensities based on maximum lactate steady state (MLSS) on a treadmill. Despite higher CD63 abundance found in the exercised groups in comparison with non-exercised rats, no differences could distinguish the groups that exercised below or above MLSS [137].

Although insightful, those results were obtained using the ExoQuick isolation method on serum samples, which may not be optimal for minimizing confounding effects of clotting and avoiding contaminants. Nevertheless, the notion that the amount of circulating ELVs can be increased by exercise but not necessarily regulated by exercise intensity is supported by this study [137]. In line with these findings, the human study from Frühbeis et al. (2015) also showed a plateau in ELV release after a given intensity [117].

Furthermore, a relatively high individual variability has been observed in individuals regarding ELV release in response to exercise [117, 128], which may be related to the methods used to partition the exercise intensity spectrum. Indeed, gold-standard methods for the determination of exercise intensity often rely on fixed-percentages of maximal HR or VO_2max, which may not correspond to exercise intensity domains [138] and subsequently do not accurately reflect the metabolic stimulus applied. While the concept of 'responders' and 'non-responders' has been previously proposed in the general exercise physiology field, it remains unclear whether this phenomenon is based on inaccuracies in assessing exercise workload intensities [139, 140]. Future work should assess differences in ELV release in response to various exercise intensities in homogenous groups of participants that are matched for gender, age, bodyweight, and exercise ability.

2.3.2 Effect of exercise modality

While most studies have examined endurance-type exercise modalities, recent work has focused on resistance exercise. Lovett et al. (2018) first examined the impact of eccentric-induced muscle damage on systemic ELV

appearance [141]. Participants performed a muscle-damaging exercise protocol that involved plyometric jumping and bouts of downhill running at 10% decline. ELVs were isolated from blood plasma using SEC, and TEM and NTA were utilized to verify ELV enrichment and determine particle concentration. ELV characterization via TEM revealed a size range of ∼30–150 nm, with MV size particles (100–1,000 nm) occurring relatively infrequently, suggesting a relatively purified population [142, 143]. Despite evidence of muscle damage (i.e., a ∼5-fold increase in creatine kinase from pre- to 24 h post-exercise), there was no evidence of an increase in ELV number or size at either the 2 or 24 h post-exercise timepoints. Based on previous findings in endurance exercise, it can be speculated that 2 h post-exercise sampling might have been already too late to observe any changes.

Two other studies from a same group investigated the impact of more classical resistance exercise on EV-related proteins and RNAs in skeletal muscle [144, 145]. In the first experiment, skeletal muscle biopsies (vastus lateralis) were taken from healthy young men prior and 1 h after a combined exercise. The 45-min session consisted of two-legged cycle ergometry at 55% of VO_2max followed by 3 sets of 8-12 repetitions of single leg knee extensor exercise at 55% of the 1-RM. When comparing pre- to 1 h post-exercise, Garner et al. (2020) did not observe any changes in the protein expression of ALIX, TSG101 or CD63 in the vastus lateralis. In addition, the expression of most genes associated with exosome biogenesis or release did not change neither. Although challenging to interpret without complementary data regarding circulating ELVs, these results suggest either a lack of exercise-induced ELV release or a rapid replenishment of skeletal muscle derived ELVs by 1 h post-exercise. In the second experiment from this group,

resistance exercise alone was used with higher loads (3 sets of 8-12 repetitions of single leg knee extension at 80% of the 1-RM) [145]. Muscle biopsies were performed before, 15 min and 3 h after exercise cessation. In contrast with their first study, Garner et al. (2022) observed a ~50% depletion of ALIX 15 min after the exercise, which could indirectly indicate EV release from the muscle. Intriguingly, this reduction was still present 3 h after the session contrasting with the results from Lovett et al. (2018). Other EV markers (CD63, TSG101) remained unchanged 15 min after the exercise, but TSG101 expression significantly increased 3 h post-exercise. Although surprising at first sight, the contradictory results regarding ALIX and TSG101 might reflect different patterns of release and/or uptake between the various EV subpopulations within skeletal muscles.

Whether or not resistance-based exercise increases the release of ELVs remains unclear. While indirect evidence suggests a possible release, additional work is needed, especially with adequate semi-quantitative analysis of circulating ELVs.

2.3.3 Effect of health status, age, and sex

Recent work examined the influence of health status on ELV appearance in response to exercise. Rigamonti et al. (2019) investigated exercise-induced ELV release in obese and normal-weight participants [146]. Following the determination of VO_2max via an incremental treadmill walking test to voluntary exhaustion, participants exercised at a constant workload corresponding to ~60% of VO_2max for 30 min or voluntary exhaustion. Plasma ELVs were isolated using differential centrifugation, filtration, and ultracentrifugation, and NTA was used to determine ELV size distribution

and concentration. The number of ELVs immediately following exercise was significantly lower compared to resting levels (returning to baseline at 3 h post-exercise), which contrasts to previous work on UC-derived ELVs [117]. Interestingly, SkMEVs ($SCGA^+$ EVs) were elevated in response to exercise, in contrast to other subpopulations such as $CD61^+$ EVs [146]. Even though no immunoblot or EM was performed to determine ELV subpopulations, this was the first study to observe that ELVs may decrease following acute exercise. Importantly, given that the perturbations in ELV content were no longer observable at 3- and 24 h- post-exercise, this study also reinforces the notion that the changes in the rate of appearance (Ra) and disappearance (Rd) of ELVs are transient. Furthermore, the authors reported that there may be a sex-based difference, with ELVs being lower in females than in their male counterparts, regardless of bodyweight. The influence of sex hormones may play a role in vesicle biogenesis [147], and while sex-based differences have been examined in MV populations previously [148, 149], the question remains open for ELV population.

Regarding aging, no study has yet investigated the potential differences in the effect of acute exercise on ELV release between young and older adults. However, some studies carried out at rest have highlighted lower circulating ELV levels in older males in comparison to their younger counterparts [150-152]. More specifically, older men (~75 y) exhibited ~50% lower CD9 and CD81 abundance when using a highly purified SEC isolation method [152]. Similar results were found with UC-derived vesicles through NTA or western blot quantification [150, 151].

2.3.4 Effect of environmental stimuli

When it comes to performance enhancement [153] or to mitigate the side effects of various pathologies [154, 155], environmental stimuli (hypoxia, heat, …) can be used to further enhance the effects of exercise. Among these stimuli, hypoxia is probably the most famous one and is often used by trainers. Hypoxia can be defined as a state of lowered oxygen availability in the tissues [156]. This state can be observed in physiological conditions like altitude and some types of exercise or in diseases such as obstructive sleep apnea [156-158]. In the context of exercise, one can distinguish local and systemic hypoxia. Local hypoxia refers to a reduction in oxygen supply limited to one limb, via, for example, occlusion band application (blood flow restriction) [159-161]. Conversely, systemic hypoxia refers to a global decrease in oxygen availability characterized by a reduction in oxygen pressure in arterial blood. This condition is found, naturally, in altitude or, artificially, in conditioned chambers/rooms [156, 162]. When reproduced artificially, hypoxia can be normobaric or hypobaric. Normobaric hypoxia is induced by reducing the proportion of oxygen in the ambient air -usually by adding nitrogen-, therefore decreasing the inspired oxygen fraction. In hypobaric hypoxia, the partial pressure of oxygen is reduced by lowering the chamber pressure as found in altitude. In the past 30 years, several hypoxic exposure and training strategies have been developed to optimize sport performance, each resulting in various adaptations [153]. Among them, the three main strategies can be described as follow: 1) “live high-train high” consisting of altitude camps where subjects live and train at moderate altitude; 2) “live high-train low” involving training at sea-level while living in moderate altitude; 3) “live low-train high” implying training under hypoxic

conditions (natural or artificial) but remaining at sea-level for the rest of the time. This last training modality is also known as “intermittent hypoxic training”. As part of this thesis, the latter modality was chosen and used with a normobaric setting (see chapter III part 1 and 2). When coupled with hypoxia, exercise can produce greater improvement in several performance components and health-related parameters than the same training performed in normoxia [153-155]. Because it has been suggested that ELVs could participate in exercise adaptations, it is tempting to hypothesize that the additional benefits observed in hypoxia might be related to those vesicles. However, no studies investigating exercise-induced ELV release under hypoxic conditions have been carried out prior to the start of this PhD thesis. Only data from in vitro models were available, particularly in cancer, endothelial and cardiac cells. Interestingly, most of the aforementioned studies on specific cellular types showed that EV release increased when hypoxia was induced [163]. Further investigation involving humans and systemic hypoxia is required to address the potential of this stimulus in increasing circulating ELVs.

2.4 Effect of chronic exercise on exosome-like vesicles release

In comparison with acute exercise, a relatively small number of studies have investigated ELV response to chronic training. Some murine studies suggested a possible increase in the number of circulating vesicles after chronic endurance intervention lasting from 2 to 8 weeks [164-166], but these results were not unanimous [167, 168]. Most importantly, heterogenous blood preparations and EV isolation techniques in addition to lack of transparency

regarding the timing of sampling after the last bout of exercise in some studies [164, 166] prevent firm conclusions [136].

Similarly, contrasting results emerge when looking at human studies, among which only two have been carried out including endurance protocols [167, 169]. In 2019, Hou et al. compared students practicing rowing for at least a year (training for 1-2 h per day, 6 days per week) to their sedentary counterparts [167]. Plasma ELVs were isolated 24 h following the last exercise session via ultracentrifugation and ExoQuick. After NTA quantification, the authors did not find any differences in particles number or size distribution between the two groups. Although observational and knowing the limitations of NTA, these results suggest that chronic endurance training might not significantly impact basal circulating ELVs. More recently, Apostolopoulou et al. (2021) recruited male participants with or without type 2 diabetes to take part in a 12-week cycle ergometer training protocol. More precisely, they participated in a 35-min HIIT session three times a week. During those sessions, they performed 4 sets of 4 min at 90% of maximal heart rate with 3 min recovery at 70%. ELVs were isolated via a combination of SEC and ultracentrifugation, which provided a relatively purified fraction. After 12 weeks of training, the authors found an increase in circulating particles without any change in particle size. However, this increase was only observable in metabolic responders displaying improved insulin resistance after training. Of note, responders exhibited lower circulating ELVs at baseline in comparison to non-responders, which were insulin sensitive. Based on the conflicting results of the aforementioned studies, it is difficult to conclude whether or not chronic endurance training is effective to increase basal circulating vesicles.

Taken together, chronic endurance exercise might lead to a higher level of basal circulating ELVs. The small number of studies, different blood preparations and the myriad of different ELV isolation techniques make firm conclusions difficult. Future training studies, whether in murine models or humans, should aim to follow ISEV guidelines [20], minimize the impact from the 'final bout' of exercise training, and stringently verify the identity of the EV subpopulation under investigation.

2.5 Exercise and training-induced modulations of exosome-like vesicles cargo

Although an increase in the amount of circulating vesicles alone can result in beneficial adaptations [164], their content is no less important [167]. Circulating vesicle cargo, such as miRNAs or proteins can regulate gene expression and metabolism of the recipient cell/tissue [170-172]. In exercise context, it is therefore important to understand how ELV cargo is modulated, as ELVs could participate in positive health adaptations by transporting exerkines.

Regarding ELV miRNAs, it has been shown that their abundance changes significantly in response to a single session of low [113] and high intensity [173] endurance exercise, and also different resistance exercise modalities [141, 174]. For instance, 1 h after a 40 min run (~80% of VO_2max), the abundance of miR-181a-5p was significantly elevated in ELVs [113]. In contrast, a single bout of flywheel-based iso-inertial resistance exercise performed by recreationally trained men (5 sets of 10 reps) resulted in an increased abundance miR-206 and miR-146a, but not miR-133b and miR-126-3p, when measured 2 h following exercise [174]. Finally, the downregulation of miR-1-3p following an eccentric-contraction induced

damage protocol (i.e., downhill running and plyometric jumping) was observed within a maximum of 24 h post-exercise [141]. Interestingly, miRNA ELV changes do not seem to be directly related to those occurring in the plasma or in the skeletal muscle, underscoring selective packaging of miRNA in ELVs during exercise [173]. Indeed, in response to high intensity interval cycling exercise (10 × 60 s at 100% of power at VO_2max), an altered timing and magnitude of changes of miRNA expression/abundance was observed amongst the three sample preparations (skeletal muscle, whole plasma and ELVs), suggesting that there is no clear inter-tissue relationship during exercise [173]. In this study, the 12 ELV miRNAs upregulated were related to angiogenesis, myogenic differentiation, glucose signaling or mitochondrial function. The above findings support the notion that exercise promotes an altered ELV miRNA and reinforce the notion that these vesicles contain a unique miRNA profile [175]. In addition to acute exercise, training status and/or health status appears to influence miRNA abundance in ELVs as well. Nair et al. (2020) compared recreationally trained to their sedentary counterparts, and observed that regular exercise increased the resting abundance of miR-486-5p, miR-215-5p, miR-941, while down-regulating miR-151b [176]. Interestingly, in response to a single bout of exercise (cycling at 70% heart rate reserve for 40 min), the recreationally active group had a distinct profile of ELV miRNAs. This observation is in line with previous evidence from murine training studies [166, 167], and it suggests that that chronic exercise training modulates ELV miRNA abundance in basal conditions. However human investigations were lacking when starting this thesis.

Similarly to miRNAs, ELV protein cargo can be modulated by both low [172] and high intensity [177] endurance exercise. The recent work from Abdelsaid and colleagues (2022) showed that a 45 min run at 50% of VO_2max was sufficient to significantly increase ELV SOD3 content by 50% (Fig. 10) [172].

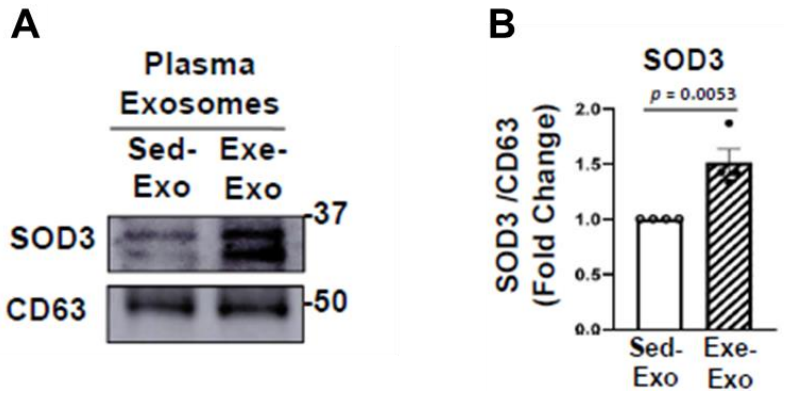

Figure 10. Effect of exercise on ELV SOD3 abundance. A) Representative western blots for SOD3 abundance in plasma ELVs before (Sed-Exo) and after a single bout of exercise (Exe-Exo). B) Averaged fold change normalized to CD63 marker level. Image and graphs from Abdelsaid et al. (2022) [172].

Importantly, additional in vitro experiments highlighted that the ELV angiogenic potential could be SOD3-dependent, which underlines the functional effect of ELV protein cargo modulation by exercise [172]. Regarding high intensity interval exercise, Kobayashi proposed a modified Tabata cycling protocol to baseball trained individuals [177]. The participants performed 8 sets of 20 s at 140% of VO_2max with 10 s of recovery. ELVs were isolated by SEC and protein composition was analyzed by mass spectrometry. Of the 558 proteins identified, 28 and 65 were unique to post-exercise and 30 min post-exercise conditions, respectively. In addition, among the shared proteins between pre- and post-exercise conditions, 20 were significantly modulated [177]. These proteins were involved in several

functions, such as platelet aggregation, coagulation cascades, acid-base homeostasis, and cellular oxidant detoxification. For instance, the content of the antioxidant enzymes catalase and peroxiredoxin II was increased directly after the session and remained elevated 30 min post-exercise [177]. Regarding ELV protein regulation by chronic exercise intervention, very little is known, as only one study has been conducted so far. In their aforementioned study from 2021, Apostolopoulou and colleagues performed a proteomic analysis of ELV content before and after 12 weeks of HIIT [169]. As for miRNAs, ELV protein content was altered according to the subject's health status. In particular, distinct profiles were identified between the subjects with type 2 diabetes (T2D) and subjects sensitive or not to insulin. In addition, ~100 proteins were modulated in each group after the training period. These proteins were related to several cellular functions, including, but not limited to, lipid and carbohydrate metabolism, signaling receptor binding, cell-to-cell interaction, free radical scavenging, and acute-phase response signaling.

Both acute and chronic exercise, whether endurance or resistance, appear to modulate the ELV cargo. However, it is important to note that some of the presented studies do not reach the international guidelines. Results from studies examining protein and miRNA abundance without verifying size, concentrations (e.g., NTA), morphological characteristics (i.e., EM) or markers of ELVs (e.g., TSG101 and ALIX) must be interpreted with caution. Without considering the ELV isolation method used, it is challenging to conclude whether the modulated miRNA signature following exercise is truly encapsulated in ELVs. Indeed, in many studies, the term exosome has been inappropriately applied to describe EVs of a small size, isolated

predominantly through the use of differential centrifugation and high-speed ultracentrifugation [19].

3. Extracellular vesicles in the context of obesity and inflammation

The previous sections have emphasized the possible role of EVs in adaptation to exercise and its beneficial effects on health. The following section will focus on the involvement of EVs in inflammation reduction by exercise in obesity.

3.1 Obesity

Obesity has been defined by the world health organization (WHO) as an "abnormal or excessive fat accumulation that may impair health". A common and easy way to identify an individual with obesity is to use the body mass index (BMI). An adult with a BMI greater than or equal to 30 will be classified as obese whereas BMI values below 30 but greater or equal to 25 refer to overweight condition [178]. Despite being useful and cost-free, BMI can sometimes be misleading as it does not differentiate fat mass and fat-free mass [179, 180]. Fat mass percentage can therefore be used to identify obese individuals with precision. A threshold of 25% and 37 % has been proposed for men and women, respectively [181] meaning that individuals with equal or greater values to these cut-offs are considered as obese. In the last 50-year, obesity prevalence has dramatically increased worldwide. With the current trend, a global prevalence could reach 18% in men and 21% in women by 2025 [182]. This represents a real epidemiologic problem, as obesity is a major risk factor for several noncommunicable diseases [178]. Indeed, due to low grade inflammation, obesity increases the risk of developing

cardiovascular diseases, diabetes, musculoskeletal disorders, and some cancers [178, 183, 184].

3.2 Role of adipose tissue in obesity-related inflammation

White adipose tissue is an important regulator of energy homeostasis [185, 186]. Adipocytes not only allow fat storage and utilization, but also act as energy sensors, secreting hormones and lipids influencing the metabolism of other organs and tissues. For instance, adipocytes are historically known to secrete leptin in response to high energy state to reduce food intake and increase energy expenditure [187]. However, when chronically exposed to high energy availability, several adaptations take place in the adipose tissue, ultimately leading to development of inflammation at both the local and systemic levels [188, 189].

3.2.1 Adipose tissue remodeling

In response to positive energy balance, dynamic changes occur in the adipose tissue. This reorganization is characterized by changes in number and/or size of mature adipocytes. While precursor cells are recruited, hypertrophic adipocytes secrete paracrine factors facilitating preadipocyte recruitment and promoting their differentiation into mature adipocytes [190]. These events are usually defined as “adipose tissue remodeling” [191]. In obese subjects, this remodeling process can be altered (Fig. 11). A sustained positive energy balance related to overnutrition leads mature adipocytes to accumulate more fat and reach a critical threshold. At this point, they cannot store more fat, and the recruitment of precursor cells leads to an increase in adipocyte number. While this number is mostly regulated and determined during childhood in normal situation [192], adipose tissue hyperplasia seems to serve as a

"recovery mechanism" to overnutrition. When adipocytes reach the critical cell size due to lipid-overloading, adipose tissue hyperplasia attempts to limit/repair metabolic alterations caused by hypertrophic adipocytes [188, 193]. In addition, when the adipose tissue storage capacity is surpassed, the excess of circulating lipids is stored as ectopic fat depot in non-adipose organs, such as liver, skeletal muscle, heart, or pancreas. When reaching critical threshold, adipocytes undergo cellular stress characterized by the initiation of an inflammatory program leading to several obesity-related complications (Fig. 11)

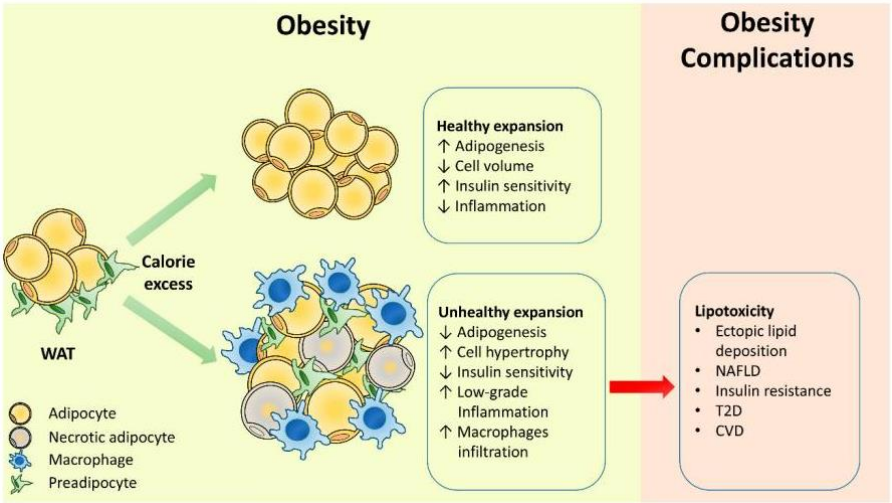

Figure 11. White adipose tissue expansion in obesity. White adipose tissue responds to caloric excess through a healthy or unhealthy expansion. Healthy expansion is characterized by adipocyte hyperplasia, which protects against metabolic complications. Unhealthy expansion involves adipocyte hypertrophy, which promotes the obesity-associated metabolic complications such as non-alcoholic fatty liver disease (NAFLD), insulin resistance, type 2 diabetes (T2D) and cardiovascular disease (CVD). Figure from Longo et al. (2019) [188].

3.2.2 Adipose tissue inflammation development

Despite the precise triggers of inflammation development in obesity remain poorly understood, several potential mechanisms have been proposed [189].

Intrinsic and extrinsic signals seem to participate in the adipose tissue inflammation induction. Gut-derived substance, dietary lipids and metabolite have been pointed out, as well as rapid adipose tissue expansion leading to cell death, hypoxia, and interactions with the extracellular matrix (Fig. 12) [189].

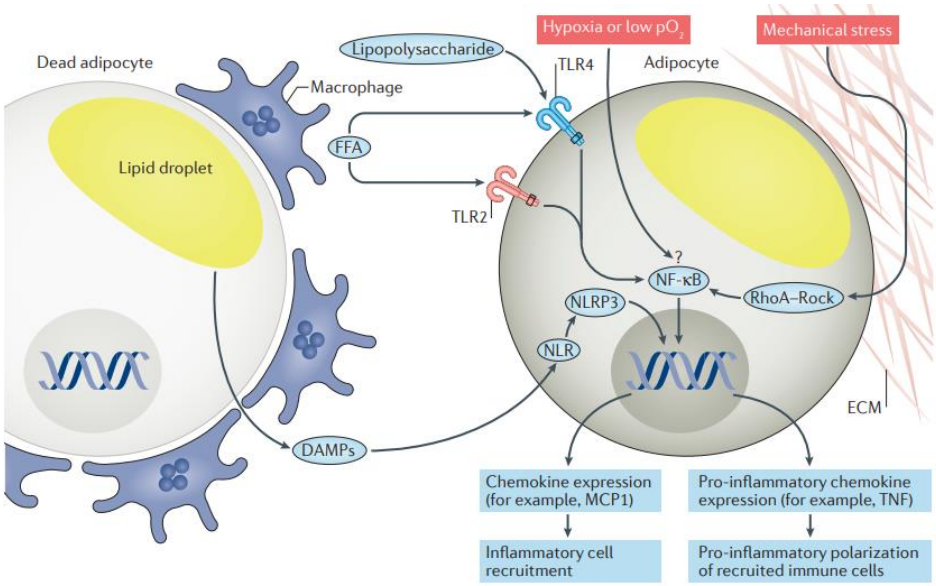

Figure 12. Initiators of obesity-related inflammation in adipocytes. Gut-derived lipopolysaccharide might stimulate inflammatory pathways by binding to the pattern recognition receptor Toll-like receptor 4 (TLR4) at the plasma membrane. Similarly, free fatty acids (FFAs) can activate inflammatory signaling through either TLR4 or TLR2. Additionally, NLRP3 activation by damage-associated molecular proteins (DAMPs), which are released from dying adipocytes and recognized by NOD-like receptors (NLRs), might also be an important initiating step in inflammation. Hypoxic conditions are also associated with inflammation in adipocytes, but the exact mechanisms responsible for this association are unclear. Finally, mechanical stress caused by adipose tissue expansion through the extracellular matrix (ECM) is sensed by the RhoA–Rock pathway, which leads to downstream inflammatory signaling. NF-κB is a signaling hub that has been suggested to be involved in inflammatory signaling downstream of all these diverse potential initiators of adipocyte inflammation in individuals with obesity. Expression of genes that encode downstream inflammatory proteins leads to expression of adipokines, including inflammatory cytokines that promote the recruitment and activation of pro-inflammatory immune cells. TNF, tumor necrosis factor. Figure and caption from Reilly et al. (2017) [189].

From an extrinsic perspective, gut-derived antigens might be involved, as intestinal permeability is increased in obesity inducing higher circulating levels of lipopolysaccharides (LPS). Those antigens could initiate an inflammatory process by activating pattern recognition receptors (PRRs), such as Toll-like receptor 4 (TLR4) in adipocytes [194]. Similarly, it seems that various lipid species, elevated because of both diet and obesity, could also contribute to the development of inflammation in the adipose tissue. For instance, free fatty acids have been shown to promote inflammation in macrophages by binding to TLR4 and TLR2 [195, 196]. Being expressed in adipose tissue of individuals with obesity, these receptors could be involved by activating the downstream NF-κB signaling pathway [197]. Because of this activation, the synthesis, and secretion of chemokines, such as MCP1, are increased, leading to macrophage infiltration into the tissue and, therefore, reinforcing the inflammation process.

As presented above, several intrinsic signals also dictate adipose tissue inflammation development. In obese individuals, dead adipocytes accumulate within the adipose tissue. Those dead or dying cells participate in recruiting immune cells to the adipose tissue, especially pro-inflammatory macrophages. These macrophages classically form a crown-like structure around the adipocytes [198, 199]. Additionally, adipocytes release damage-associated molecular patterns (DAMPs) that can be sensed by NOD-like receptor (NLR). In macrophages, when activated, these receptors activate the inflammasome, which induces the production of pro-inflammatory cytokines, such as IL-1β or IL-18 [200]. Apart from that mechanism, adipose tissue expansion seems also to induce local hypoxia because of decreased perfusion. This reduction in oxygen availability might be an additional initiator of

inflammation within the tissue by an increased level of hypoxia-inducible factor 1α (HIF-1α), ultimately leading to NF-κB pathway activation [201]. Of note, macrophages seem to accumulate preferentially in hypoxic area of the tissue [202]. While substantial evidence links hypoxia to inflammation, it is less clear whether hypoxia is a consequence of adipose tissue expansion or a direct cause of obesity-related consequences [189]. Finally, a last trigger seems to be the mechanical stress applied to the adipocytes when increasing their volume. Indeed, increased triglycerides storage in adipocytes in the extracellular matrix-fixed environment can induce mechanical stress on the cells [189]. Although the exact mechanism has not been identified, this mechanical stress could be sensed by the RhoA-Rock pathway, which could lead to activation of downstream inflammatory signaling [203].

Through these various mechanisms, the altered adipose tissue remodeling induced by obesity results in the activation of NF-κB signaling [188, 189]. The expression of genes encoding for inflammatory proteins is thus increased. Finally, those adipokines, including inflammatory cytokines, participate in the recruitment and the activation of immune cells leading to systemic inflammation [188, 189, 204].

3.2.3 Immune response in adipose tissue

Immune cells residency in adipose tissue is a usual phenomenon required to maintain the integrity and hormonal sensitivity of adipocytes. However, the balance between the different immune cells is rapidly impaired with overfeeding and obesity [189].

In lean individuals, the adipose tissue is characterized by the presence of T helper 2 (Th2) and T regulatory (Treg) cells. These cells produce several

cytokines, such as IL-4, IL-10 and Il-13, allowing macrophages to stay in a M2-polarized state through peroxisome proliferator-activated receptor (PPAR) γ and δ activation [205-207]. M2 macrophages are associated with tissue remodeling and inflammation resolution [208]. For instance, these cells release anti-inflammatory molecules, including transforming growth factor β (TGF-β) and IL-10 [209]. Thus, under a balanced energetic state, adipocytes and immune cells coordinate to regulate the storage and utilization of energy, which preserves insulin sensitivity (see 3.2.4), and, consequently, ensures a certain metabolic flexibility.

In contrast, the adipose tissue of obese individuals has a higher number of Th1 cells relative to the number of Th2 and Treg. Th1 cells secrete pro-inflammatory cytokines such as interferon gamma (IFN-γ), which stimulate and recruit M1 macrophages [209]. An increase in total macrophage infiltration and an increased ratio of M1 to M2 macrophages take place within the tissue. This M1-polarization is considered to be a pro-inflammatory phenotype as these macrophages secrete cytokines, such tumour necrosis factor alpha (TNF-α) or IL-1β [209]. In this context, the local inflammation initiated in adipocytes is sustained by immune cells, particularly macrophages, which ultimately leads to metabolic dysfunctions.

3.2.4 Low-grade inflammation and metabolic dysfunction

As stated above, obesity is a major risk factor for several noncommunicable diseases, including but not limited to diabetes, non-alcoholic fatty liver disease (NAFLD) and cardiovascular diseases. Our current understanding suggests that the development of these complications is closely related to inflammation.

Within adipose tissue, insulin resistance seems to be induced through the activation of NF-κB and c-Jun N-terminal kinase (JNK) signaling pathways [210]. Indeed, when activated by the various mechanisms presented above (see 3.2.2), the NF-κB pathway allows the release of pro-inflammatory cytokines, such as TNFα or IL-1β. In turn, these cytokines activate JNK, which induces an inhibitory phosphorylation of insulin receptor substrate 1 (IRS1) [211]. That phosphorylation impairs insulin-stimulated IRS1 tyrosine phosphorylation, reducing its interaction with phosphatidylinositol 3-kinase (PI3K), and thus inhibits the signal transduction that normally occurs when insulin interacts with its receptor [211].

Due to the systemic inflammation state and ectopic fat accumulation, metabolic dysfunctions are triggered in other organs [188]. In the liver, an abnormal hepatic lipid accumulation, commonly defined as NAFLD, seems to lead to insulin resistance via different lipid products. The accumulation of diacylglycerides has been shown to activate protein kinase C epsilon (PKCε), which binds to and inhibits insulin receptor tyrosine kinase activity. Thus, IRS2 and Akt2 phosphorylation is reduced, ultimately leading to a diminished glycogen synthesis [212]. In a similar way, elevated lipid products within the skeletal muscle seem to impair insulin signaling. Diacylglycerols and ceramides have been shown to activate PKC, which prevents IRS1 and PI3K to ensure insulin message transduction [213, 214]. Skeletal muscles being one of the main tissues responsible for whole-body glucose homeostasis, insulin-resistant skeletal muscles are a major determinant in developing type 2 diabetes [215]. In addition to liver and skeletal muscles, ectopic lipid deposition can also occur in the heart [188]. While fatty acids can be used for energy metabolism in the mitochondria, β-oxidation might not match the

excessive lipid delivery induced by obesity [188]. In this situation, different lipid intermediates, such as diacylglycerols and ceramides, end up accumulating. As in skeletal muscles, these molecules can activate PKC, which is implicated in the development of several myocardial diseases [216]. The cardiac lipotoxicity observed in obesity is characterized by cardiomyocytes insulin resistance and apoptosis, and also contractile dysfunction [216, 217].

3.3 Potential of extracellular vesicles in inflammation reduction

To reduce obesity-induced inflammation and its related metabolic dysfunctions, application of lifestyle interventions, such as caloric restriction and exercise training, have shown efficacy [218, 219]. While caloric restriction might become less effective as a long-term intervention [220], regular exercise seems to reduce inflammation independently of its effect on adiposity [221]. The potential mechanisms through which exercise training reduces inflammation involve mainly skeletal muscles, the adipose tissue, endothelial cells and immune cells (Fig. 13) [221].

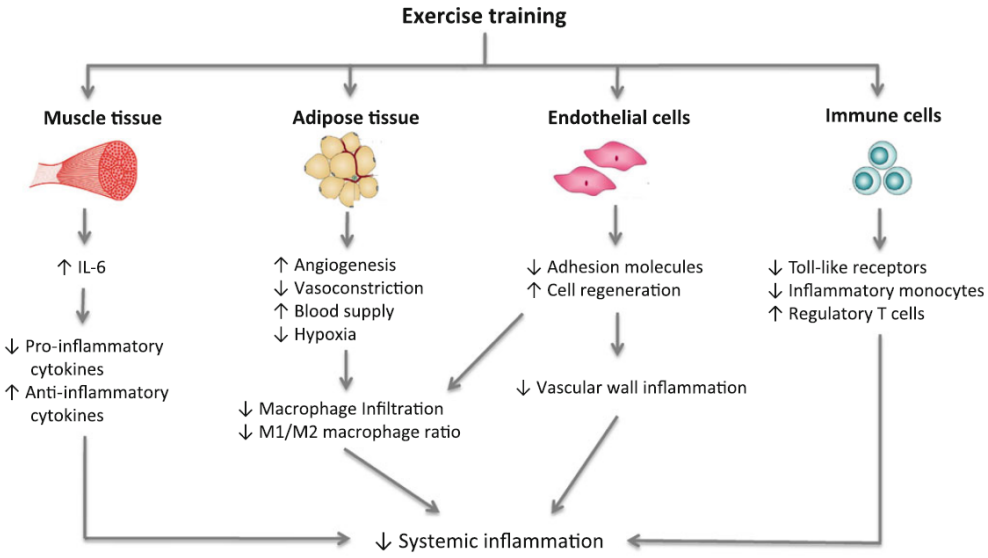

Figure 13. Potential mechanisms through which exercise training reduces inflammation in obesity. Acute exercise stimulates skeletal muscle release of IL-6, which further inhibits actions of proinflammatory cytokines and increases levels of anti-inflammatory cytokines in other tissue/cells and systemically. Exercise training may increase angiogenesis, alleviate vasoconstriction, and increase blood flow, thereby reducing hypoxia and the associated chronic inflammation in adipose tissue. Exercise also reduces adhesion molecule production by endothelial cells and stimulates the regeneration of these cells and lowers vascular wall inflammation. Furthermore, exercise training may reduce the expression of toll-like receptors and production of pro-inflammatory cytokines in monocytes, lower the number of pro-inflammatory cells, and increase the number of regulatory T cells. All these mechanisms may contribute to the anti-inflammatory effects of exercise training, independent of its effects on weight loss. IL interleukin. Figure and caption from You et al. (2013) [221].

When contracting, skeletal muscles induce a large but transient increase in IL-6 in the circulation. On one hand, this change could increase the release of anti-inflammatory cytokines, such as IL-10 and IL-1 receptor antagonist (IL-1RA). On the other, this augmentation in IL-6 levels might also inhibit the effects of pro-inflammatory cytokines, such as TNF-α [222]. At the adipose tissue level, exercise training induces several significant adaptations. The increased angiogenesis [223, 224] and the possible decrease in

vasoconstriction allow a greater blood flow [225], which in turn tends to reduce local hypoxia [221]. Together, these adaptations may result in a reduction in macrophage infiltration, as well as to a switch from the pro-inflammatory phenotype M1 to the anti-inflammatory phenotype M2 [221, 226]. During obesity, the endothelium can be damaged, which leads the endothelial cells to release adhesion molecules. These molecules, such as ICAM-1, VCAM-1 and selectins, attract leucocytes participating in the local inflammatory response [227]. By increasing the regeneration of endothelial cells and reducing the production of adhesion molecules, exercise training could reduce inflammation in the vascular wall and decrease macrophage infiltration within tissues [221]. Finally, exercise training seems also to modulate immune cell function. A reduction in TLR expression and pro-inflammatory monocytes but also an increase in circulating regulatory T cells might lead to diminished systemic inflammation [228-230].

Beyond those mechanisms, ELVs have been recently proposed to be actors in the anti-inflammatory inter-cellular crosstalk induced by exercise training [231]. Indeed, in addition to the effects induced by IL-6 release, skeletal muscles could reduce inflammation by the release of ELVs. In 2022, Sullivan and his colleagues highlighted that a single week of concurrent training was sufficient to modulate muscle-derived EV cargo in obese individuals [232]. In response to the training, miRNA ELV content related to IL-10, IL-6, TLR and NF-κB signaling pathways was altered. Despite no effect on systemic inflammation markers due to the very short intervention, these results suggest that miRNA ELV content might contribute to inflammation reduction in longer protocols. Regarding adipose tissue adaptation, a recent study conducted in mice has also suggested a possible implication of ELVs [233].

In response to 8 weeks of endurance treadmill training, ELV miR-22-3p, -122 and -192 levels were reduced in obese mice. Being associated with impaired adipogenesis, increased insulin and increased fat mass, these downregulated miRNAs might, in part, explain the positive outcomes measured at the adipose level. After the training period, obese mice exhibited an increased number of small adipocytes and a reduced number of larger adipocytes, increased expression of adipogenesis markers, and higher activity of lipogenesis enzymes [233]. Although several associations have been highlighted in the study, the actual link between ELV content modulation and adipose tissue adaptation to exercise remains to be elucidated. Based on this last study and knowing the mechanisms of inflammation development in adipose tissue, ELVs released during exercise could reduce inflammation by promoting adipogenesis. Finally, ELVs have been shown to interact with macrophages in various inflammatory diseases [234]. In obesity, adipocytes seem to secrete ELVs inducing M1 polarization, while adipose-derived stem cells (ADSC) would rather release ELVs promoting a M2 polarization. As endurance training could stimulate ADSC [235], the ELV/macrophage axis might be another mechanism linking exercise to inflammation reduction. Taken together, the above results suggest the involvement of ELVs in the exercise-mediated anti-inflammatory process. However, more studies are needed to better identify their content modulation by exercise/training as well as the direct mechanisms of action of those vesicles.

This overview of the literature has introduced the notions required to understand the results of the present work. The objectives of this PhD thesis will be described in the following chapter.

Chapter II: Aims of the work

Exercise confers remarkable health benefits, which can be partly attributed to the release of bioactive molecules called exerkines. While initially thought to be released by classical secretory pathways, recent evidence suggests that these molecules could also be released and transported to their target by ELVs. These vesicles could thus participate in the adaptive process to exercise by allowing communication between cells at both local and remote levels.

Despite growing interest in the scientific community in recent years, the regulation of these vesicles during exercise remains poorly understood. Studies following the ISEV guidelines are rare, which prevents strong conclusions. Regarding endurance exercise, the effects of submaximal continuous exercise on circulating ELVs were unknown when starting this thesis, as were the effects of adding an environmental stimulus such as hypoxia. The effects of chronic exercise performed either in normoxic or hypoxic conditions remained as well to be elucidated. Finally, most of the studies have investigated ELV regulation in healthy populations, which does not allow to extend the findings to a pathological population, such as individuals with obesity or prediabetes.

Chapter III will explore these various conditions in the context of ELV biogenesis and release. The results presented in that chapter come from three studies conducted during my PhD. The first two have been published [236, 237], while the third one is in preparation for submission to the Journal of Extracellular Vesicles. Abstracts of published articles from other projects carried out during the thesis can be found in appendix.

Knowing the limitations of numerous studies in the field due to inadequate methodologies, it was essential to conduct our studies in accordance with the ISEV guidelines. In this view, we combined different tools (see methods chapter III part 1, 2 and 3) to obtain pure ELV isolation with sufficient yield to performed protein, miRNA, and mass spectrometry analyses. As recommended, the characterization of the vesicles included particle analysis combined with the quantification of specific markers and contaminants, and visual confirmation by transmission electron microscopy. These robust methods allowed us to investigate the following points:

ELV release during submaximal endurance exercise in normoxic and hypoxic conditions in healthy and prediabetic subjects is addressed in **part 1**. In this first study, blood samples were taken before, during and directly after exercise to evaluate the kinetics of release. Skeletal muscle biopsies were also taken to analyze ELV biogenesis pathway in such conditions.

Since content is just as important as quantity, the regulation of basal circulating ELVs and their miRNA content by chronic exercise is presented in **part 2**. In this second study, endurance trained athletes were recruited to participate in a 6-week training protocol at different simulated altitudes (sea-level, 2000 m, 3000 m or 4000 m). ELV amount and content were analyzed from blood collected priori and after the training intervention.

Finally, ELV regulation in individuals with obesity and low-grade inflammation is addressed in **part 3**. The impact of both acute and chronic exercise on ELV amount and proteome was studied to investigate the link between ELV content and exercise-related inflammation reduction.

Chapter III: Results

Part 1: Effects of an acute exercise bout in hypoxia on extracellular vesicle release in healthy and prediabetic subjects

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ABSTRACT

The purpose of this study is to investigate exosome-like vesicle (ELV) plasma concentrations and markers of multivesicular body (MVB) biogenesis in skeletal muscle in response to acute exercise. Seventeen healthy [body mass index (BMI): 23.5 ± 0.5 $kg \cdot m^{-2}$] and 15 prediabetic (BMI: 27.3 ± 1.2 $kg \cdot m^{-2}$) men were randomly assigned to two groups performing an acute cycling bout in normoxia or hypoxia (FiO_2 14.0%). Venous blood samples were taken before (T0), during (T30), and after (T60) exercise, and biopsies from m. vastus lateralis were collected before and after exercise. Plasma ELVs were isolated by size exclusion chromatography, counted by nanoparticle tracking analysis (NTA), and characterized according to international standards, followed by expression analyses of canonical ELV markers in skeletal muscle. In the healthy normoxic group, the total number of particles in the plasma increased during exercise from T0 to T30 (+313%) followed by a decrease from T30 to T60 (-53%). In the same group, an increase in TSG101, CD81, and HSP60 protein expression was measured after exercise in plasma ELVs; however, in the prediabetic group, the total number of particles in the plasma was not affected by exercise. The mRNA content of TSG101, ALIX, and CD9 was upregulated in skeletal muscle after exercise in normoxia, whereas CD9 and CD81 were downregulated in hypoxia. ELV plasma abundance increased in response to acute aerobic exercise in healthy subjects in normoxia, but not in prediabetic subjects, nor in hypoxia. Skeletal muscle analyses suggested that this tissue did not likely play a major role of the exercise-induced increase in circulating ELVs.

Keywords: ALIX; cycling; exosomes; prediabetes; TSG101

INTRODUCTION

Exercise exerts its systemic effects partly by cross talk between organs that release molecules into the bloodstream called “exerkines” [136]. Exerkines may be carried as cargo inside extracellular vesicles (EVs) that are released by different secretory cell and have emerged as a mechanism for intercellular communication [113, 238]. Currently, three primary classes of EVs are distinguished according to their size and formation mode. Apoptotic bodies are the largest EVs (500–4,000 nm) and are generated upon programmed cell death [23]. Microvesicles, with a size of 100–1,000 nm, are released into the extracellular environment by budding and pinching of the plasma membrane [24]. Finally, exosomes, the smallest EVs (40–120 nm) are generated from the endosomal pathway and released by the fusion of multivesicular bodies (MVBs) with the plasma membrane and release into the extracellular fluid/plasma [239]. Exosomes have been found to be transporters of a variety of cellular cargoes [238], such as microribonucleic acids (miRNAs) [240], messenger ribonucleic acids (mRNAs), deoxyribonucleic acid (DNA), and proteins [241] that can ultimately be transferred to adjacent cells or distant organs. The proteins involved in the formation of exosomes are often used as markers. Among them, the most often used are MVB-forming proteins (X-interacting protein ALG2, ALIX, and tumor susceptibility gene 101, TSG101), heat shock proteins (chaperones; HSP), and vesicle adhesion proteins (tetraspanins; CD9, CD81, and CD63). Because of significant overlap in size and markers of EV subpopulations, it remains difficult to distinguish between the different classes of EVs with the current isolation techniques. Therefore, the term exosome-like EV (ELV) is an appropriate

term of usage encompassing the smallest EVs (i.e., exosomes and small microvesicles) [242].

ELVs have been found to be released following a single bout of exercise as well as after exercise training, inducing changes in ELV plasma concentrations, markers, and cargoes. Frühbeis et al. [117] observed a rapid increase of ELVs into the circulation in response to a short-duration cycling incremental test in recreationally active men. Moreover, HSP/HSC70 and flotillin 1 (Flot1), two ELVs markers, were elevated directly after exercise. Those results were corroborated in other studies in both human [128, 243] and murine models [137]. Despite the increased number of studies analyzing the effect of exercise on EV release, few of them studied the implication of skeletal muscle in this process [113, 128].

More recently, ELVs were proposed to be involved in the beneficial effects of exercise in type 2 diabetes (T2D) [244]. Treatment of sedentary mice with ELVs isolated from the plasma of trained mice improved glucose tolerance, insulin sensitivity, and reduced plasma triglycerides to levels similar to those of the trained group itself [245]. In addition, acute exercise and exercise training have been shown to regulate the levels of circulating miRNAs involved in insulin resistance and obesity [233] and angiogenesis [173] in rodents or humans. In summary, it appears that ELVs enriched with bioactive molecules induced by exercise could counteract the development of T2D, but the exact mechanisms remain to be explained.

Since exercise under hypoxic conditions appears to have an additive effect on glucose tolerance and insulin sensitivity compared with normoxic conditions in healthy [246], obese [247], and T2D [248] individuals, we hypothesized that hypoxia could modulate the release of ELVs during and after exercise in

those populations. Therefore, we investigated ELV plasma concentrations and MVB biogenesis markers in skeletal muscle in response to an acute bout of exercise performed in normoxic or hypoxic conditions in healthy and prediabetic subjects.

MATERIALS AND METHODS

Participants

Seventeen healthy (H, 28.8 ± 2.4 yr; 23.5 ± 0.5 kg·m^{-2}) and 15 prediabetic (P, 44.6 ± 3.9 yr; 27.3 ± 1.2 kg·m^{-2}) men gave their consent to participate in this study (Table 1). The study was approved by the Ethical Committee of the Université catholique de Louvain (Belgium) and conducted in accordance with the Declaration of Helsinki. The inclusion criterion was nonsmoking men aged 20–65 yr. Exclusion criteria were as follows: contraindication to intense physical exercise, exposure to an altitude >1,500 m the month preceding the experiment, physical activity >3 h/wk, drug treatment for insulin resistance, and sleep apnea syndrome. The healthy group was defined by fasting glucose levels lower than or equal to 100 mg·dL^{-1} and blood glucose levels lower than 140 mg·dL^{-1} at the 120th min during an oral glucose tolerance test (OGTT). The prediabetic group was defined by fasting glucose levels between 100 and 126 mg·dL^{-1} and/or blood glucose levels between 140 and 200 mg·dL^{-1} at the 120th min during an oral glucose tolerance test (OGTT) according to the American Diabetes Association. In addition, glycated hemoglobin (HbA1c) was measured by chromatography (ADAMS; Menarini; Ha-8180V) and was found to be higher in the prediabetic group than in the healthy group (5.3 ± 0.07 vs. 5.0 ± 0.06%, P = 0.01). Here, we used the remaining skeletal muscle and blood samples from a previous study [249] to investigate the effects of an acute hypoxic cycling exercise on the release

of ELVs. Detailed morphological and clinical characteristics of the subjects as well as the detailed protocol can be found in De Groote et al. [249]. All results reported here are original.

Table 1: Subjects characteristics

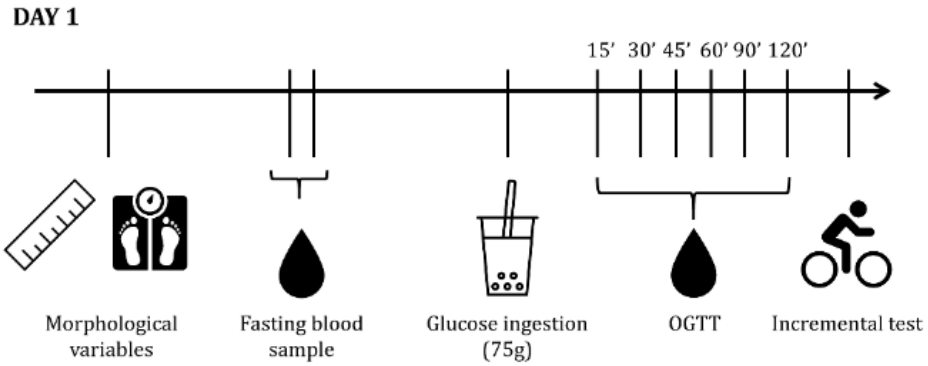
	Healthy	**Prediabetic**	***p-value***
Age (y)	28.8 ± 2.4	44.6 ± 3.9	$P = 0.001$
Body mass (kg)	73.8 ± 1.2	90.0 ± 4.5	$P = 0.001$
BMI ($kg \cdot m^{-2}$)	23.5 ± 0.5	27.3 ± 1.2	$P = 0.005$
VO_2max ($mL \cdot kg^{-1}min^{-1}$)	45.1 ± 1.8	30.8 ± 2.5	$P < 0.0001$
Fasting Plasma Glucose ($mg \cdot dL^{-1}$)	94.3 ± 1.1	107.3 ± 1.8	$P < 0.0001$
Oral Glucose Tolerance test 120min ($mg \cdot dL^{-1}$)	102.9 ± 6.9	137.6 ± 10.4	$P = 0.007$

Values are means ± SEM. BMI: Body mass index. $p<0.05$; $p<0.01$; $p<0.001$ *vs* healthy group.

Protocol

In a parallel exercise trial design, the healthy (H) and prediabetic (P) subjects were divided, respectively, into two groups matched according to physical fitness ($\dot{V}O_2max$; NE, normoxic exercise; HE, hypoxic exercise; n = 8 in H NE; n = 8 in P NE; n = 9 in H HE and n = 7 in P HE). Normobaric hypoxia was achieved after nitrogen injection in a confined room to dilute oxygen content in the air (high-altitude system B-Cat, Tiel, The Netherlands). Morphological, physiological, and basal blood variables were determined

before experiments, allowing for ∼1-wk "wash-out" period (Fig. 1). Blood samples were taken from an antecubital vein in 10-mL tubes containing lithium heparin, which were immediately centrifuged at 4°C for 10 min at 3,000 g. Plasma was transferred to microtubes and stored at −80°C. Maximal oxygen uptake ($\dot{V}O_2max$, Ergocard, Medisoft) was determined during a maximal incremental exercise test on a cycle ergometer. On the day of the experiment, subjects came to the laboratory in the morning in a fasted state (12 h). After blood collection, a first muscle sample was taken from the m. vastus lateralis under local anesthesia (1–2 mL lidocaine) through a 5-mm incision in the skin. Muscles samples were immediately frozen in liquid nitrogen and stored at −80°C. Then, the exercise bout began directly either in normoxic (NE; FiO_2: 20.9%) or hypoxic (HE; FiO_2: 14%) conditions and consisted of cycling for 60 min at an HR corresponding to 55% $\dot{V}O_2max$. This workload was chosen to ensure that the relative exercise intensity was comparable between normoxia and hypoxia. During exercise, venous blood samples were drawn at 30 min and 60 min. Directly at the end of the exercise, a second muscle sample was taken in normoxic conditions.



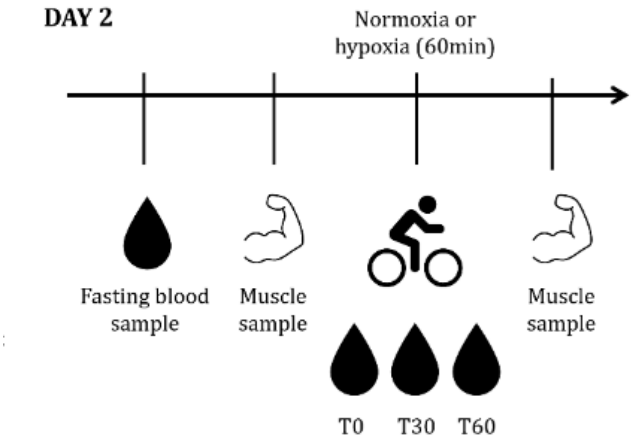


Figure 1. Experimental protocol. Morphological variables, oral glucose tolerance test (OGTT), and maximal incremental exercise test were performed at day 1. One week later, venous blood samples were taken before (T0), during (T30), and after (T60) exercise and biopsies from m. *vastus lateralis* before and after exercise at day 2.

Plasma ELV isolation

The isolation and characterization of the ELVs were performed in accordance with International Society for Extracellular Vesicles recommendations [20].

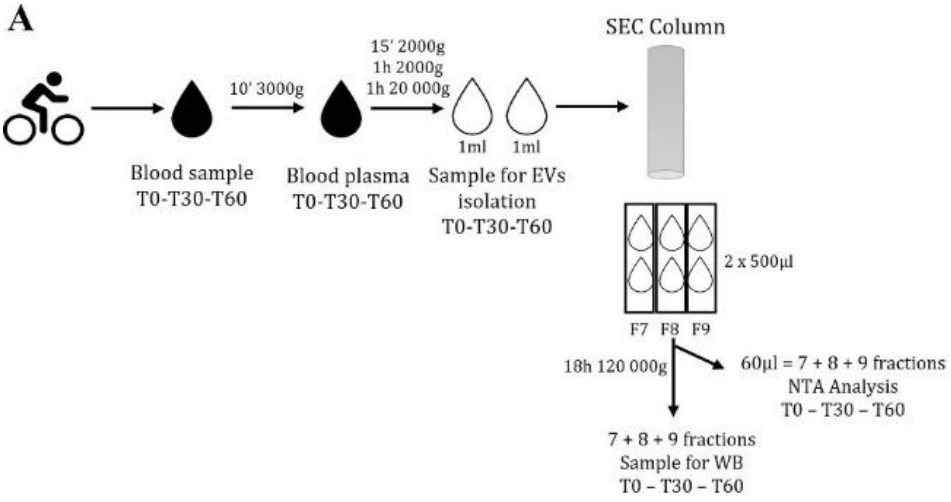
Venous plasma preparation

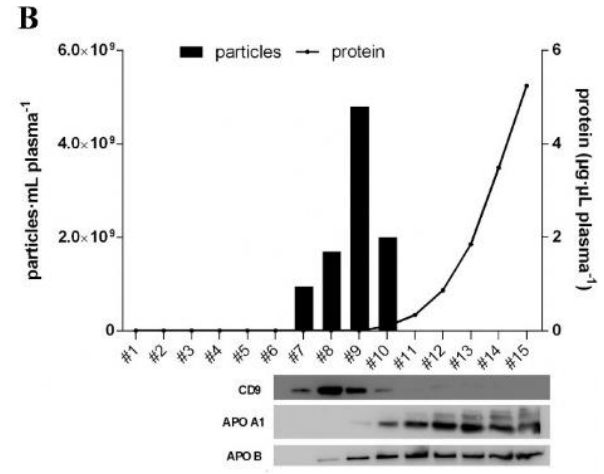
Plasma samples were slowly thawed on ice and centrifuged at 4°C for 15 min at 2,000 g to remove platelets, then 60 min at 2,000 g to remove apoptotic bodies, and finally 60 min at 20,000 g to remove pelleted large EVs. Supernatant was used for downstream experiments. Plasma-derived ELVs appear to be stable during freeze-thaw cycles in terms of number and size assessed by NTA [250, 251].

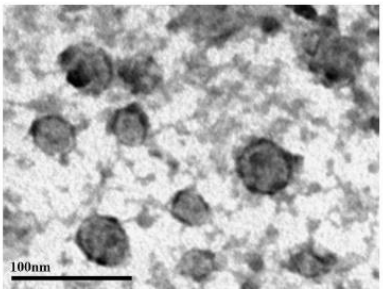
Results

Size exclusion chromatography

EV isolation by size exclusion chromatography (SEC) was performed using Izon qEV70nM columns according to the manufacturer (Izon). Preliminary experiments were performed to determine which of the 15 fractions were EV rich. Briefly, after the column was washed with PBS, 1 mL of plasma was loaded on the column and 500-µL fractions were collected. For each sample, two isolations by SEC were performed (2 × 1 mL of plasma). Fractions 1 to 15 were analyzed for particle count using nanoparticle tracking analysis (NTA), for protein concentrations using the DC (detergent compatible) protein assay (Bio-Rad), and for the presence of vesicular (CD9) and nonvesicular markers (APO B and APO A1) by Western blotting (see Electrophoresis and immunoblotting). Based on the results presented in Fig. 2, fractions 7 to 9 were considered EV rich since they were enriched in particles positive for CD9 and contained a lower amount of proteins and lipoproteins.







D

Particles diameter (nm)

	T0	T30	T60
HNE	107.2 ± 4.1	109.5 ± 3.1	110.9 ± 3.9
PNE	106.5 ± 3.2	104.3 ± 3.1	101.4 ± 4.4
HHE	102.8 ± 1.9	107.6 ± 3.1	104.9 ± 4.1
PHE	104.5 ± 5.2	103.7 ± 3.1	107.1 ± 3.6

Figure 2. Isolation of plasma ELVs [20]. A: outline of the experimental protocol to characterize ELVs released during exercise; NTA (bars) and protein concentrations (line) on the 15-SEC, fractions, combined with WB analysis using EV-markers (CD9) and apolipoprotein A1 (APO A1) and B (APO B) as non-EV markers on fractions 7 to 15 (B). Fractions 7 to 9 are considered ELV rich and protein- and lipoprotein-poor and were pooled; transmission electron microscopy image of the pooled fractions of SEC-EVs (negative staining), scale bar refers to 100 nm (C); median ± SE of the particle diameter of the pooled fractions of SEC-ELVs (F7 + F8 + F9) collected at T0, T30, and T60 in HNE (n = 8), PNE (n = 8), HHE (n = 9), and PHE (n = 7) (D). ELV, exosome-like vesicle; EV, extracellular vesicles; HHE, healthy hypoxic exercise group; HNE, healthy normoxic exercise group; NTA, nanoparticle tracking analysis; PHE, prediabetic hypoxic exercise group; PNE, prediabetic normoxic exercise group; SEC, size exclusion chromatography; WB, Western blotting.

Results

Ultracentrifugation

The duplicated (2 × 500 µL) fractions 7, 8, and 9 obtained after SEC were pooled and ultracentrifuged at 4°C for 18 h at 100,000 g (TLA55 rotor, k-factor 66, Beckman). The supernatant was removed to concentrate the samples 10× and the three pellet fractions (fractions 7, 8, and 9) were pooled to obtain one sample per subject for further Western blot analyses (see below).

Nanoparticle tracking analysis

Before ultracentrifugation, a small volume of samples (20 µL) from fractions 7, 8, and 9 were pooled for nanoparticle tracking analysis (NTA) analysis. ELVs were counted by the Zetaview (Particle Metrix), which captures Brownian motion through a laser scattering microscope combined with a video camera to obtain size distribution (50–1,000 nm) and concentrations. Samples were diluted in UltraPure DNase/RNase-Free Distilled Water (UDW) at 1:150–1:6,000 to reach 50–200 particles/frame corresponding to $\sim 2.10^{7}$–10.10^{8} particles·mL−1. Sensitivity was set to 80 and camera shutter to 100 to detect less than 5–10 particles/frame as background when UDW alone was injected. Measurements were performed with medium resolution for two cycles at 11 different positions (or frames) of the cell chamber containing the sample. The Zetaview software was used to generate results in text files.

Negative staining in transmission electron microscopy

Cupper grids (300 mesh) were cleaned by sonification for five times 5 min in ethanol and covered with a 1% formvar film. After overnight drying, a 5-nm carbon layer was electron beamed on top of the formvar film. A 50-µL 1%

uranyl acetate solution in freshly bidistillate water was placed on the prepared grids for 5 min. Then the excess of liquid was removed with a Whatman filter paper (N° 415) and overnight dried in a dry chamber. The next day the prepared sample was validated in transmission electron microscopy (CM12, Philips) at 80 kV. The film had to be intact and uniformly stained. Biological samples were diluted in freshly bidistillate water (1×) and a 20-µL diluted sample was put on the formvar-carbon-coated grids for 5 min. Then 20 µL of 2% uranyl acetate was added. After 5 min, the excess of liquid was removed with a Whatman filter paper (N° 415) and samples were overnight dried in a dry chamber. The next day the samples could be studied in the electron microscope (CM12, Philips) at 80 kV. Pictures were taken at ×60,000 magnification.

Skeletal Muscle Collection and Analysis

Muscle biopsies

Muscle biopsies were taken in the m. vastus lateralis according to the modified Bergström technique with suction [252]. After local anesthesia (xylocaine 2% without epinephrine, Astra Zeneca, Cambridge, UK), a 5-mm incision was made with a scalpel and pressure was applied for 10 min to reduce bleeding. The Bergström needle was then inserted into the incision, at least 1 cm beyond the fascia, to reach the muscle and three successive cuts were performed to obtain 150–200 mg of sample. The samples were immediately frozen in liquid nitrogen and stored at −80°C.

Real-time PCR

Approximately 15 mg of muscle biopsy sample was homogenized in 1 mL TRIzol reagent (Thermo Fisher Scientific) with a TissueLyser II (Qiagen).

Results

RNA isolation was achieved according to the manufacturer's instructions. RNA quality and quantity were assessed by Nanodrop (Thermo Fisher Scientific) spectrophotometry. Reverse transcription was performed from 1 μg RNA using the iScriptcDNA Synthesis kit (Bio-Rad Laboratories), following the manufacturer's instructions. The following primers were used: tumor susceptibility 101 protein (TSG101), forward sequence CATTCCCACAGCTCCCTTATAC, and reverse sequence CTCAAGGCTTCTCCCAAGTAAA; ALG-2-interacting protein X (ALIX), forward sequence GGTGCTAGCTTCCCTTAATC, and reverse sequence GATGCCTCCCTGTTCAATC; CD9, forward sequence GATTGCTGTCCTTGCCATTG, and reverse sequence CTCCTGTGTAGAAGCTGGAATTA; CD81, forward sequence CCTGCTCTTCGTCTTCAATTTC, and reverse sequence TCTCCCAGCTCCAGATACA; and ribosomal protein L19 (RPL19), forward sequence CGCTGTGGCAAGAAGAAGGTC, and reverse sequence GGAATGGACCGTCACAGGC. The PCR was run using the following conditions: 3 min at 95°C, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C using the CFX Connect Real-Time System (Bio-Rad Laboratories). All samples were run in duplicate, and each reaction was processed in a 10-μL volume containing 4.8 μL SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories), 0.1 μL of each primer (100 nM final), and 5 μL cDNA at the appropriate dilution. Melting curves were systematically analyzed as quality control. Quantification was assessed using the delta CT method, relative mRNA expression levels were normalized to ribosomal protein L19, the expression of which was unaffected by conditions or exercise.

Results

Protein extraction

A portion (~20 mg) of muscle biopsies was freeze-dried for 48 h and dissected free of visible fat, blood, and connective tissue. Muscle tissue was homogenized with stainless steel beads 2 × 30 s at 30 Hz using a TissueLyser II (Qiagen) in 50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM sodium orthovanadate, 1 mM EDTA (pH 8.0), 1 mM EGTA (pH 8.0), 1% NP-40, 10% glycerol, 2 mM phenylmethanesulfonyl fluoride, 10 $\mu g \cdot mL^{-1}$ leupeptin, 10 $\mu g \cdot mL^{-1}$ aprotinin, and 3 mM benzamidine. After rotation end-over-end for 30 min, lysate supernatants were collected by centrifugation (10,000 g) for 20 min at 4°C. Lysate protein concentrations were measured using the bicinchoninic acid (BCA) method using BSA standards and BCA assay reagents (Pierce Biotechnology, Rockford, IL).

Electrophoresis and immunoblotting

For the muscle samples, total protein levels of relevant proteins were determined by standard immunoblotting techniques loading equal amounts of protein. For blood samples (see Ultracentrifugation for samples preparation), an equal volume of vesicles was loaded for all samples. The primary antibodies used were apolipoprotein B (APO B, 1:1,000 dilution, Santa Cruz; sc-376818), apolipoprotein A1 (APO A1, 1:1,000 dilution, Santa Cruz; sc-393636), cluster of differentiation (CD) 9 (1:500 dilution, Cell Signaling; D3H4P), tumor susceptibility 101 protein (TSG101, 1:500 dilution, Abcam; Ab30871), cluster of differentiation (CD) 81 (1:500 dilution, Cell Signaling; D3N2D), heat-shock protein 60 (HSP60, 1:1,000 dilution, Cell Signaling; D307), ALG-2-interacting protein X (ALIX, 1:500 dilution, Cell Signaling;

E6P9B), and actin (1:1,000 dilution, Sigma Aldrich, Cat. No. A2066). Polyvinylidene difluoride membranes (Immobilon Transfer Membrane; Millipore) were blocked in TBS-Tween containing 5% skimmed milk for 60 min at room temperature. Membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Membranes were thereafter scanned, and proteins quantified with GeneSnap software and tools (Syngene, Cambridge, UK).

Statistics

Statistical analyses were performed using GraphPad Prism 7.00, and data are reported as means ± SE. A two-way ANOVA (mix model) within each environmental condition (normoxia and hypoxia) was used with the subjects as a random variable and groups (healthy and prediabetic) and exercise (pre and postexercise) as fixed independent variables. When the ANOVA test was significant ($P < 0.05$) or tended to be significant ($P < 0.1$) for the groups, environmental conditions, exercise and/or interaction main effects, Fisher least-significant different post hoc tests were performed to compare mean values. For Table 1, a Student's t test was used to determine differences in subject's characteristics between the healthy and prediabetic group. Statistical significance was set at $P < 0.05$. Differences ($P < 0.05$) between pre and postexercise values are indicated by asterisks (*), differences between healthy and prediabetics are indicated by hatch signs (+), differences versus T0 are indicated by (#), and differences versus T30 are indicated by ($). Grubb's test was performed to detect outliers. When the Grubb's test was significant ($P < 0.05$), the value was considered statistically abnormal and removed from analysis.

RESULTS

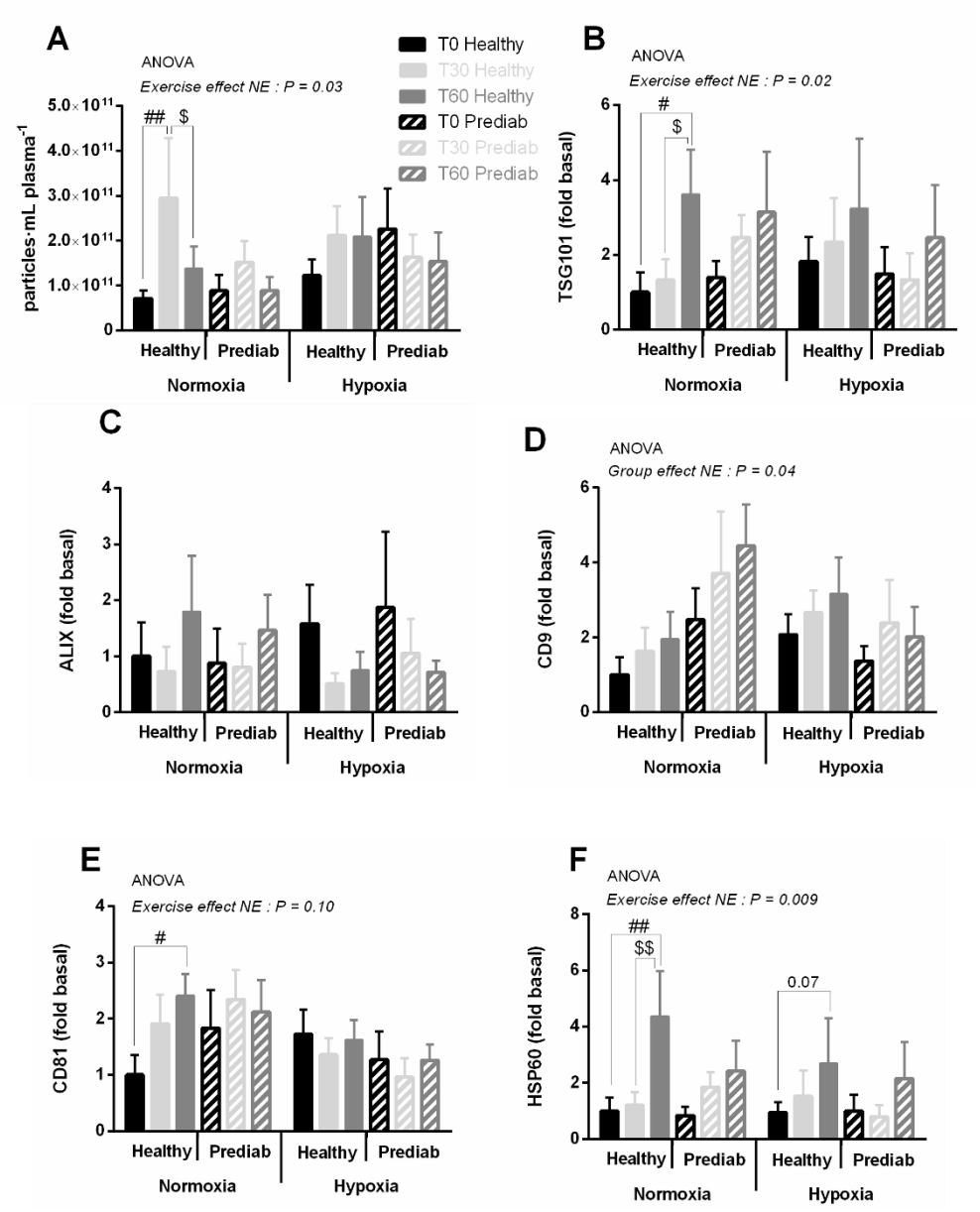
NTA Analyses of ELVs in Plasma – Exercise Effects

NTA analysis of the ELVs-enriched pooled fractions isolated by SEC from the plasma revealed a median particle size ranging from 101.4 ± 4.4 nm to 110.9 ± 3.9 nm for the four groups at the three time points, which is entirely compatible with ELVs (Fig. 2D). The total number of particles remained constant during exercise from T0 to T30 and T60, except for the healthy normoxic exercise group for which an increase at T30 (+313%, P = 0.007) followed by a decrease at T60 (−53%, P = 0.05) was observed (Fig. 3A). Of note, a large interindividual variability was observed in the total number of particles.

Canonical Markers of ELVs in Plasma – Exercise Effects

NTA analysis is not the most appropriate technique to assess the number of ELVs due to a potential background from non-EVs particles in the plasma. Hence, Western blot (WB) analysis of known EVs biomarkers was performed on pooled ELV-enriched fractions to get a complete interpretation of the effects of exercise on vesicle release into the blood (Fig. 3, B–G). Increases in TSG101 (T60 vs. T0: +261%, P = 0.01; T60 vs. T30: +172%, P = 0.03; Fig. 3B), CD81 (T60 vs. T0: +140%, P = 0.02; Fig. 3E), and HSP60 (T60 vs. T0: +336%, P = 0.005; T60 vs. T30: +263%, P = 0.007; Fig. 3F) protein expression in the healthy normoxic exercise group and a tendency to an increase in HSP60 (T60 vs. T0: +180%, P = 0.07; Fig. 3F) in the healthy hypoxic exercise group were detected during exercise.

Results


A
ANOVA
Exercise effect NE : P = 0.03
T0 Healthy
T30 Healthy
T60 Healthy
T0 Prediab
T30 Prediab
T60 Prediab
particles·mL plasma-1
5.0×10^11
4.0×10^11
3.0×10^11
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1.0×10^11
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##
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Healthy
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Exercise effect NE : P = 0.02
TSG101 (fold basal)
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Group effect NE : P = 0.04
CD9 (fold basal)
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Exercise effect NE : P = 0.10
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Exercise effect NE : P = 0.009
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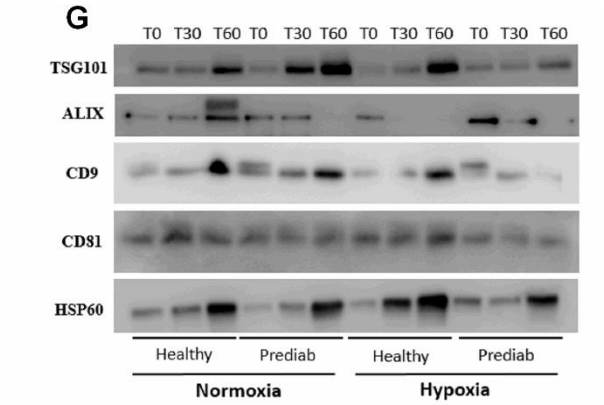
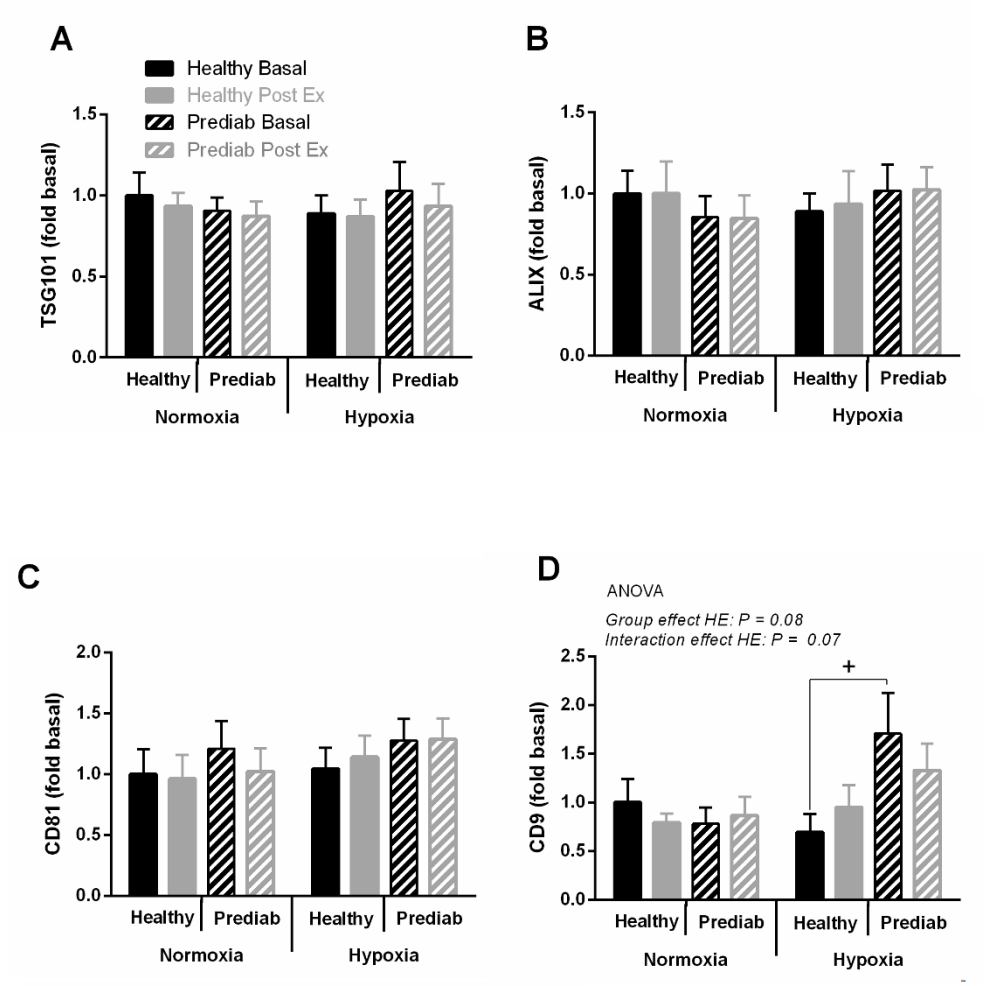


Figure 3. Effects of an acute exercise in hypoxia on SEC-isolated ELVs biomarkers from blood samples in healthy and prediabetic adults. Healthy normoxic exercise group (n = 8), prediabetic normoxic exercise group (n = 8), healthy hypoxic exercise group (n = 9), and prediabetic hypoxic exercise group (n = 7), except when specified. A: nanoparticle tracking analysis (number of particles) of SEC-isolated ELVs; tumor susceptibility 101 (TSG101; B); X-interacting protein ALG2 (ALIX; C); cluster of differentiation (CD) 9 (healthy normoxic exercise group n = -1; healthy hypoxic exercise group n = -1; prediabetic normoxic exercise group n = -2; prediabetic hypoxic exercise group n = -1) (D); cluster of differentiation (CD) 81 (E); heat shock protein 60 (HSP60) protein expression at T0, T30, and T60 during exercise in the four groups (F); and representative Western blots (G). Values are presented as means ± SE. #P < 0.05, ##P < 0.01 vs. T0; $P < 0.05, $$P < 0.01 vs. T30. B, basal; P, postexercise. "Fold basal" means fold changes compared with the T0 value from the healthy group in normoxia. ELV, exosome-like vesicle; SEC, size exclusion chromatography.

Protein Markers of MVB Biogenesis in Skeletal Muscle – Exercise Effects

Knowing the crucial role of skeletal muscle during exercise by releasing myokines potentially carried by ELVs, we aimed to examine the MVB biogenesis pathway in skeletal muscle. TSG101 (Fig. 4A), ALIX (Fig. 4B), and CD81 (Fig. 4C) protein expression was not modified by exercise or by group condition. The protein expression of CD9 was higher in the prediabetic compared with the healthy hypoxic exercise group at basal (+124%; P = 0.02; Fig. 4D).

Results


A
Healthy Basal
Healthy Post Ex
Prediab Basal
Prediab Post Ex
TSG101 (fold basal)
1.5
1.0
0.5
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Healthy
Prediab
Healthy
Prediab
Normoxia
Hypoxia
B
ALIX (fold basal)
1.5
1.0
0.5
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Healthy
Prediab
Healthy
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Normoxia
Hypoxia
C
CD81 (fold basal)
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ANOVA
Group effect HE: P = 0.08
Interaction effect HE: P = 0.07
+
CD9 (fold basal)
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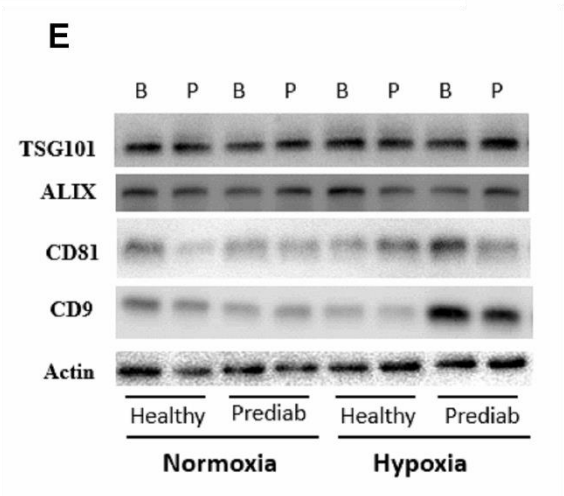
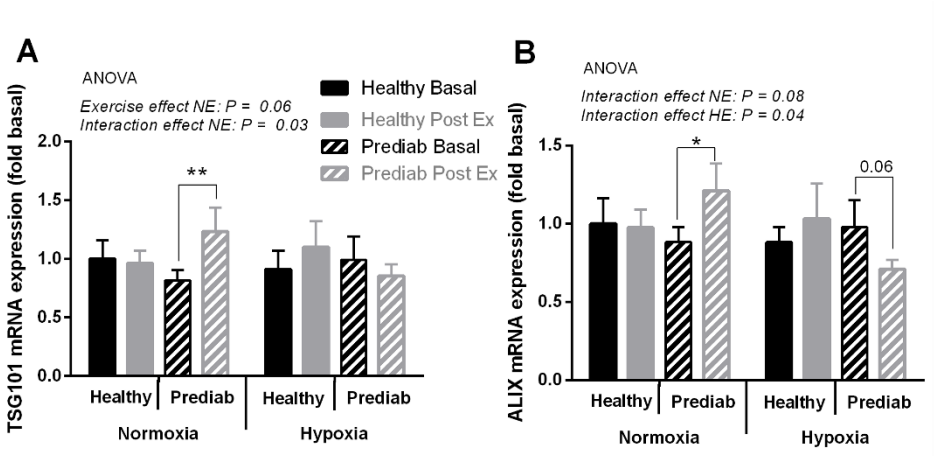


Figure 4. Effects of an acute exercise in hypoxia on the protein expression of components of the MVB biogenesis pathway in skeletal muscle of healthy and prediabetic adults. Healthy normoxic exercise (n = 8), prediabetics normoxic exercise (n = 8), healthy hypoxic exercise (n = 7), and prediabetics hypoxic exercise (n = 7). A: tumor susceptibility 101 (TSG101); X-interacting protein ALG2 (ALIX; B); cluster of differentiation (CD) 9 (C); cluster of differentiation (CD) 81 protein expression at basal and postexercise in the four groups (D); representative Western blots (E). Values are presented as means ± SE. +$P < 0.05$ vs. healthy; B, basal; P, postexercise. "Fold basal" means fold changes compared with the mean basal value from the healthy group in normoxia. MVB, multivesicular body.

Transcriptional Markers of the MVB Biogenesis in Skeletal Muscle – Exercise Effects

In the prediabetic normoxic exercise group, exercise induced an increase in TSG101 (+59%, P = 0.008, Fig. 5A), ALIX (+27%, P = 0.02, Fig. 5B), and CD9 (+78%, P = 0.03, Fig. 5C) gene expression compared with the basal state. In the prediabetic hypoxic exercise group, exercise tended to decrease ALIX (−27%, P = 0.06) and decreased CD9 (−24%, P = 0.006) and CD81 (−38%, P = 0.0004; Fig. 5D) gene expression compared with the basal state. CD9 and CD81 gene expression was higher in the basal state in the prediabetic hypoxic exercise group compared with the healthy hypoxic exercise group (CD9: +44%, P = 0.009; CD81: +28%, P = 0.02).



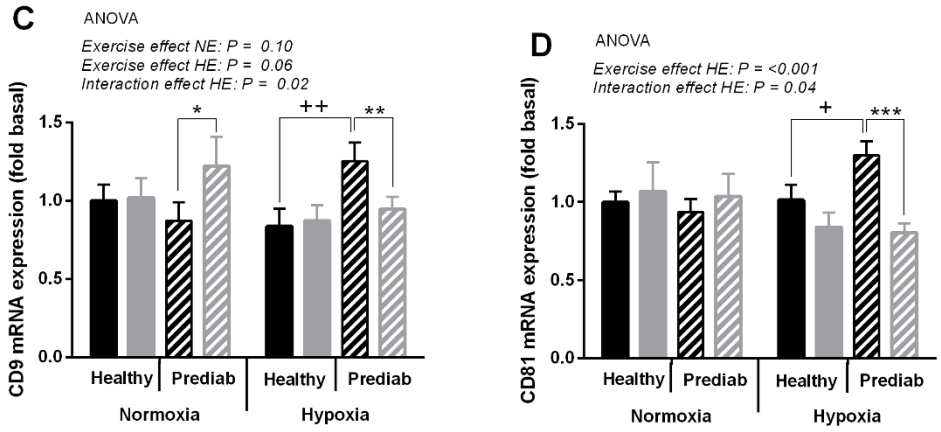


Figure 5. Effects of an acute exercise in hypoxia on the gene expression of components of the MVB biogenesis pathway in skeletal muscle of healthy and prediabetic adults. Healthy normoxic exercise group (n = 8), prediabetic normoxic exercise group (n = 8), healthy hypoxic exercise group (n = 7), and prediabetic hypoxic exercise group (n = 7). A: tumor susceptibility 101 (TSG101); X-interacting protein ALG2 (ALIX; B); cluster of differentiation (CD) 9 (C); and cluster of differentiation (CD) 81 gene expression at basal and postexercise in the four groups (D). Values are presented as means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 vs. basal; +P < 0.05, ++P < 0.01 vs. healthy; B, basal; P, postexercise. "Fold basal" means fold changes compared with the mean basal value from the healthy group in normoxia. MVB, multivesicular body.

DISCUSSION

The aim of this study was to assess ELVs concentrations in plasma and abundance of proteins involved in MVBs biogenesis in skeletal muscle in response to an acute bout of exercise performed in normoxic or hypoxic conditions in healthy and prediabetic subjects. We isolated and characterized ELVs-enriched fractions and found by NTA an increase in circulating particles during low intensity endurance exercise. This increase was exclusive to the healthy normoxic exercise group and transient as the exercise-induced increase in particles after 30 min dropped close to the baseline values at the end of the 60-min exercise. This result goes in line with previous work showing an increase of circulating particles in response to exercise when measured by NTA [117, 137]; however, is not consistent with other data showing either no change [128, 141] or even a decrease [146]. Because of the many different isolation methods, exercise modalities and timings of blood collection, a firm conclusion is difficult to make. More specifically, when it comes to ELVs quantification, NTA has shown some limits since it detects lipoprotein particles in addition to ELVs [253, 254]. Despite that the fractions isolated in our study contained abundant CD9 signal and lower apolipoprotein signals, and were considered ELV-enriched, the methodological weakness of NTA calls for caution. Brahmer et al. [128] indeed showed that NTA was unable to highlight exercise-induced increase in ELVs. The authors concluded that NTA was probably not a suitable technique to evaluate ELVs dynamics during exercise and that semiquantitative methods such as immunoblotting should be preferably used because of the lower interferences with contaminating factors [128]. We therefore decided to further investigate ELVs plasma levels by immunoblotting. Protein markers TSG101, CD81,

and HSP60 were increased in the EVs-isolates after 60 min of exercise. Similar to what we observed using NTA, these changes were only observable in the healthy subjects who performed their session in normoxia; however, the timing and consistency of the results were not congruent. For example, we did not find a significant change in circulating ELVs during exercise (30 min) but only at the end of it (60 min) which confirms that other elements than ELVs may confound the NTA analysis. This increase in ELVs after cycling corroborates previous human [117, 128, 243] and murine [137] studies showing an increase in ELVs protein markers in response to aerobic exercise. Flot1 and HSP70 [117] as well as CD63, CD9, and CD81 [128] have been shown to increase in ELVs isolates after a cycling incremental test. Similarly, Flot1 [117, 243] and HSP70 [243] increased after a treadmill incremental test. Finally, after a 40-min run at low [20% below maximal lactate steady state (MLSS)], moderate (MLSS), and high (20% above MLSS) intensity, the canonical ELVs marker CD63 was increased in ELVs isolates from Wistar rat serum [137]. Taken together, the above results indicate that both cycling and running exercise are able to increase the release of ELVs into the circulation.

To the best of our knowledge, no human study has investigated the effects of environmental hypoxia on the release of ELVs into the circulation. Since hypoxia enhances the beneficial effects of exercise on whole body glucose homeostasis in T2D subjects [248], ELVs might mediate the effects of exercise and hypoxia on targeted organs via enrichment in bioactive molecules. Moreover, EVs release has been shown to be increased upon hypoxia but most of the studies have been performed in cancer, endothelial, and cardiac cells in vitro (see Bister et al. for a review [163]). Here, the

addition of hypoxia to exercise did not increase the release of ELVs. If anything, this was rather a downregulation of exercise-induced release of ELVs. It is likely that the lack of effect in the healthy hypoxic exercise group was attributed to the lower absolute exercise intensity. We chose to work with an equal relative intensity between normoxia and hypoxia, i.e., at a HR corresponding to 55% $\dot{V}O_2$max, as exercise performed at the same absolute intensity is perceived to be more difficult in hypoxia than in normoxia [255]. The Borg scale, measuring the rate of perceived exertion (RPE), was collected immediately after exercise and confirmed that the perception of exercise intensity was equal between the hypoxic and normoxic groups (∼RPE 12). Since the absolute exercise intensity was lower in hypoxia than in normoxia, it is possible that the intensity was not sufficient to increase ELVs concentrations in the hypoxic groups. It has previously been shown that a continuous moderate exercise at 60% $\dot{V}O_2$max, very close to the intensity used here, even decreased ELVs concentrations postexercise [146]. However, ELVs were counted using NTA solely in the latter study. An increase in the concentration of ELVs after exercise in human has been reported after an incremental maximal exercise [117, 128, 243] based on NTA and/or protein expression of ELVs markers. Therefore, we cannot exclude that the exercise intensity chosen here was too low to induce detectable release of ELVs into the circulation in the groups exercised in hypoxia. Further studies are required to determine which exercise and hypoxia intensities would be able to induce changes in ELV release and to determine what causes those changes at the systemic and cellular levels.

In a similar way as hypoxia, our data suggest that prediabetic conditions might blunt the release of ELVs in response to an acute bout of exercise.

Although the majority of studies have focused on obesity, T2D, and microvesicles [244], no study has looked at the effect of exercise on ELV release in prediabetic subjects. Nevertheless, in 2019, Rigamonti et al. [146] investigated the effect of a moderate intensity treadmill exercise (30 min at ~60% $\dot{V}O_2max$) on ELV release in obese- and normal-weight subjects [body mass index (BMI) = 40 and 24 $kg \cdot m^{-2}$, respectively]. Despite the inherent limitations of using NTA and flow cytometry without Western blotting or electron microscopy confirmation, this study highlights the differences in ELV release between several cell types in obese- and normal-weight subjects. No association between BMI and ELVs was found. Regarding the EV origin, adipose tissue is of primary interest in the context of obesity and prediabetes as EVs have been proposed to mediate the interaction between adipocytes and macrophages, leading to inflammation [256]. In the study of Rigamonti et al., fatty acid binding protein (FABP+) vesicles (adipose tissue-derived vesicles) did not change with exercise and showed no association with BMI either. However, because of the difference in the targeted populations, it seems difficult to compare and to draw conclusions between the latter and the present study. Of note, our prediabetic group fulfilled the criteria for prediabetes from the American Diabetes Association for glucose levels after an OGTT but not for HbA1c, probably indicating a moderate prediabetic status. More studies are needed to elucidate the implication of BMI and adipose tissue on ELV release in response to exercise, specifically in a prediabetic population.

Knowing the crucial role of skeletal muscle in the release of myokines [90], potentially carried by ELVs, during exercise, we decided to analyze the MVB biogenesis pathway in skeletal muscle. Indeed, several in vitro investigations

showed that both myoblasts and myotubes had ELVs releasing activity [242]. Despite the few in vivo studies, the release of ELVs by skeletal muscle into the circulation has been supported by labeling studies [136]. The current identification of skeletal muscle derived-ELVs (SkM-ELVs) in the circulation is based on the enrichment of the ELVs pool with the glycoprotein alpha-sarcoglycan (SGCA). SGCA-positive ELVs represent up to 5% of the circulating vesicles [113]. Although it becomes increasingly clear that ELVs can be released by skeletal muscle, ELVs regulation by exercise remains unclear. Only one study investigated the effect of aerobic exercise (60% $\dot{V}O_2$max) on SGCA-positive ELVs [146]. After exercise, despite a decrease in the total amount of ELVs, SkM-ELVs were found to be increased in the circulation [146]. Another method to indirectly determine the contribution of skeletal muscle to circulating ELVs is to measure the formation and amount of ELVs in this tissue. Only two studies have investigated the biogenesis and the release of ELVs from skeletal muscle [114, 144]. In the first one, the authors observed that mice muscles incubated in serum-free culture media for 24 h were able to release ELVs but more specifically that oxidative muscles released a higher amount of ELVs than glycolytic muscles. This observation suggests that SkM-ELVs release might be fiber-type specific [114]. In the second study, the authors investigated the MVB pathway in response to acute exercise by measuring ELVs protein and gene expression in the vastus lateralis muscle [144]. The protein expression of ALIX, CD63, and TSG101 remained unchanged 1 h after aerobic exercise (45 min, 55% $\dot{V}O_2$max) and after aerobic combined with resistance exercise (leg extension, 3×8–12 repetitions at 55% 1-RM). In addition, the gene expression of most of the components of MVB biogenesis was not affected by exercise, except for ALIX which was increased after the combined exercise only [144]. Here, the

gene expression of components of MVB biogenesis in the vastus lateralis was differentially regulated between the healthy and prediabetic subjects after exercise. In the healthy subjects, the mRNA levels of ALIX, TSG101, CD9, and CD81 were not affected by exercise. In the prediabetic subjects, the mRNA levels of TSG101, ALIX, and CD9 were upregulated after exercise in normoxia, whereas CD9 and CD81 were downregulated in hypoxia. However, those modifications in mRNA abundance were not corroborated at the protein levels in any conditions, which could reflect a real lack of effect at the protein levels or possibly a delayed response at the protein compared with the mRNA levels. Furthermore, using endogenous bone marrow mitochondrial DNA haplogroup labeling (bone marrow transplant patient), it was found that the vast majority of exosome enriched particles at rest were bone marrow derived, and although the proportion of ELVs derived from bone marrow declined following exercise, they remained the predominant source [257]. Altogether, it is unlikely that skeletal muscle contributed to a large extent to the increase we observed in circulating ELV-enriched fractions in the healthy subjects [136].

Finally, several limitations should be raised. First, the healthy and prediabetic groups were not age-matched, whereas a decrease in ELV concentrations with age has been reported, likely due to greater internalization with advancing age [258]. However, prediabetes affects the elderly more than the young, and our groups represent the global reality. Another limitation of this study is that we did not determine the amount of circulating ELVs coming from skeletal muscle by directly labeling SGCA. Coupling the latter with the analysis of MVB biogenesis in skeletal muscle is the most valuable strategy to determine the proportionate contribution of skeletal muscle to the release of ELVs into

the circulation (myokines). However, due to the limited amount of biological material, labeling ELVs for SGCA was not possible. Similarly, analysis of the cargo transported in the ELVs, which would have required twice as much as the material available in this study, was not possible. Knowing that some miRNAs play a key role in the interorgan communication, it would be valuable to analyze their expression in both circulating and muscle ELVs. In the present context of glucose intolerance, it seems that miR-22 [233], miR-126 [173], miR-133a,b, and miR-206 [259, 260] would be of particular interest.

Perspectives and Significance

Our results confirmed previous findings on ELVs release in response to acute aerobic exercise in healthy subjects. In contrast, the typical ELVs increase observed after aerobic exercise was not found in prediabetic subjects nor in hypoxic conditions. Additional skeletal muscle analyses suggested that this tissue was not likely a dominant source of exercise-induced increase in the amount of circulating ELVs. Thus, our plasma ELV analyses are reflective of the total exerkine response and we cannot imply a specific tissue of origin. Future experiments should focus on the cargo found inside/transported by ELVs after exercise, which could be as critical, if not more, as the total amount of ELVs.

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DISCLOSURES

Exerkine Corporation is a biotechnology company that develops and commercializes therapies based on nutritional supplements, exercise-derived factors (“exerkines”), and extracellular vesicles to treat and diagnose genetic disorders, chronic diseases, and aging. M. A. Tarnopolsky is the founder, C.E.O., and C.S.O. of Exerkine Corporation, which provided support in the form of salaries for M. I. Nilsson. M. A. Tarnopolsky and M. I. Nilsson are shareholders in the company. Exerkine holds numerous patents that may be used for commercial development. The other authors have no conflict of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

E.D.G., F.A.B., and L.D. conceived and designed research; G.W., E.D.G., and F.A.B. performed experiments; G.W. and E.D.G. analyzed data; G.W., E.D.G., O.D., J.P.N., M.I.N., C.E.P., and M.A.T. interpreted results of experiments; G.W. and E.D.G. prepared figures; G.W., E.D.G., and L.D. drafted manuscript; G.W., E.D.G., F.A.B., O.D., J.P.N., M.I.N., C.E.P., M.A.T., and L.D. edited and revised manuscript; G.W., E.D.G., F.A.B., O.D., J.P.N., M.I.N., C.E.P., M.A.T., and L.D. approved final version of manuscript.

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Part 2: Effects of a 6-week sprint interval training protocol at different altitudes on circulating extracellular vesicles

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ABSTRACT

Purpose: This study aimed to investigate the modulation of circulating exosome-like extracellular vesicles (ELVs) after 6 wk of sprint interval training (SIT) at sea level and at 2000, 3000, and 4000 m.

Methods: Thirty trained endurance male athletes (18–35 yr) participated in a 6-wk SIT program (30-s all-out sprint, 4-min 30-s recovery; 4–9 repetitions, 2 sessions per week) at sea level (n = 8), 2000 m (fraction of inspired oxygen (FiO_2) 0.167, n = 8), 3000 m (FiO_2 0.145, n = 7), or 4000 m (FiO_2 0.13, n = 7). Venous blood samples were taken before and after the training period. Plasma ELVs were isolated by size exclusion chromatography, counted by nanoparticle tracking analysis, and characterized according to international standards. Candidate ELV microRNAs (miRNAs) were quantified by real-time polymerase chain reaction.

Results: When the three hypoxic groups were analyzed separately, only very minor differences could be detected in the levels of circulating particles, ELV markers, or miRNA. However, the levels of circulating particles increased (+262%) after training when the three hypoxic groups were pooled, and tended to increase at sea level (+65%), with no difference between these two groups. A trend to an increase was observed for the two ELV markers, TSG101 (+65%) and HSP60 (+441%), at sea level, but not in hypoxia. Training also seemed to decrease the abundance of miR-23a-3p and to increase the abundance of miR-21-5p in hypoxia but not at sea level.

Conclusions: A 6-wk SIT program tended to increase the basal levels of circulating ELVs when performed at sea level but not in hypoxia. In contrast,

ELV miRNA cargo seemed to be modulated in hypoxic conditions only. Further research should explore the potential differences in the origin of ELVs between normoxic and local and systemic hypoxic conditions.

Keywords: EXOSOMES, HYPOXIA, CYCLING, MIRNA

INTRODUCTION

Recently, growing interest has emerged regarding the potential role of extracellular vesicles (EVs) in the multisystemic adaptive process to exercise [136]. EVs have been proposed to be part of the multisystemic response to exercise as carriers of bioactive molecules called “exerkines,” namely, the different molecules released in response to exercise into the circulation. To date, EVs have been classified into three different subpopulations based on their size and their release mechanism: apoptotic bodies, microvesicles, and exosomes. Apoptotic bodies, the largest vesicles (1–5 μm), are generated upon the cell apoptosis and are released by large budding of the plasma membrane [261]. Microvesicles also form by budding from the plasma membrane, but they differ from apoptotic bodies by their smaller size (~100–1000 nm) and their content [24]. Finally, exosomes, the smallest subtype (~50–150 nm), are generated from the endosomal pathway and released in the extracellular space by the fusion of intracellular multivesicular bodies with the plasma membrane [67]. Because of significant contamination of exosomes with other small vesicles and because it remains difficult to distinguish the different classes of EVs with the current isolation techniques, the term exosome-like EV (ELV) will be used to consider heterogeneity in the smallest EVs, that is, exosomes and small microvesicles (<200 nm) [242].

During acute exercise, the release of ELVs into the blood circulation is increased [136]. In 2015, Frühbeis et al. [117] were the first to highlight a rapid release of ELVs in response to both a cycling and a running incremental exercise to exhaustion in recreationally active men. Since then, similar release kinetics in response to exercise have been observed in both human [128, 243,

262] and murine [137, 167, 263] models. In addition to the quantitative changes in circulating vesicles abundance, modifications of the ELV microRNA (miRNA) content seems to occur after resistance exercise [141, 174], moderate-intensity endurance exercise [137, 176, 264], and high-intensity interval training (HIIT) [173]. Regarding the regulation of ELVs by chronic training, few studies have been conducted in various models using different training modalities [150, 164-168]. It seems that chronic exercise training could lead to higher levels of basal circulating vesicles. However, in the aforementioned studies, heterogenous blood preparations and EV isolation techniques were used, and several methodological concerns prevent firm conclusions [136]. Training studies that follow International Society for Extracellular Vesicles (ISEV) guidelines are currently lacking, especially in humans. In terms of cargo, chronic exercise training could modulate vesicular miRNAs, as it is the case after acute exercise [167, 168, 176].

To date, most of the studies investigating the modulation of ELV release by exercise has been conducted at sea level (SL). However, when it comes to sport performance enhancement in trained athletes, the use of environmental stressors such as hypoxia or heat is common practice, as it seems to offer additional benefits [153, 265]. In this perspective, intermittent hypoxia is often associated with very high-intensity exercises such as sprint interval training (SIT), which is characterized by repeated maximal exercise bouts (usually 30 s) with 2–4 min of recovery [266, 267]. In a previous study, we observed a possible blunting effect of hypoxia on ELV release during an acute aerobic exercise in hypoxia [236]. Based on this finding, we hypothesized that a training intervention in intermittent hypoxia might downregulate the circulating ELV amount and/or content compared with an identical training

at SL. The present study aims to test this hypothesis of changes in abundance and content of circulating ELVs by comparing the effect of a 6-wk SIT program at SL and at 2000, 3000, and 4000 m.

METHODS

Participants

Thirty-one trained endurance male athletes (amateur competing cyclists or triathletes) were recruited based on their age (18–35 yr) and their training volume (4–10 h·wk−1). All participants gave their written consent to voluntarily participate to the experiment, which was approved by the ethical committee of the UCLouvain (B403201939034) and conducted in accordance with the Declaration of Helsinki. A medical checkup was organized, and exclusion criteria for participation were set as follows: smoking, exposure to an altitude above 1500 m during the month before the experiment, and any health risk that could compromise the participant's safety during training and/or hypoxic exposure. The participants were asked to maintain their usual dietary habits and training load throughout the experiment. The participants were also asked to avoid strenuous workout the day before the experimental training sessions. Of the 31 participants involved, a single one had to stop the study because of a fall that occurred during a training session aside from the study.

Experimental protocol

In the present study, we used the remaining blood samples from a previous experimental trial to investigate the effects of a 6-wk SIT protocol at different altitudes on circulating ELVs. Detailed information about the experimental

protocol can be found in the study by Warnier et al. [268]. Briefly, pretest and posttest were composed of a blood sampling, a hemoglobin mass measurement, an incremental fitness test, a Wingate test, and a 600-kJ time trial. Blood sampling was performed during the first pretest session, 2 wk before the first training session of the program. All cycling tests and training sessions were performed using a cycle ergometer (Cyclus II; RBM Electronics, Leipzig, Germany) and the participant's personal bike. After the pretest period, the participants were semirandomly assigned to one of the four experimental groups based on the peak power output (PPO) measured during an incremental exercise test to obtain in each group, similar values of fitness level, age, and body weight (Table 1). The four groups participated to a 6-wk supervised cycling training program involving SIT at different altitudes: SL (FiO_2 0.209, n = 8), 2000 m (FiO_2 0.167, n = 8), 3000 m (FiO_2 0.145, n = 7), and 4000 m (FiO_2 0.13, n = 7). Normobaric hypoxia was achieved by nitrogen injection in a confined room to dilute oxygen content in the air (high-altitude system; B-Cat, Tiel, the Netherlands). After the training intervention, all participants participated to the posttest period. Because miRNA modulation in ELVs has been shown to occur up to 24 h after exercise [141], whereas longer follow-ups are lacking, we collected posttraining samples 72 h after the last session to ensure a return to baseline values. Blood sampling was performed 3 d after the last training session, and the first cycling test was performed 5 d after the last session to allow a complete recovery.

Results

Table 1. Participants characteristics.

	Sea-level (=8)	2,000m (n=8)	3,000m (n=7)	4,000m (n=7)
Age (y)	26.0 ± 1.7	25.4 ± 1.7	25.4 ± 1.6	25.1 ± 1.7
Weight (kg)	67.0 ± 1.5	71.2 ± 2.9	72.4 ± 3.7	72.6 ± 4.7
BMI ($kg \cdot m^{-2}$)	21.2 ± 0.7	21.1 ± 0.7	22.5 ± 1.2	21.9 ± 1.5
Basal PPO (W)	313 ± 12	318 ± 11	335 ± 13	307 ± 12

Values are means ± SEM for the sea-level group (F_iO_2 0.209) and altitudes groups (2,000 m, F_iO_2 0.167; 3,000 m, F_iO_2 0.145; 4,000 m, F_iO_2 0.13).

Training sessions

During the whole experiment, all participants were blinded to the training conditions. They participated to a supervised SIT session twice a week for 6 wk based on Puype et al. [269]. The sessions started with a 10-min warm-up at ~150 W (80–90 rpm, 17.5 N·m) and two 5-s sprints. Thereafter, the participants had to repeat a series of 30-s sprints with a 4-min 30-s recovery at ~75 W (80–90 rpm, 8.75 N·m). The first and last sprints of each session were set as a Wingate test (30-s maximal cycling exercise with a resistance set to 7.5% of the participant's body weight (0.075 kg·kg bw−1)). The other sprints were set at 80% of the Wingate load. The participants were asked to go all-out on all sprints and try to produce the highest possible power output. The number of sprints per session was progressively increased from four in week 1 to nine in week 6.

Blood collection and EV isolation

Blood drawing from an antecubital vein of the forearm was performed in EDTA tubes. Blood was centrifuged for 15 min at 2000g at 4°C to obtain plasma. An extra 15-min centrifugation at 2000g was performed to remove the remaining platelets. Platelet-free plasma fraction was frozen at −80°C until ELV isolation. Apoptotic bodies and large vesicles were removed by differential centrifugation with 1 h at 2000g and 1 h at 20,000g, respectively. The supernatant was then filtered through 0.22-µm syringe filters and ELV isolation was performed by size exclusion chromatography (SEC) using qEVoriginal SEC columns (Izon Science). One milliliter of platelet-free plasma was loaded on the column and 500-µL fractions were collected according to the manufacturer's recommendations. For each sample, two separate isolations by SEC were performed (2 × 1 mL of plasma; Fig. 1). Fractions 7 to 9 were considered ELV rich as described in our previous work [236]. These fractions were pooled for further analysis.

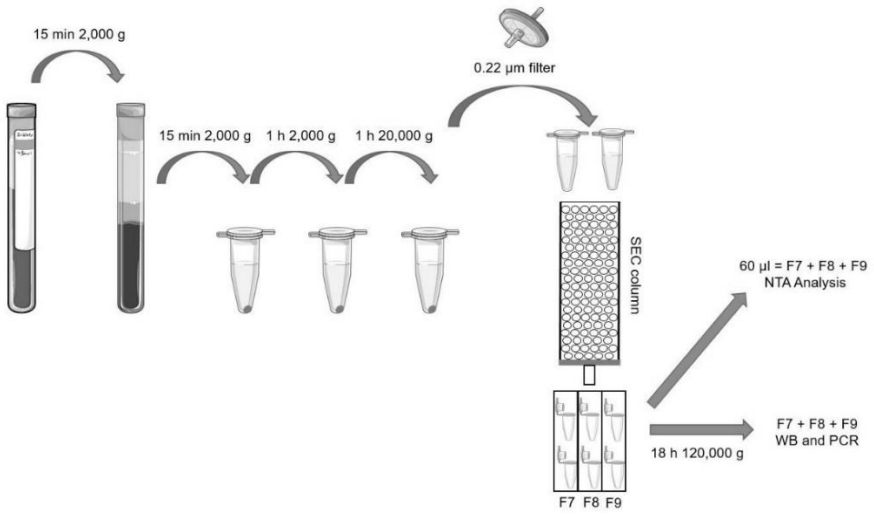


Figure 1. ELV isolation method from plasma samples. NTA, nanoparticle tracking analysis; WB, western blot; PCR, polymerase chain reaction.

Concentration by ultracentrifugation

The duplicated (2 × 500 μL) fractions 7, 8, and 9 obtained after SEC were pooled and ultracentrifuged at 4°C for 18 h at 100,000g (TLA55 rotor, k-factor 66; Beckman). The supernatant was removed to concentrate the samples 10×, and the three pellet fractions (7–9) were pooled to obtain one sample per participant for further Western blot and polymerase chain reaction (PCR) analyses (see discussion hereinafter).

Nanoparticle tracking analysis

Before ultracentrifugation, a small volume of samples (20 μL) from fractions 7, 8, and 9 were pooled for nanoparticle tracking analysis (NTA) analysis. ELVs were counted by the Zetaview (Particle Metrix, GmbH), which captures Brownian motion through a laser scattering microscope combined with a video camera to obtain size distribution (50–1000 nm) and concentrations. Samples were diluted in UltraPure™ DNase/RNase-free distilled water at 1:150–1:6000 to reach 50–200 particles per frame corresponding to ~2.10^7–1.10^8 particles·mL−1. Sensitivity was set to 80 and camera shutter to 100 to detect less than 5–10 particles per frame as background when UltraPure™ water alone was injected. Measurements were performed with medium resolution for 2 cycles at 11 different positions (or frames) of the cell chamber containing the sample. The Zetaview software was used to generate results in text files. Data are reported as percentage changes compared with pretraining according to the formula [(post − pre)/pre] × 100, with a value of 0 meaning no change between posttraining and pretraining. To allow comparison with other studies, absolute pretraining and posttraining mean values are also

presented in the Results section (individual absolute values are available in Supplementary Fig. 1, see in appendix)

Western blotting

An equal volume of vesicle isolates was loaded for all samples as usually performed with ELV protein homogenates [117, 128, 137, 236]. The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Immobilon Transfer Membrane; Millipore). Membranes were blocked in Tris-buffered saline–Tween containing 5% skimmed milk for 60 min at room temperature (RT). Membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with horseradish peroxidase–conjugated secondary antibody for 1 h at RT. Membranes were thereafter scanned, and signals quantified with GeneSnap software and tools (Syngene, Cambridge, United Kingdom). The following antibodies were used: ALIX (1:500 dilution, Cell Signaling Technology (CST), E6P9B), CD9 (1:1000 dilution, CST, D3H4P), CD81 (1:1000 dilution, CST, D3N2D), HSP60 (1:1000 dilution, CST, D307), and TSG101 (1:1000 dilution, Abcam, Ab30871). Data are reported as percentage changes compared with pretraining according to the formula [(post − pre)/pre] × 100, with a value of 0 meaning no change between posttraining and pretraining. As for circulating particles, individual absolute values are available in Supplementary Fig. 1 (see in appendix).

RNA extraction and real-time quantitative PCR

RNA extraction was performed using the miRNeasy Mini Kit (Qiagen). Seven hundred microliters of QIAzol lysis reagent and 10 μL of cel-miR-54-

5p spike-in (20 pM) were added to 100 µL of ELV isolates, and the samples were incubated for 5 min at RT. After the addition of 140 µL of chloroform, the samples were vortexed for 15 s and incubated for 3 min at RT. After incubation, the mixture was centrifuged at 12,000g for 15 min at 4°C. The upper aqueous phase was transferred to a fresh tube and vortexed briefly after the addition of 1.5 volume of ethanol. Samples were then transferred to miRNeasy Mini spin columns that were centrifuged at ≥8000g for 15 s at RT, and flow-through was discarded. After wash steps with the kit buffers, RNA pellets were dried by centrifugation at ≥8000g for 2 min at RT and eluted in 20 µL of water. RNA was quantified using Quant-iT™ RiboGreen™ RNA Assay Kit (Thermo Scientific). Pulsed reverse transcription was performed on 6 µL of plasma-derived EV-RNA using M-MLV Reverse Transcriptase (Invitrogen, Waltham, MA) and a mix of specific miRNA stem–loop primers [270]. One microliter of cel-miR-39-5p at 20 pM was added to the RT mix as second spike-in. Real-time quantitative PCR was performed as described, in the presence of 300 nM of universal reverse primer, 300 nM of specific forward primer, 250 nM of probe with Takyon Probe Master Mix (Eurogentec, Seraing, Belgium) on a CFX96 touch real-time PCR Detection System (Bio-Rad, Watford, United Kingdom). Primers and probes sequences are listed in Supplementary Table 1 (see in appendix). Candidate miRNAs were selected based on their presence in ELVs and their response to high-intensity cycling exercise [173]: miR-126-3p, miR-16-5p, miR-208b-3p, miR-208a-3p, miR-1-3p, miR-23a-3p, miR-23b-3p, miR-486-5p, miR-378a-5p, miR-222-3p, miR-451a, miR-21-5p, miR-148b-3p and miR-150-5p (Supplementary Table 1, see in appendix). Data are presented according to the ΔΔCT method using the geometric mean of the two spikes-in as reference

miRNAs and pretraining as the reference condition, with a value of 0 meaning no change between posttraining and pretraining.

Transmission electron microscopy

ELV images were taken by negative staining in transmission electron microscopy (TEM). Cupper grids (300 mesh) were cleaned by sonification for five times 5 min in ethanol and covered with a 1% formvar film. After overnight drying, a 5-nm carbon layer was electron beamed on top of the formvar film. Fifty microliters of a 1% uranyl acetate solution in freshly bidistilled water was placed on the prepared grids for 5 min. Then the excess of liquid was removed with a Whatman filter paper (No. 415) and dried overnight in a dry chamber. The next day, the prepared sample was validated in TEM (CM12, Philips) at 80 kV. The film had to be intact and uniformly stained all over. Biological samples were diluted in freshly bidistilled water (1×), and a 20-μL diluted sample was put on the formvar-carbon–coated grids for 5 min. Then 20 μL of 2% uranyl acetate was added. After 5 min, the excess of liquid was removed with a Whatman filter paper (No. 415), and samples were dried overnight in a dry chamber. The next day, the samples were analyzed with the electron microscope (CM12, Philips) at 80 kV. Pictures were taken at 60,000 times magnification.

Statistics

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS v.25.0; IBM, Armonk, NY), and data are reported as mean changes ± SEM between posttraining and pretraining as described in the Western blotting and real-time quantitative PCR sections. The data distribution was assessed with the Shapiro–Wilk test. Because of nonnormal

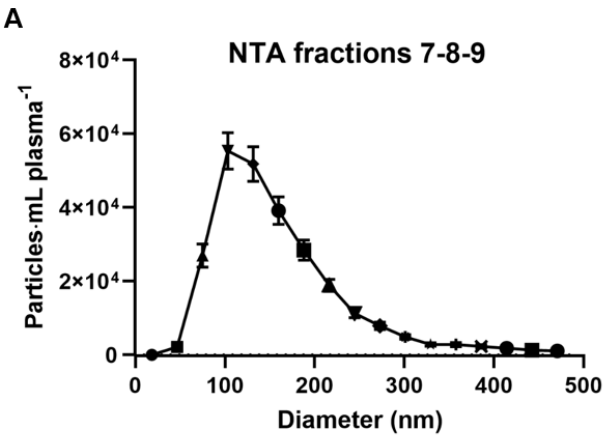
distribution, nonparametric analyses were applied. A Kruskal–Wallis test was used to compare the four groups' pretraining values and showed no statistical differences except when indicated. A nonparametric one-sample t-test comparing mean change with 0 was used to assess the training effect in each group. In a separated analysis, the global training effect was evaluated with all four conditions grouped using a nonparametric one-sample t-test comparing mean change with 0. A Kruskal–Wallis test was used to compare the four different altitudes, and a Mann–Whitney test was applied if a significant difference was observed to confirm the difference between two groups. The hypoxia effect was assessed comparing the group training at SL with the three altitude groups pooled. Statistical significance was set at $P < 0.05$. Interquartile range (IQR) method was used to detect outliers in each group. A value greater than third quartile + 1.5IQR or less than 1st quartile − 1.5IQR was considered statistically abnormal and removed from the analysis. Samples with undetectable values in Western blot or PCR were removed as well. The number of samples included in the statistical analyses is given in the legend of the figures. Graphs were created using GraphPad Prism 8.4.0.

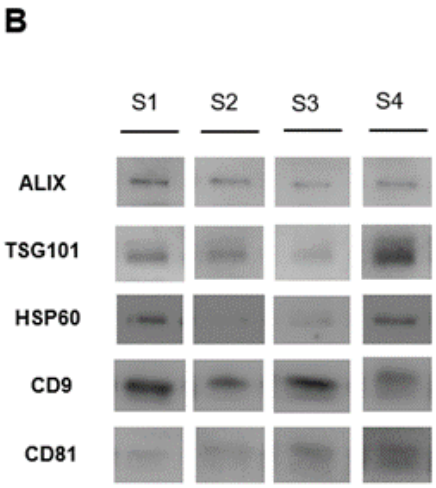
RESULTS

ELV characterization

As per ISEV recommendations [20], NTA, Western blot and TEM analyses were conducted to confirm the presence of ELVs in our samples (pooled fractions 7–9; Fig. 2). NTA analysis showed a peak of particle concentration at 100 nm and most particles less than 200 nm (Fig. 2A). Median diameter of particles was measured at 144 ± 3.5 nm. These two results fit with the ISEV definition of small EVs (<200 nm) [20]. Detection of ELV markers ALIX,

TSG101, HSP60, CD9, and CD81 is presented in Figure 2B in four participants. The presence of those specific markers indicates the presence of small EVs in our fractions. Finally, vesicular structures within the size range of small EVs were identified by TEM (Fig. 2C). Taken together, the aforementioned results demonstrated the successful isolation of ELVs from our human plasma samples.





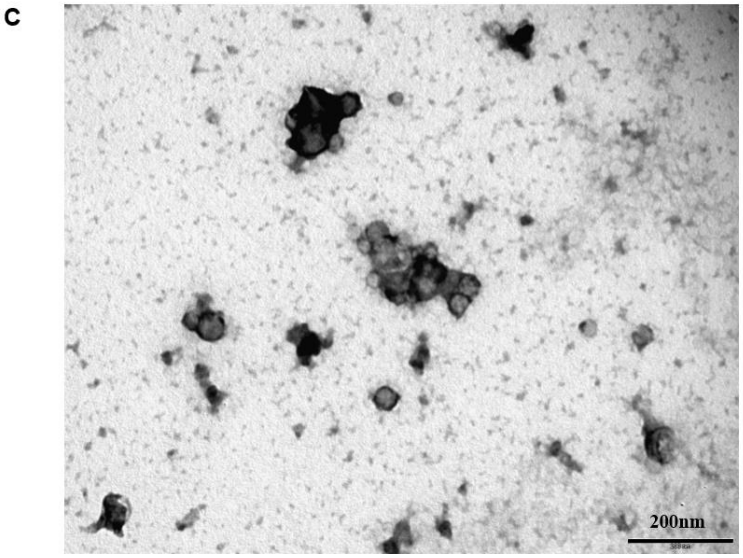


Figure 2. ELV characterization. A, Particle size distribution in pooled fractions analyzed by NTA. B, WB bands showing ELV markers in four participants (ALIX, TSG101, HSP60, CD9, and CD81). C, TEM image of the pooled fractions (negative staining). Scale bar refers to 200 nm. WB, Western blotting

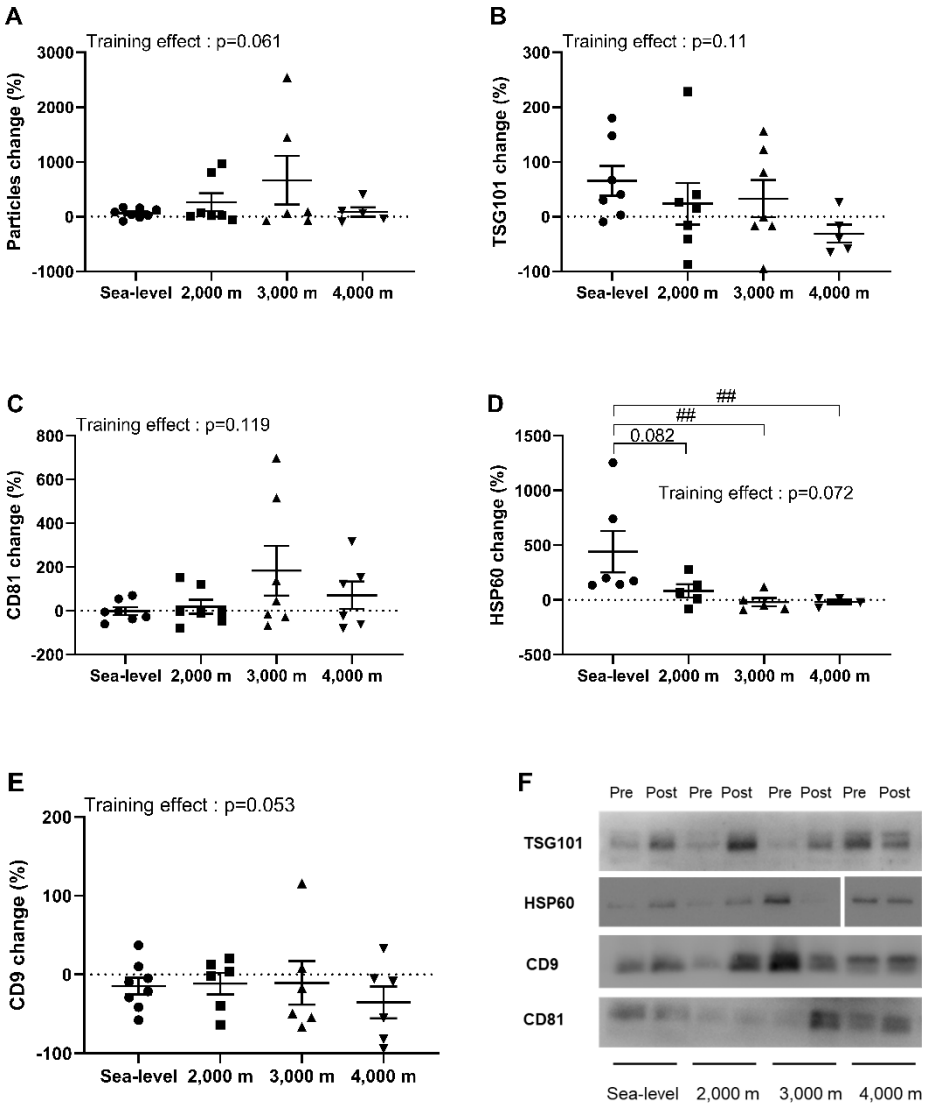
Circulating ELVs

We first compared the levels of circulating particles by NTA before and after the training in all the participants. This comparison showed a trend to increase after 6 wk of training (+47%, P = 0.061). However, training did not increase the number of particles when each training altitude group was analyzed individually (pretraining vs posttraining (± SEM); SL: 3.7 ± 1.1 × 1010 vs 5.1 ± 1.7 × 1010; 2000 m: 3.1 ± 1.4 × 1010 vs 3.2 ± 1 × 1010; 3000 m: 3 ± 1.5 × 1010 vs 5.6 ± 2.2 × 1010; 4000 m: 3 ± 1.6 × 1010 vs 4.1 ± 3.1 × 1010). No difference between the four groups could be detected either (Fig. 3A). With

regard to particle size, the median diameter did not change after the training period without any difference between the groups.

Knowing the limitations and the lack of specificity of NTA analysis, Western blot analysis of known ELV markers was performed to assess the changes in circulating vesicles. When all participants were considered as a whole, training did not globally change the protein abundance of TSG101 (+27%, P = 0.11; Fig. 3B) and CD81 (+31%, P = 0.119; Fig. 3C), but a trend was observed for HSP60 (+50%, P = 0.072; Fig. 3D) and CD9 (−18%, P = 0.053; Fig. 3E). When each altitude group was analyzed separately, training did not significantly change the abundance of the aforementioned ELV markers. In addition, no difference was found between the groups for those proteins except for HSP60 (P = 0.01; Fig. 3D). For HSP60, the changes observed at SL were larger than the change at 3000 m (P = 0.004) and at 4000 m (P = 0.01) and tended to be larger than the change at 2000 m (P = 0.082).

Because we lacked significance but sometimes observed trends, we decided to consider the three altitude training groups together (hypoxia) and reanalyze the training effect in hypoxia and compare the effect of hypoxia to SL. We found that the number of circulating particles was increased by the training program when it occurred in hypoxia (+262%, P = 0.028), whereas it only showed a trend to increase at SL (+65%, P = 0.077), with no difference between the two groups (Fig. 3G). Regarding the two ELV markers TSG101 (+65%, P = 0.053; Figs. 3B, H) and HSP60 (+441%, P = 0.066; Figs. 3D, I), which showed a trend to increase after the SIT at SL, their abundance was not increased in the pooled hypoxia group. In addition, change of TSG101 abundance seemed lower (P = 0.053; Fig. 3H), and change of HSP60 abundance was lower (P = 0.002; Fig. 3I) in hypoxia as compared with SL.


A
Training effect : p=0.061
Particles change (%)
3000
2000
1000
0
-1000
Sea-level
2,000 m
3,000 m
4,000 m
B
Training effect : p=0.11
TSG101 change (%)
300
200
100
0
-100
Sea-level
2,000 m
3,000 m
4,000 m
C
Training effect : p=0.119
CD81 change (%)
800
600
400
200
0
-200
Sea-level
2,000 m
3,000 m
4,000 m
D
##
##
0.082
Training effect : p=0.072
HSP60 change (%)
1500
1000
500
0
-500
Sea-level
2,000 m
3,000 m
4,000 m
E
Training effect : p=0.053
CD9 change (%)
200
100
0
-100
Sea-level
2,000 m
3,000 m
4,000 m
F
Pre
Post
Pre
Post
Pre
Post
Pre
Post
TSG101
HSP60
CD9
CD81
Sea-level
2,000 m
3,000 m
4,000 m

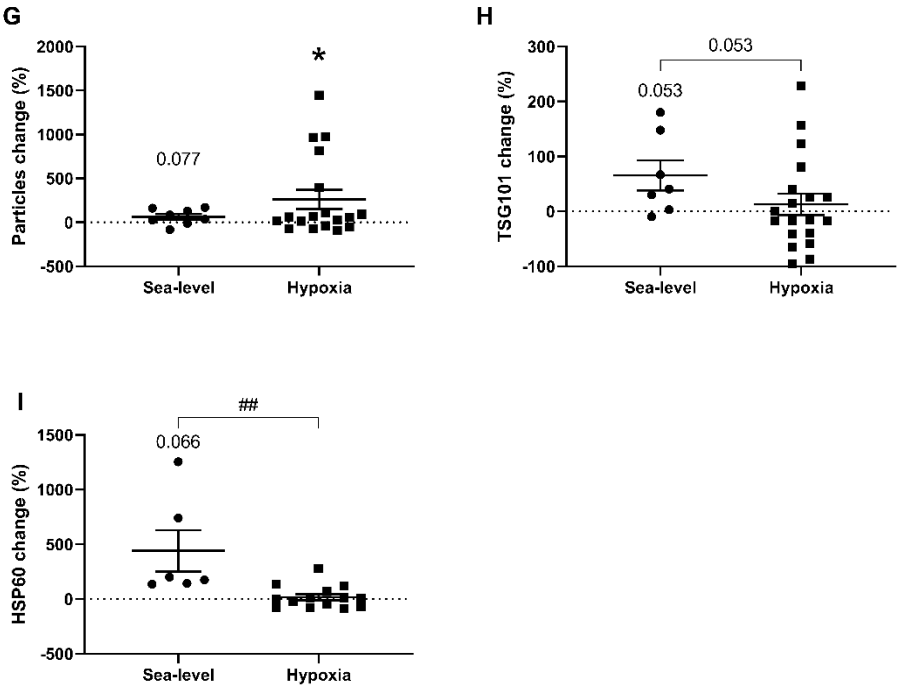
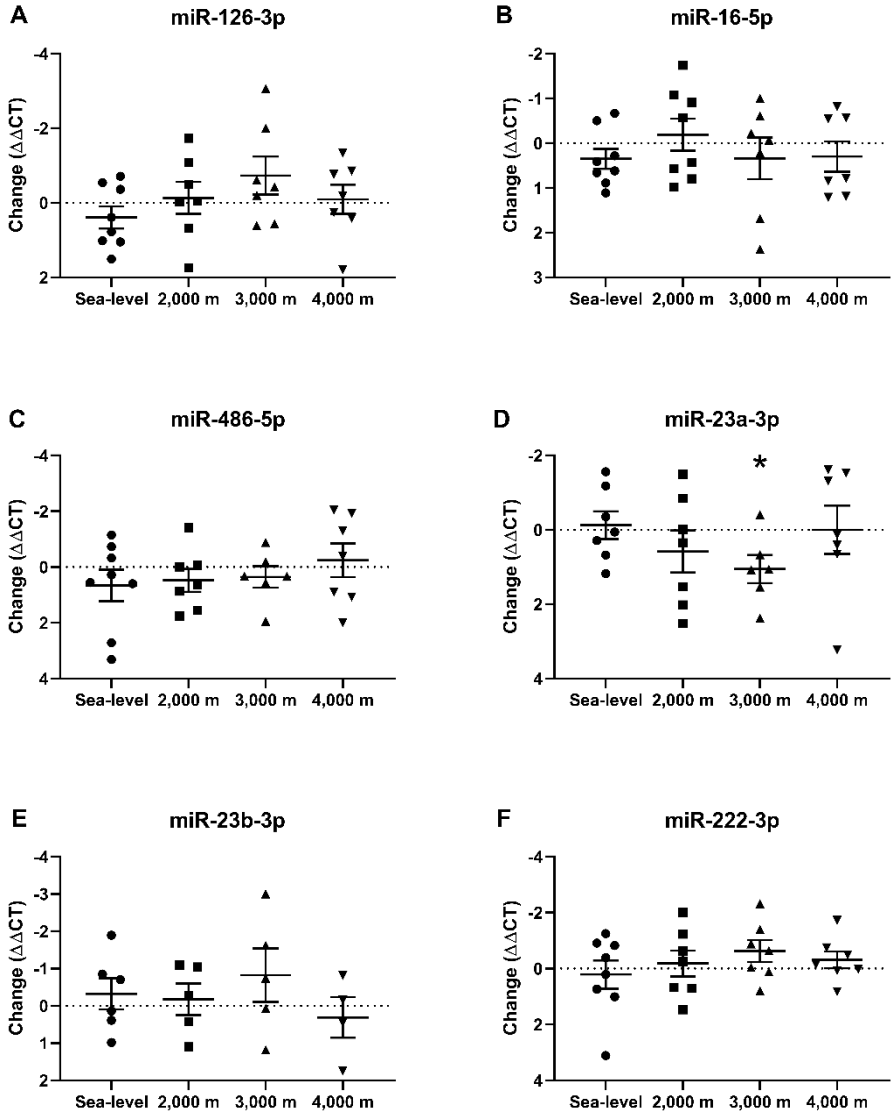


Figure 3. Circulating ELVs. SL (n = 8), 2000 m (n = 8), 3000 m (n = 7), 4000 m (n = 7), and hypoxia (n = 22), except when specified. A, Circulating particles change between pretraining and posttraining (2000 m: n = −1; 3000 m: n = −1; 4000 m: n = −2). B, TSG101 abundance change (SL: n = −1; 2000 m: n = −1; 4000 m: n = −2). C, CD81 abundance change (SL: n = −1; 2000 m: n = −1; 4000 m: n = −1). D, HSP60 abundance change (SL: n = −2; 2000 m: n = −3; 3000 m: n = −2; 4000 m: n = −3). E, CD9 abundance change (2000 m: n = −2; 3000 m: n = −1; 4000 m: n = −1). F, Representative WB analysis of SEC-ELVs (pooled fractions 7–9) using ELV markers. G, Circulating particles change, SL vs hypoxia (hypoxia: n = −4). H, TSG101 abundance change, SL vs hypoxia (SL: n = −1; hypoxia: n = −3). I, HSP60 abundance change, SL vs hypoxia (SL: n = −2; hypoxia: n = −8). Data are reported as percentage changes compared with pretraining according to the formula [(post − pre)/pre] × 100, with a value of 0 meaning no change between posttraining and pretraining. Values are mean changes ± SEM. *P < 0.05 vs pretraining, ## P < 0.01 between groups.

miRNA content of ELVs

Besides the abundance of circulating ELVs, their cargo is also an important parameter to consider. We thus investigated the miRNA content of the isolated ELVs. Among the 14 miRNAs analyzed, six were not detectable (miR-208a-3p, miR-208b-3p, miR-1-3p, miR-451a, miR-378a-5p, and miR-148b-3p). Of the remaining eight (Figs. 4A–H), only miR-23a-3p and miR-21-5p showed a differential abundance induced by SIT. The abundance of miR-23a-3p in ELV was lower after 6 wk of training, but only at 3000 m ($P = 0.039$; Fig. 4D), with no difference between the four groups. We found that the abundance of miR-21-5p in ELVs was affected by the different training conditions ($P = 0.027$; Fig. 4G). More specifically, only the participants trained at 3000 m had a higher abundance of ELV miR-21-5p after the training ($P = 0.011$). In addition, the change at 3000 m was different ($P = 0.008$) and the change at 4000 m tended to be different ($P = 0.073$) from the change at SL. Finally, the change observed at 3000 m tended also to be different from the change at 2000 m ($P = 0.052$). Of note, miR-21-5p abundance tended to be different between our four groups at baseline ($P = 0.051$). Ultimately, we analyzed the differences in miRNA abundance in ELVs between SL training and hypoxia training as a whole, regardless of the altitude levels. A trend to a lower abundance of miR-23a-3p was found in hypoxia only ($P = 0.064$), with no difference between normoxia and hypoxia (Fig. 4I). Conversely, level of miR-21-5p in ELVs was higher after 6 wk of training in hypoxia ($P = 0.006$), and this increased abundance was significantly higher when compared with ELVs from participant trained at SL ($P = 0.02$; Fig. 4J).


A
miR-126-3p
Change (ΔΔCT)
-4
-2
0
2
Sea-level
2,000 m
3,000 m
4,000 m
B
miR-16-5p
Change (ΔΔCT)
-2
-1
0
1
2
3
Sea-level
2,000 m
3,000 m
4,000 m
C
miR-486-5p
Change (ΔΔCT)
-4
-2
0
2
4
Sea-level
2,000 m
3,000 m
4,000 m
D
miR-23a-3p
*
Change (ΔΔCT)
-2
0
2
4
Sea-level
2,000 m
3,000 m
4,000 m
E
miR-23b-3p
Change (ΔΔCT)
-4
-3
-2
-1
0
1
2
Sea-level
2,000 m
3,000 m
4,000 m
F
miR-222-3p
Change (ΔΔCT)
-4
-2
0
2
4
Sea-level
2,000 m
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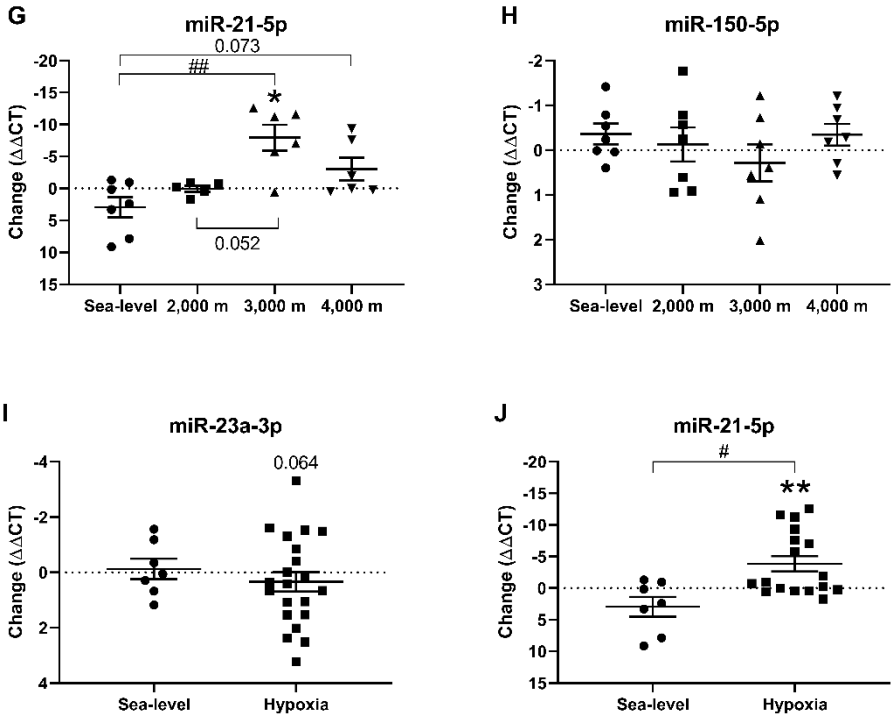

Figure 4. miRNA content of ELVs. SL (n = 8), 2000 m (n = 8), 3000 m (n = 7), 4000 m (n = 7), and hypoxia (n = 22), except when specified. A, miR-126-3p abundance change between pretraining and posttraining (2000 m: n = −1). B, miR-16-5p abundance change. C, miR-486-5p abundance change (2000 m: n = −1; 3000 m: n = −1). D, miR-23a-3p abundance change (SL: n = −1; 2000 m: n = −1; 3000 m: n = −1). E, miR-23b-3p abundance change (SL: n = −2; 2000 m: n = −3; 3000 m: n = −2; 4000 m: n = −3). F, miR-222-3p abundance change (2000 m: n = −1). G, miR-21-5p abundance change (SL: n = −1; 2000 m: n = −3; 3000 m: n = −1; 4000 m: n = −1). H, miR-150-5p abundance change (SL: n = −1; 2000 m: n = −1). I, miR-23a-3p abundance change, SL vs hypoxia (SL: n = −1; hypoxia: n = −1). J, miR-21-5p abundance change, SL vs hypoxia (SL: n = −1; hypoxia: n = −5). Data are presented according to the ΔΔCT method using the geometric mean of the two spikes-in as reference miRNAs and pretraining as the reference condition, with a value of 0 meaning no change between posttraining and pretraining. Values are mean changes expressed in ΔΔCT ± SEM. *P < 0.05, **P < 0.01 vs pretraining; # P < 0.05, ## P < 0.01 between groups.

DISCUSSION

The aim of the present study was to assess the impact of a 6-wk SIT protocol at different altitudes on basal plasma ELV levels and content in trained endurance athletes. To our knowledge, this is the first study analyzing the effect of exercise training on human ELVs isolated and characterized according to ISEV guidelines. The main results were the following: 1) the levels of circulating particles after training increased in hypoxia and tended to increase at SL, with no difference between the two groups; 2) the two ELV markers TSG101 and HSP60 tended to be more abundant after training at SL, but not in hypoxia; 3) SIT tended to decrease the abundance of miR-23a-3p and to increase the abundance of miR-21-5p in hypoxia but not at SL.

First, we found an upward trend in circulating particles after the training intervention. This result is consistent with previous studies conducted in mice showing an increase in basal particles levels after chronic training [164, 168], but not with another study in rats reporting no change [167]. In accordance with the three aforementioned studies [164, 167, 168], we found no change in the particle size after 6 wk of training. To our knowledge, only two studies have investigated the chronic effect of training on basal circulating ELVs in human, with conflicting findings [167, 169]. On the one hand, Hou et al. compared students involved in rowing practice for at least a year (training for 1–2 $h \cdot d^{-1}$, 6 $d \cdot wk^{-1}$) to their sedentary counterparts [167]. They did not find any differences in particles number or size distribution between the two groups suggesting no effect of chronic training on basal circulating ELVs. Although this result must be considered with caution as the study was purely observational. On the other hand, Apostolopoulou et al. [169] set up a training program in male participants with or without type 2 diabetes. They found an

increase in circulating particles after 12 wk HIIT without any change in particle size. Of note, this increase was only observable in metabolic responders displaying improved insulin resistance after training. Because NTA analysis is unspecific and cannot always characterize the ELV response to exercise [128, 136], Western blotting was used to assess the changes in basal circulating ELVs, as recommend by ISEV. When analyzing the global effect of training, we only found trends to upregulation (HSP60) or downregulation (CD9). In rodents, ELV markers have been shown to increase [164-166] or not [167, 168] after chronic exercise. In human, the two studies having investigated the chronic effects of exercise on basal circulating ELVs did not analyze ELV markers before and after training [167, 169]. In addition, of the five studies on rodents, two [164, 166] did not report the blood sampling timing, which does not allow to differentiate between acute and chronic adaptations. Additional studies including both NTA and Western blot analysis are required, especially in human, to conclude about the effects of chronic exercise on basal circulating ELV levels and markers.

We were then interested in analyzing the potential differences between training at SL and the three hypoxic groups. Indeed, only minor differences could be detected in the levels of circulating particles, ELV markers, and miRNA between the SL and the three hypoxic groups. It was then opted for pooling the three hypoxic groups, regardless of the altitudes. At SL, the abundance of circulating particles as well as of TSG101 and HSP60 tended to increase after training. In hypoxia, the levels of circulating particles increased after training, but the abundance of TSG101 and HSP60 remained unchanged. Because, according to the ISEV guidelines, changes in both the amount of particles and canonical markers are needed to confirm a change in

circulating ELVs, our results suggest a potential increase in circulating ELVs after a 6-wk SIT intervention when performed at SL, but not in hypoxia. However, it might be that the SIT induced change in the expression of ELV markers. To date, hypoxic stress impact on EV release has been mainly studied at a cellular level in the context of cancer and in various tissues. In these conditions, most publications report an increase in ELV release upon hypoxia [163]. In humans, a single study investigated the effect of intermittent hypoxia on ELV release in the context of an acute cycling exercise [236]. In this previous work from our laboratory, we did not observe any increase in ELV release in response to exercise when performed in hypoxia (FiO_2: 0.14, ~3000 m). Even though the absence of increase may have been related to a lower absolute exercise intensity in hypoxia versus normoxia, the data of the present work corroborate the idea of a different regulation of the levels of circulating ELVs in hypoxic versus normoxic conditions. Further studies will be needed to confirm those findings and to explore the cellular mechanisms involved in this process.

Although the increase in the number of circulating vesicles alone may provide beneficial adaptations [164], their content is no less important [167]. Circulating vesicle cargo such as miRNAs can regulate gene expression and metabolism of the recipient cell/tissue [170, 171]. Here, we selected candidate miRNAs based on the findings of D'Souza et al. [173] highlighting several exercise-responsive miRNAs in ELVs. Several miRNAs were upregulated or downregulated in ELVs after a cycling HIIT session (10 × 60 s at PPO). In our study, among the selected responsive miRNAs, eight were detected, but only the abundance of miR-23a-3p and miR-21-5p was modulated after the training program. Intriguingly, the training effect was only observed for miR-

21-5p (upward trend) and for miR-23a-3p (downregulation) when the training program was performed under hypoxia and specifically at 3000 m. The downregulation of miR-23a-3p seems consistent with a recent human pilot study in which this miRNA was shown to be reduced in ELV cargo with both short- (6 months) and long-term (25 yr) physical activity [271]. Being associated with several cancers [272], a low abundance of miR-23a-3p reflects a positive adaptation with health benefits. Because our participants were trained adults, the rather small decrease at 3000 m and the lack of change in the other groups might be explained by an already low abundance of ELV miR-23a-3p. Contrarily to miR-23a-3p, miR-21-5p increased after training, but similarly, this change was observed in hypoxia only. This last element suggests a different effect of chronic exercise when performed in hypoxia in comparison to SL. Although the implications of miR-21-5p seem to be diverse, ELV miR-21-5p could mediate cardioprotective effects by targeting gene products involved in apoptosis [273] but also neuroprotective effects via the Akt pathway [274]. In addition, it seems that endurance training may positively regulate lipid metabolism in the liver via an upregulation of this miRNA [275]. Therefore, an increase in circulating miR-21-5p might participate in adaptations to endurance training. Previous human [167, 176] and murine [168] studies provided evidence that chronic exercise can modulate the basal circulating ELV content, but our study is the first to suggest that this modulation could be different when training in hypoxia. Recently, the impact of an acute blood flow–restricted (BFR) resistance exercise on ELV miRNA cargo and surface markers was investigated [276]. A modulation of 12 ELV miRNAs was measured after the BFR resistance exercise session. In addition, ELVs isolated after the BFR session tended to enhance muscle satellite cell proliferation when incubated in vitro, which

offers a functional insight. Finally, the authors observed an alteration in the surface ELV markers, suggesting a modification in terms of ELV source during exercise. The release of ELVs was increased mainly in bone marrow, immune system, endothelial cells, and blood cells. Unfortunately, this study did not include a nonexercising control group, which prevent to evaluate the effect of the ischemic condition per se [276]. Taken together, the previous and our results show that exercising with limiting oxygen supply alters ELV miRNA content in an acute and chronic manner. Additional research will be required to confirm that this modulation is different from the one observed when training at SL and to determine potential differences between systemic hypoxia and BFR, as those strategies are known to differ somewhat and to induce subtle different physiological adaptations [277].

Limitations and perspectives

Some limitations must be considered in regard of the presented results. The main limitation of our experiment resides in the relatively low number of participants. Although this number was sufficient to detect some differences in training-induced changes in ELV abundance and specific ELV miRNA between SL and hypoxia, which was the purpose of the present study. Second, when it comes to chronic exercise adaptations, other variables than training per se can come into play. Variables such as nutrition and sleep are of primary importance. Even though participants were asked to maintain their dietary habits, they did not fill dietary diary during the training program. Considering that very little is known about this, future research should investigate the impact of the different macro and micronutrients on ELV biogenesis. In addition, the participants were trained endurance athletes. Knowing the interindividual heterogeneity of adaptations to exercise and hypoxia [278-

280], caution should be taken to extend the results to other populations. Finally, the ELV release origin was not addressed because it was beyond the scope of the present study and would have required more material than the one in our possession. This is an important point to be investigated in the perspective of developing exercise mimetics and more specifically exosome-based therapy.

CONCLUSION

Previous human [167, 176] and murine [168] studies provided evidence that chronic exercise can modulate the basal levels and content of circulating ELVs, but our study is the first to suggest that this modulation could be different when training occurs under hypoxia. A 6-wk SIT program tended to increase the basal levels of circulating ELVs when performed at SL, but not in hypoxia. In contrast, ELV miRNA cargo seemed to be modulated in hypoxic conditions only. Further studies will be needed to confirm those findings and to explore the cellular mechanisms involved in this process. Moreover, further research should explore the potential differences in the origin of ELVs between normoxic and local and systemic hypoxic conditions.

GRANTS

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Results

ACKNOWLEDGMENTS

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DISCLOSURES

Exerkine Corporation is a biotechnology company that develops and commercializes therapies based on nutritional supplements, exercise-derived factors ("exerkines"), and extracellular vesicles to treat and diagnose genetic disorders, chronic diseases, and aging. Exerkine Corporation provided support in the form of salaries for M. I. N., who is a shareholder in the company. Exerkine holds numerous patents that may be used for commercial development. The other authors have no conflict of interest, financial or otherwise, to disclose. The results of the present study do not constitute endorsement by American College of Sports Medicine. The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

AUTHOR CONTRIBUTIONS

Author Contributions: L. D. had primary responsibility for the final content. G. W., M. F., and L. D. designed the study. G. W. conducted the experiment. C. E. P., O. D., J. P. N., and M. I. N. helped with technical assistance. G. W., E. D. G., O. D., and D. N. M. performed the laboratory analysis, and analyzed and interpreted the data. G. W. and L. D. wrote the manuscript. All authors commented on and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Chapter IV: General discussion, limitations, perspectives, and conclusions

This last chapter of the thesis aims to discuss the results of the three studies presented in chapter III. Some points addressed in the discussion section of the different articles will be taken up and discussed here with a global approach. In addition, our results will be compared to the most recent data of the field.

1. General discussion

The release of active biomolecules such as exerkines seems to be an important component of the adaptive process to exercise [281]. In the recent years, it has been suggested that these exerkines could be transported by EVs to reach their target. Although some studies have demonstrated the involvement of EVs in the transport of exerkines, the regulation of these vesicles by exercise remains poorly understood [136]. In this view, we decided to focus our work on the impact of both acute and chronic exercise intervention on the release of ELVs in the circulation and on their miRNA and protein content. While most of the papers included only healthy participants, we extended our studies to obese and prediabetic subjects for whom exercise has beneficial effect on various health parameters. Finally, the effect of hypoxic condition was addressed in both populations, as this additional stimulus can potentiate the effects of exercise not only for performance enhancement but also for its therapeutic effects [153-155].

Does continuous submaximal endurance exercise increase circulating ELVs?

When starting this thesis, we had clear evidence that an incremental test to exhaustion led to an increased in circulating ELVs. The well-designed studies from Frühbeis (2015) and Brahmer (2019) highlighted that those maximal efforts triggered an increase in the release of ELVs in both recreationally active and trained men [117, 128]. In addition, this increased release was observed in different endurance modalities, such as cycling and running [117]. Although some data suggested that ELV concentration could start to rise at lower intensity in these maximal tests [117], no human studies with complete vesicle characterization investigated the effect of a continuous submaximal endurance exercise on ELV release. In our first study [236], we collected blood samples before, after 30 min and at the end of a 60-min low intensity cycling exercise (~55% of VO_2max). While circulating particles were elevated after 30 min before returning to baseline, the protein markers TSG101 (+261%), CD81 (+140%), and HSP60 (+336%) were elevated after 60 min of exercise only [236]. This discrepancy between these two quantification methods confirms the previous findings from Brahmer et al. (2019) showing that NTA was unable to detect the exercise-induced increase in ELVs [128]. Together with Brahmer's results, our data suggest that NTA is not a suitable to assess ELV kinetic during exercise. Semiquantitative methods such as western blot should be preferably used because of the lower interferences with contaminating factors. Interestingly, the samples collected after 30 min of exercise did not reveal any significant alterations in the different protein markers, suggesting a slower ELV release kinetic in comparison with incremental tests. The results of our last study corroborate this hypothesis, as no changes were observed in CD9, CD81, TSG101 and

ALIX abundance after 30 min of cycling at an intensity corresponding to 60% of VO_2peak (unpublished data). Recently, three other studies investigated the effect of a submaximal continuous cycling exercise on ELV release [282-284]. After a 20-min cycling exercise at 70% of VO_2max, Chong et al. (2024) found a significant increase in TSG101 and syntenin-1 abundance [282]. Additionally, a separated analysis from the same group revealed that the exercise effect was identical in young and older men independently of fitness level [283]. Although significant, it should be noted that the observed increase in circulating ELVs was relatively modest (25-50%) when compared with the elevation found after incremental tests [117, 128] or after our 60-min continuous exercise (~200%) [236]. After 45 min of cycling at an equivalent intensity (70% of VO_2max), Doncheva and colleagues (2022) observed a slightly higher increase in CD9 abundance (~70%) [284]. Taken together, the above results show that at moderate intensity (70% of VO_2max) cycling exercise triggers modest increase in ELV release as soon as 20 min after the start of the exercise. Moreover, this release seems to be gradually greater when extending the duration. At lower intensity ($\leq$ 60% of VO_2max), the kinetic seems a little bit different, as no changes are observed after 30 min while maximal ELV accumulation seems to occur after an hour [236]. Overall, it seems that both intensity and duration influence ELV release. At submaximal intensities, higher intensity induces a quicker increase in ELV release, but longer durations seem required to induce large effects as found with maximal efforts. Figure 1 illustrates the current understanding of the ELV release kinetic during endurance exercise.

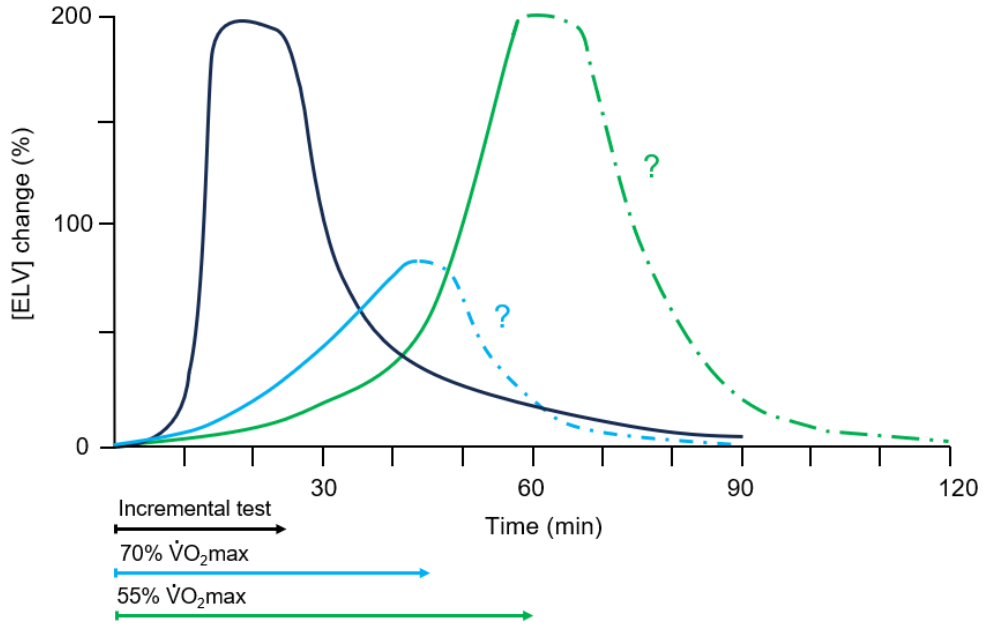


Figure 1. ELV release kinetic with different exercise intensity and duration. ELV: exosome-like vesicle. Blue, black, and green arrows represent the duration of each exercise. Solid lines represent ELV kinetics based on available data. Dash-dotted lines represent a speculative kinetics after exercise cessation for continuous submaximal efforts.

From a molecular perspective, the homeostasis disturbance through energetic and oxidative stresses has been shown to regulate ELV release. Despite the exact links are missing, energetic sensors such as mTORC1, AMPK and sirtuins but also ROS production seem to modulate ELV release [285]. For instance, mTORC1 and sirtuins inhibition and high levels of reactive oxygen species (ROS) seem to trigger an increase in ELV release [286-290]. Despite being a known antagonist of mTOR, the effects of AMPK are less clear and could be cell type-, dose and time-dependent [285]. Although each of the above-mentioned pathways are associated with ELV release regulation, further work is required to establish their specific molecular target.

While the ELV regulation by exercise presented above is valid for an exercise performed in normoxia by healthy individuals, the effect of hypoxia or prediabetic conditions on ELV release was unknown at the beginning of this thesis. Compared to chronic exposure, intermittent hypoxic training typically leads to muscular rather than systemic adaptations [153]. However, based on data from cell culture models showing greater ELV release in hypoxia [163], it was tempting to hypothesize that its additional benefits could also be ELV-related. Surprisingly, we did not observe an increase in ELV release when combining the exercise to hypoxic stimulus. We were the first to show that adding a hypoxic stimulus to low intensity cycling exercise could blunt the typical exercise-induced increase in circulating ELVs [236]. After 60 min of cycling at a simulated altitude of ~3000 m, the different vesicular markers were unchanged. Although the majority of studies have measured an increase in vesicle release by cancer, cardiac and mesenchymal cells under hypoxic conditions [163], Liu et al. have shown that the opposite is also possible in endothelial cells [291]. They observed a reduction in ELV concentration when cells were incubated under hypoxic conditions for 72 h. One of the potential underlying mechanisms involved the nSMase2 enzyme, which participates in ILV formation and was reduced under hypoxic conditions. Given that endothelial cells are known to contribute to the exercise-triggered ELV release [128], it is plausible that a decrease in ELV biogenesis in these cells have regulated the circulating pool. Adopting a macroscopic perspective, the decision to maintain equal relative intensity (internal load, heart rate corresponding to 55% $\dot{V}O_2$max) across both normoxic and hypoxic conditions in our study may also have exerted an influence [236]. Given the lower absolute exercise intensity (external load) in hypoxia, there is a possibility that the intensity did not reach a threshold conducive to elevate

ELV concentrations. It is then possible that the absence of a clear increase can be ascribed to this lower absolute intensity, rather than being solely attributable to the hypoxic environment per se. In the absence of subsequent studies conducted on the topic following the publication of our article, it is difficult to draw definitive conclusions. It remains unclear how systemic hypoxia impacts the release of ELVs during exercise and by which cellular mechanisms. Other investigations will be necessary to explore these unanswered questions.

In a similar way to what we observed with hypoxia, our first study suggested that prediabetes could also impact ELV release during exercise [236]. Indeed, no changes were observed in prediabetic subjects after either 30 or 60 min of exercise. In our third study, obese subjects, also displaying a certain degree of insulin resistance, were recruited. In the latter, no differences between lean and obese individuals were highlighted, and no increased ELV release was observed either (unpublished data). Based on what is known about ELV kinetics (see above), the exercise duration was too short to detect any group difference as ELV release begins to rise significantly beyond 30 min of exercise. Beyond our own studies, 2 other reports included obese or dysglycemic individuals [146, 284]. In the first one, Rigamonti et al. (2019) investigated the effect of a 30-min treadmill exercise (~60% of VO_2max) on ELV release in obese and normal-weight adults [146]. Contrary to our results, the authors did not identify any association between ELV response and BMI. However, as stated in the introduction section, this study only included particle analysis to assess ELV release changes, which has been shown to be inadequate to evaluate ELV response during exercise. In the second study, Doncheva et al. (2022) recruited sedentary men with or without dysglycemia

and overweight and measured ELV markers before and directly after 45 min of cycling [284]. As mentioned above, a global effect of exercise was found with a moderate increase (~70%) in ELV release. Unfortunately, no additional statistics were performed to analyze the two subgroups independently. As for hypoxia, the lack of literature on these pathological conditions prevents to extend the interpretation of our results. Further studies will be needed to clarify the impact of those specific conditions on exercise-induced ELV release.

Does chronic exercise increase basal circulating ELVs?

Contrary to acute exercise for which well-designed studies on incremental tests were available, the understanding of how long-term exercise regulate basal circulating vesicle was far from clear when starting this work. Contrasting results from rodent studies indicated that chronic interventions (2 to 8 weeks) could either result in an increase [164-166] or no changes [167, 168] in basal circulating vesicles. However, among these studies, very heterogenous methods were applied, collection period was not always stated and the ISEV guidelines were rarely entirely followed [136]. In this context, the conduction of our second and third studies allowed us to better understand how basal circulating ELV pool is regulated by regular exercise. In the first of the two studies, young, trained cyclist and triathletes were recruited to participate in a 6-week sprint interval training (SIT) protocol at different simulated altitudes [237]. In the second one, lean and obese sedentary adults (~40 y) were enrolled to follow a low intensity cycling program for 12 weeks (unpublished data). After the sprint interval training protocol, ELV abundance remained unchanged regardless of the altitude used [237]. Similarly, in response to the 12-week endurance program, we did not observe

significant changes in the different ELV marker abundance (unpublished data). However, particle diameter and CD81 abundance were respectively lower (8 nm) and higher (65%) in obese individuals in comparison with their lean counterparts suggesting the release of specific ELV subpopulations in this pathology (unpublished data). While the relatively low number of participants might have limited our statistical power in both studies, other elements could explain the lack of effect observed in our studies. First, the fitness level of the athletes involved in the sprint interval study might have been already too high to allow significant variations. Indeed, in a recent study, Darragh et al. (2023) observed that there was no differences in ELV marker abundance between recreationally active, endurance-trained (5 km running in 16 min 30 s) and strength-trained males (bench press 148 kg, back squat 228 kg and deadlift 253 kg) [292]. In this study, the recreationally active participants were engaged in ~4 h of training per week, while endurance and strength participants were training ~9 h per week. These results suggest that regular exercise without being involved in intense or sport-specific training might be enough to reach a ceiling in basal circulating ELV amount. Our cyclists and triathletes being involved in competition and practicing between 4 and 10 h per week, it is conceivable that they already reached their ceiling before taking part to the study. Secondly, in our third study, participants only trained 90 min per week at a low exercise intensity (60% of VO_2max) (unpublished data). Sport performance and several physiological adaptations being closely related to training volume and/or training intensity [293-295], the same may be true for vesicles. Our low volume, low intensity intervention might not have been sufficiently stimulating to induce marked changes. Aside from our publications, two other groups investigated the effects of chronic endurance exercise on basal circulating ELV amount in humans [167, 169].

On the one hand, the longitudinal study of Hou et al. (2019) showed no effect of regular training on vesicle quantity [167]. On the other, Apostolopoulou et al. (2021) observed an increase, but exclusively in metabolic responders displaying improved insulin resistance after training [169]. In both cases, ELVs were solely quantified by particle analysis, which limits the interpretation of the results.

While our work and previous studies focused on endurance exercise [164-169, 237], two recent studies have been conducted with a resistance modality [150, 152]. In the first one, untrained older adults (males and females, 70-85 y) participated in a resistance program twice a week for 8 weeks [150]. The participants performed 3 sets (8-12 repetitions) of 8 different exercises (6 upper and 2 lower body) with a load corresponding to 40% of 1-RM. The loads were adjusted each week (+5%) to allow optimal stimulus. Blood samples were obtained 5-6 days before and after the completion of the training program. ELVs were then isolated with ultracentrifugation. After the 8 weeks, the amount of circulating ELVs remained unchanged as assessed by abundance of EVs markers CD9, CD63, CD81 or Flot-1 [150]. In the second study, Xhuti et al. (2023) proposed a home-based resistance exercise program to older men (~75 y) [152]. The intervention lasted 12 weeks and comprised 3 sessions per week (3 sets of 10-15 repetitions for each of the 12 exercises). Different elastic bands were used to adjust and progressively increase the resistance force to ensure that adaptations occur. Blood sampling was performed prior and after the program, having not performed structured exercise for ~96 h. In response to this 3-month program, the abundance of ELV marker TSG101 was increased (~40%), suggesting an increased amount of circulating ELVs. While the load prescription is difficult to compare

between the 2 studies, the total volume (16 sessions of 24 sets vs 36 sessions of 36 sets) might explain in part the differences observed on ELV response. Indeed, the training volume was almost 4 times greater in Xhuti's study (2023) in comparison with Estebanez's experiment (2021).

Taken together, it would appear that chronic endurance or resistance exercise might lead to a higher level of basal circulating ELVs or at least of certain ELV subpopulations. Whereas young and trained individuals might be less responsive, older adults displaying lower circulating vesicles or deconditioned subjects might experience an increase after several weeks of training. Contrasting with acute exercise, if such increase does occur, the magnitude of change might be limited to a few dozen of percent [152]. In addition, a minimal threshold of training volume and intensity might be required to induce significant changes. Ultimately, similarly to what we observed for acute exercise, hypoxia and metabolic pathologies could interact with regular exercise, inhibiting the effects of the latter on basal ELV levels.

Does exercise and training modulate ELV miRNA content and proteome?

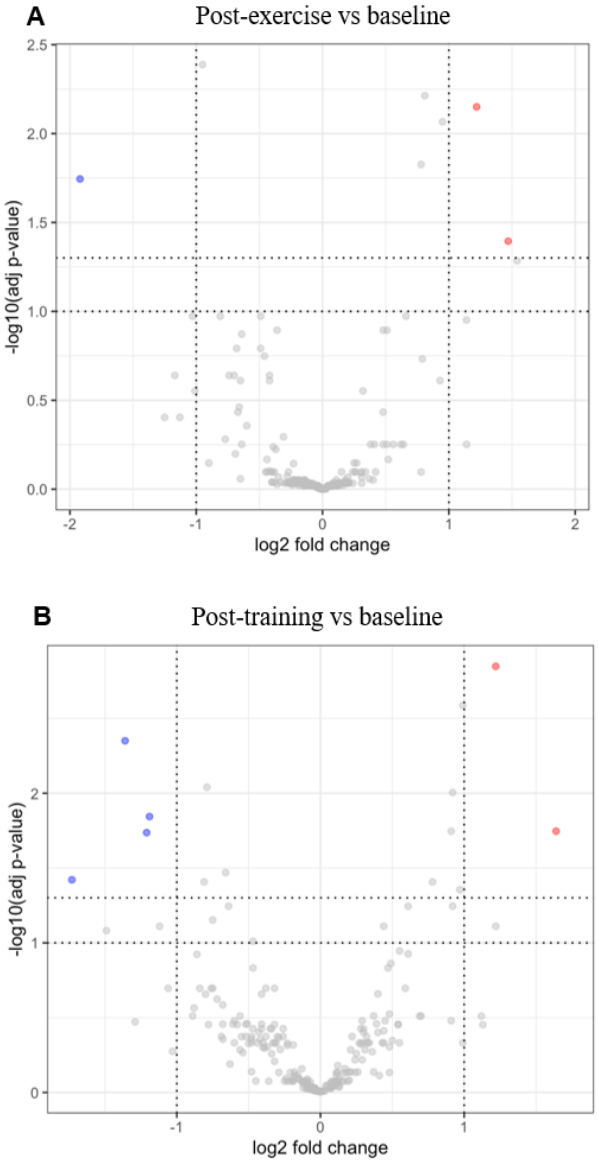
Our comprehension of how exercise and training modulate gene expression and, thus, promote health has evolved and now includes ELVs. Clear evidence indicate that vesicular content can be internalized and can regulate the metabolism of the target cell [13, 114, 115, 296]. Among the different molecules contained in ELVs, miRNAs have been extensively studied. These short-chain non-coding RNAs play a major role in translational repression by regulating post-transcriptional gene expression. In both observational and interventional studies, ELV miRNA content was shown to be regulated by exercise. Although such modulation was observed in several acute studies, human chronic interventions were lacking [136]. Moreover, the effect of

combining exercise with an environmental stimulus, such as hypoxia, was unknown. In our second study, trained cyclists and triathletes participated in a 6-week SIT protocol at different altitudes [237]. Blood samples were taken prior the intervention and 72 h after the last training session to avoid any acute effects. When analysing ELV content, we targeted a selection of miRNAs known to be responsive to acute HIIT exercise [173]. Among these RNAs, none were regulated after the 6 weeks when performing the SIT protocol at sea-level. When looking at the training performed in hypoxia, we observed heterogeneous effects, as significant abundance modulation was found at a simulated altitude of 3000 m (FiO_2=14.5%), but not at 2000 m (FiO_2=16.7%) or 4000 m (FiO_2=13.0%). The abundance of miR-21-5p was increased by the 6-week intervention, whereas the abundance of miR-23a-3p was lowered. As stated in chapter 3 (part 2), the implications of miR-21-5p seem to be diverse. This miRNA could mediate cardioprotective [273] and neuroprotective [274] effects, and also positively regulate lipid metabolism in the liver through endurance training [275]. However, the interpretation of miRNA regulation is likely a matter of context. While an increase in miR-21-5p abundance through exercise and/or training seems beneficial for health, an upregulation of this same miRNA has also been reported in a wide variety of disease states ranging from cardiovascular disease to cancer [297]. Similarly, miR-23a-3p abundance modulation leads to conflicting results depending on the model analyzed. Being associated with several cancers, the downregulation of this miRNA observed at 3000 m in our study, despite rather small, could reflect additional health benefits after training at that altitude. This finding goes in line with the results of Garai and colleagues (2021) showing that miR-23a-3p was reduced in ELV cargo with both short- (6 months) and long-term (25 y) physical activity [271]. However, the recent study from Xhuti et al. (2023)

highlighted that miR-23a-3p regulation by exercise was probably not as binary as we presented in our second article [152, 237]. First, the authors observed that miR-23a-3p abundance was lowered with aging (~75 y vs ~22 y). Secondly, rather than reducing abundance, training increased significantly miR-23a-3p, returning to levels comparable to that in young participants [152]. While miR-23a has been shown to target atrophy-related genes and inhibit their translation in skeletal muscle [298, 299], Zhang et al. (2018) also reported that miR-23a in isolated circulating ELVs can attenuate skeletal muscle atrophy by reducing the expression of TRIM63/MuRF1 and FBXO32/atrogin-1 [299]. In their study, Xhuti and colleagues observed reduced levels of miR-23a-3p in both old participant skeletal muscle biopsies and old myotubes (passage 30) [152]. Knowing that a down-regulation of miR-23a-3p has also been observed in conditions of muscle wasting [111, 298, 300] and given that sarcopenia is an age-related condition of muscle loss, the deleterious effects of reduced miR-23a-3p may contribute to sarcopenia [152]. In this view, strength training could limit sarcopenia by maintaining adequate levels of miR-23a-3p. As stated above, except for miR-21-5p and miR-23a-3p at 3000 m, no change in miRNA abundance was observed after our SIT program [237]. This lack of changes observed in most of the groups in our study might be explained by both training status and/or the miRNA selection. On the one hand, our participants were already trained (4-10 h/week) for several years before the study, which may have prevented further adaptations, especially in such a short period of time (6 weeks). When more marked effects are observed in the literature, sedentary or older individuals are recruited [152, 232]. On the other hand, the miRNA candidates were selected based on their responsiveness to an acute HIIT cycling session [173]. As often in exercise physiology, different effects can be observed between

acute and chronic exercise. For instance, a typical increase in IL-6 is observed during an acute exercise where long-term interventions rather lower the levels of this cytokine [301]. It is then conceivable that some miRNAs are regulated in response to acute exercise only while the abundance of others are modulated by chronic exercise.

Beyond the genetic cargo of ELVs, thousands of proteins have been found to be contained and transported by those vesicles [302, 303]. Despite no change was observed in circulating ELV abundance after 30 min of cycling in our third study, this short low intensity exercise was sufficient to modulate the ELV proteome. Similarly, ELV proteome was significantly modified after the whole procedure (12 weeks) without changes in circulating ELV amount (unpublished data). Interestingly, in acute and chronic configuration, the ELV proteome was not only modulated in term of abundance (Fig. 2A-B) but also in term of diversity (Fig. 2C). In lean participants, acute exercise regulated the abundance of three proteins (Fig. 2A) whereas six proteins were regulated after 12 weeks of training (Fig. 2B).


A
Post-exercise vs baseline
-log10(adj p-value)
log2 fold change
B
Post-training vs baseline
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log2 fold change

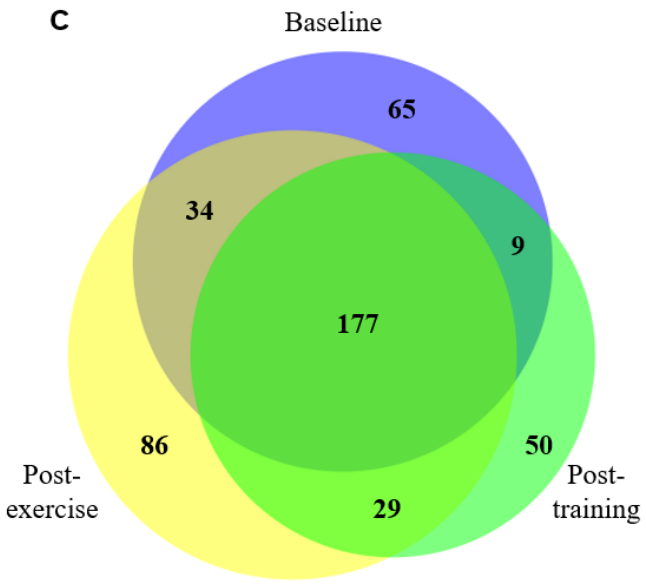

Figure 2. ELV proteome regulation by acute and chronic exercise in lean individuals. The vesicles were isolated before, directly after the first session of exercise (30 min at 60% of VO_2peak) and at the end of the 12-week protocol. A) Volcano plot of up- and downregulated proteins after acute exercise in lean group; B) Volcano plot of up- and downregulated proteins after chronic exercise in lean group. Red dots represent upregulated proteins (> 1-fold), and blue dots represent downregulated proteins (< -1-fold). C) Venn diagram of proteome diversity at baseline, post-exercise, and post-training in lean group.

Among the 450 proteins identified in this group, ~180 proteins were found in the three conditions (baseline, post-exercise, and post-training), while the remaining were specific to one or two conditions (Fig. 2C). Together, these results confirm previous findings showing that different modalities of acute [172, 177, 282, 304] and chronic [169, 305] exercise can induce significant changes in ELV protein cargo loading. While it is not surprising to see significant modulation following a HIIT session [177], the recent publication of Chong and colleagues (2024) showed that a cycling exercise as short as 20 min was sufficient to up-regulate the abundance of several proteins in ELVs. After the exercise, 13 proteins were upregulated, with a majority of them

involved in the activation of the immune system [282]. Although ELV origin was not addressed in this study, the results reinforce the notion that immune cells significantly contribute to the release of ELVs during exercise as previously described by Brahmer et al. (2019) [128]. When comparing the ELV proteome of our lean individuals with the one from obese participants, we observed significant differences in protein diversity, not only at baseline, but also after the acute exercise and the whole program (see section results part 3 and Fig. 3). This element confirms that ELV content can be altered by a metabolic impairment state, such as obesity. ELV content modulation by exercise and training seems also to be altered, as several proteins found in lean participants were absent from obese proteome (Fig. 3). In addition, less proteins were regulated after the whole program in obese participants in comparison with the lean ones (unpublished data, see section results part 3).

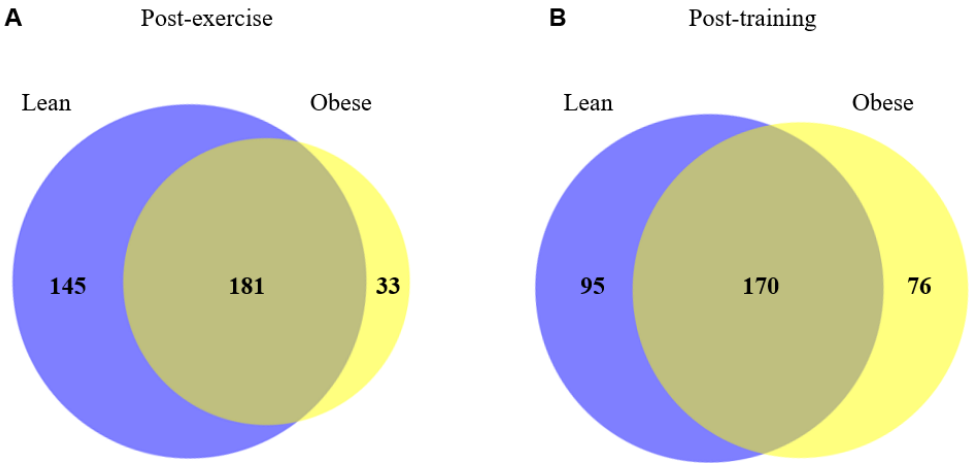

Figure 3. Differences in ELV proteome diversity between lean and obese individuals after acute and chronic exercise. A) Venn diagram of proteome diversity in post-exercise; B) Venn diagram of proteome diversity in post-training.

These results corroborate those from Apostolopoulou et al. (2021) [169]. In their study, the authors found that the proteins up- or down-regulated after 12 weeks of HIIT were different based on the metabolic status. Specific patterns of modulation were found in T2D, insulin-resistant and insulin-sensitive participants [169]. In a similar way, in Chong's paper (2024), fit and unfit participants exhibited different responses to exercise. More proteins were upregulated in ELVs after the exercise in fit individuals in comparison with their unfit counterparts [282].

Based on our data and the available literature, it is now clear that both pathological conditions and exercise can modulate the cargo transported in ELVs. In addition, interactions seem to occur, as diabetes, insulin resistance and obesity states impact the ELV response to exercise in term of cargo selection and transport. The extent to which these modifications in ELV cargo contribute to the adaptive process to exercise remains to be determined.

Do extracellular vesicles participate in exercise-mediated inflammation reduction?

Low grade inflammation being involved in several complications related to obesity, the objective of the third project was to reduce this inflammatory state in our participants through endurance training. While the involvement of ELVs in this process was suggested in the literature [231], we investigated whether the change in ELV proteome could be related to the inflammatory process.

After a single session of exercise (30 min at 60% of VO_2peak), we observed a decrease in the phosphorylation state of NF-κB in adipose tissue samples of our obese participants, but not in the skeletal muscle. After 12 weeks, NF-κB

phosphorylation state was not significantly modulated in both tissues. Additional analysis revealed that the levels of several pro-inflammatory cytokines, such as IL-1β, IL-6 and IFNγ were reduced in adipose tissue as well. Finally, the systemic inflammatory status, assessed by CRP levels, was reduced (-57%), showing that our low volume, low intensity endurance program was efficient to reduce low-grade inflammation. This last result goes in line with a recent meta-analysis conducted in overweight and obese adults showing that endurance programs of different durations (8 weeks to 2 years) lead to a reduction in CRP ranging from -9% to -53% [219]. In some of the studies included in this review, this reduction was concomitant to a fat mass diminution. In our study, it was not the case, suggesting an indirect effect of exercise training to reduce CRP levels, possibly through ELVs.

When looking at the proteins regulated by acute and chronic interventions in our obese participants, we found none specifically involved in the inflammation process. However, both configurations increased the abundance of antioxidant enzyme peroxiredoxin (PRDX) family members (unpublished data). Those results go in line with several recent studies showing an upregulation of proteins involved in the antioxidant pathway after acute and chronic exercise [169, 172, 283, 304, 306, 307]. For instance, Abdelsaid et al. (2022) showed that 45 min at only 50% of VO_2max was sufficient to increase the abundance of ELV superoxide dismutase 3 (SOD3) (~50%) in sedentary adults. Additionally, when comparing sedentary and exercised (2 weeks) mice, the authors observed higher levels of SOD3 in the latter (~100%) [172]. This effect of chronic exercise is also corroborated by two other studies. In the first one, Lisi and colleagues (2023) highlighted higher ELV SOD2 abundance in trained young males when compared to their untrained

counterparts [306]. In the second one, Apostolopoulou et al. (2021) found that a 12-week HIIT program upregulated the abundance of ELV peroxiredoxin-1 and 2 as well as SOD2 [169]. Taken together those results highlight a consistent antioxidant response within ELVs with both acute and chronic exercise. Knowing that ROS can activate gene transcription-related to inflammation [308], it is plausible that ELVs could participate in the inflammation reduction through the control of oxidative stress via antioxidant enzymes such as SOD and PRDX. In addition, both SOD and PRDX family proteins have been shown to act directly and/or indirectly on the NF-κB or inflammasome pathways, ultimately inhibiting the latter [309-314]. However, as the different isoforms of these enzymes are active in specific cellular compartments, it remains unclear how they can reach these sites. While PRDX 1, 2 and 6 have cytosolic actions, SOD3 regulates extracellular superoxide and SOD2 is present in the mitochondria [315-317]. Regarding SOD3, dynamic uptake and release through endosomal compartment have been highlighted in macrophages [318]. After ELV internalization, SOD3 could be stored in intracellular vesicles before further release at the plasma membrane to modulate extracellular space redox state. In their study, Abdelsaid and colleagues (2019), observed an increase in H_2O_2 in endothelial cells incubated with ELVs overexpressing SOD3 [172]. Conversely, the functional role of ELV SOD2 remains to be determined as this enzyme typically reaches mitochondria through specific signal sequence [319, 320]. Apart from the ELV proteome modulation, other elements present in ELVs might be involved in the exercise-related inflammation reduction. As described above and in the introduction section, exercise and training have been shown to regulate specific ELV miRNAs. More specifically, after a single week of concurrent training, Sullivan and colleagues (2022) observed

a significant modulation of muscle-derived ELV miRNAs in obese individuals. These miRNAs are known to target mRNAs related to inflammation processes, suggesting that the potential anti-inflammatory effects of ELVs could occur at a translational level [232]. In a more indirect way, chronic endurance intervention has also been shown to reduce ELV miRNAs associated with impaired adipogenesis, increased insulin and increased fat mass [233]. Such downregulation might be one of the mechanisms explaining the positive outcomes measured in the adipose tissue after 8 weeks of training. The authors observed an increased number of small adipocytes and a reduced number of larger adipocytes, an increased expression of adipogenesis markers, and an increased activity of lipogenesis enzymes [233]. These positive adaptations might in turn participate in the reduction of NF-κB activity, as excessive fat storage can trigger the inflammatory process [188, 189]. Together, the above findings indicate that acute and chronic exercise seem to regulate antioxidant and miRNA ELV content rather than ELV pro- and anti-inflammatory proteins. After the ELV internalization, these molecules could act on the target cell inflammatory pathway and thus reduce its activation (Fig. 4).

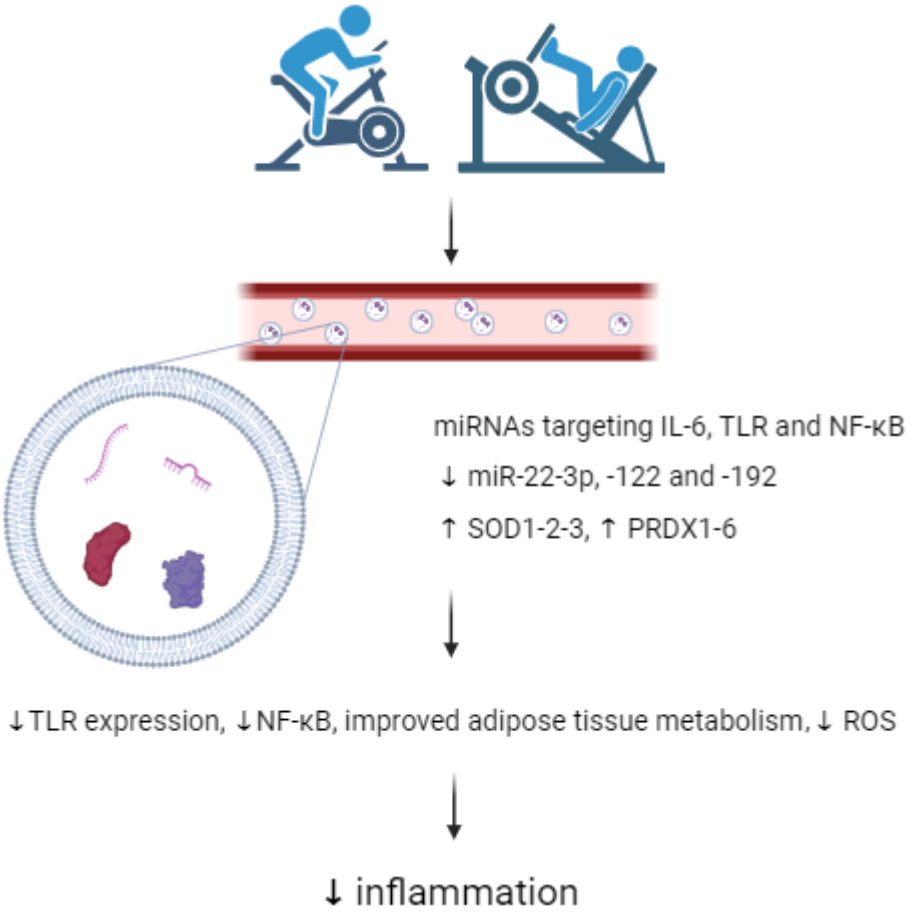

Figure 4. Exercise and potential anti-inflammatory effects of ELVs. TLR, Toll-like receptor; NF-κB, nuclear factor-kappa; SOD, superoxide dismutase; PRDX, peroxiredoxin; ROS, reactive oxygen species.

2. Limitations and perspectives

Our main goal was to explore the effect of physical exercise on ELV release. Across our 3 studies, we recruited just over 80 participants, comprising trained, sedentary, obese, and pre-diabetic individuals. However, only male participants were included. Knowing that both sex and menstrual phase seem

to influence MV release [147-149], it would be interesting to investigate whether it is also the case for smaller vesicles as ELVs. Although some studies suggest differences in ELV release during exercise between men and women [146, 321], robust studies following international guidelines are still required.

As the question of relative versus absolute intensity is inherent to the assessment of the effects of hypoxia, it could be interesting to compare a normoxic control condition with two different hypoxic conditions. A first one with an equal relative intensity (internal load) as found in our first study. A second one with an equal absolute intensity (external load). Such experiment could unravel whether it is hypoxia per se or the reduced power output that blunts the release of ELVs during endurance exercise when performed in systemic hypoxia.

Whereas our first two studies were more descriptive, mainly investigating quantitative changes in ELV release, the third was more integrative. Inflammatory pathways were addressed in different tissues, at the systemic level and in ELVs. However, causality between the changes observed in the vesicle proteome and the systemic and local inflammation reduction could not be established. While it was planned to finalize the project with in vitro experiments on human myotubes and 3T3-L1 adipocytes, these could not be conducted by the redaction of this thesis. The purpose was to investigate the potential anti-inflammatory effects of exercise-derived vesicles on TNF-α-induced inflammation in differentiated myotubes and adipocytes. Future studies combining human and cellular experiments are needed to better understand the mechanisms by which ELVs reduce inflammation.

3. Conclusion

While previous publications highlighted that incremental maximal efforts could increase the circulating ELVs, we showed that it was also the case with low intensity endurance exercise in a duration/intensity-dependent manner. Moreover, this typical increase in ELVs by exercise seemed to be blunted in prediabetes and in hypoxic conditions. Contrasting with the acute effects, the impact of long-term training is less clear and might be limited to a smaller magnitude of change, especially in already trained populations. Here as well, hypoxia and metabolic pathologies could interact with regular exercise, inhibiting the effects of the latter on basal ELV levels. Beyond purely quantitative changes, exercise and regular training modulate ELV cargo as well. A single session of 30 min was sufficient to regulate ELV proteome diversity and abundance. Similarly, a whole endurance program of 12 weeks further regulated ELV protein cargo in lean men and in obese individuals with low-grade inflammation. In this context, it seems that the potential anti-inflammatory effects of ELVs could be related to changes in antioxidant enzymes abundance rather than a modulation of pro- or anti-inflammatory proteins in these vesicles.

Overall, while much work remains to be done, our three studies have contributed to a better understanding of both the acute and chronic effects of exercise on ELV regulation.

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Appendix

Appendix 1:

Supplementary Table 1. RNA primers.

Gene	Forward primer	Reverse primer
CD68	TGGGCAAAGTTTCTCCTG	GGATGATGCAGAAAGCAATAAG
PGC-1α	CACTTACAAGCCAAACCAACAACT	CAATAGTCTTGTTCTCAAATGGGGA
PDCD6IP	GGTGCTAGCTTCCCTTAATC	GATGCCTCCCTGTTCAATC
TSG101	CATTCCCACAGCTCCCTTATAC	CTCAAGGCTTCTCCCAAGTAAA
CD9	GATTGCTGTCCTTGCCATTG	CTCCTGTGTAGAAGCTGGAATTA
CD81	CCTGCTCTTCGTCTTCAATTTC	TCTCCCAGCTCCAGATACA
B2M	ATGAGTATGCCTGCCGTGTGA	GGCATCTTCAAACCTCCATG
PPIA	GCTGGACCCAACACAAAT	TCCATGGCCTCCACAATA

Appendix 2:

Review > Front Physiol. 2021 Feb 1:11:604274. doi: 10.3389/fphys.2020.604274. eCollection 2020.

Extracellular Vesicles and Exosomes: Insights From Exercise Science

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Affiliations + expand

PMID: 33597890 PMCID: PMC7882633 DOI: 10.3389/fphys.2020.604274

Free PMC article

Abstract

The benefits of exercise on health and longevity are well-established, and evidence suggests that these effects are partially driven by a spectrum of bioactive molecules released into circulation during exercise (e.g., exercise factors or 'exerkines'). Recently, extracellular vesicles (EVs), including microvesicles (MVs) and exosomes or exosome-like vesicles (ELVs), were shown to be secreted concomitantly with exerkines. These EVs have therefore been proposed to act as cargo carriers or 'mediators' of intercellular communication. Given these findings, there has been a rapidly growing interest in the role of EVs in the multi-systemic, adaptive response to exercise. This review aims to summarize our current understanding of the effects of exercise on MVs and ELVs, examine their role in the exercise response and long-term adaptations, and highlight the main methodological hurdles related to blood collection, purification, and characterization of ELVs.

Keywords: EV isolation; aerobic exercise; circulation; exerkine; exosome; extracellular vesicle; resistance exercise; size-exclusion chromatography.

Appendix 3:

› Sports (Basel). 2020 Nov 18;8(11):148. doi: 10.3390/sports8110148.

Effects of Sprint Interval Training at Different Altitudes on Cycling Performance at Sea-Level

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PMID: 33217937 PMCID: PMC7698804 DOI: 10.3390/sports8110148

Free PMC article

Abstract

Background: Benefits of sprint interval training performed in hypoxia (SIH) compared to normoxia (SIN) have been assessed by studies mostly conducted around 3000 m of simulated altitude. The present study aims to determine whether SIH at an altitude as high as 4000 m can elicit greater adaptations than the same training at 2000 m, 3000 m or sea-level.

Methods: Thirty well-trained endurance male athletes (18-35 years old) participated in a six-week repeated sprint interval training program (30 s all-out sprint, 4 min 30 s recovery; 4-9 repetitions, 2 sessions/week) at sea-level (SL, n = 8), 2000 m (F_iO_2 16.7%, n = 8), 3000 m (F_iO_2 14.5%, n = 7) or 4000 m (F_iO_2 13.0%, n = 7). Aerobic and anaerobic exercise components were evaluated by an incremental exercise test, a 600 kJ time trial and a Wingate test before and after the training program.

Results: After training, peak power output (PPO) during the incremental exercise test increased (~6%) without differences between groups. The lactate threshold assessed by Dmax increased at 2000 m (+14 ± 12 W) and 4000 m (+12 ± 11 W) but did not change at SL and 3000 m. Mean power during the Wingate test increased at SL, 2000 m and 4000 m, although peak power increased only at 4000 m (+38 ± 38 W).

Conclusions: The present study indicates that SIH using 30 s sprints is as efficient as SIN for improving aerobic and anaerobic qualities. Additional benefits such as lactate-related adaptations were found only in SIH and Wingate peak power only increased at 4000 m. This finding is of particular interest for disciplines requiring high power output, such as in very explosive sports.

Keywords: Wingate test; cycling; hypoxia; lactate threshold; repeated sprint training; time trial.

Appendix 4:

› FASEB J. 2021 Aug;35(8):e21773. doi: 10.1096/fj.202100654RR.

Higher strength gain after hypoxic vs normoxic resistance training despite no changes in muscle thickness and fractional protein synthetic rate

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PMID: 34324735 DOI: 10.1096/fj.202100654RR

Abstract

Acute hypoxia has previously been suggested to potentiate resistance training-induced hypertrophy by activating satellite cell-dependent myogenesis rather than an improvement in protein balance in human. Here, we tested this hypothesis after a 4-week hypoxic vs normoxic resistance training protocol. For that purpose, 19 physically active male subjects were recruited to perform 6 sets of 10 repetitions of a one-leg knee extension exercise at 80% 1-RM 3 times/week for 4 weeks in normoxia (FiO_2 : 0.21; n = 9) or in hypoxia (FiO_2 : 0.135, n = 10). Blood and skeletal muscle samples were taken before and after the training period. Muscle fractional protein synthetic rate was measured over the whole period by deuterium incorporation into the protein pool and muscle thickness by ultrasound. At the end of the training protocol, the strength gain was higher in the hypoxic vs the normoxic group despite no changes in muscle thickness and in the fractional protein synthetic rate. Only early myogenesis, as assessed by higher MyoD and Myf5 mRNA levels, appeared to be enhanced by hypoxia compared to normoxia. No effects were found on myosin heavy chain expression, markers of oxidative metabolism and lactate transport in the skeletal muscle. Though the present study failed to unravel clearly the mechanisms by which hypoxic resistance training is particularly potent to increase muscle strength, it is important message to keep in mind that this training strategy could be effective for all athletes looking at developing and optimizing their maximal muscle strength.

Keywords: deuterium; hypoxia; muscle thickness; myogenesis; protein synthesis.

Appendix 5:

› Med Sci Sports Exerc. 2024 May 15. doi: 10.1249/MSS.0000000000003485. Online ahead of print.

Repeated Sprint Training in Hypoxia Improves Repeated Sprint Ability to Exhaustion Similarly in Active Males and Females

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PMID: 38767990 DOI: 10.1249/MSS.0000000000003485

Abstract

Purpose: The aim of this study was to compare the physiological adaptations of males and females to repeated sprint training in hypoxia.

Methods: Active males and females completed 7 weeks of repeated sprint training in normoxia (RSN, FiO2 = 0.209, males: n = 11, females: n = 8) or hypoxia (RSH, FiO2 = 0.146, males: n = 12, females: n = 10). Before (Pre-) and after (Post-) training, a repeated sprint ability test (RSA) was performed (10 s cycle sprints with 20 s recovery between sprints, until exhaustion), and aerobic and anaerobic qualities were evaluated in normoxia.

Results: The number of sprints during RSA increased after training in HYP from 11 to 21 in males and from 8 to 14 in females ($p < 0.001$, CI = [5, 11]), without significant changes after RSN (10 vs 14 and 8 vs 10 in males and females, respectively). No improvements in mean or peak power output were found in either group. Total work during RSA improved after training in all groups (+9 ± 2 kJ, $p < 0.001$). Tissue saturation index (TSI) during the repeated sprints was higher in females than males (+10 ± 2 %, $p < 0.001$). The difference in TSI between the recovery and sprint phases remained unchanged after training. VO2peak during an incremental exercise test increased in all groups (+3 ± 1 ml·kg-1·min-1, $p = 0.039$). Mean power output during a Wingate test also increased in both males and females in RSN and RSH (+0.38 ± 0.18 W·kg-1, $p = 0.036$). No changes were observed in hematological parameters after training.

Conclusions: Seven weeks of RSH further increased the number of repeated sprints performed to exhaustion compared to RSN in females, in the same order of magnitude as in males.

Appendix 6:

› Front Bioeng Biotechnol. 2020 Nov 5:8:565679. doi: 10.3389/fbioe.2020.565679. eCollection 2020.

Acute and Chronic Effects of High Frequency Electric Pulse Stimulation on the Akt/mTOR Pathway in Human Primary Myotubes

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PMID: 33224929 PMCID: PMC7674644 DOI: 10.3389/fbioe.2020.565679

Free PMC article

Abstract

Electrical pulse stimulation (EPS) has been suggested to be a useful method to investigate the mechanisms underlying the adaptations of human skeletal muscle to both endurance and resistance exercise. Although different myotube stimulation protocols mimicking acute and chronic endurance exercise have been developed, no convincing protocol mimicking resistance exercise exists. Adaptations to resistance exercise mainly ensue via the Akt/mTOR pathway. Therefore, the aim of this study was to develop a high frequency EPS protocol mimicking resistance exercise both acutely (100 Hz, 15 V, 0.4 ms with 4 s rest between each contraction for 30 min) and chronically (acute EPS protocol repeated on three consecutive days) on human myotubes. Compared to control conditions, the acute EPS protocol increased the phosphorylation of Akt^{Ser473} at 0 h (+91%, p = 0.02) and 3 h (+95%, p = 0.01), and $mTOR^{Ser2448}$ at 0 h (+93%, p = 0.03), 1 h (+129%, p = 0.01), and 3 h (+104%, p = 0.0250) post-stimulation. The phosphorylation of $ERK1/2^{Thr202/Tyr204}$ was increased at 0 h (+69%, p = 0.02) and 3 h (+117%, p = 0.003) post-stimulation compared to control conditions. In addition, both $S6K1^{Thr389}$ (+157%, p = 0.009) and $S6^{Ser240/244}$ (+153%, p = 0.003) phosphorylation increased 1 h after EPS compared to control conditions. Chronic EPS protocol increased the phosphorylation of $S6K1^{Thr389}$ 1 h (+105%, p = 0.03) and 3 h (+126%, p = 0.02) and the phosphorylation of $S6^{Ser240/244}$ 1 h (+32%, p = 0.02) after the end of the last stimulation. In conclusion, the present work shows that human muscle cells subjected to EPS can be used as an *in vitro* model of acute and chronic resistance exercise.

Keywords: Akt; ERK1/2; cell culture; high frequency; mTOR.

Appendix 7:

Randomized Controlled Trial > Am J Physiol Endocrinol Metab. 2021 Jan 1;320(1):E43-E54.
doi: 10.1152/ajpendo.00263.2020. Epub 2020 Oct 26.

Effect of hypoxic exercise on glucose tolerance in healthy and prediabetic adults

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Free article

Abstract

This study aimed to investigate the mechanisms known to regulate glucose homeostasis in human skeletal muscle of healthy and prediabetic subjects exercising in normobaric hypoxia. Seventeen healthy (H; 28.8 ± 2.4 yr; maximal oxygen consumption ($\dot{V}O_{2max}$): 45.1 ± 1.8 mL·kg^{-1}·min^{-1}) and 15 prediabetic (P; 44.6 ± 3.9 yr; $\dot{V}O_{2max}$: 30.8 ± 2.5 mL·kg^{-1}·min^{-1}) men were randomly assigned to two groups performing an acute exercise bout (heart rate corresponding to 55% $\dot{V}O_{2max}$) either in normoxic (NE) or in hypoxic (HE; fraction of inspired oxygen [Formula: see text] 14.0%) conditions. An oral glucose tolerance test (OGTT) was performed in a basal state and after an acute exercise bout. Muscle biopsies from m. vastus lateralis were taken before and after exercise. Venous blood samples were taken at regular intervals before, during, and after exercise. The two groups exercising in hypoxia had a larger area under the curve of blood glucose levels during the OGTT after exercise compared with baseline (H: +11%; P: +4%). Compared with pre-exercise, an increase in p-TBC1D1 Ser237 and in p-AMPK Thr172 was observed postexercise in P NE (+95%; +55%, respectively) and H HE (+91%; +43%, respectively). An increase in p-ACC Ser212 was measured after exercise in all groups (H NE: +228%; P NE: +252%; H HE: +252%; P HE: +208%). Our results show that an acute bout of exercise in hypoxia reduces glucose tolerance in healthy and prediabetic subjects. At a molecular level, some adaptations regulating glucose transport in muscle were found in all groups without associations with glucose tolerance after exercise. The results suggest that hypoxia negatively affects glucose tolerance postexercise through unidentified mechanisms.**NEW & NOTEWORTHY** The molecular mechanisms involved in glucose tolerance after acute exercise in hypoxia have not yet been elucidated in human. Due to the reversible character of their status, prediabetic individuals are of particular interest for preventing the development of type 2 diabetes. The present study is the first to investigate muscle molecular mechanisms during exercise and glucose metabolism after exercise in prediabetic and healthy subjects exercising in normoxia and normobaric hypoxia.

Keywords: AMPK; GLUT4; OGTT; cycling; diabetes.

Appendix 8:

FASEB J. 2020 Jan;34(1):1885-1900. doi: 10.1096/fj.201902244R. Epub 2019 Dec 10.

Acute environmental hypoxia potentiates satellite cell–dependent myogenesis in response to resistance exercise through the inflammation pathway in human

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PMID: 31914659 DOI: 10.1096/fj.201902244R

Abstract

Acute environmental hypoxia may potentiate muscle hypertrophy in response to resistance training but the mechanisms are still unknown. To this end, twenty subjects performed a 1-leg knee extension session (8 sets of 8 repetitions at 80% 1 repetition maximum, 2-min rest between sets) in normoxic or normobaric hypoxic conditions (FiO2 14%). Muscle biopsies were taken 15 min and 4 hours after exercise in the vastus lateralis of the exercised and the non-exercised legs. Blood samples were taken immediately, 2h and 4h after exercise. In vivo, hypoxic exercise fostered acute inflammation mediated by the TNFα/NF-κB/IL-6/STAT3 (+333%, +194%, + 163% and +50% respectively) pathway, which has been shown to contribute to satellite cells myogenesis. Inflammation activation was followed by skeletal muscle invasion by CD68 (+63%) and CD197 (+152%) positive immune cells, both known to regulate muscle regeneration. The role of hypoxia-induced activation of inflammation in myogenesis was confirmed in vitro. Acute hypoxia promoted myogenesis through increased Myf5 (+300%), MyoD (+88%), myogenin (+1816%) and MRF4 (+489%) mRNA levels in primary myotubes and this response was blunted by siRNA targeting STAT3. In conclusion, our results suggest that hypoxia could improve muscle hypertrophic response following resistance exercise through IL-6/STAT3-dependent myogenesis and immune cells-dependent muscle regeneration.

Keywords: exercise; hypoxia; inflammation; muscle; myogenesis.