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**Detrimental effects of environmental
pollutants on adult and induced pluripotent
stem cells-derived myogenic progenitor cells.**

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ABSTRACT

Environmental pollutants have been proven to exert detrimental effects on organisms and their burden on public health is nowadays a real concern. Among all the pollutants, ozone (O₃) and polystyrene nanoparticles display diffusive behaviour responsible for accumulation and toxicity in many tissues both at the foetal level and in the adult organism. Notably, pollutants have been associated to alterations in microRNAs (miRNAs) expression, including miRNAs involved in muscle function and tissue regeneration known as myo-miRNA. Thus, the aim of this study is to evaluate the effect of O₃ and polystyrene nanoparticles on human myogenic cells using two distinct models: adult myogenic progenitor cells (hMuSCs) and induced pluripotent stem cell-derived myogenic progenitor cells (iPSC-hMuSCs), as foetal model. Regarding the first model, hMuSCs were treated with O₃ (120 ppb) and polystyrene nanoparticles of different sizes (100, 200, 600 and 800 nm) at two dilutions (1:1000, 1:2000), used either alone or in combination. Then, measurements of cells viability, differentiation capacity and miRNAs expression (miR-1, miR-133a/b, miR-206, and miR-23a/b) offered insights into the harmful effects of pollutants on hMuSCs. Additionally, superoxide anion levels were assessed, considering that pollutants are known to enhance the oxidative stress. For the foetal model, iPSC-hMuSCs were exposed to O₃ (120 ppb) and 100 nm nanoparticles diluted 1:1000, tested both alone and in combination. Cell viability, differentiation capacity, and miRNAs (miR-1, miR-133a/b, miR-206, and miR-23 a/b) expression were evaluated similarly to the first project. The results showed a negative impact of both O₃ and nanoparticles on myogenic progenitors, highlighting the detrimental effects of these pollutants on skeletal muscle tissues both on the adult and foetal muscles.

Detrimental effects of environmental pollutants on adult and induced pluripotent stem cells-derived myogenic progenitor cells.

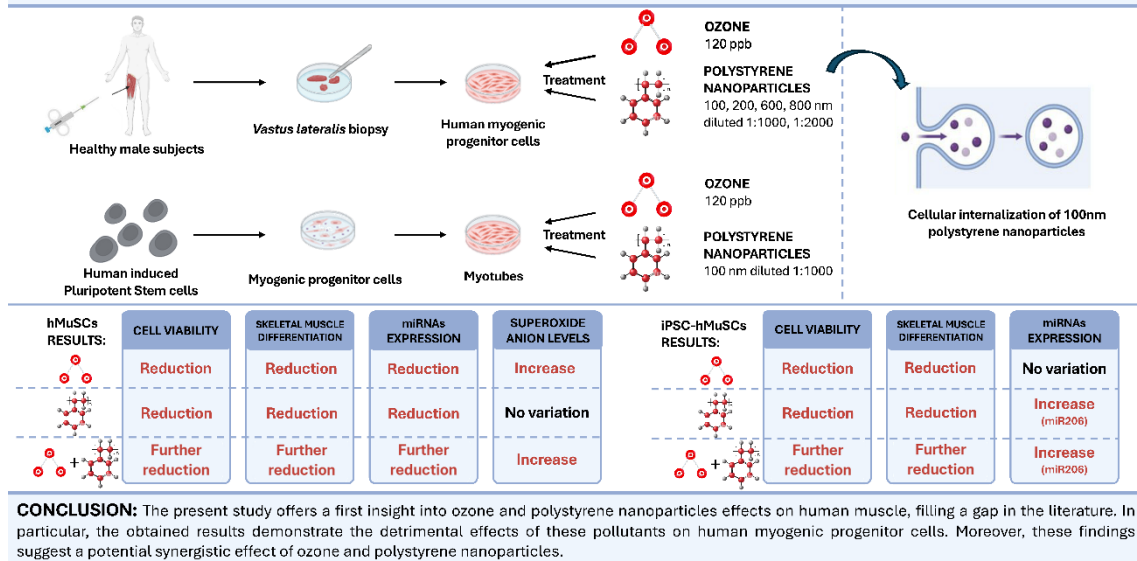


Fig. 0 Graphical Abstract.

CHAPTER 1

INTRODUCTION

1.1 Environmental pollutants

The literature of the last decades rose the attention of the scientific community toward chemicals and hazardous compounds capable to threaten human health. Referring to them as “environmental pollutants”, researchers all around the world are describing everywhere the presence of diffusive and persistent compounds correlating them to clinical evidence. Environmental pollutants are generated by human activity as well as natural events. Events such as volcanic eruptions, natural oil seeps, methane and radon emissions, dust storms, and wildfires are few examples of the involvement of nature in the spreading of environmental pollutants. Considering the nature-dependant diffusion of pollutants as the baseline, human activities strongly exacerbate the effects of these naturally occurring events bringing the levels of environmental pollutants far beyond safety thresholds. Indeed, anthropogenic sources of environmental pollutants are the most abundant in the world, contaminating soil, air, and water with no exceptions [1]. Such contamination can occur because of normal human activity as well as due to carelessness of developed countries in which there is often no interest in adopting precautionary conditions. On the contrary, poor developed countries are not always able to implement new technologies for waste management, as it can require funds. At any rate, pollutants are spread worldwide in the end [2]. For instance, wastewater, pesticides, industries’ byproducts, chemicals used in industrial activities, petroleum-derived products, and domestic livestock represents few sources of soil contaminants. Such contaminants can be spread directly across the soil or precipitate on it from atmosphere. In fact, incomplete combustion of substances, smelting, nuclear accidents, radionuclide deposition from weapon testing can lead to pollutants deposition onto the soil. Generally, soil pollutants can release harmful molecules after degradation, among which heavy metals like mercury, arsenic, and lead, polychlorinated biphenyls (PCBs), phthalates, dioxins, bisphenol A, polycyclic aromatic hydrocarbons (PAHs). As soon as exposure to such soil occurs, both through direct contact or indirect (e.g. crops), soil pollutants can interact with the human body entering the organism via nose, mouth or skin, leading to a plethora of health

problems [3,4]. Air pollutants are equally harmful. Sulphur dioxide (SO₂), ozone (O₃), nitrogen oxides (NO_x), heavy metals, carbon monoxide (CO) and many other are pollutants released into the air by industries and power plants, vehicle emissions, burning wastes and so on. Inhalation, but also ingestion and skin contact, are main ways for their absorption causing detrimental health issues [5,6]. Similarly to soil and air, water pollution is a major concern due to the relevance of water for both artificial and organic activities. Wastes from industrial, agricultural, radioactive, domestic activities are released in the water, spreading detergents, organic industrial compounds, other cleaning substances, toxins, pesticides with chloroacetanilides and triazines, heavy metals and many others [4,7]. New pollutants that can be found in soil, air, and water are personal care products, endocrine disruptors, industrial chemicals, pharmaceuticals, viruses, bacteria, and so on. Such pollutants are non-monitored “emerging pollutants” that can enter the environment in many ways, ending in undesired accumulation in the ecosystem and eventually in the food chain. Although they are unprovided of a regulatory framework, the impact of emerging pollutants on human health can be harmful [8,9]. Overall, addressing the issue of environmental pollution requires a multidisciplinary approach for a proper detection of pollutants, their identification, regulation, and prevention of further spreading.

Among all the cited pollutants, polystyrene nanoparticles and O₃ are known for their diffusive behaviour and toxicity when inside the human body, playing a detrimental role on human health. In the following section such pollutants will be described in depth, highlighting their effects on specific tissues and organs. Finally, an overall depiction of environmental pollutants and their effect on human health will be displayed. Overall, the narrative will proceed in order to dissect the involvement of pollutants’ toxicity in human skeletal muscles, aiming to fill the literature gap about this detrimental scenario. Moreover, an in-depth functional and molecular study of the skeletal muscles system will be provided, followed by a description of the models chosen for the present study and a state-of-the-art picture of skeletal muscle-pollutants harmful interactions.

1.2 Polystyrene nanoparticles

Accounting for at least the 70% of water pollution, plastics are widespread synthetic organic polymers produced in greater amounts year-by-year. Among all the plastic,

polystyrene displays interesting properties. It is an aromatic polymer produced starting from styrene monomers, which are in their turn obtained from catalytic dehydrogenation of benzene and ethylene [10]. Thanks to its thermoplastic properties, with high durability and translucency, polystyrene ended to be used in producing many objects for everyday use, among which CDs, toothbrushes, cups, plates, but also toys, trays, containers and office supplies [11]. Although polystyrene shows many interesting and versatile properties, such plastic is not bio-degradable ending to cause widespread environmental pollution. Only a small fraction of the total polystyrene produced is recycled by re-using it with unchanged form or by incineration at 1000°C. The only way to handle polystyrene biologically requires the involvement of microorganisms which decompose such plastic, but with low efficiency and productivity [12–14]. When released into the environment, polystyrene may undergo fragmentation caused by abrasion, biodegradation, or UV rays, spreading smaller particles of micrometric and nanometric sizes [15–17]. Such small particles can be identified starting from their source in primary and secondary. For instance, primary sources can be industries and pharmaceuticals which produce polystyrene-based objects like cosmetics or electronic devices. On the other hand, secondary sources are based on improper waste management of primary plastics which disintegrate them into smaller units under chemical or physical modalities [18]. In particular, polystyrene nanoparticles, due to their size, easily enter the human body by mouth or skin, but evidence of hair passive absorption have been found as well [19–21]. Their toxicity is strictly linked to accumulation levels and nanoparticles size, causing oxidative stress, inflammation, immunotoxicity, and cytotoxicity [20]. Once inside the cells, polystyrene nanoparticles alter structure and function. An example of this is the impairment of mitochondria stability by nanoparticles, causing increased levels of Reactive Oxygen Species (ROS) and release of proinflammatory and proapoptotic factors, eventually ending in cell death [22,23]. The entrance of polystyrene nanoparticles inside cells can occur via pinocytosis, phagocytosis, or passive transport through the cell membrane [24,25]. Moreover, such nanoparticles can bind proteins thereby forming the protein corona which defines the biological identity of nanoparticles helping them to remain dissolved in biological fluids. In this way, complexes protein-nanoparticle will be recognized by cells, tissues, and organs [26]. Of particular interest is the impact of

polystyrene nanoparticles on the skeletal muscle system. However, it will be discussed in the last chapter.

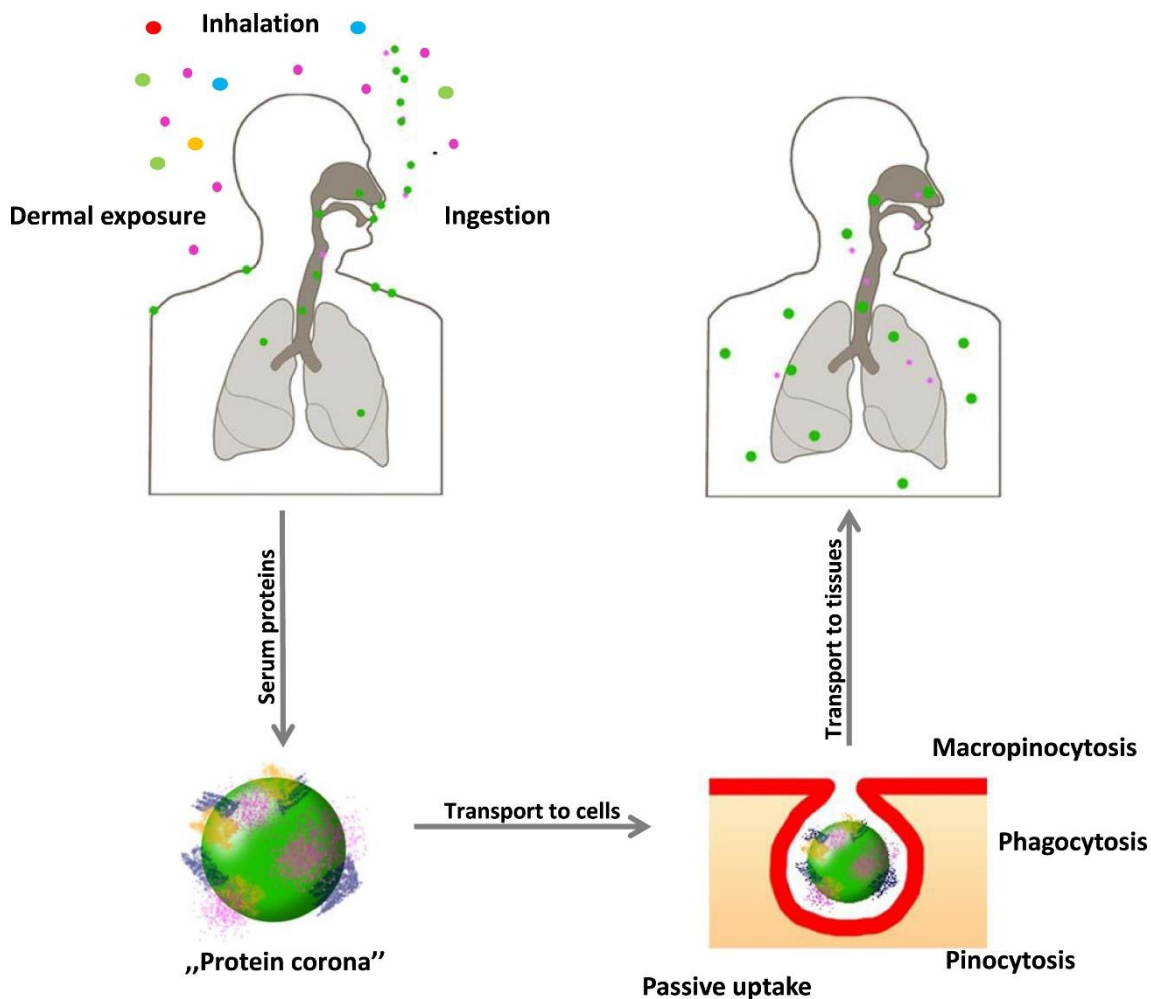


Fig. 1 Nanoparticles absorption by humans [11].

1.3 Ozone (O₃)

O₃ is an extremely reactive gas which poses a relevant health hazard worldwide. Exposure to such pollutants has been linked to increased morbidity and mortality, with children and elderly greatly sensitive to O₃ toxic effects [27,28]. Chronic diseases, mucosal irritation, systemic inflammation, hypersensitivity, decrease of function in lungs, enhanced fibrinolysis, cardiovascular features, and dyslipidemia are only few examples of the detrimental effects of O₃ exposure [29]. In polluted environments, the action of UV rays on volatile organic compounds or nitric dioxide (NO₂) leads to the formation of nitric oxide (NO) and a highly reactive singlet oxygen, which will react with oxygen thereby producing O₃, a major pollutant in urban areas [29]. Thus, O₃ can be found naturally. For

instance, atmospheric O_3 is responsible for the formation of the O_3 layer in the stratosphere which offers a reliable barrier for harmful UV rays. However, O_3 spreading at lower altitudes like at tropospheric or ground levels can cause all the detrimental effects that occur after direct contact with such reactive gas damaging all the living beings. Furthermore, O_3 can be produced and released in massive amounts by human activities like industrial processes or vehicles exhaust. O_3 can be also produced exploiting the volatile organic compounds released by biomass and fossil fuel combustion, gasoline evaporation or gaseous fuels, pesticides, painting inks, cleaning agents and so on. Solar intensity is pivotal for volatile organic compounds conversion and O_3 formation, with levels of O_3 increased where the release of volatile organic compounds is high [29]. Thus, the worldwide spreading of O_3 and its raising levels made such pollutant a great challenge for the environment [30]. O_3 absorption can vary based in airways size, with children and women displaying the higher levels of absorption [31]. Once inside the human body, O_3 induces oxidative stress and inflammation in the upper airways: its toxicity is strictly dependent on concentration and length of exposure [32]. Indeed, O_3 is a powerful oxidant and most of its toxicity is mediated via reactive free radicals, even though O_3 is not a radical species [33,34]. Due to O_3 low solubility in water, most of the inhaled pollutant reaches the airways lower tract where the epithelial lining fluid is present. Here, reactions between O_3 and lipids, proteins and antioxidant compounds contained in the epithelial lining fluid cause the pollutant to dissolve releasing reactive byproducts responsible for spreading of oxidative stress and inflammation in the respiratory tract and to tissues and organs beyond [35,36].

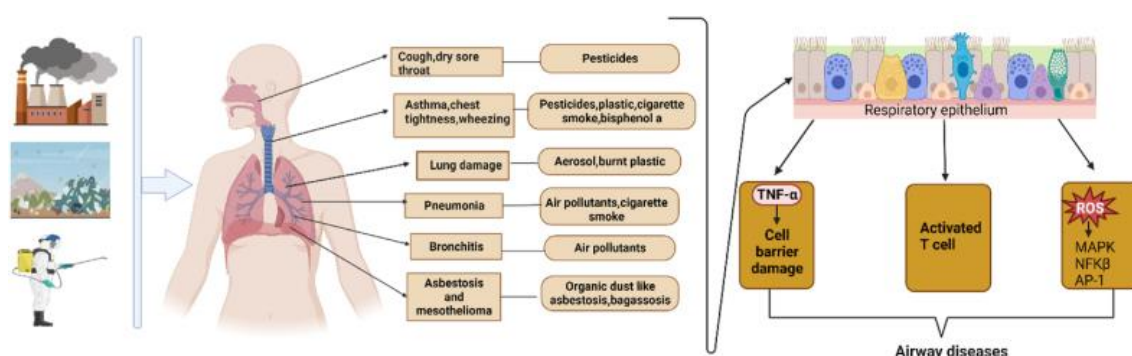


Fig. 2 Environmental pollutants impact on the respiratory system [2].

CHAPTER 2

SKELETAL MUSCLE: STRUCTURE AND FUNCTION

2.1 Skeletal muscle

As one of the major constituents of the human body mass (40%), skeletal muscle is pivotal not only for movement but also for metabolism and immune regulation. It is one of the three major muscles in the body, and it operates all the voluntary movements by attaching to the bones via tendons. Skeletal muscle is evolutionarily conserved, keeping structure and function unaltered through millions of years, as confirmed by investigations on fossils from 560 million years ago [37]. The skeletal muscle is formed by the association of myocytes, also called myofibers, which represent the first functional unit in the tissue. Myofibers tie together to form muscle bundles which are complementary in length. Such association occurs by wrapping the perimysium on the surface. The mature skeletal muscle is then formed by the gathering of more muscle bundles. In particular, this process requires the formation of physiological syncytia, that is to say myofibers, without which skeletal muscles cannot form [38]. Myomakers and myomergers are the muscle-specific fusogen proteins responsible for myofibers formation [39–42]. Fusogens mediate the anchoring between cells involved in the fusion process. Indeed, the anchored membranes are pushed toward each other allosterically by the fusogen. At this point, the outer layer of such complex fuse, forming the hemifusion state. In this state the convergence of the inner layers promotes the formation of a fusion pore and subsequent cytoplasm exchange, reaching the irreversible membranes fusion. Specifically, myomakers facilitate the processes that occur before the formation of the fusion pore, while myomergers trigger the subsequent steps [43]. Myomaker is a membrane protein formed by seven transmembrane domains and an intracellular C-terminal tail with a cysteine rich section essential for the fusion process. Such fusogen is a limiting factor for the fusion [39,44]. Indeed, myomaker expression is strictly regulated and the fusion process can properly occur when this fusogen is expressed during regeneration and muscle hypertrophy on differentiated muscle stem cells (MuSCs) while the fusion is not affected by myomaker produced on myofibers [45–47]. Such interesting behaviour suggests that a physiological

skeletal muscle formation relies on appropriate location and timing of myomaker expression. Regarding myomerger, it is a single-pass transmembrane small protein of which two isoforms exist. In fact, the myomerger gene encodes a short one of 84 amino acids and a longer one of 108 amino acids [41,48]. The shorter isoform is the most expressed during myoblast differentiation. Few domains of such isoform have been identified, among which the one involved in the membrane anchoring with an N-terminal hydrophobic region and a C-terminal domain with two α -helices (helix-1 and helix-2), the second of which considered as a fusion peptide, that are pivotal for fusion pore formation [42,48,49]. The fusion between cells is pivotal to discriminate myofibers from other cells with single nucleus. Each myofiber is composed of myofibrils, and each myofibril displays the sarcomere as functional unit. The sarcomere is confined between Z-bands, which separate each sarcomere and bisects the I-band of neighboring sarcomeres. In here, thin and thick filaments, nebulin, and titin, work together to perform the contractile and relaxing functions of the skeletal muscle [50]. In particular, thick filaments are formed by myosin, whose ATP-dependent activity provide the energy needed for the cross-bridge cycle, whereas thin filaments are constituted by actin isoforms like troponin and tropomyosin [51]. Regarding nebulin, it is important for the stabilization of thin filaments by interaction with three actin subunit and the indirect regulation of myosin [52]. Titin, or connectin, is a big protein with a specialized N2A domain mainly involved in the assembly and fixation of myosin [53,54]. The regulation of the excitation-contraction coupling is up to the skeletal muscle substructure called “triad”. Triads are located on the Z-bands and are formed by the apposition of the T-tubule and the terminal sarcoplasmic reticulum. In particular, a triad is composed by a central T-tubule, terminal cisternae on the sides, and specialize proteins like calcium buffering proteins, calcium channel regulators, the ryanodine receptor 1 (RyR1) which is coupled with the dihydropyridine receptor (DHPR) on the T-tubule, and sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) [55]. Thus, muscle contraction is promoted by both excitation–contraction coupling and the sliding of the filaments. Such complex process starts with the generation and transmission of action potential to the triads after activation of the postsynaptic fast sodium voltage-gated channel α subunit 4 (SCNA4), causing depolarization of DHPR and induction of extracellular Ca^{2+} influx. The rising levels of Ca^{2+} trigger the release of Ca^{2+} from the cisternae via RyR1. The released Ca^{2+} interacts

with the calcium binding subunit (TnC) of the troponin causing its conformational change and the removal of tropomyosin, exposing myosin-binding sites on actin. At this point, the energized head of myosin binds to actin at an angle of 45°. After the binding, the myosin changes conformation reaching a 90° angle with ADP release. Then, new ATP attaches to the head of myosin inducing its detachment from actin. The hydrolysis of such ATP is responsible for energizing the myosin head. Finally, sarcomeres can start a new cross-bridge cycle. Sustained muscle contraction will be maintained as much as the Ca^{2+} keeps binding the TnC on troponin. If Ca^{2+} influx stops, and troponin returns to its original conformation the skeletal muscle will undergo relaxation [51,56].

From a molecular perspective, many pathways are activated in order to preserve muscle homeostasis regulating synthesis and degradation of muscle proteins [57]. For instance, the activation of phosphoinositide 3-kinase (PI3K) mediated by the insulin-like growth factor I (IGF-I) brings to the phosphorylation of protein kinase B (Akt) and thereby the Akt-mediated phosphorylation and inhibition of the tuberous sclerosis complex 2 (TSC2). Inhibition of TSC2 causes the activation of the mammalian target of rapamycin (mTOR) which is the major player in skeletal muscle homeostasis and protein synthesis. Activation of mTOR brings to activation of mTORC1 complex (mTORC1) which by phosphorylating downstream proteins such as S6 ribosomal protein (S6) and 70 kDa ribosomal S6 protein kinase (p70S6K) will begin transcription and translation of muscle proteins to increase muscle fiber size [58]. As described for the Akt/mTOR/S6 axis, the synthesis of muscle proteins can be also promoted by Akt via inhibition of glycogen synthase kinase 3- β (GSK3- β) and activation of the eukaryotic initiation factor 2B (eIF2b), which will promote translation of specific genes [59]. Interestingly, protein degradation and catabolism can be regulated by IGF-I/Akt as well. Via PI3K/Akt signaling the forkhead box protein O (FOXO) undergoes phosphorylation which causes FOXO to be blocked in the cytoplasm unable to promote the transcription of catabolic genes such as atrogen 1, muscle atrophy F box protein (MAFbx), and muscle ring 1 (MuRF1) [60]. If activated, such genes would cause protein degradation via autophagy-lysosome system or ubiquitin-proteasome system [61,62]. However, catabolism could be activated via other pathways. An example of this is the activation of the catabolic gene MuRF1 via inflammatory pathway of nuclear factor kappa B (NFkB) in which the

production of inflammatory cytokines such as interleukins 1 β (IL-1 β) and 6 (IL-6), and tumor necrosis factor α (TNF- α) can cause decrement of the muscle fiber size [63,64].

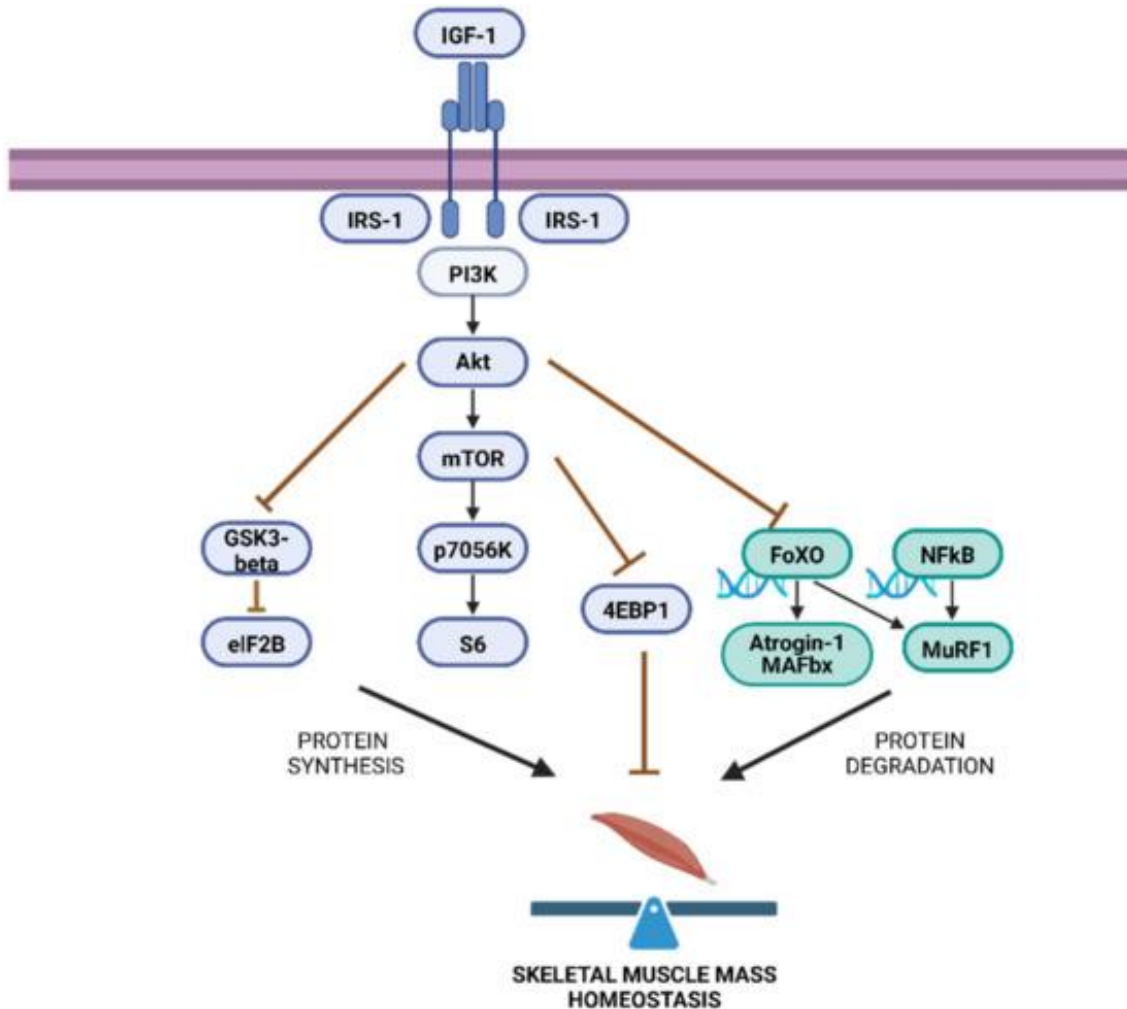


Fig. 3 Pathways involved in skeletal muscle synthesis and degradation [58].

2.2 Myogenesis

The development of the skeletal muscle system occurs in a life-long multi-step process called myogenesis, in which the strict control proliferation, differentiation, and fusion of myogenic cells leads to generation of multinucleated and functional muscle fibers able to contract and relax. The formation of a new muscle starts in the primary myogenesis (embryonic phase) with the formation of the myoblasts during development in uterus. Here, from epiblast differentiation firstly mesoderm progenitors are produced and then anterior presomitic mesoderm (aPSM)-dermomyotomal progenitor cells. Myogenic progenitors on peripheral edge of the dermomyotome extend downward to form the

myotome. In such migration myogenic progenitors differentiate into spindle mononuclear myoblast which then migrate to the site of muscle formation to form mature myofibers [65]. As hallmarks of this process, Paired-box 3 and 7 (Pax3/7) and myogenic factor 5 (Myf5) are transcription factors sequentially expressed to initiate myogenesis [66]. In particular, aPSM-dermomyotomal progenitor cells expressing Pax3 fuse with each other to form primary myotubes [67]. Then, a subset of such progenitors downregulates Pax3 and upregulate Pax7 inducing the secondary myogenesis (foetal phase) in which Pax7⁺ progenitors produce foetal myotubes by fusing each other or with primary myotubes [66]. New myotubes in this phase can be produced by overstimulation of myoblasts by laminin and fibronectin, main components of the basal lamina which is assembled during secondary myogenesis as well as neuromuscular and myotendinous junctions [67]. Regarding the tertiary myogenesis (neonatal phase), type I (slow-twitch) and type II (fast-twitch) fibers, and respective subtypes (i.e. IIa, IIb, and IIx), forms following specialization of existing myofibers. Moreover, in the neonatal phase a stem cell niche is established with MuSCs which originate from a subset of progenitors expressing Pax7 and are pivotal for maintaining the regenerative capability of skeletal muscle tissue [65,68]. Interestingly, proliferating MuSCs appear to be able to express Pax7 whereas Pax7 is downregulated during their differentiation [66,69]. Thus, asymmetrically proliferating myoblasts show Pax7 expression and can thereby distinguished in subtypes depending on the expression on Myf5: that is to say Pax7⁺/Myf5⁺ MuSCs and Pax7⁺/Myf5⁻ MuSCs [70]. The first type of MuSCs exploit M-cadherin to joint with myofibers and are distributed on the apical surface. The seconds, on the other hand, connects via laminin receptor integrin $\alpha 7\beta 1$ to the basal lamina surrounding myofiber [70,71]. In the last phase of myogenesis (adult phase) the body tries to keep the physiology of the skeletal muscles as stable as possible. However, as soon as physiology is lost the skeletal muscles undergo changes in which repair mechanisms and myofiber regeneration will try to restore the normal environment. Interestingly, mature myofibers can be expanded by MuSC-dependent cell-cell fusion, by which more nuclei are added to the existing myotube and then to myofiber [72]. After the fusion process, newly generated myotubes show myonuclei located at the center of the structure. The migration of myonuclei is a key event for the proper transition from myotubes to myofibers. Indeed, myonuclei in a mature myofiber are strategically and uniformly organized to minimize

the transport distance, to reduce reciprocal inhibition, and to enhance their activity [73]. In order to do so, myonuclei have to cluster under the traction of the cytoskeleton, to spread across the long axis of the forming myotube, and to migrate to the plasma membrane where the nuclei are fixed [74,75]. Many proteins are involved in these processes such as microtubules, microtubule-associated proteins (MAPs), actin and actin-associated proteins, which form a track for myonuclei movements. The latter are performed over the described track by microtubules motor protein like kinesin and dynein, which bind to the KASH domain of the linkers of the nucleoskeleton and cytoskeleton (LINC) complex. In particular, dynein forms a complex with dynactin which is recruited to the nuclear envelope of newly fused nuclei under the stimulation of the Rho-GTPase CDC42, Par6, and Par3. Here, the binding with Par6 β makes the complex to move on the microtubules emitted from the nuclei in the cluster at the center of the myotube and pulls the new nuclei toward the cluster. Similarly, myonuclei in the cluster can move on the microtubules emitted from the incoming nuclei exploiting the same motor system to shorten the distance by reaction force [76–78]. In this way, myonuclei will end to be arranged strategically along the myotubes. Subsequently, the organized myonuclei gradually disperse to the lower part of the sarcolemma, forming the typical nuclear distribution of myofibers. Their dispersion appears to be mediated by the centripetal force resulting from contraction and crosslinking of myofibrils [79]. Indeed, local accumulation of fibronectin produced by myofibroblast triggers the activation of Src and the focal adhesion kinase (FAK) via α 5-integrin in the cytoplasm, which results in the phosphorylation of paxillin and engagement of β -Pix. The latter starts a cascade in which Cdc42, N-Wasp, and actin-related proteins-2/3 (Arp2/3) are sequentially activated stimulating the remodeling of desmin, an intermediate filament protein located near the Z-bands that binds myofibrils to the sarcomere. Once the crosslink is made, the tightening of myofibrils during the contraction of myotubes pushes away myonuclei to the periphery. Indeed, the traction force produced during myofiber contraction and relaxation can be harmful for the nuclei. With the myonuclei in periphery damage to them can be avoided. Moreover, LINC complex and cytoskeleton grant nuclear stability and protection by forming the perinuclear barrier [79–81]. The complex nuclear disposition is essential to preserve myofibers and skeletal muscles during formation but also regeneration.

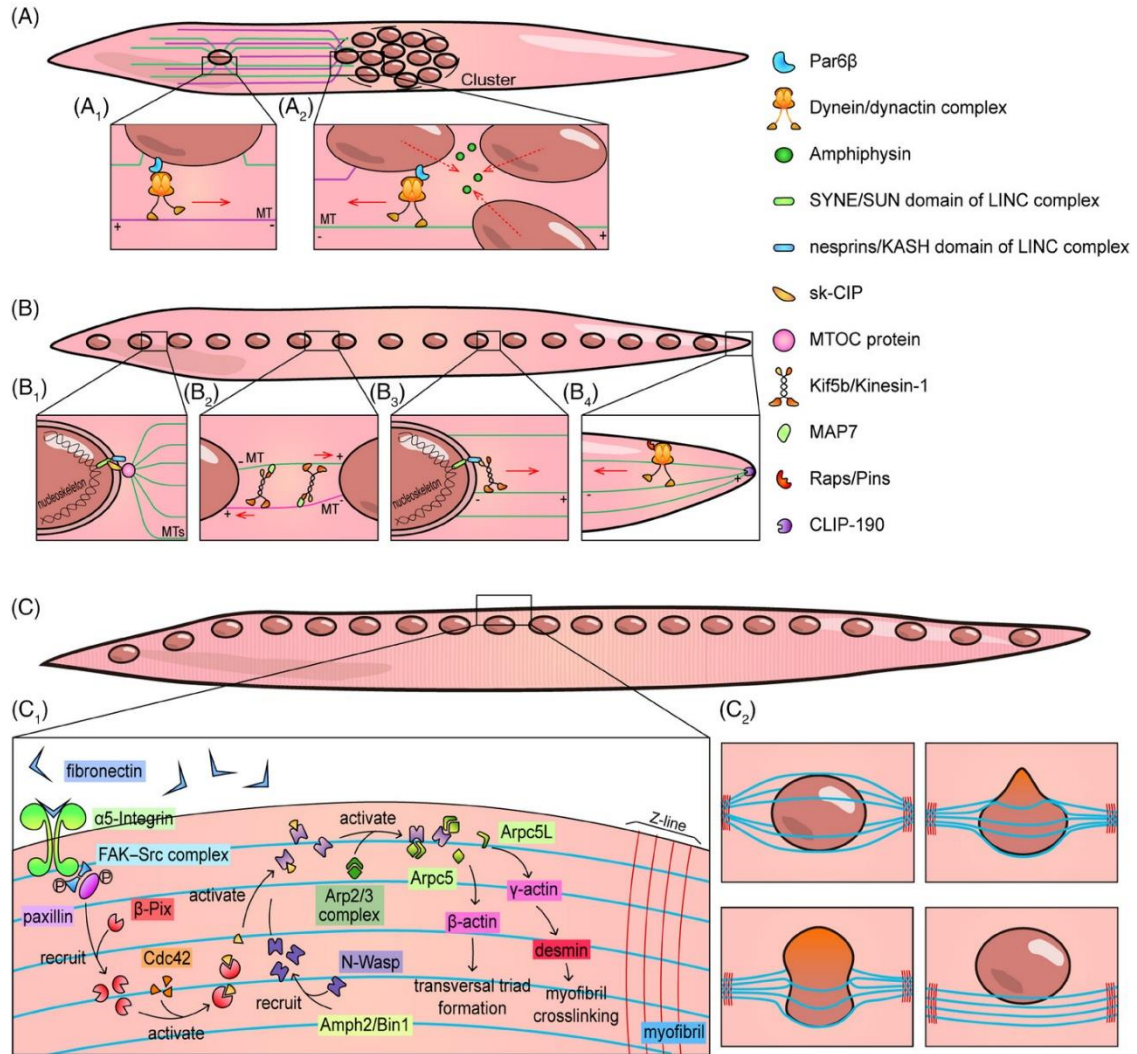


Fig. 4 Molecular basis of myonuclear migration [43].

2.2.1 Expression of miRNA and myogenesis

Behind the complex mechanism of myogenesis a more complex molecular regulation takes place to assure the proper muscle development. Indeed, muscle specific transcription factors families like myogenic regulatory factors (MRFs), among which myoblast determination protein 1 (MyoD), and myocyte enhancer factor-2 (Mef2) define the skeletal myogenesis regulating gene expression. Nonetheless, other factors are involved too. For instance, micro-RNAs (miRNAs) have been described, within myogenesis, as a new level of control. MiRNAs are small non-coding RNAs first discovered in 1993 in *C. Elegans* which control gene expression at the post-transcriptional level [82–84]. The transcription of miRNAs genes, mainly mediated by RNA polymerase II but also polymerase III, brings to the production of long primary

transcripts called pri-miRNAs, which are subsequently processed by the nuclear RNase III Drosha/DGCR8, a nuclear RNase III, to generate shorter pre-miRNAs of around 70 base pair (bp). Interestingly, the Drosha-dependent processing can be avoided when the splicing machinery starts forming pre-miRNAs (mirtrons) [85–87]. Pre-miRNAs are then exported by Exportin-5 via nuclear pore complex to the cytoplasm. Exportin-5 is, at first, complexed with pre-miRNAs and Ran-GTP forming the ternary complex. The latter pass the pore complex thanks to Ran-GTP. [88–90]. Once in the cytoplasm, Ran-GAP hydrolyzes the GTP to GDP starting a conformational change which permits the pre-miRNAs release in the cytosol. Here, the RNase III called Dicer further process pre-miRNAs shortening them to around 22 nucleotides [89]. Mature miRNAs are pivotal regulators of numerous cellular and molecular events. Indeed, miRNAs regulate gene expression by binding via partial sequence complementarity to the 3'UTR of specific mRNAs, causing their translational inhibition and decay [91]. In particular, after Dicer processing mature miRNAs are double-stranded molecules. While one strand, called non-guide strand, is prone to degradation, the other strand, called guide strand, is composed by nucleotide sequences for specific genes. Indeed, via guide strands miRNAs guides the RNA-induced silencing complex (RISC) containing Argonaute 2 (Ago2) and TAR RNA binding protein (TARBP2) modulating target gene expression [92,93]. Thus, by decreasing mRNA levels miRNAs cause gene expression repression or inhibition [94]. Nonetheless, miRNA-mediated translational activation has been reported too [95,96]. In the context of myogenesis, miRNA-mediated regulation is essential. Indeed, issues in the miRNA production machinery can be harmful. Researchers described *in vivo* the importance of miRNAs, and the molecules involved in their production, showing how mice with muscular Dicer deleted can die perinatally with small muscle mass, atypical myofiber morphology, and increased muscle cell death [97]. Similarly, mice with DGCR8 gene knocked out showed severe dilated cardiomyopathy and heart failure, suggesting once again the important role played by miRNAs in the muscles [98]. Among the miRNAs involved in myogenesis the following can be found: miR-1, miR-23a/b, miR-24, miR-26a, miR-27b, miR-29b/c, miR-125b, miR-133a/b, miR-181, miR-206, miR-208b/499, miR-214, miR-221/222, miR322/424, miR-486, and miR-503 [99–101]. All the cited miRNAs display myogenic functions even though not all of them are muscle-restricted miRNAs but ubiquitous miRNAs. For instance, miR-23a/b is upregulated in

C2C12 cells during early stage of differentiation, and regulates the expression of thioredoxin reductase 1 (TrxR1), which is downregulated during skeletal muscle differentiation, promoting myogenesis [102]. MiR-214 production is stimulated by MyoD and myogenin, and de-repressed by enhancer of zeste 2 homolog 2 (EZH2) during differentiation [103]. Moreover, miR-214 further promote myogenic differentiation by targeting N-Ras thereby facilitating cell cycle exit [104]. During myoblast differentiation miR-24 and miR-29b/c are both upregulated. While the upregulation of miR-24 exploits the de-repression action of transforming growth factor beta (TGF- β)/Smad signaling, miR-29b/c is stimulated by serum response factor (SRF) and Mef2 [105–107]. In particular miR-29b/c is normally repressed in myoblast by the transcription factor Yin Yang 1 (YY1) which release miR-29b/c during differentiation and muscle regeneration [106]. Other ubiquitous miRNAs upregulated during myoblast differentiation are miR-26a, miR-27b, miR-181, miR-322/424, and miR-503 [108–111]. In particular, miR-26a targets EZH2, similarly to miR-214, avoiding the EZH2/YY1-dependent negative regulation of the skeletal myogenesis [109,112]. MiR-27b is able to downregulate Pax3 promoting the differentiation thereby suppressing the Pax3-dependent muscle stem cell-maintenance and migration [110]. Again, miR-181 is involved in the muscle development in which de-repress the expression of MyoD by targeting the homeobox protein Hox-A11 [108]. Regarding miR-322/424 and miR-503, they share the phosphatase CDC25A as common target, which is involved in the inhibition of CDK2 with cell cycle exit thereby promotion of myogenesis [111]. Unfortunately, the regulators of such upregulated miRNAs are still unknown even though their involvement in myogenesis has been described. On the other hand, fewer ubiquitous miRNAs are downregulated during myogenic differentiation. For instance, miR-125b by targeting insulin-like growth factor II (IGF-II) is a suppressor of myogenic differentiation and muscle regeneration under the negative regulation of mTOR signaling [113,114]. Similarly, miR-221/222 are downregulated during myoblast differentiation, as described in quail myoblast and mouse C2C12 myocytes [115]. Moreover, their expression seem to be related to inflammatory events and cell infiltration in injured muscles [116,117]. Regarding the muscle-restricted miRNAs, also known as myo-miRNAs, the most studied families are miR-1, miR-133a/b, and miR-206 [57]. The expression of such miRNAs is under the control of MyoD, Mef2, and SRF and they can be specifically found in both cardiac and skeletal muscles. Their

activity is pivotal for the proper muscle development. Indeed, miR-1, miR-133a/b, and miR-206 have been found expressed at incredibly high levels during myoblast differentiation [109,118,119]. Regarding miR-1, mTOR is responsible for the regulation of the expression of miR-1 by controlling MyoD stability promoting an mTOR-MyoD axis [119]. Similarly, MyoD is involved in the regulation of the expression of miR-133a/b and miR-206 [120,121]. Indeed, MyoD is strategically placed upstream of many myogenic miRNAs, suggesting the importance of its regulation during myogenesis [99]. Interestingly, the upregulation of miR-1, miR-133a/b, and miR-206 during myogenesis and muscle regeneration is preceded by a transient drop of their levels immediately after muscle injury or functional overload, as described *in vivo* [117,119,122,123]. Moreover, such downregulation can follow muscle damage and fibrosis but also correlated, specifically of miR-1 and miR-206, with the anti-proliferative effects of these miRNAs on MuSCs. Indeed, miR-1 and miR-206 target Pax6 inhibiting MuSCs proliferation and promoting differentiation [124]. Among the other targets of miR-1 and miR-206, the downregulation of connexin 43, a gap junction protein, is essential for myoblast fusion while the histone deacetylase 4 (HDAC4), which is a transcriptional repressor of the expression of muscle genes, is involved with miR-206 in the stimulation of neuromuscular synapses regeneration, as described in mice [125–127]. Again, HDAC4 play a role in skeletal myogenic fusion by regulating the fusion-promoting factor follistatin, which is under the regulation of miR-1, establishing the mTOR-MyoD-miR-1-HDAC4-follistatin pathway [119]. Regarding miR-133a/b, it shows several targets among which SRF, with promotion of myoblast proliferation thereby decreasing myogenic differentiation [118]. Moreover, miR-133a/b targets the uncoupling protein 2, which is involved in the regulation of energy consumption and thermogenesis, downregulating myoblast differentiation [128]. On the other hand, the downregulation by miR-133a/b of the neuronal polypyrimidine tract-binding protein (nPTB), an alternative splicing factor, promote differentiation [129]. Other muscle-restricted miRNAs are miR-208b/499 and miR-486. In the regard of miR-208b and miR-499, they are expressed in cardiac and skeletal muscles and are involved in the regulation of myofiber specification by targeting repressors of the transcription of genes related to slow myofibers, and muscle maintenance [130]. MiR-486, on the other hand, shows a myogenic function and its activation depends of MyoD, SRF, and myocardin-related transcription factor-A (MRTF-

A) [131]. Indeed, miR-486 is highly stimulated during myoblast differentiation and its main targets appeared to be the phosphatase and tensin homolog (PTEN) and FOXO1a involved in the PI3K signaling, which is pivotal in skeletal myogenesis [131,132].

Thus, the regulatory activity of miRNAs is crucial for proper skeletal muscle development. Considering the possibility for miRNAs to share targets and co-operate for the regulation of the molecular machinery, it is possible to define them as powerful instruments to dissect the molecular regulation behind myogenesis.

2.3 Skeletal muscle function

Skeletal muscles are responsible for homeostasis maintenance in the human body. Indeed, they exert many functions such as movement, thus force generation, but also metabolic and endocrine functions, thereby regulating body movement and postures, organ protection, thermogenesis and temperature modulation, and secretion of regulatory molecules [56,133,134].

While the mechanism of contraction and relaxation has been already described above, it is worth discussing muscle fibers in major detail. As previously said, different types of myofiber exist in the skeletal muscle: type I (slow-twitch) and type II (fast-twitch) fibers, and respective subtypes (i.e. IIa, IIb, and IIx). In particular, type I myofibers are characterized by a slow but lasting contraction determined by great amounts of mitochondria and aerobic metabolic enzymes and low amounts of ATPase making them perfect for endurance movements. Regarding type II myofibers, type IIb fibers are rich in glycolytic enzymes and active ATPase with glycogen stocks. Thus, type IIb fibers display poor aerobic function with the generation of strong force and fast contraction which are perfect characteristics for executing powerful but short movements. Type IIa and type IIx share common characteristics with type I and type IIb. Indeed, while type IIa is in the middle between type I and type IIb from points of view of metabolism and contraction, type IIx is more similar to type IIb fibers with oxidative metabolism and slow contraction speed. From a structural perspective, each type of fiber can be distinguished by differences in myosin heavy chain (MyHC), essential for proper contraction, of which four subtypes exist: MyHC I, MyHC IIa, MyHC IIb, and MyHC IIx. Thus, the classification of myofibers types is based on the specific MyHC component. Nonetheless, myofibers can switch type depending on the requirements of the external environment:

type I can switch to type IIa; type IIa can go back to type I or change to type IIx; type IIx can change back to type IIa or diverge to type IIb; type IIb can only go back to type IIx [135,136]. As mentioned above, the metabolic pattern among myofiber types shows changes. Indeed, glucose consumption gradually diverges from oxidative to glycolytic metabolism between types I and IIb, with different rates of ATP generation and speed of contraction. In particular, fiber type IIb shows the higher amounts of ATP followed, in a decreasing trend, by fiber type IIx, fiber type IIa, and fiber type I. On the other hand, fiber type I shows the best properties for fatigue endurance followed, in decreasing trend, fiber type IIa, fiber type IIx, and fiber type IIb. Overall, all the myofiber types store glucose in the form of glycogen which can be directly involved in excitation and contraction, even though its storage and consumption are specific for each fiber type [136,137]. Glucose metabolism is pivotal for skeletal muscle as well as for the entire body, and it is made possible by the glucose uptake which is mediated by the insulin-regulated glucose transporter 4 (GLUT4) and the glucose gradient. GLUT4 is located intracellularly while the muscle is in resting state and under the control of muscle contraction and insulin it migrates to the membrane thereby increasing the glucose uptake, consumption, and storage [138,139]. In this way, more ATP will be produced to support skeletal muscle contraction via, for instance, glycolysis but also oxidative phosphorylation, and oxidation of fatty acids [140]. The more muscular metabolism raises the stronger the thermogenesis will be. Indeed, skeletal muscle is the major producer of heat in the human body. For example, repetitive autonomous contractions when the body is exposed to cold environments can cause the so called “shivering thermogenesis” in which ATP hydrolysis produces heat in a not so efficient way. In fact, shivering thermogenesis consumes glucose and can be responsible for muscle fatigue due to glucose depletion over time [141]. Thus, skeletal muscles rely on “non-shivering thermogenesis” in long-term cold adaptation, in which the sarcolipin binds to SERCA uncoupling the SERCA-mediated ATP hydrolysis from Ca^{2+} transport to the sarcoplasmic reticulum thereby increasing the futile cycling of Ca^{2+} and heat production without causing muscle contraction and in a less energetically costly way [142].

Regarding the secretion of regulatory molecules, skeletal muscles are able to produce and release hundreds of molecules, referred to as “myokines”, via myofibers determining paracrine, autocrine, and endocrine effects [143]. Among the myokines, myostatin and

myostatin can modulate skeletal muscle mass promoting or repressing muscle hypertrophy [144]. Myokines like IL-6 can modulate muscle metabolism by promoting GLUT4 migration to cell membrane and glucose uptake via insulin signaling, but also stimulating lipolysis of the visceral fat and promote bone formation [144–146]. As for the local effects, myokines exert distant effects on other organs. Evidence of muscle-derived myokines produced during exercise like cathepsin B and irisin in the brain enhancing cognitive functions by improving hippocampal neurogenesis via brain-derived neurotrophic factor (BDNF) have been found [147,148]. Moreover, myokines can reach the gastrointestinal tract and the islet β cells affecting insulin production [149]. Thus, skeletal muscles are linked to every part of the body influencing positively and negatively the local and distant physiology.

CHAPTER 3

STEM CELLS TO MODEL THE SKELETAL MUSCLE

3.1 Stem cells: an overview

Stem cells are cells able to indefinitely proliferate, self-renew producing more stem cells, and differentiate into any cell type of which the organism is composed. In order to do so, stem cells exploit symmetrical and asymmetrical division [150]. While the symmetrical division produce identical clones (self-renew), asymmetrical division can produce daughter cells with different fates due to asymmetric inheritance of cellular component thereby producing a stem cell clone and a diverged and more differentiated cell [150,151]. Stem cells can be classified in relation to their potency in totipotent, pluripotent, multipotent, oligopotent, and unipotent stem cells, with each state characterized by a certain level of plasticity [152]. Indeed, totipotent stem cells, like the zygote, are the most potent stem cells able to differentiate into any cell type. Such cells originate as soon as the sperm cell fertilizes the oocyte and can form embryonic and extra-embryonic structures. Around the fifth day the embryo reaches the state of blastocyst in which an inner cell mass and the trophectoderm can be distinguished. While the latter is responsible for the development of placenta and other extra-embryonic structures, the former contain cells which remain in an undifferentiated state and by which first the epiblast and then the fetus originate. Indeed, the inner cell mass contains Pluripotent Stem Cells (PSCs), such as Embryonic Stem Cells (ESCs), that can differentiate in cells from all the three germ layers (i.e. ectoderm, mesoderm, endoderm) but not produce the extra-embryonic structures. From the sum of all the germ layers, all the specialized cells and tissues of the human body are produced. Starting from the ectoderm, brain and skin are generated while blood, bone, and muscle originate from the mesoderm, and gut, lungs, and liver from the endoderm. Once a pluripotent cell starts to genetically diverge, it can differentiate into a Multipotent Stem Cell (MSCs) which is specifically committed to one of the three germ layers. MSCs show a narrow spectrum of differentiation compared to the pluripotent ones, specializing in well-distinguished cell lineages (i.e. adult stem cells, like hematopoietic stem cells). When MSCs further differentiate, Oligopotent Stem Cells (OSCs) are generated. An example of OSCs can be found in myeloid progenitors which are enough specialized to produce white cells and not the red ones. Furthermore, OSCs can

differentiate in Unipotent Stem Cells (USCs) that show the narrowest spectrum of differentiation among stem cells being able to produce only one cell type [153,154]. The differentiation process can be influenced or even driven by mechanical stimuli of surrounding cells as well as the chemical composition of the local environment. Moreover, specific internal signals originated by overexpression of key genes can be found too [154].

3.2 Adult stem cells

Known as resident or somatic stem cells, adult stem cells are low-abundant undifferentiated cells within the differentiated cells that organize tissues and organs. Being stem cells, adult stem cells are characterized by abilities such as self-renew and potency, even if the latter shows a narrow spectrum of differentiation [155]. Indeed, such cells can be multipotent and able to differentiate into more specialized cells in dependance of the tissue of origin. Adult stem cells can be found in organized structures called “niche”. The niche is pivotal in the maintenance of the local environment in which the niche assures homeostasis and regulates growth and structural and functional regeneration after injuries. In order to do so, stemness is kept as constant in the niche in an orchestration between chemically specialized microenvironment and resident proliferative stem cells, among which adult stem cells. Adult stem cells are essential for reaching local homeostasis. Normally, such cells are quiescent. To replenish loss of cells, which can occur both physiologically and pathologically, adult stem cells start to proliferate and differentiate in the required types of cell [155,156]. Many tissues contain adult stem cells, among which skin, intestine, blood, brain, and muscle [155,157,158].

3.2.1 Muscle Stem Cells (MuSCs)

Skeletal muscles possess resident adult stem cells, known as progenitors or satellite cells, which are involved in the regeneration process. Cited cells are generally in a quiescent state but, when activated, they are capable of both proliferating and differentiating. They are also generally referred to as muscle stem cells (MuSCs) [158, 162]. During daily activities, functional skeletal muscles remain inevitably injured when are unable to handle a too important tensile strength with loss of post-mitotic cells which cannot undergo cell division. Indeed, MuSCs attempt to restore the injured tissue regulating regeneration and

growth, and in the end promoting homeostasis in the skeletal muscles [159]. Such cells, as per the other adult stem cells, are rare in muscles, representing almost the 6% of all the muscle fiber-associated nuclei [159]. Moreover, MuSCs are normally quiescent and keep the G₀ phase of cell cycle. Interestingly, such cells express Pax7 whose role in regulating self-renew of MuSCs has already been reported [66,69]. In particular, Pax7 expression indicates proliferative activity in MuSCs [160]. Their proliferation is important for replenishment of stem cell niche in the skeletal muscle as well as production of committed myoblast. In the latter case, expression of MyoD is crucial for myoblast differentiation. In fact, MyoD is an early marker of commitment for MuSCs differentiation [161]. Thus, Pax7 and MyoD are both expressed in MuSCs, but their levels change over time. While MuSCs exploit their Pax7-dependent proliferation to replenish the niche, the levels of MyoD expression are low and symmetric cell division is predominant, producing more MuSCs expressing Pax7 at high levels [162]. On the other hand, asymmetric division starts when cell loss occurs producing both a cell expressing Pax7 at high levels and a diverged and committed cell which will start to express MyoD at high levels and reduced levels of Pax7. Such cells will differentiate into myoblasts and then in myofibers, in the first case expressing MyoD, MRF4 and Myf5 with low levels of Pax7, and in the second case expressing lower levels of Pax3/7 and Myf5 but also high levels of myogenin and MyHC [163,164].

3.3 Induced pluripotent stem cells (iPSCs)

The ability to produce iPSCs artificially from somatic cells represented a great scientific achievement: in 2006, Kazutoshi Takanashi and Shinya Yamanaka were the first in generating the so called “induced Pluripotent Stem Cells” (iPSCs) [165]. By regulating human stemness factors such as OCT3/4, SOX2, KLF4, and c-MYC, somatic cells can be driven to iPSCs. Such cells display molecular characteristics near to those of ESCs, with common transcriptional and epigenetic profiles, and pluripotency [165,166]. Their usage is incredibly promising for regenerative medicine and biomedical sciences. Indeed, iPSCs can differentiate in any kind of cells in the body starting from somatic cells, making possible the production of specific tissues and organs but also exploiting the patient itself as source of somatic cell thereby removing immunogenicity and ethical issues. Moreover, such patient-specific materials can be used in order to reach a personalized medicine by

testing specific drugs or therapeutic strategies, finding the best solution for each patient [166]. Regarding iPSCs differentiation, many *in vitro* protocols exist for myogenic induction where starting from key signaling factors of developmental and regenerative myogenesis researchers try to recapitulate in the laboratory the biochemical events which bring to production of MuSCs and subsequently other specialized actors such as myotubes [167–170]. In order to do so, iPSCs can be exploited in two main strategies in which myogenic differentiation is achieved by transgenic approaches based on the overexpression of myogenic factors such as Pax3/7 and MyoD, and eventually with epigenetic regulators like Jumonji domain-containing protein D3 (JMJD3) and BRG1/BRM-associated factor 60 (BAF60), or by direct differentiation mediated by growth factors and small molecules as transgene-free approaches [171–176]. iPSCs-derived myotubes can be used as 2D model or organized in 3D structures to further develop physiological or pathological skeletal muscles [177,178]. In the latter case, a test for depolarization ability or contractility levels could be required for a proper characterization [179,180]. Even though such cells are promising, several challenges need to be addressed to safely exploit them. Indeed, iPSCs production can be troublesome. Transgene-based protocols which use viruses can lead to unwanted mutations in DNA with higher risk of tumor formation. Also, both transgene-based and transgene-free approaches can produce differentiated populations in which residual undifferentiated cells can be found. Undifferentiated iPSCs represent a serious risk for teratoma formation due to their pluripotency [181]. Thus, standardized protocols for iPSC generation and differentiation are necessary to ensure the safety and efficacy of new models and therapies.

CHAPTER 4

ENVIRONMENTAL POLLUTANTS AND SKELETAL MUSCLES: IS THERE AN INTERACTION?

4.1 Mechanisms of toxicity

Exposure to environmental pollutants such as plastic particles and O₃ can be responsible for local and eventually systemic oxidative stress and inflammation affecting tissues and organs, among which skeletal muscles [182]. Indeed, the adverse effects of environmental pollutants exposure can impair the integrity and the functioning of the skeletal muscles in a detrimental interplay between oxidative and inflammatory molecular actors, and mitochondria appeared to be in the middle of such dynamics [183]. For instance, pollutants are able to increase ROS levels causing oxidative damage to mitochondria, impairing their structural integrity, altering membrane potential and mitochondrial dynamics, and disordering the ATP and Ca²⁺ balances [184]. Mitochondrial dysfunction leads to more oxidative stress, unbalancing the pivotal oxidant/antioxidant equilibrium, and eventually to apoptosis promotion. Moreover, released mitochondrial content can trigger inflammatory response. For instance, high ROS production can cause activation of both NFκB and nuclear factor erythroid 2-related factor 2 (Nrf2), the latter subsequently partially inactivated by the oxidative environment [185,186]. Moreover, mitochondrial DNA and ROS could activate NOD-like receptor thermal protein domain-associated protein 3 (NLRP3) inflammasome raising inflammation [186]. Thus, pollutants can activate pro-inflammatory cascades, with release of cytokines and chemokines. As previously described, inflammation is detrimental in the peculiar context of the skeletal muscle [63,64]. Activation of NFκB can be responsible for catabolism promotion and amplification of the pre-existent inflammation [63,64]. On the other hand, oxidative stress, under a certain threshold, could be considered a physiological characteristic of the skeletal muscle. Indeed, skeletal muscle represent a hypoxic environment in which the MuSCs niche take place among myofibers and basal lamina, with a small (around 15%) resident MuSCs population [187]. Such resident MuSCs are quiescent and display low metabolic activity due to low amounts of ATP and mitochondria [188]. In this way, these MuSCs can prevent the increment of ROS levels to preserve their regenerative ability [189]. Thus, pollutant-dependent oxidative stress can be detrimental

to MuSCs and skeletal muscle regeneration by altering an oxidative equilibrium which is responsible for in-depth damage, till MuSCs niche physiology [190].

Even though so much has been discovered around environmental pollutants' effects on tissues and organs, it is still not enough to fully describe such a complex interplay in which, among others, skeletal muscles surely play a role. Therefore, the current evidence of skeletal muscles interacting with pollutants such as polystyrene particles and O₃ will be discussed in the following section.

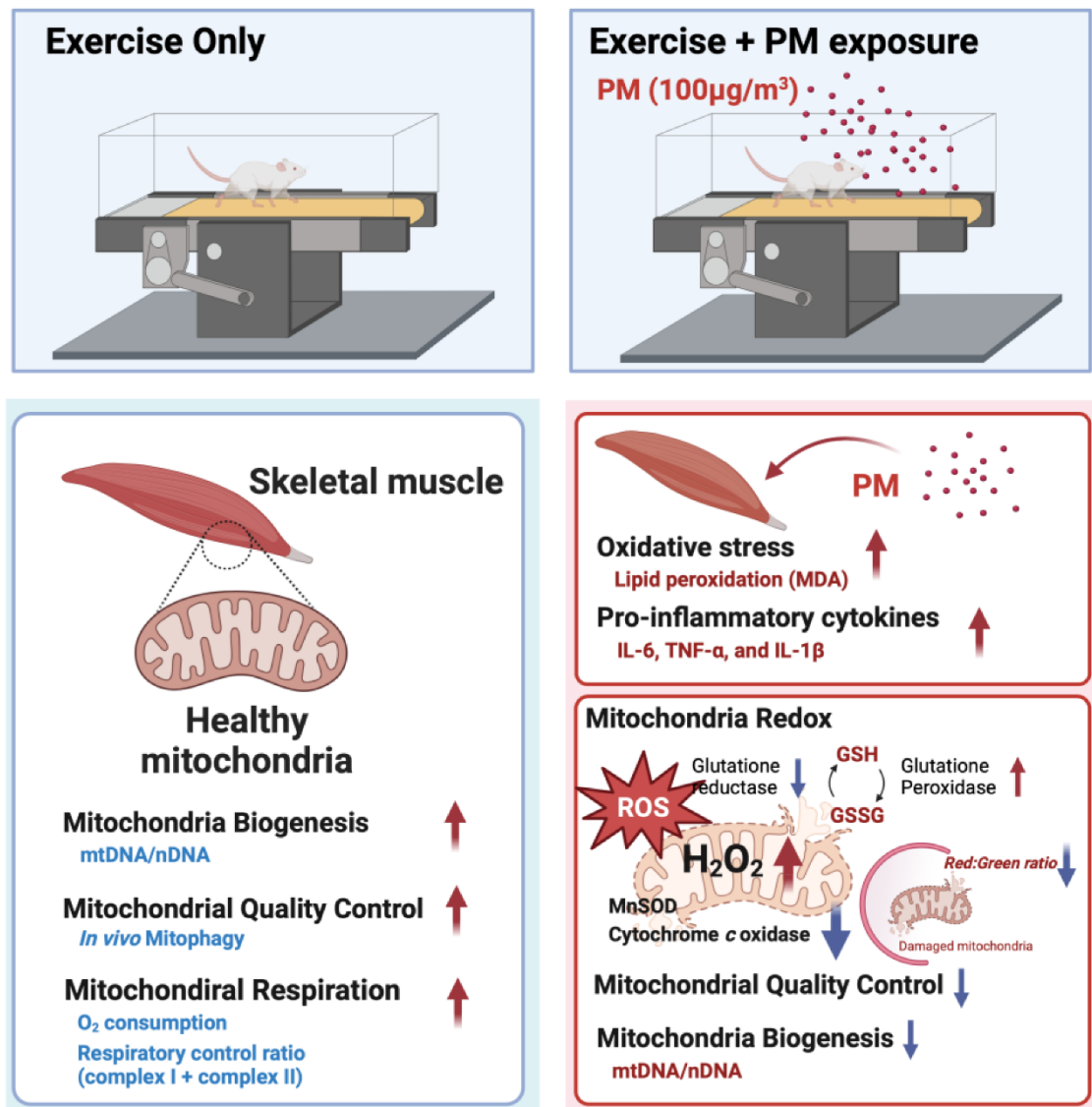


Fig. 5 Particulate matter (PM) effects on skeletal muscle and mitochondrial function [183].

4.2 Evidence of interaction between environmental pollutants and skeletal muscles

The burden of environmental pollutants over the human health is enormous [2]. As mentioned above, environmental pollutants, in particular polystyrene particles and O₃, are able to exert toxic effects on all the major systems in the human body, among which the muscle system. For instance, Shengchen and its collaborators carefully described the effects of polystyrene particles on muscular regeneration in mice, highlighting the skeletal muscles involvement in environmental pollutants-mediated toxicity [191,192]. They found oxidative stress in satellite cells and inflammation during regeneration as expected, and disruption of the equilibrium between myogenic and adipogenic differentiation. Thus, the regeneration of the muscle fibers was impaired, suggesting the deep impact of such pollutant on skeletal muscles [191]. In another study, polystyrene particles of various sizes orally administered to mice caused damage to skeletal muscle and impaired muscle cells differentiation and muscle development, with the stronger effects the smaller the size. Interestingly, myogenic markers resulted downregulated in the quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) and western blot analyses, with upregulation of lipid differentiation-related factors, suggesting the ability of the administered pollutant to induce lipid accumulation [193]. Similarly, Chen and collaborators assessed the effects and the diffusive behavior of polystyrene nanoparticles via drinking water in mice observing adverse effects on pregnancy and foetal development. Indeed, the researchers found that the pollutant limited the foetal growth altering the skeletal muscle and lipid metabolism, highlighting the invasiveness and the detrimental effects that such pollutant can exert [194].

Regarding O₃, only a few studies explored the link between it and the skeletal muscles. For instance, Liu and collaborators evaluated the toxic effects of O₃ exposure during exercise on rats [195]. Their results showed pathological changes in the rat quadriceps with reduced ATP levels, inflammation, oxidative stress, and mitochondria-dependent apoptosis. Such toxic effects intensified accordingly to exercise intensity increment negatively affecting the sport performance of the rat on the treadmill compared to controls trained without O₃ exposure. Interestingly, the toxic effects of O₃ on skeletal muscle were present in rats regardless of the activity on the treadmill and the level of O₃ used, underscoring the great risk that follows the exposure to such environmental pollutant even at mild levels [195]. Similarly, a Chinese group performed a national cross-sectional study

to assess the role of air pollutants like O₃ in increasing the risk for sarcopenia in Chinese people [186]. Their findings produced evidence for increased prevalence of sarcopenia in association with air pollutants, among which O₃, exposure. In particular, for each increment of 1µg/m³ of O₃ a corresponding increase of 9,3% in risk of sarcopenia was found, with stronger association in females [196]. Lastly, evidence for O₃-dependent metabolic impairment in the skeletal muscle was proposed by Vella and colleagues [197]. By exploiting O₃ at 0,8ppm, rats exposed to such pollutant displayed oxidative and endoplasmic reticulum stresses, insulin resistance, and c-Jun N-terminal kinase (JNK) activation causing impaired insulin signaling whose role has already been described. Such results were confirmed *in vitro* by treating C2C12 cells with ozone-exposed rats-derived bronchoalveolar fluids, highlighting the key role of the lungs in the establishment of the O₃ toxicity. The described results are of great interest considering that not only describes O₃ molecular and metabolic toxicity on skeletal muscle in rats but also propose a strong linkage between O₃ and metabolic diseases like type 2 diabetes among which skeletal muscles play a pivotal metabolic role [197].

The literature of the last decades points the finger toward the detrimental role of environmental pollutants on human health describing the involvement of many systems of the human body, including the muscle system. Even if so much has been proposed and described so far, the knowledge regarding such pollutants, like polystyrene nanoparticles and O₃, and their effects on the skeletal muscle are still far from being fully addressed. Hopefully, future research will further describe not only toxicity mechanisms but also localization of and pathways in the tissues and organs in order to better understand their behavior and to potentially limit their spreading in the human body.

CHAPTER 5

AIM OF THE THESIS

5.3 Aim hMuSCs

In the last decades several studies have been conducted on environmental pollutants, highlighting their detrimental effects on human health both at short and long term [8,9]. Among the most widespread pollutants it is possible to find the O₃ and polystyrene nanoparticles, as mentioned above. Regarding O₃, it is broadly released by human activities like industrial processes [30]. In relation to polystyrene nanoparticles, polystyrene is largely used to produce many objects for everyday use due to its thermoplastic properties. However, polystyrene is not a bio-degradable plastic and consequently it is easily released into the environment [11–15]. The great impact of these pollutants on the human body has been proposed but not so much addressed. There is a general lack in detailed studies describing the impact of environmental pollutants in the human body's systems. For instance, the existing literature provides limited insights into the effects of these pollutants on muscles, and the available studies have been mainly conducted on animal models.

In light of this, the aim of the present thesis was to evaluate the interactions between environmental pollutants and human muscle, using biopsies-derived hMuSCs as human *in vitro* model. Considering the described extensive spreading of O₃ and polystyrene nanoparticles, these two pollutants have been selected for the experiments to analyze their effects on hMuSCs. To achieve this goal, the pollutants were tested firstly alone to better understand the impact of the single pollutant on hMuSCs. Subsequently, O₃ and polystyrene nanoparticles were tested in combination to evaluate their possible synergistic action, given that multiple pollutants are present in the environment simultaneously.

To gain an initial understanding of pollutants impact on hMuSCs, alterations in the fundamental parameters of cellular physiology, such as cell viability and differentiation capacity, were assessed. Furthermore, the expression of miRNAs involved in the proliferation and differentiation pathways of myogenic cells was examined.

Since the results described in literature about the correlation between pollutants and increased oxidant levels and considering, in particular, the O₃ capability to easily produce

the superoxide anion, superoxide anion levels in hMuSCs exposed to pollutants was also assessed [198].

In summary, this work was expected to provide a better understanding of O₃ and polystyrene nanoparticles' effects on human muscles, filling the literature gap.

5.2 Aim iPSCs-hMuSCs

Exposure to environmental pollutants during pregnancy has been proved to exert harmful effects for foetal development. Indeed, several studies demonstrated that pollutants exposure is associated with negative outcomes in the newborns, such as intrauterine growth restriction, preterm birth, decreased birth weight, and alterations in lung development and function [199].

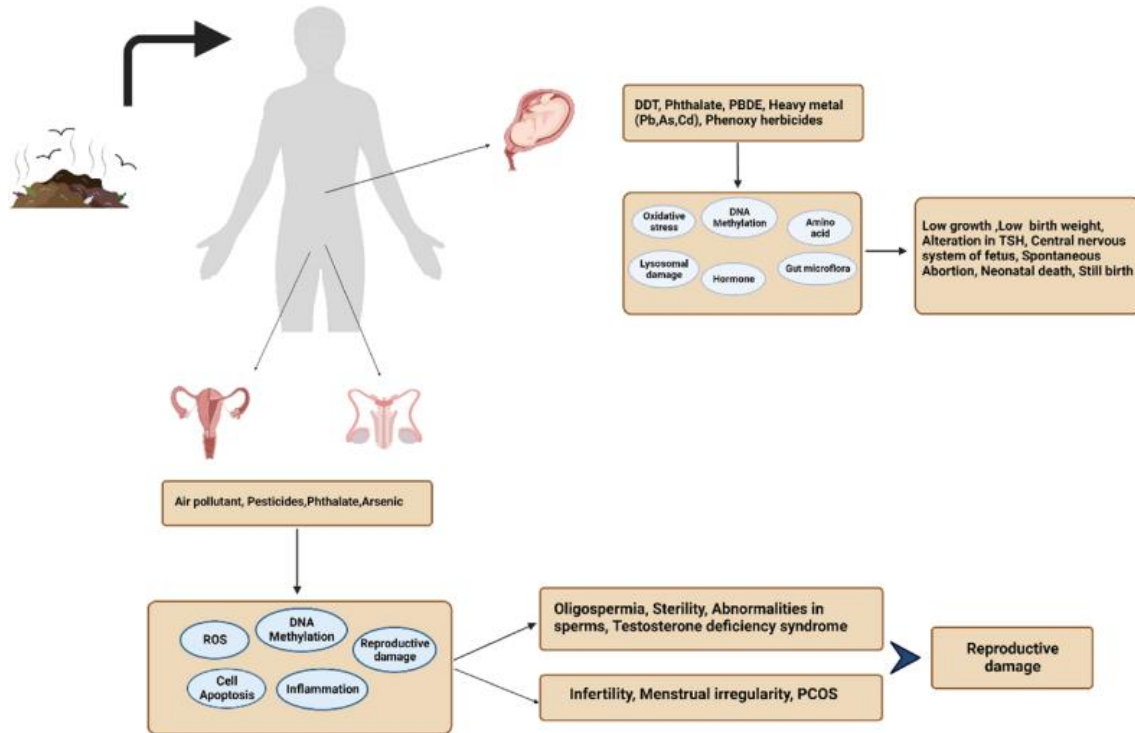


Fig. 6 Environmental pollutants effects on reproductivity and prenatal health [2].

To date, the impact of pollutants on foetal muscles has not been extensively explored. Thus, the aim of the study was to evaluate their effects on iPSC-hMuSCs. In fact, iPSCs represent a useful cell model to assess the impact of pollutants at foetal level [200].

In light of the diffusive behaviour of O₃ and polystyrene nanoparticles and their capability to harm human health, as previously described, these two pollutants were selected for the

experiments [8,9,11–14,30]. Firstly, they were used alone and then in combination, to explore both their singular impact and their potential synergistic effect on iPSC-hMuSCs. As for hMuCSs, to acquire a preliminary understanding of O₃ and polystyrene nanoparticles action on iPSC-hMuSCs, cell viability and differentiation capability were analyzed. Furthermore, the expression of miRNAs involved in myogenic proliferation and differentiation pathways was evaluated.

Through this work, it was expected to enhance the knowledge about the O₃ and polystyrene nanoparticles' effects on human muscle, particularly foetal muscle.

CHAPTER 6

MATERIALS AND METHODS

6.1 hMuSCs

6.1.1 Human subjects' recruitment and muscle biopsies sampling

Healthy male subjects (n=18) between 20 and 80 years old were recruited to undergo muscle biopsies of *Vastus Lateralis* in order to obtain hMuSCs. Before the study all the subjects signed written informed consent and the Ethics Committee for Biomedical Research, University of Chieti (PROT 1884 COET), ensured the adherence of the study to the Declaration of Helsinki (as amended in 2000). Furthermore, the health status of all subjects was assessed before the biopsy to avoid the presence of exclusion criteria, such as pathologies linked with muscle, bone or heart. ECG and blood pressure were also measured to guarantee they were within the standard ranges.

The tiny percutaneous needle technique was applied to perform the muscle biopsies in the area between the superior margin of the patella and the greater trochanter, which is characterized by absence of relevant neuro-vascular structures [201,202]. After anesthesia performed with 2 ml of xylocaine (1-2% Vol/Vol) the sterile disposable percutaneous needle was inserted directly into the muscle and the muscle fragment was collected.

Following the biopsy, muscle tissues were collected in a 10 ml tube with HAM's F-10 medium (#ECB7503SL, Euroclone, Italy) plus 50 mg/ml Gentamicin (#ECM0011D, Euroclone, Italy) in a sterile environment. The biopsies were stored first at 4°C, for up to 4 hours, then in liquid nitrogen at -196°C with 1 ml of defined Foetal Bovine Serum (FBS, #CHA1111L, Euroclone, Italy) and 10% dimethyl sulfoxide (DMSO, #D5879, Sigma-Aldrich, USA).

6.1.2 Culture of hMuSCs

hMuSCs were obtained from muscle biopsies following the procedure described by Fulle et al. [203]. Muscle biopsies were quickly defrosted at 37°C in the water bath and were placed in cell culture dishes with FBS after DMSO removal. Then, the biopsies were cut in small pieces with sterile scissors and each piece was plated with a drop of FBS to increase the adherence to the dish surface. At this point all the dishes with the biopsies were positioned in the incubator at 37°C with 5% of CO₂. 48 hours later, the Growth

Medium (GM) was added in the muscle biopsies dishes. The GM consists of the following components (% vol/vol):

- HAM's Nutrient Mixture F10,
- 20% FBS,
- 1% Penicillin-Streptomycin 100X (#ECB3001D, Euroclone, Italy),
- 1% Stable Glutamine 200mM (#ECB3004D, Euroclone, Italy),
- 0.1% Gentamicin.

After 7-14 days the first mononucleated cells migrated out of explants. Thus, the latter were removed, and cells were transferred to a new plate.

When cells reached 70-80% of confluence, they were split according to the following procedure: the GM was removed and two washes with Phosphate Buffered Saline (PBS, #ECB4004L, Euroclone, Italy) were carried out in order to remove the waste substances produced by cellular metabolism. Subsequently, trypsin-EDTA (#ECB3052D, Euroclone, Italy) was added to the dish and the latter was incubated at 37°C for 5 minutes. Once completely detached from the dish surface, cells were recovered in tubes and centrifugated for 5 minutes at 1200 rpm. Cells' pellet was resuspended with fresh GM to allow the cells count in the Burker's chamber and according to the number, cells were plated in new dishes. The population doubling level (pdl) was calculated after each split procedure, given that first cells derived from biopsies have 1 pdl, using the following formula:

$$\text{formula: } \frac{\log_{10}\left(\frac{N_t}{N_i}\right)}{\ln 2}.$$

· N_t = number of cells counted during the split procedure.

· N_i = number of cells seeded during the previous split procedure.

6.1.3 Differentiation of hMuSCs

The differentiation of hMuSCs into multinucleated myotubes was obtained as follows: cells were split and were plated in new dishes with GM. After one day of incubation, GM was removed and replaced with Differentiation Medium (DM), which consist of the following components:

- Dulbecco's Modified Eagle Medium High Glucose (DMEM, #ECB7501L, Euroclone, Italy),
- 5% Vol/Vol Horse Serum (HS, #ECS0091L, Euroclone, Italy),

- 1% Vol/Vol Glutamax 100X,
- 1% Vol/Vol Penicillin-Streptomycin 100X,
- 10 µg/ml of Insulin (#I-5500, Sigma-Aldrich, USA),
- 50 µg/ml Gentamicin,
- 100 µg/ml human Apo-Transferrin 99 % powder (#T-2036, Sigma-Aldrich, USA).

Regarding the HS, it was deactivated at 56°C for 30 minutes before use.

Cells were cultured in DM medium until the complete differentiation into myotubes, which occurs after 7 days.

6.1.4 Characterization of hMuSCs

To assess the myogenic purity and the effectiveness of differentiation of cell culture an immunocytochemical assay was performed using the antibodies Desmin and MF20 for the detection of Desmin and Myosin Heavy Chains proteins, respectively. Thus, cells were seeded in a 48-wells plate at a density of 5300 cells/cm² and after 7 days of incubation with DM the assay was conducted as described below. Firstly, the medium was removed and two washes with PBS 1X were carried out. Then cells were fixed in 70% ethanol for 1 minute and after one wash in PBS 1X for 5 minutes and one wash in Tris-Buffered Saline (TBS 1X) for another 5 minutes, the saturation of the non-specific sites with a solution of 2% FBS in TBS 1X for 30 minutes was performed. Subsequently, cells were incubated for 1 hour with one of the primary antibodies:

- Monoclonal Mouse primary antibody anti-human Desmin, clone D33 (#M0760, DAKO, USA), diluted 1:40 in TBS + 1% Bovine Serum Albumin (BSA, #A9647, Thermo Fisher Scientific, USA),
- Monoclonal Mouse primary antibody anti-Myosin Heavy Chain, clone MF20 (Developmental Studies Hybridoma Bank, University of Iowa, USA), diluted 1:50 in TBS + 1% BSA.

As negative control, only TBS + 1% BSA without antibodies was added to one well.

After the incubation with the primary antibodies and two washes in TBS 1X (5 minutes each), cells were incubated first with the link biotinylated secondary antibody (#K5005, DAKO, USA) for 15 minutes and then with the Streptavidin Horseradish Peroxidase (HRP, #K5005, DAKO, USA) for an additional 15 minutes. Finally, a solution of DAB + Chromogen with HRP Substrate Buffer (1:50) (#K5007, DAKO, USA) was added and

the reaction was immediately halted with water addition. Myogenic cells and differentiated cells can be detected due to their brown staining, with haematoxylin used for counterstaining.

To determine the myogenic purity and differentiation rate, 10 random pictures of each well were taken using a digital camera (Canon EOS 350D, Japan) connected to the microscope (Leica Microsystems CMS GmbH, Germany) at 20X magnification. Then, the number of cells positive for desmin antibody was quantified using the ImageJ software and the percentage of myogenic purity was calculated as the ratio of the number of desmin-positive cells to the total number of cells multiplied by 100. Instead, regarding the differentiation rate, it was calculated as Fusion Index percentage (FI%) as the ratio of the number of nuclei in the myotubes to the total number of nuclei multiplied by 100.

6.1.5 Treatment: pollutants exposure protocols

hMuSCs were treated with O₃ and polystyrene nanoparticles. Regarding O₃, it was tested at a concentration of 120 ppb, which is two times above the threshold limit defined by EU legislation as safe for human health [204]. The O₃ was directly injected into the incubator at the selected concentration using the device Ozone Calibration Source™—Model 306 (OCS™) from 2B Technologies Inc. (USA) connected to the incubator.

Concerning the polystyrene nanoparticles, nanoparticles of different sizes (100, 200, 600 and 800 nm) were purchased (10% solids suspension, #43302, #69057, #5067 and #65984, Sigma-Aldrich, USA) and diluted 1:1000 and 1:2000 in GM or DM for treatment. The cited dilutions were selected accordingly to previous studies where their cytotoxic effects have been demonstrated on various cell lines, including human cells [205].

The two pollutants were tested firstly alone and then in combination. hMuSCs cultured in a standard incubator without any treatment were used as controls. Indeed, in this case the incubator was not linked with the ozone device, and polystyrene nanoparticles were not added to hMuSCs medium.

6.1.6 Viability

Cell viability was evaluated through a colorimetric assay that measured a blue formazan product reduced from tetrazolium-based compound by living cells (MTT assay). To

perform the assay, hMuSCs were seeded in 96-well plate and, after treatment, 20 μ L of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide at a concentration of 5 mg/mL (#475989, Sigma-Aldrich, USA) were added to each well. Subsequently, hMuSCs were incubated for 3 hours at 37°C and then centrifuged at 2000 rpm for 15 minutes. The supernatant was removed, the formazan crystals at the bottom of the well were dissolved in 200 μ L of DMSO and the plates were incubated for another 30 minutes at 37°C. Finally, a reading on the spectrophotometer Synergy H1 BioTek (Agilent, USA) was performed at a wavelength of 540 nm.

MTT assay was carried out after 24, 48 and 72 hours of hMuSCs incubation with O₃ and polystyrene nanoparticles of different sizes (100, 200, 600 and 800 nm) at two different dilutions (1:1000, 1:2000) tested alone. Subsequently, MTT was performed at the same timepoints (24, 48, 72 hours) after exposing hMuSCs to O₃ in combination with 100 and 200 nm polystyrene nanoparticles diluted 1:1000 and 1:2000.

6.1.7 Differentiation capability

The differentiation capability of hMuSCs was assessed through an immunocytochemistry assay using MF-20 antibody for the Myosin Heavy Chain protein detection, as previously described in the hMuSCs characterization paragraph. hMuSCs were cultured for 4 and 7 days in DM while being incubated with O₃ and nanoparticles of different sizes (100 and 200 nm) diluted 1:1000 and 1:2000 either alone or in combination before performing the protocol for differentiation capability.

6.1.8 miRNAs expression

The total RNA isolation was performed on hMuSCs pellets after 24 hours of treatment with O₃ and polystyrene nanoparticles of different sizes (100 and 200 nm) at 1:1000 dilution, firstly tested alone and then in combination. To achieve this goal, miRNeasy Micro Kit (#217084, Qiagen, Netherlands) was performed according to the manufacturer's protocol, and the total amount of RNA was quantified using the NanoPhotometer® NP80 (Implen, Germany). The absorbance was measured at 2 different wavelengths λ : 260 nm, indicative of nucleic acid presence, and 280 nm, indicative of protein presence. The A260/A280 ratio was also evaluated as a quality index for the obtained RNA.

After RNA extraction, the reverse transcription of RNA into cDNA was carried out through the High-Capacity cDNA Reverse Transcription kit (#4368814, Applied Biosystems, USA). Finally, the qRT-PCR was performed in 96-well plates with the TaqMan probes and the specific TaqMan Universal Master Mix II, no UNG (#4440040, Applied Biosystems, USA) using the QuantStudio™ 7 Pro Real-Time PCR System (Thermo Fisher Scientific, USA). Specific miRNA sequence probes (Applied Biosystems, Life Technologies, Italy) were used to evaluate the expression of miR-1, miR-206, miR-133a, miR-133b, miR-23a and miR-23b: hsa-miR-1 (#002222), hsa-miR-133a (#002246), hsa-miR-133b (#002247), hsa-miR-206 (#000510), hsa-miR-23a (#000399) and hsa-miR-23b (#000400). The ubiquitously expressed miR-16, hsa-miR-16-5p (#000391, Applied Biosystems, USA), was used as an internal control. Furthermore, the relative quantification of miRNA targets was performed using the ΔC_t formula ($C_{t\text{miRNA of interest}} - C_{t\text{miR-16}}$), following the Ct method.

6.1.9 Superoxide anion levels

Mitochondrial superoxide levels were assessed through the Mitochondrial Superoxide Detection Kit (Fluorometric, #ab219943, Abcam, UK), in according to the manufacturer's instructions. hMuSCs were seeded in a 96-well black-bottom plate and were treated for 24 hours with O₃ and polystyrene nanoparticles of different sizes (100 and 200 nm) diluted 1:1000, tested both alone and in combination. After exposure to environmental pollutants, two washes with PBS 1X were carried out and hMuSCs were incubated with the fluorogenic reagent of the kit for 30 minutes at 37°C in the dark. Finally, the fluorescence was measured at an excitation wavelength of 540 nm and an emission wavelength of 590 nm using the spectrophotometer Synergy H1 BioTek.

6.1.10 Statistical Analysis

The statistical analysis was performed through the Software GraphPad Prism, using version 9.3.1 (GraphPad Software, USA). The data are reported as the mean $\pm$ SEM, for the MTT assay and the Superoxide Assay kit, and mean $\pm$ SD for differentiation assay and miRNAs expression. Furthermore, unpaired *t*-test was conducted in order to identify statistical differences, with a p-value < 0.05 considered statistically significant.

6.2 iPSC-hMuSCs

6.2.1 Culture of iPSCs

The human healthy iPSCs lines were generated using the Sendai Virus to reprogramme male donor fibroblast cells at the Stem Cell Institute Leuven, KU Leuven, in the laboratory of Prof. M. Sampaolesi. Undifferentiated iPSCs were cultured in Essential 8 Flex Basal Medium (E8, #A1517001, Thermo Scientific, USA) supplemented with Essential 8 Flex Supplement (50x, #A2858501, Thermo Scientific, USA) on plates coated with Geltrex LDEV-Free hESC-Qualified Reduced Growth Factor Basement Membrane Matrix (#A1413201, Thermo Scientific, USA). The penicillin–streptomycin 0.1% (#15140122, Thermo Scientific, USA) was also added to the medium. iPSCs were incubated under normoxic parameters (21% O₂ and 5% CO₂) at 37°C and were split approximately every two or three days using 0.5 mM EDTA (#AM9912, Thermo Scientific, USA) in Phosphate-Buffered Saline (PBS, #10010023, Thermo Scientific, USA).

6.2.2 Differentiation of iPSCs

iPSCs differentiation into skeletal muscle was assessed following the protocol of Caron et al. in a 5% O₂/5% CO₂ incubator [206]. Before inducing the differentiation, iPSCs were split using TrypLE (#12604013, Thermo Scientific, USA), were seeded onto Growth Factor Reduced (GFR) Matrigel (#354230, Corning, USA)-coated plates and were incubated at 37°C with 5% of CO₂ for 24 hours with E8 medium supplemented with RevitaCell (#A2644501, Thermo Scientific, USA) diluted 1:100. The following day, the medium was replaced by the first differentiation medium, namely Skeletal Muscle Induction Medium, until the day 10 of differentiation. Cited medium consisting of the following component:

- Essential 6 (E6, #A1516401, Thermo Scientific, USA),
- 5% horse serum (#16050122, Thermo Scientific, USA),
- 3 µM CHIR99021 (#4423, Bio-technique, USA),
- 2 µM SB431542 (#M1794, Bio-connect, AbMole, USA),
- 10 ng/ml human recombinant epidermal growth factor (hr-EGF, #AF-100-15, PeproTech, USA),
- 10 µg/ml insulin (#I9278, Sigma-Aldrich, USA),

- 0.4 µg/ml dexamethasone (#D4902, Sigma-Aldrich, USA),
- 200 µM ascorbic acid (#A4403, Sigma-Aldrich, USA).

At day 10, cells were split using 0.05% trypsin-EDTA (#25300054, Thermo Scientific, USA), plated again onto GFR Matrigel-coated plates, and maintained for 8 days in Skeletal Myoblast Medium. This second differentiation medium was composed of:

- E6,
- 5% Vol/Vol horse serum,
- 10 µg/ml insulin,
- 10 ng/ml hr-EGF,
- 20 ng/ml human recombinant hepatocyte growth factor (hr-HGF, #100-39, PeproTech, USA),
- 10 ng/ml human recombinant platelet-derived growth factor (hr-PDGF, #100-14A, PeproTech, USA),
- 20 ng/ml human recombinant basic fibroblast growth factor (hr-bFGF, #AF-100-18B-100, PeproTech, USA),
- 20 ng/ml recombinant human oncostatin M (#300-10, PeproTech, USA),
- 10 ng/ml insulin-like growth factor 1 (IGF-1, # 100-11-100, PeproTech, USA)
- 2 µM SB431542,
- 200 µM ascorbic acid.

After 18 days of differentiation, the previous medium was replaced by the third differentiation medium, the Myotube Medium, containing:

- E6,
- 10 µg/ml insulin,
- 20 ng/ml oncostatin,
- 50 nM necrosulfonamide (#74054, Cell Signaling, USA),
- 200 µM ascorbic acid.

Cells were cultured in Myotube Medium for 7 days until the fully differentiation into myotubes.

6.2.3 Treatment: pollutants exposure protocols

iPSC-hMuSCs were exposed to O₃ and polystyrene nanoparticles. Concerning O₃, it was tested at twice the safety limit defined by EU legislation for human health, i.e. 120 ppb

[204]. The device Ozone Calibration Source™—Model 306 (OCS™) from 2B Technologies Inc. (USA) was connected to the incubator to directly inject the O₃ at the cited concentration.

Relating to the polystyrene nanoparticles, 100 nm nanoparticles (10% solids suspension, #43302, Sigma-Aldrich, USA) at 1:1000 dilution were added to the DM.

iPSC-hMuSCs were treated firstly with O₃ and polystyrene nanoparticles alone and then with the combination of the two.

Controls were represented by untreated iPSC-hMuSCs cultured in standard conditions. Thus, the ozone device was not connected to the incubator and the medium was polystyrene nanoparticles-free.

6.2.4 Viability

iPSC-hMuSCs viability was assessed using the MTT assay, a colorimetric test that measures the ability of living cells to reduce tetrazolium-based compound into a blue formazan product. To perform the MTT, iPSC-hMuSCs were seeded in 96-well plate and once completely differentiated into myotubes, were treated with pollutants. Subsequently, to each well were added 20 µL of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide at a concentration of 5 mg/mL (#475989, Sigma-Aldrich, USA). Cells were incubated at 37°C for 3 hours and then centrifuged for 15 minutes at 2000 rpm. After supernatant removal, 200 µL of DMSO were added to each well to dissolve the formazan crystals at the bottom of the plates. The latter were incubated for an additional 30 minutes at 37°C and the absorbance at a wavelength of 540 nm was measured using the spectrophotometer Synergy H1 BioTek.

MTT assay performed after 24 hours of iPSC-hMuSCs exposure to O₃ and polystyrene 100 nm nanoparticles diluted 1:1000, tested both alone and in combination.

6.2.5 Differentiation capability

iPSC-hMuSCs differentiation capability was evaluated using an immunocytochemistry assay with MF-20 and MyoD1 antibodies to detect Myosin Heavy Chain and Myogenic Differentiation 1 proteins, respectively. Before performing the test, iPSC-hMuSCs were seeded in 6-well plates and fully differentiated into myotubes. On day 26 of differentiation, cells were incubated for 24 hours with O₃ and 100 nm nanoparticles

diluted 1:1000, tested either alone or in combination. After treatment, cells were fixed for 15 minutes using 4% paraformaldehyde (#441244, Sigma-Aldrich, USA) and were permeabilized for 30 minutes with 1% bovine serum albumin (BSA, #A7979-50ML, Sigma-Aldrich, USA) in PBS + 0.2-0.5% Triton™ X-100 (#T8787-50ML, Sigma-Aldrich, USA). Subsequently, a 10% donkey serum (#S2170, Biowest, France) solution was added to each well to block non-specific binding and cells were incubated at 4°C overnight with the two primary antibodies:

- Monoclonal Mouse primary antibody anti-Myosin Heavy Chain, clone MF20 (Developmental Studies Hybridoma Bank, University of Iowa, USA), diluted 1:20 in PBS + 1% BSA,
- Monoclonal Rabbit primary antibody anti-human MyoD1, clone D8G3 (#13812S, Bioké, Cell Signaling Technology, USA), diluted 1:400 in PBS + 1% BSA.

The next day, the secondary antibodies Alexa Fluor™-conjugated Alexa 594 (#A32744, Invitrogen, USA) and Alexa 488 (#A32790, Invitrogen, USA) diluted 1:500 were added to the wells to reveal MF20 and MyoD1, respectively. Finally, nuclei were marked using the Hoechst 33342 Solution diluted 1:3000 (#62249, Thermo Scientific, USA). To acquire pictures the Nikon Eclipse Ti microscope was used in combination with the NIS-Elements software (Nikon, Japan). Furthermore, the differentiation rate was calculated as the ratio of the nuclei number in the myotubes to the total number of nuclei multiplied by 100, i.e. FI%.

6.2.6 miRNAs expression

iPSC-hMuSCs were fully differentiated into myotubes and on day 26, were treated for 24 hours with O₃ and 100 nm polystyrene nanoparticles diluted 1:1000, firstly tested alone and then in combination. Subsequently cell pellets were obtained from which the total RNA isolation was performed with the TRIzol™ reagent (#15596026, Invitrogen, USA), following to the manufacturer's instructions. Briefly, Trizol and Chloroform (#11352878, Thermo Scientific, USA) were added to the cell pellet and samples were centrifuged at 2000 g for 15 minutes at 4°C. The aqueous phase was recovered and transferred into new tubes. Then, isopropanol 100% (#10215390, Thermo Scientific, USA) was added to each tube, followed by centrifugation for 10 minutes at 4°C and 12000 g. After pellet washing with ethanol 75% (#11924051, Thermo Scientific, USA) and centrifugation for 7 minutes

at 4°C and 7400 g, 20 µL of RNase-free water were added to the pellet. Nanodrop™ 1000 (Thermo Scientific, USA) was used to quantify the total amount of RNA. The absorbance at 260 nm, to evaluate the presence of nucleic acid, and 280 nm, to evaluate the presence of proteins. The A260/A280 ratio was also measured to assess the quality of the obtained RNA.

Once quantified, the RNA extraction was reverse transcribed into cDNA using the miRCURY LNA RT Kit (#339340, Qiagen, Netherlands), in agreement with the manufacturer's instructions. As a final step, the quantitative real-time PCR was performed in 384-well plates with the miRCURY LNA SYBR® Green PCR Kit (#339346, Qiagen, Netherlands) using the ViiA 7 System. To evaluate the miRNAs expression specific miRNA sequences the following probes (#339306, Qiagen, Netherlands) were used: hsa-miR-1, hsa-miR-133a, hsa-miR-133b, hsa-miR-206, hsa-miR-23a and hsa-miR-23b. To calculate the relative miRNAs expression was used the ΔC_t method ($C_{t\text{miRNA of interest}} - C_{t\text{miR-16}}$), normalizing to the internal control UniSp6 spike-in (#339346, Qiagen, Netherlands).

6.2.7 Statistical Analysis

The GraphPad Prism software, version 9.3.1 (GraphPad Software, USA), was used for the statistical analysis. The MTT data are shown as mean $\pm$ SEM, while differentiation capability and miRNAs expression data are reported as mean $\pm$ SD. To evaluate statistical differences, unpaired *t*-test was performed considering a *p*-value < 0.05 as statistically significant.

CHAPTER 7

RESULTS

7.1 hMuSCs

7.1.1 hMuSCs viability

Measurements of hMuSCs viability depicted the impact of the tested pollutants on these cells. Indeed, hMuSCs exposed to O₃ and polystyrene nanoparticles showed reduced viability. In detail, hMuSCs exposed to O₃ for 24, 48 and 72 hours showed a statistically significant reduction of viability compared to controls, already after 24h of incubation (**Figure 7**). Similarly, hMuSCs exposed for 24, 48 and 72 hours to polystyrene nanoparticles of different sizes (100, 200, 600 and 800 nm) and dilutions (1:1000, 1:2000) showed a reduced viability, with a stronger effect related to smaller size and higher concentration of nanoparticles (**Figure 8**). Based on the results obtained, only the 100 and 200 nm nanoparticles were selected for the subsequent study and tested in combination with O₃. Interestingly, hMuSCs exposed for 24, 48 and 72 hours to a combination of O₃ and nanoparticles of different size (100 and 200 nm) and dilutions (1:1000, 1:2000) showed reduced viability in comparison to control cells, particularly when the dilution of nanoparticles is lower (**Figure 9**).

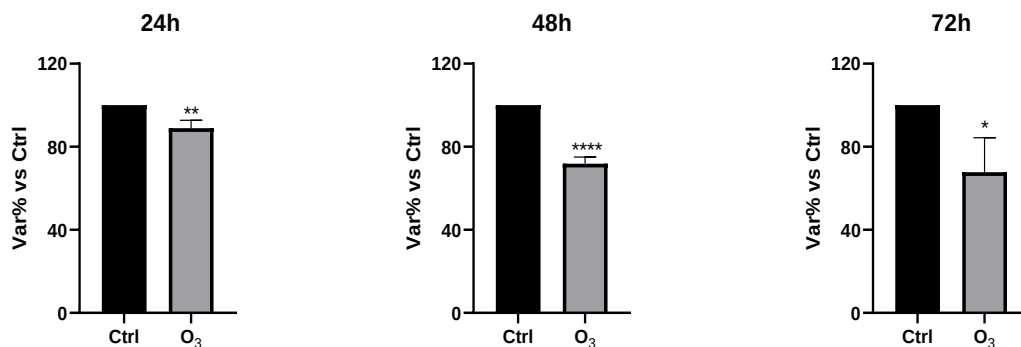


Fig.7 MTT assay of hMuSCs: Viability of hMuSCs exposed to O₃ (120 ppb) for 24h, 48h and 72h. Data are expressed as Var% vs Ctrl. The means $\pm$ SEM of $n=40$ and were analyzed using unpaired Student's *t*-tests **** $P<0.0001$, ** $P<0.005$, * $P<0.05$ vs Ctrl.

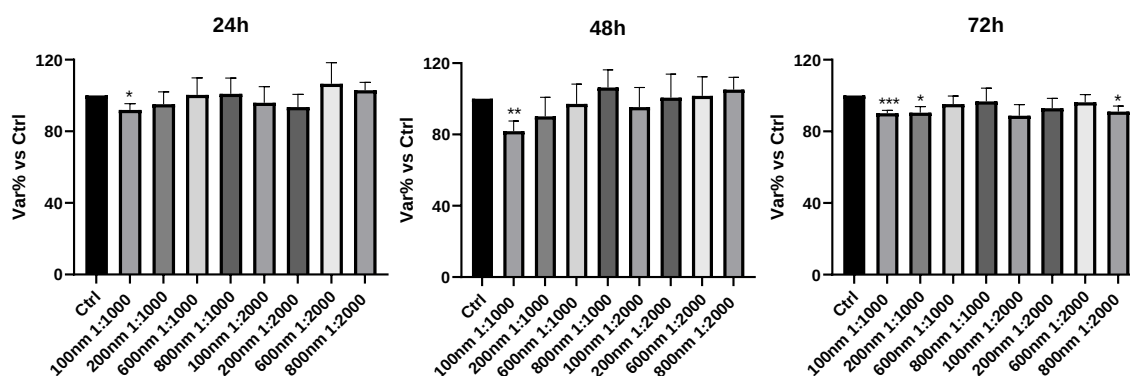


Fig.8 MTT assay of hMuSCs: Viability of hMuSCs treated for 24h, 48h and 72h with nanoparticles of different size (100, 200, 600 and 800 nm) and dilutions (1:1000, 1:2000). Data are expressed as Var% vs Ctrl. The means $\pm$ SEM of $n=40$ and were analyzed using unpaired Student's *t*-test. *** $P<0.0005$, ** $P<0.005$, * $P<0.05$ vs Ctrl.

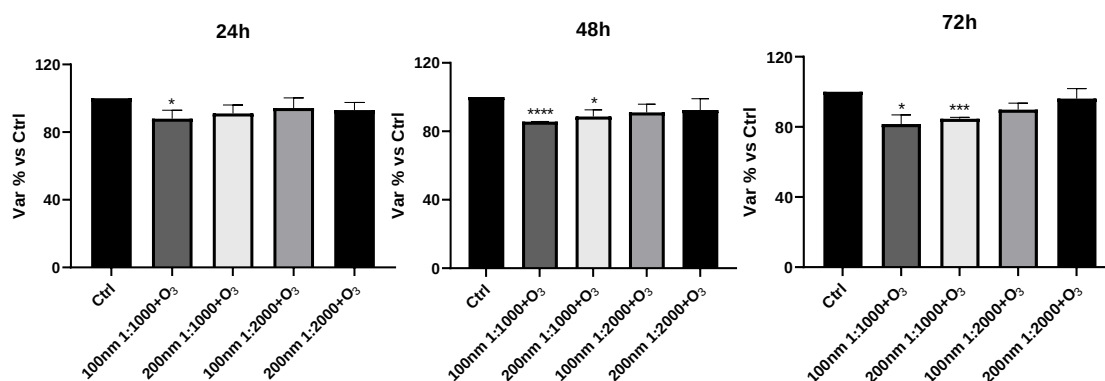


Fig.9 MTT assay of hMuSCs: Viability of hMuSCs treated for 24h, 48h and 72h with nanoparticles of different size (100 and 200 nm) and dilutions (1:1000, 1:2000) upon O₃ (120 ppb) exposure. Data are expressed as Var% vs Ctrl. The means $\pm$ SEM of $n=40$ and were analyzed using unpaired Student's *t*-test **** $P<0.0001$, *** $P<0.0005$, * $P<0.05$ vs Ctrl.

7.1.2 hMuSCs differentiation

The exposure to the chosen environmental pollutants impacted on the differentiation of hMuSCs as well. In fact, hMuSCs showed a reduced FI% after exposure to O₃ and nanoparticles both at 4 and 7 days of differentiation (**Figure 10 and 11**), corresponding to an early and a standard differentiation timepoint, respectively.

In detail, on day 4 a general reduction in FI% was observed in hMuSCs exposed to O₃ and polystyrene nanoparticles (100 and 200 nm; dilutions 1:1000, 1:2000), both alone and in combination, compared to controls. Notably, the worst and statistically significant result was recorded after treatment with O₃ in combination with 100 nm nanoparticles at 1:1000 dilution, as shown in **Table 1**.

On day 7, the FI% of hMuSCs treated with both O₃ and nanoparticles of different size (100 and 200 nm) and dilutions (1:1000, 1:2000) was significantly reduced, both when the pollutant were used alone or in combination. When combined, O₃ and 100 nm nanoparticles at 1:1000 dilution, demonstrated an even more detrimental effect, further reducing the FI% compared to pollutants tested alone (**Table 2**).

Furthermore, on both days 4 and 7, the number of unfused desmin-positive cells was higher in treated cells, either with pollutants used alone or in combination, compared to controls (**Table 1 and 2**).

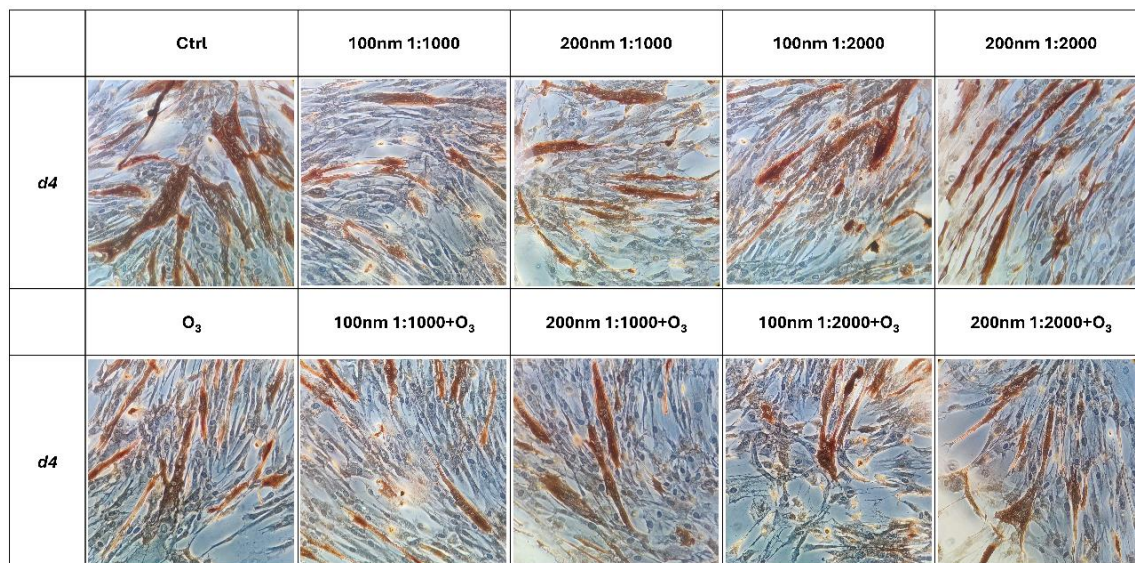


Fig.10 Immunocytochemistry on hMuSCs with MF-20. hMuSCs treated with O₃ (120 ppb) and nanoparticles of different size (100 and 200nm) and dilutions (1:1000, 1:2000), tested both alone and in combination. Day 4 (d4) of differentiation.

Sample	Fusion Index (%)	Unfused desmin+ cells (%)
Ctrl	38,70 ± 16,72	38,43 ± 05,62
100nm 1:1000	27,82 ± 14,09	71,09 ± 13,48 ***
200nm 1:1000	29,22 ± 15,92	74,30 ± 14,34 ***
100nm 1:2000	31,05 ± 18,79	49,69 ± 10,90 *
200nm 1:2000	33,54 ± 14,52	53,88 ± 20,92
O ₃	30,21 ± 14,39	62,11 ± 26,01
100nm 1:1000+O ₃	24,62 ± 16,99 *	81,14 ± 13,87 ****
200nm 1:1000+O ₃	28,66 ± 19,85	46,59 ± 26,20
100nm 1:2000+O ₃	27,75 ± 21,58	45,33 ± 08,47
200nm 1:2000+O ₃	28,06 ± 13,44	81,19 ± 08,86 ****

Tab.1 Immunocytochemistry on hMuSCs with MF-20. Fusion Index percentage of hMuSCs treated with O₃ (120 ppb) and nanoparticles of different size (100 and 200 nm) and dilutions (1:1000, 1:2000), tested both alone and in combination. Day 4 of differentiation. Data are shown as: percentage of Fusion Index (mean ± SD) of n=15; percentage of unfused cells positive to Desmin antibody (mean ± SD) of n=15. Student's t-test. ****P<0.0001, ***P<0.0005, *P<0.05 vs Ctrl.

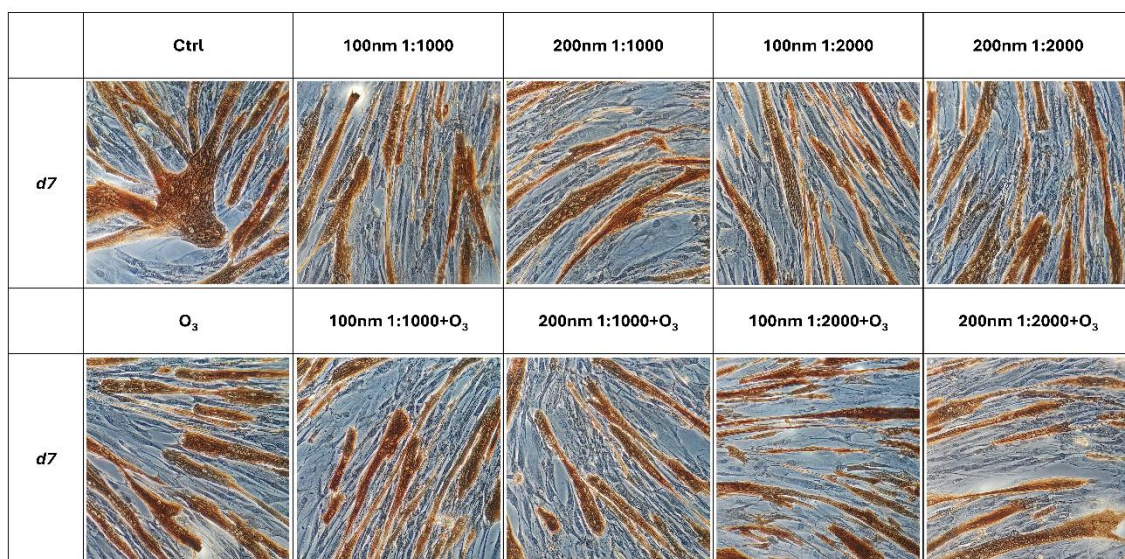


Fig.11 Immunocytochemistry on hMuSCs with MF-20. hMuSCs treated with O₃ (120 ppb) and nanoparticles of different size (100 and 200 nm) and dilutions (1:1000, 1:2000), tested both alone and in combination. Day 7 (d7) of differentiation.

Sample	Fusion Index (%)	Unfused desmin+ cells (%)
Ctrl	61,92 ± 11,16	11,29 ± 05,13
100nm 1:1000	48,95 ± 13,38 **	25,77 ± 10,58 **
200nm 1:1000	50,09 ± 13,09 **	19,71 ± 09,48 *
100nm 1:2000	49,73 ± 11,87 **	21,43 ± 06,40 **
200nm 1:2000	49,77 ± 13,15 **	27,24 ± 20,43
O ₃	50,26 ± 07,36 **	29,37 ± 12,19 **
100nm 1:1000+O ₃	45,82 ± 09,58 ***	19,46 ± 06,73 *
200nm 1:1000+O ₃	46,51 ± 09,83 ***	28,55 ± 10,19 ***
100nm 1:2000+O ₃	48,66 ± 09,10 ***	32,04 ± 12,67 ***
200nm 1:2000+O ₃	47,01 ± 07,60 ***	33,12 ± 12,36 ***

Tab.2 Immunocytochemistry on hMuSCs with MF-20. Fusion Index percentage of hMuSCs treated with O₃ (120 ppb) and nanoparticles of different sizes (100 and 200 nm) and dilutions (1:1000, 1:2000), tested both alone and in combination. Day 7 of differentiation. Data are shown as: percentage of Fusion Index (mean ± SD) of n=15; percentage of unfused cells positive to Desmin antibody (mean ± SD) of n=15. Student's t-test. ***P<0.0005, **P<0.005 *P<0.05 vs Ctrl.

7.1.3 hMuSCs miRNAs expression

Alterations in miRNAs expression were also observed. In particular, myo-miRNAs, including miR-1, miR-133a, miR-133b, and miR-206 [57], as well as miR-23a and miR-23b, were evaluated. When treated with O₃ and nanoparticles of different sizes (100 and 200 nm) at 1:1000 dilution, hMuSCs displayed alteration in cited miRNAs levels. In detail, **Figure 12** highlights a general trend of down-regulation of miRNA expression after 24 hours of treatment with nanoparticles compared to control cells. Furthermore, 24 hours of exposure to O₃ also lead to a general trend of down-regulation. This trend was further confirmed in the combined treatment with both pollutants. Notably, the results revealed a statistically significant downregulation of all analyzed miRNAs, particularly for miR-23a and miR-23b, following incubation with the combination of O₃ and polystyrene nanoparticles.

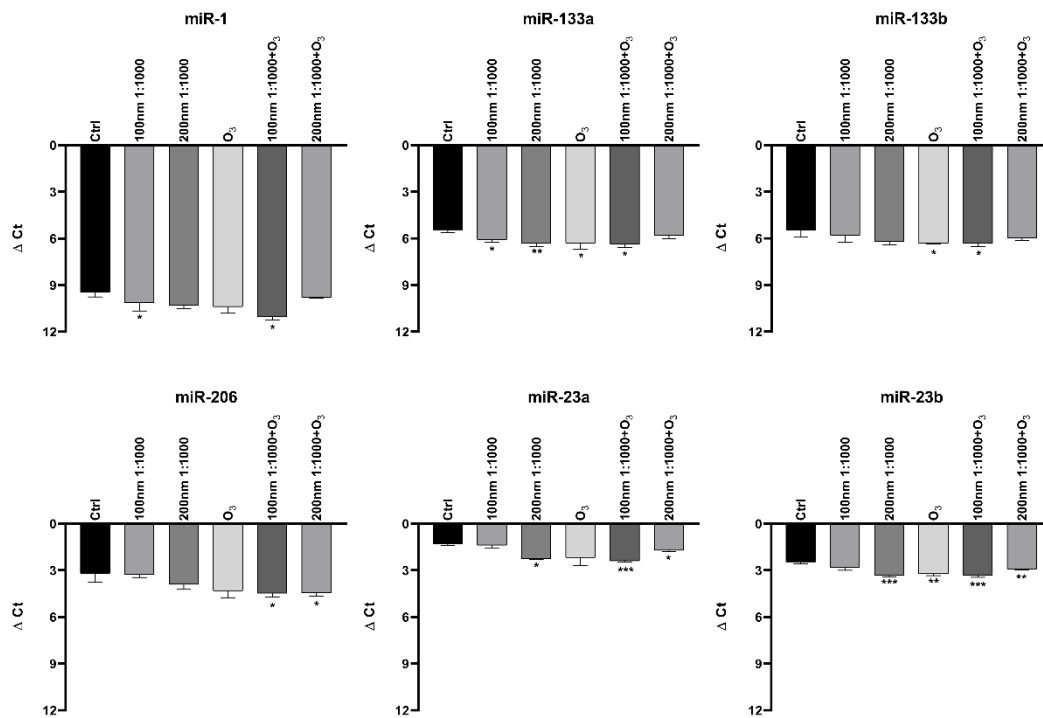


Fig. 12 Modulation of miRNA expression (ΔCt) levels in hMuSCs upon exposure to environmental pollutants. qRT-PCR on hMuSCs: miRNA expression treated with O₃ at 120 ppb, nanoparticles (100 and 200 nm) diluted 1:1000, and the combination of nanoparticles and O₃. Student's *t*-test. Data are shown as mean $\pm$ SD of *n*=3. ****P*<0.0005, ***P*<0.005, **P*<0.05 vs Ctrl.

7.1.4 hMuSCs superoxide anion levels

Intracellular levels of superoxide anion were measured to evaluate oxidative damage induced by O₃ and polystyrene nanoparticles. Thus, hMuSCs treated for 24 hours with O₃ and nanoparticles of different size (100 and 200 nm) at 1:1000 dilution, tested both alone and in combination. The results described a significant increment of superoxide anion levels upon pollutants exposure compared to controls. In particular, O₃ alone caused a stronger effect on hMuSCs compared to nanoparticles alone. Nonetheless, the combination of the two pollutants exerted a potent synergistic effect, with significantly higher levels of superoxide anion found in hMuSCs exposed to O₃ and 100nm nanoparticles at 1:1000 dilution, as shown in **Figure 13**.

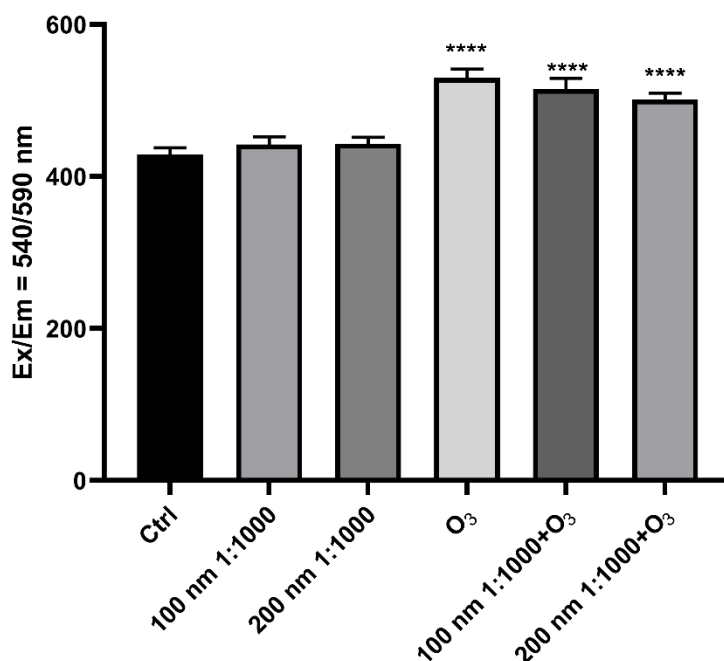


Fig.13 Levels of superoxide anion in hMuSCs after 24h of O₃ and nanoparticles exposure. Mitochondrial Superoxide Assay kit on hMuSCs: Intracellular levels of Superoxide anion of hMuSCs treated for 24h with O₃ at 120 ppb and nanoparticles (100 and 200 nm) diluted 1:1000, tested alone and in combination. Student's *t*-test. Data are shown as mean ± SEM of *n*=15. *****P*<0.0001 vs Ctrl.

7.2 iPSC-hMuSCs

7.2.1 iPSC-hMuSCs viability

MTT assays for iPSC-hMuSCs demonstrated the impact of O₃ and polystyrene nanoparticles pollutants on these cells' viability (**Figure 14**). In fact, the cells were exposed to pollutants alone, as well as the combination of the two. When used alone, a small decrease in viability was observed in cells treated with either O₃ at alone or 100 nm nanoparticles at 1:1000 dilution alone, if compared to control cells. Interestingly, the combination of O₃ and nanoparticles (100 nm at 1:1000) caused the worst and statistically significant reduction in iPSC-hMuSCs viability compared to controls.

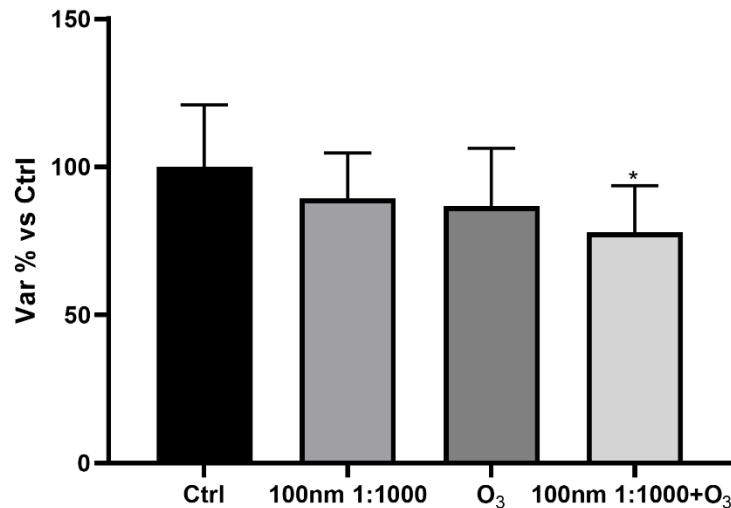


Fig. 14 iPSC-hMuSCs viability exposed to O₃ and nanoparticles. MTT assay of iPSC-hMuSCs: Viability of iPSC- hMuSCs treated with nanoparticles 100nm and 1:1000 dilution upon O₃ (120 ppb) exposure. Data are expressed as Var% vs Ctrl. The means $\pm$ SEM of $n=5$ and were analyzed using unpaired Student's t -test. * $P<0.05$ vs Ctrl.

7.2.2 iPSC-hMuSCs differentiation

The exposure to O₃ and polystyrene nanoparticles also altered the differentiation of iPSC-hMuSCs. Cells were treated with the two pollutants alone or in combination and changes in FI% were analyzed (**Figure 15**). Notably, it is possible to observe in **Table 3** statistically significant reduced scores of FI% in iPSC-hMuSCs (d26) exposed to both nanoparticles diluted 1:1000 at 100 nm tested alone and the combination of O₃ and nanoparticles 100 nm diluted 1:1000, compared to controls. O₃ also led to a reduction in cells' FI%, even though the reduction was not significant. The most pronounced outcome, with the lowest FI%, was recorded for the combination of the two pollutants.

Moreover, an increase of unfused nuclei was detected for iPSC-hMuSCs treated with O₃ and nanoparticles, both alone and in combination (**Table 3**).

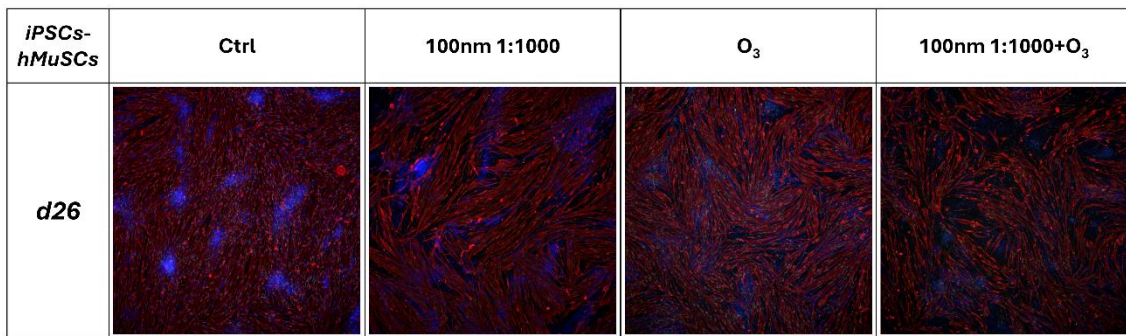


Fig. 15 Immunocytochemistry on iPSC-hMuSCs for MF-20 (Red) and Nuclei (DAPI). iPSC-hMuSCs treated with O₃ (120 ppb) and nanoparticles of 100nm diluted 1:1000, tested both alone and in combination. Day 26 (d26) of differentiation.

Sample	Fusion Index (%)	Unfused nuclei (%)
Ctrl	45,21 ± 8,83	54,79 ± 8,83
100nm 1:1000	28,23 ± 3,65 **	72,40 ± 3,61 ***
O ₃	33,96 ± 14,71	66,04 ± 14,71
100nm 1:1000+O ₃	25,93 ± 5,36 **	69,32 ± 8,82 *

Tab. 3 Fusion Index percentage after exposure of iPSC-hMuSCs to O₃ and nanoparticles. Fusion Index and Unfused nuclei percentages of iPSC-hMuSCs treated with O₃ at 120 ppb, nanoparticles (100 nm) diluted 1:1000, and the combination of nanoparticles and O₃ at day 26 of differentiation evaluated by immunofluorescence for MF20 and Nuclei (DAPI). Data are shown as: percentage of Fusion Index; percentage of unfused nuclei positive to DAPI of n=5. Student's t-test. **P<0.005.

7.2.3 iPSC- hMSCs miRNAs expression

The expression of miR-1, miR-133a, miR-133b, miR-206, miRNA-23a and miRNA-23b was evaluated in iPSC-hMuSCs exposed to pollutants, and alterations were observed. In particular, iPSC-hMuSCs at day 26 of differentiation were treated with O₃ alone, nanoparticles 100 nm diluted 1:1000 alone, and a combination of the two pollutants. A general alteration in all the analyzed miRNAs was detected. Interestingly, the only statistically significant result was the up-regulation of miR-206 in iPSC-hMuSCs under exposure to O₃ alone and the combination of O₃ and polystyrene nanoparticles, as shown in Figure 16.

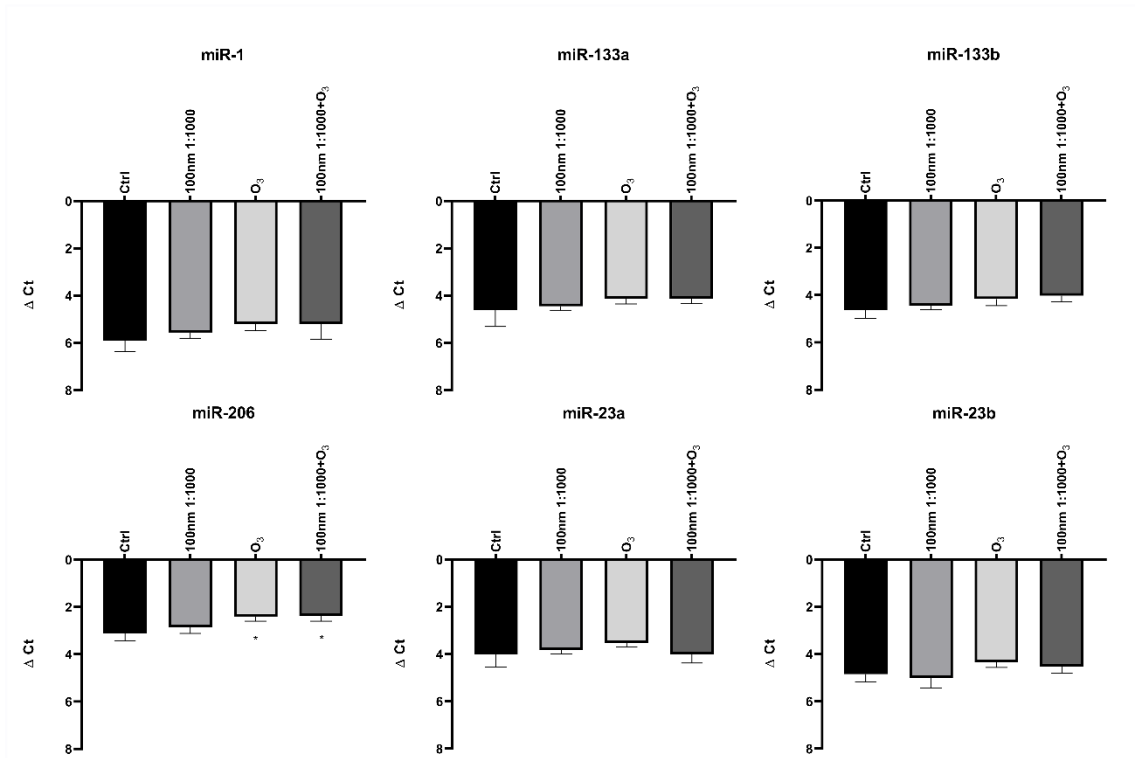


Fig. 16 Modulation of miRNAs expression levels (ΔCt) in iPSC-hMuSCs upon exposure to environmental pollutants. qRT-PCR on iPSC-hMuSCs: miRNA expression in iPSC-hMuSCs treated with O₃ at 120 ppb, nanoparticles (100nm) diluted 1:1000, and the combination of nanoparticles and O₃. Student's t-test. Data are shown as mean $\pm$ SD of n=9. *P<0.05 vs Ctrl.

CHAPTER 8

DISCUSSION

8.1 hMuSCs discussion

Considering the diffusive behavior of pollutants, in particular polystyrene nanoparticles and O₃, with their known adverse effects on human health and in light of the absence of specific study on human muscle, aim of the project was to examine environmental pollutant interaction with hMuSCs [182]. The obtained results highlight a negative impact of pollutant on hMuSCs viability. In fact, in **Figure 7** and **Figure 8** is possible to observe a reduction of treated cells viability compared to controls, which is significant already after 24 hours of incubation with O₃ or polystyrene nanoparticles tested alone. Concerning O₃, the concentration of 120 ppb was selected, as it represents twice the safe threshold for human health established by EU legislation, and it corresponds to levels repeatedly recorded in various regions of the US and Europe [204]. Studies in literature demonstrate how 120 ppb O₃ are able to exert toxic effects on several human cell types, however data on muscle cells were lacking [207,208]. Our findings demonstrate that the same harmful effect also occurs in hMuSCs. Regarding polystyrene nanoparticles, several studies in literature compare micro- and nano-particles effects on cells reporting their size-dependent toxic impact. Indeed, smaller size particles are able to lead to the worst damage. This effect is linked with the significantly higher accumulation of smaller size particles, i.e. nanoparticles, founded in several organs in animal models [209,210]. Our outcomes confirm the same effect also on human muscle: hMuSCs treated with polystyrene nanoparticles showed a significantly reduced viability compared to controls, in particular when treated with the smallest nanoparticles (100 nm) diluted 1:1000 in all three time points considered. Instead, by using 600 and 800 nm nanoparticles, no relevant differences were found in relation to controls (**Figure 8**). Thus, it is possible to assume that there is a higher accumulation of 100 and 200 nm, compare to 600 e 800 nm, in organs such as muscles, causing more pronounced damage. In view of the evidence in the literature and the results obtained for cell viability, only 100 and 200 nm nanoparticles were selected for the subsequent studies.

Indeed, cell viability was evaluated after hMuSCs incubation with O₃ in combination with the selected nanoparticles at different dilutions. The results exhibited also in this case a

reduction in cells viability with the most significant effect linked to the treatment with the combination of O₃ and nanoparticles at 1:1000 dilution. A significant result was already detected after 24 hours for cells treated with O₃ and 100 nm nanoparticles at 1:1000 dilution as illustrated in **Figure 9**. Taken together, these results demonstrated how O₃ and nanoparticles affected hMuSCs viability compared to controls, both alone and in combination.

Pollutants are also able to affect the differentiation capacity of hMuSCs. In fact, in the early differentiation timepoint, i.e. 4 days, the results reported in **Figure 10** and **Table 1** demonstrated a general trend of reduction in treated cells FI%, compared to controls. In particular, the significant and worst result was recorded for hMuSCs incubated with O₃ in combination with 100 nm nanoparticles used at 1:1000 dilution. Once fully differentiated, after 7 days, hMuSCs showed a significant reduction of FI% for all the tested conditions evaluated compared to controls, as showed **Figure 11** and **Table 2**, with the worst effect still related to the combination of O₃ with 100 nm nanoparticles used at 1:1000 dilution. Interestingly, the morphology of cells seems to be affected by pollutants as well. Indeed, treated hMuSCs form smaller myotubes with a reduced number of nuclei compared to the control cells, as observed in **Figure 10** and **Figure 11**. Interestingly, the worst effect was detected after treatment with O₃ in combination with 100 nm nanoparticles used at 1:1000 dilution, still, confirming what we have seen after 4 days. These results demonstrate a negative effect of cited pollutants on hMuSCs differentiation capability, highlighting a possible synergistic effect of O₃ and nanoparticles. Furthermore, in the light of viability and differentiation capability outcomes linked to nanoparticles treatment of cells, the lower dilution was found to have a more damaging effect. Such data agree with the reported literature that underline the higher toxicity of plastic particles when at higher concentrations [211]. For this reason, only cited dilution was selected for the following studies. Considering, also, that the detrimental effects of pollutants were already evident after 24 hours of treatment, only one time point was selected for the subsequent analysis, namely 24 hours.

To further evaluate pollutants interaction with hMuSCs, the expression of miRNAs was analyzed. As previously described, myo-miRNAs miR-1, miR-133a, miR-133b and miR-206, are known to be associated with proliferation and myogenic differentiation pathways [99–101]. Their activity is pivotal for the proper muscle development as they have been

found expressed at high levels during myoblast differentiation [109,118,119]. In addition, recent studies demonstrated the involvement of miR-23a and miR-23b in myogenic differentiation pathways [102]. In consideration of this, all the cited miRNAs were analyzed to further evaluate pollutants interaction with hMuSCs. The results in **Figure 12** demonstrated a trend of down-regulation for all miRNAs analyzed, using pollutants alone or in combination. The impact appears to be more pronounced in cells treated with O₃ in association with 100 nm polystyrene nanoparticles. Indeed, the down-regulation effect after cited treatment is significant for all miRNAs analysed, and mainly for miR-23a and miR-23b. Therefore, these data confirm our findings previously described, which indicated an impaired proliferation and differentiation following treatment with pollutants and, particularly, with the combination of O₃ and 100 nm nanoparticles diluted 1:1000. Finally, levels of superoxide anion were investigated. Indeed, as previously mentioned, plastic particles and O₃ can be responsible for oxidative stress, increasing ROS levels and causing oxidative damage to mitochondria [101,184]. In particular, O₃ is a powerful oxidant and most of its toxicity is mediated via reactive free radicals, especially superoxide anion, which O₃ is able to generate as it rapidly decomposes in the aqueous phase [33,34,198,212]. The results, reported in **Figure 13**, exhibited an increase in superoxide anion levels after treatment with O₃ alone and with the combination of O₃ and nanoparticles compared to controls, while nanoparticles alone seemed to not affect such ROS levels at all. Interestingly, these results suggest a combined mechanism of toxicity in which O₃-dependent oxidative stress is exacerbated by the co-presence of polystyrene nanoparticles, significantly at their lowest size and dilution.

Taken together, all the results obtained demonstrate the detrimental effect of both O₃ and polystyrene nanoparticles on hMuSCs. These pollutants are able to affect cells viability and differentiation capability, also leading to a miRNA expression alteration and to an increase in superoxide anion levels. Regarding nanoparticles, the effect is related to the dimension and the dilution tested. In detail, the smallest size nanoparticles (100 nm) used at lower dilution (1:1000) showed the worst effect on all the parameters considered. Moreover, the data suggest a synergistic detrimental effect of O₃ and nanoparticles on hMuSCs, since the most significant and worst effect is in all the case related to the combination of the two pollutants. Finally, the pollutants effects are detectable in the short-term since damage were recorded already after 24 hours of incubation.

8.2 iPSC-hMuSCs discussion

Since previous studies conducted on hMuSCs highlighted the detrimental effect of O₃ and polystyrene nanoparticles on adult muscle, the same pollutant were tested on iPSC-hMuSCs. Regarding polystyrene nanoparticles, 100 nm nanoparticles at 1:1000 dilution demonstrates the worst effects in hMuSCs. For this reason, only cited nanoparticle was selected for studies on iPSC-hMuSCs.

The obtained results well depicted the effects that such harmful pollutants exerted over iPSC-hMuSCs. Even though there is no significant evidence for the effects caused by the pollutant tested alone, a general decreasing trend can be observed for treated iPSC-hMuSCs compared to controls, suggesting that mechanisms of toxicity are working against physiology in such model. In particular, a synergistic activity of O₃ and polystyrene nanoparticles caused a decrement in cell viability (**Figure 14**). As previously described, the tested pollutants are responsible for establishing an oxidative and inflammatory environment both at local and distant levels [101,184]. These results agree with the previously obtained data on hMuSCs, where a reduction in cell viability was observed too.

Furthermore, the toxicity manifested by the tested pollutants impacted on the iPSC-hMuSCs differentiation, as shown in **Figure 15** and **Table 3**. The data obtained by the tested pollutants are consistent between each condition compared to control cells, suggesting once again the detrimental effects of the tested pollutants on human health. Moreover, as seen earlier for hMuSCs, the worst effect seems to be related to the exposure of nanoparticles and O₃ in combination.

To gain a better understanding of the molecular profile of iPSC-hMuSCs upon pollutants treatment, the same myo-miRNAs tested on hMuSCs were evaluated also in this case (**Figure 16**). Interestingly, the up-regulation found for such miRNAs is inconsistent with the data obtained on hMuSCs, as already stated. Indeed, a general trend of down-regulation was not recorded for iPSC-hMuSCs, as instead seen earlier for hMuSCs. Moreover, of all the assessed miRNAs, only miR-206 displayed significant results with raised levels under the effect of O₃ alone and the combination of the tested pollutants compared to controls. Although in contrast to previously obtained data, an increment in miR-206 levels can reflect a deep damage upon iPSC-hMuSCs as it is known that such

miRNA is up-regulated in satellite cells after injury [213,214]. Furthermore, the up-regulation of mir-206 was also recorded in several pathologies, including Duchene Muscular Dystrophy and Amyotrophic Lateral Sclerosis, indicating that it can be considered as biomarker of muscle dysfunction [215].

Taken together the obtained results highlight how both O₃ and polystyrene nanoparticles can affect iPSC-hMuSCs, reducing their viability and differentiation capacity, and altering miRNA expression, above all miR-206. The impact is particularly significant when O₃ and polystyrene nanoparticles are used in combination, suggesting a potential synergistic effect of the two. These data also confirm the outcomes derived from previous experiment on hMuSCs, drawing attention to the detrimental effect of such pollutants at foetal levels and, thus, during pregnancy. Indeed, recent studies highlight the toxic effect of environmental pollutant exposure during pregnancy for foetal development leading to intrauterine growth restriction, premature birth and reduced birth weight. It can also alter lung development and function of neonates [199]. Considering also that findings on damages to muscle related to environmental pollutants exposure during pregnancy are currently lacking and that iPSC can represent a cell model useful to study effect of pollutant at foetal level, the data collected in this study offer a first insight into the detrimental effects of O₃ and polystyrene nanoparticles [200].

CHAPTER 9

CONCLUSIONS

9.1 Conclusions

The results obtained in the present study demonstrate the detrimental effect of O₃ and polystyrene nanoparticles on both adult and foetal muscle. Indeed, the outcomes highlight how already after 24 hours of exposure to pollutants alterations in cells viability, differentiation capability and miRNAs expression can be found in treated cells compared to controls. In detail, in both the models used, i.e. hMuSCs and iPSC-hMuSCs, a significant reduction in cells viability and differentiation capability was recorded after treatment with O₃ and polystyrene nanoparticles tested both alone and in combination. Regarding miRNAs expression, in treated hMuSCs was observed a general trend of down-regulation of all the miRNA analyzed and, in particular, of miR-133a, miR-206, miR-23a and miR-23b, while in treated iPSC-hMuSCs was identified an up-regulation of miR-206. In both instances, the alternations in miRNAs expression caused by pollutants led to a damage to muscle physiology. Moreover, the hMuSCs exposure to pollutants caused an increase in superoxide anion levels, that can result in higher oxidative stress and consequent damage to muscle.

The experiments conducted on hMuSCs prove that smaller size nanoparticles 100 nm are the most harmful, probably due to an increased invasiveness linked to their small size as suggested in the discussion. The dilution is another parameter linked to polystyrene nanoparticles impact: in fact, the worst effect was recorded after treatment with nanoparticles diluted 1:1000.

Finally, the obtained results demonstrate a potential synergistic effect of O₃ and polystyrene nanoparticles, specifically 100 nm nanoparticles diluted 1:1000, since in all the assays performed on hMuSCs and iPSC-hMuSCs the cited combination led to the worst outcome, highlighting the most harmful effect.

To conclude, the present study offers a first insight into the pollutants negative impact on human muscle. In the future, it will be worthwhile to deepen the knowledge about the molecular mechanisms of action of the cited pollutants to better understand how O₃ and polystyrene nanoparticles exert their toxic effects. As future perspective of the study, it will be also interesting evaluate the impact of such pollutants on more complex in human

in vitro models, namely 3D models, to see if the results obtained in hMuSCs and iPSC-hMuSCs are confirmed. In fact, evidence of evaluation of environmental pollutants and their effects on more complex models, such as *in vivo* animal models, can be found in literature. Therefore, new human 3D *in vitro* models can pave the way to more in-depth studies to describe molecular and toxicological impacts of environmental pollutants on human biology avoiding, where scientifically and ethically possible, the animal usage, even though the complexity of an *in vivo* system will be not recapitulated *in vitro*.

At the clinical level, the next step would involve validating whether the observed cytotoxicity and dysregulation of critical myogenic miRNAs are recapitulated in human subjects with high environmental exposure to O₃ and polystyrene nanoparticles. Prospective clinical studies should aim to correlate exposure biomarkers with muscle function metrics and oxidative stress indices, thereby establishing a link between the *in vitro* observations and potential human pathology.

From an epidemiological perspective, these findings support the initiation of large-scale, longitudinal studies to assess the prevalence of pollutant-induced muscle impairments across diverse populations. Such investigations should integrate environmental monitoring data with clinical phenotyping to establish exposure-response relationships. Moreover, further investigation of the apparent synergism between O₃ and polystyrene nanoparticles through advanced mechanistic studies would be valuable for elucidating the molecular cascades underlying this synergistic toxicity. Gaining mechanistic insight is crucial not only for refining exposure risk models but also for developing targeted interventions to mitigate pollutant-induced muscle damage.

In summary, integrating these preclinical findings into a translational framework holds significant promise for elucidating the public health implications of environmental pollutants on muscle physiology, ultimately guiding both therapeutic and regulatory strategies.

CHAPTER 10

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