



**“G. d’Annunzio” University of
Chieti-Pescara**



“G. D’Annunzio” School of Advanced Studies
Doctorate school in
INNOVATIVE TECHNOLOGIES IN CLINICAL MEDICINE AND
DENTISTRY
XXXVIII CYCLE

***In Vitro Assessment Of The Cytotoxic Effects Of Pesticides On Human Gingival
Mesenchymal Stem Cells (hGMSCs) And Human Umbilical Vein Endothelial
Cells (HUVECs)***

Department of Innovative Technologies in Medicine & Dentistry

Disciplinary Area MEDS-26/A

Ph.D. Student

Dr. Antonella Mazzone

Coordinator

Prof. Sergio Caputi

Tutor

Prof. Emanuela Mazzon

Academic Years 2022/2025



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Mazzone A.

*Ai miei nonni,
stelle del mio cielo.*

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ABSTRACT

The widespread use of pesticides in agriculture and industry has emerged as a critical global health concern, as chronic exposure to these chemical agents poses significant risks to human physiology and environmental safety. The oral cavity and the vascular endothelium represent two primary targets for xenobiotics; in particular, the high vascularization of the sublingual and oral mucosa facilitates the systemic absorption of lipophilic pesticides, allowing them to bypass first-pass metabolism and enter the bloodstream directly. This study evaluates the molecular and biological effects of exposure to Boscalid, Pyraclostrobin, Propamocarb, and Lambda-cyhalothrin on inflammatory and senescence pathways using a dual-model approach: Human Gingival Mesenchymal Stem Cells (hGMSCs), representing the oral regenerative potential, and Human Umbilical Vein Endothelial Cells (HUVECs), representing the systemic vascular barrier. Analysis through MTS assays, SEM, and immunofluorescence, alongside gene expression of NALP3, CASP1, IL-1 β , p53, p21, and TERT, revealed that pesticide exposure—particularly in synergistic combinations like BPYPR and BPYLC—induces significant morphological alterations, triggers ROS production, and activates the NLRP3 inflammasome. Furthermore, treated cells exhibited accelerated cellular senescence characterized by telomere shortening and mitochondrial DNA (mtDNA-CN) imbalances, demonstrating that pesticides simultaneously compromise the progenitor pool essential for tissue repair and the vascular infrastructure necessary for systemic homeostasis, posing a generalized systemic risk.

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1. INTRODUCTION

1.1 OVERVIEW OF PESTICIDES' USE AND RELEVANCE TO PUBLIC HEALTH

Pesticides have played a crucial role in agricultural production, preservation, pest control, and disease management. Their widespread adoption over the past century has largely resulted from the development and commercialization of highly effective synthetic organic chemical agents (Tudi et al., 2021). While their intrinsic toxicity is advantageous for controlling pests and pathogens, it raises significant concerns regarding their potential adverse effects on human and animal species (Aktar, Sengupta, & Chowdhury, 2009). Extensive literature links pesticide exposure to a wide spectrum of adverse health outcomes, including neurotoxicity, mutagenicity, carcinogenicity, teratogenicity, and endocrine disruption (Mnif et al., 2011; S. Mostafalou & Abdollahi, 2013; Nicolopoulou-Stamati, Maipas, Kotampasi, Stamatis, & Hens, 2016).

Despite an increase in regulatory awareness and sustainability goals, pesticide use remains extensive. In the European Union (EU), around 350,000 tons of pesticides were sold each year between 2011 and 2020, with no decrease in consumption (<https://ec.europa.eu/eurostat/web/products-eurostat-news/-/ddn-20220502-1>).

In addition, in the last few years there has been a rise in demand for food that has the consequence of the growth of human exposure to pesticides. Exposure may occur through multiple pathways, including professions associated with pesticide production, transportation, application, and distribution that lead to heightened pesticide residue, and the proliferation and accumulation of pesticides within the food chain (Asghar & Mf, 2016; Gangemi et al., 2016). Beyond direct human effects, pesticide use has profound ecological consequences. According to the European Environment Agency (EEA), more than 80% of EU agricultural soil and 22% of surface waters are contaminated with at least one pesticide compound (European Environment, 2021). Such contamination disrupts microbial diversity, harms pollinators and aquatic organisms, and facilitates the transfer of pesticide residues into the human food chain via bioaccumulation (Aktar et al., 2009; C. Shekhar et al., 2024).

Furthermore, reports from the European Food Safety Authority (EFSA) and PAN Europe consistently demonstrate the frequent detection of pesticide residues in staple food products, raising serious concerns regarding chronic, low-dose exposure among the general population (Carrasco Cabrera, Di Piazza, Dujardin, Marchese, & Medina Pastor, 2024) (<https://www.pan-europe.info/blog/pesticide-residues-eu-food-nothing-applaud>).

1.2 CLASSIFICATION OF PESTICIDES

Pesticides are classified according to their target organisms:

- **Herbicides** (e.g. glyphosate, triazines), used to control weeds and invasive plants
- **Insecticides**, including pyrethroids, neonicotinoids, organophosphates and carbamates, target essential components of the insect nervous system, such as ion channels, neurotransmitter receptors, or enzymes critical for nerve signal transmission
- **Fungicides** such as succinate dehydrogenase inhibitors (SDH) and strobilurins targeting fungi and oomycetes
- **Other categories** include rodenticides, acaricides, nematocides, and biocides.

Each functional category encompasses multiple subclasses defined by their chemical structure and action mechanism (Garud, Pawar, Patil, Kale, & Patil, 2024).

Representative mechanisms include:

- **Pyrethroids** which target voltage-gated sodium channels in insect neurons, prolonging channel opening and inducing continuous nerve firing that leads to paralysis and death (Matsuda & Sattelle, 2004).
- **Neonicotinoids** which act as agonists of nicotinic acetylcholine receptors, causing persistent neuronal overstimulation due to resistance to enzymatic degradation (Thany, 2025).
- **Organophosphates and carbamates**, which inhibit acetylcholinesterase, resulting in acetylcholine accumulation and sustained neuromuscular excitation (Vale & Lotti, 2015).
- **SDHI fungicides** which inhibit succinate dehydrogenase (mitochondrial complex II), impairing cellular respiration and ATP production (Duarte Hospital et al., 2025).

- **Strobilurins**, which block the cytochrome bc₁ complex (complex III) of the mitochondrial respiratory chain, thereby disrupting energy generation (Feng, Huang, Zhan, Bhatt, & Chen, 2020).

Importantly, many of these molecular targets are conserved across species, raising concerns about unintended toxic effects in non-target organisms, including humans.

1.3 SELECTED PESTICIDES: CHARACTERISTICS, USAGE, AND TOXICOLOGICAL RELEVANCE

The present study focuses on 4 widely used pesticides: Boscalid, Pyraclostrobin, Propamocarb, and Lambda-Cyhalothrin. These compounds represent distinct chemical categories, including fungicides and insecticides, and are extensively applied to protect fruit, vegetable, and cereal crops. (Djouaka et al., 2018; Lv et al., 2022). Beyond their extensive use in agriculture and their recurrent presence as residues in food products, they hold significant regulatory relevance within the European Union as well as by the World Health Organization (WHO) ([https://www.who.int/groups/joint-fao-who-meeting-on-pesticide-residues-\(jmpr\)/publications/reports](https://www.who.int/groups/joint-fao-who-meeting-on-pesticide-residues-(jmpr)/publications/reports); https://food.ec.europa.eu/plants/pesticides/eu-pesticides-database_en). In addition, all 4 pesticides are regulated under EU Regulation (EC) No 396/2005, which establishes Maximum Residue Limits (MRLs), the highest legally permitted concentration (in mg/kg) of pesticide residue in or on food and feed. Specific MRLs are defined for hundreds of pesticide–commodity combinations, reflecting the widespread potential for dietary exposure. (European Food Safety, 2024). Increasing experimental evidence indicates that these pesticides may exert cytotoxic, genotoxic, and mitochondrial effects in human non-cancerous cell models, even at low or sub-cytotoxic concentrations. (Carbone, Mathieu, Vandensande, & Gallez, 2023; Çobanoğlu, Coskun, & Cayir, 2019; Jin et al., 2021; saleh, Al-Masri, & Ezzedin, 2024). These findings highlight potential health risks extending beyond traditional carcinogenic endpoints and underscore the importance of investigating their cellular and molecular effects in relevant human cell systems.

1.3.1. BOSCALID

Boscalid is a fungicide belonging to the succinate dehydrogenase inhibitor (SDHI) class (Stammler, 2008) and has been approved for use in the European Union since 2008 (Directive 2008/44/EC) (Figure 1). It is applied to control a broad spectrum of fungal pathogens affecting crops such as grapes, nuts, tomatoes, and leafy vegetables.

The European Food Safety Authority (EFSA) conducted a comprehensive review of boscalid's maximum residue levels (MRLs) in 2014, leading to updates for specific crops such as pomegranates and legumes (Authority, 2015). According to recent EFSA monitoring data, boscalid remains among the most frequently detected pesticide in plant- and animal-derived food samples (Cabrera, Di Piazza, Dujardin, Marchese, & Pastor, 2024).

From a toxicological perspective, recent *in vitro* studies have demonstrated that boscalid induces oxidative stress, mitochondrial dysfunction, and apoptotic cell death in normal human cells, even at sub-cytotoxic concentrations (d'Hose, Isenborghs, Brusa, Jordan, & Gallez, 2021). Notably, Faita et al. (2024) reported that the exposure to boscalid and its mixture with pyraclostrobin significantly reduced oxygen consumption rates (OCR) and increased reactive oxygen species (ROS) levels in Pheriphal blood mononuclear cells (PBMCs) and human fibroblasts, highlighting concerns regarding mixture toxicity and mitochondrial liability (Carbone et al., 2023).

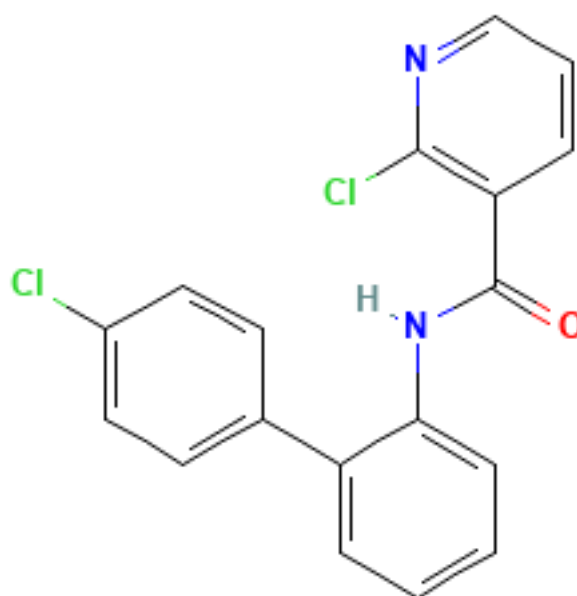


Figure 1: Boscalid chemical representation
(<https://pubchem.ncbi.nlm.nih.gov/compound/Boscalid>)

1.3.2. PYRACLOSTROBIN

Pyraclostrobin is a broad-spectrum strobilurin fungicide used extensively in European agriculture, particularly in cereals, vegetables, and orchard crops (Figure 2). Its primary mechanism involves inhibition of mitochondrial complex III, which may lead to damage in ATP synthesis and, thus, of the entire cell (Carbone et al., 2023).

While pyraclostrobin is not one of the most frequently detected residues in European monitoring programs (Cabrera et al., 2024), its persistence and potential for cumulative toxicity, especially in combination with SDHI fungicides, have raised increasing concern. In fact, it has been reported its synergistic mitochondrial toxicity when combined with other fungicides such as boscalid, emphasizing the need to investigate realistic exposure scenarios involving pesticide mixtures in human exposure scenarios (Carbone et al., 2023).

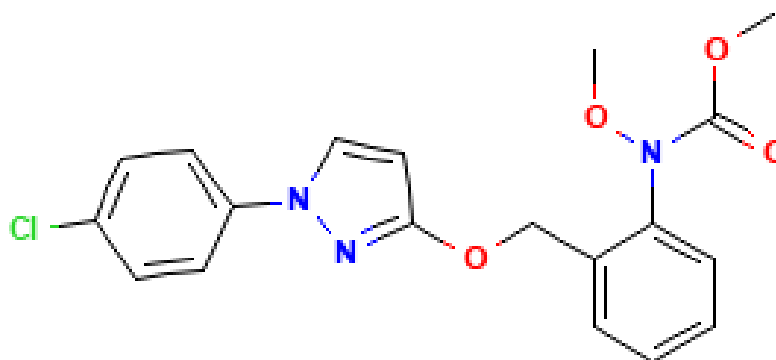


Figure 2: Pyraclostrobin chemical representation
(<https://pubchem.ncbi.nlm.nih.gov/compound/Pyraclostrobin>)

1.3.3 PROPAMOCARB

Propamocarb is a carbamate fungicide widely used for the control of oomycetes (e.g., *Phytophthora*, *Pythium*) in vegetable crops, including lettuce, cucumbers, and potatoes (Figure 3). Owing to its systemic properties, propamocarb is readily absorbed and translocated within plant tissues, providing effective protection against both root and foliar diseases (Hu, Hong, Stromberg, & Moorman, 2007).

The EFSA peer review of propamocarb was initially conducted in 2006 (Authority, 2006). More recently, updated MRL assessments were completed in 2025, including revised evaluations for leafy vegetables such as lettuce (European Food Safety, 2025).

Although propamocarb does not rank among the most frequently detected pesticide residues in food according to monitoring reports conducted by EFSA in the years 2021–2023 (Cabrera et al., 2024), its extensive and systemic use justifies further toxicological investigation. As a carbamate compound, propamocarb may interfere with cellular redox balance and mitochondrial function, particularly after chronic or repeated exposure. In vitro studies have reported mitochondrial dysfunction and increased oxidative stress in neuronal and non-neuronal cell models, suggesting potential subcellular toxicity even at non-lethal concentrations. (S. N. Li et al., 2022).

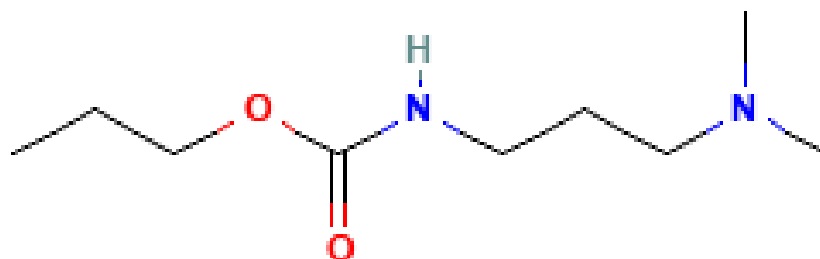


Figure 3: Propamocarb chemical representation (<https://pubchem.ncbi.nlm.nih.gov/compound/Propamocarb>)

1.3.4 LAMBDA-CYHALOTHRIN

Lambda-cyhalothrin is a synthetic pyrethroid insecticide extensively applied in both agricultural and public health fields (Figure 4). Its primary mechanism of action involves the disruption of voltage-gated sodium channels in insect nerve cells, leading to prolonged depolarization, paralysis, and death. Due to the conserved nature of these channels, similar interactions may occur in mammalian cell membranes, especially in excitable tissues (Field, Davies, O'Reilly, Williamson, & Wallace, 2017).

In a report published in May 2024, EFSA highlighted analytical limitations in distinguishing between the two cyhalothrin isomers, lambda- and gamma-cyhalothrin, under current residue detection methods (Authority et al., 2017). A previous report of EFSA, dated 2021, already indicated the presence of lambda/gamma-cyhalothrin residues in 46 food samples, with some acute exposure exceeding the acute reference dose (ARfD) for fruits such as bananas and table grapes (Cabrera, Pastor, & EFS, 2021).

Toxicologically, lambda-cyhalothrin has been associated with increased oxidative stress, membrane damage, and inflammatory responses in mammalian cells. Given the high vascularization of the sublingual and oral mucosa, oral exposure may facilitate rapid systemic absorption of lipophilic insecticides. Consequently, oral epithelial and endothelial cell models represent biologically relevant systems for the *in vitro* assessment of lambda-cyhalothrin-induced cytotoxicity and vascular effects (Giray, Gurbay, & Hincal, 2001; saleh et al., 2024).

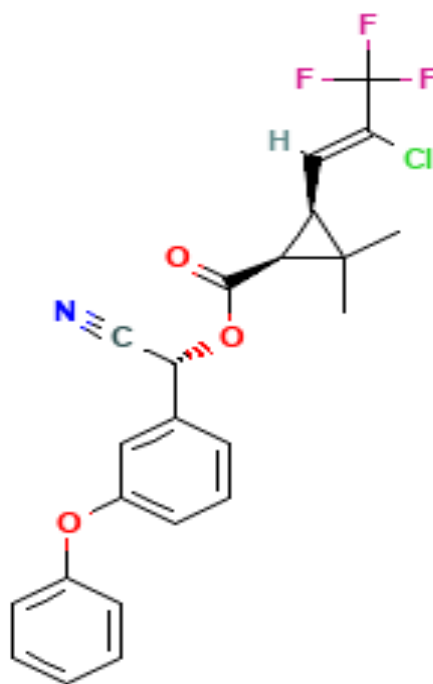


Figure 4: Lambda-Cyhalothrin chemical representation (<https://pubchem.ncbi.nlm.nih.gov/compound/Lambda-Cyhalothrin>)

1.4 ROUTES OF HUMAN EXPOSURE

Human exposure to pesticides can occur through multiple routes, depending on the nature of the pesticide, its formulation, method of application, environmental persistence, and individual behavioural factors (Sara Mostafalou & Abdollahi, 2017). The primary routes include dermal contact, inhalation, oral ingestion, and ocular exposure, each with distinct toxicokinetic profiles and health implications. A major concern related to pesticide exposure is their ability to cross biological barriers and accumulate in mucosal tissues, thereby contributing to systemic distribution and long-term health effects (Richardson, Fitsanakis, Westerink, & Kanthasamy, 2019; Roberts & Karr, 2012).

1.4.1 DERMAL EXPOSURE: THE PRIMARY OCCUPATIONAL ROUTE

Dermal exposure represents one of the most significant routes of pesticide uptake among individuals involved in agricultural and horticultural sectors. Exposure may occur during pesticide mixing, loading, spraying, and equipment cleaning, particularly when personal protective equipment (PPE) is insufficient or improperly used. Factors influencing dermal absorption include skin integrity, pesticide lipophilicity, ambient temperature, and duration of contact (Ahmad et al., 2024; Badea, Trifu, Darabont, & Țărnău, 2022; Sullivan et al., 2017).

Studies have shown that up to 90% of total pesticide exposure among subjects who manipulate pesticides may occur via the dermal route (Aggarwal et al., 2014). Lipophilic compounds, such as organochlorines, can penetrate the stratum corneum, accumulating in subcutaneous adipose tissue, leading to prolonged systemic exposure (Dewailly et al., 1999). Field biomonitoring studies have demonstrated strong correlations between dermal exposure biomarkers, such as urinary pesticide metabolites and work intensity, and improper PPE usage (Barr, 2008).

1.4.2 INHALATION EXPOSURE

Inhalation exposure occurs through contact with pesticides aerosols, mists, vapors, or dust during their application, in greenhouses, closed environments, or in urban vector control operations (Mamane, Baldi, Tessier, Raherison, & Bouvier, 2015).

The respiratory tract constitutes a highly efficient route for systemic absorption due to its extensive surface area and rich vascularization (Gonda, 2006). Fine droplets (diameter <10 µm) can reach the alveoli, therefore flowing into the bloodstream (Ou, Hang, & Deng, 2020).

Epidemiological investigations have linked chronic inhalation of pesticides to asthma, bronchitis, and pulmonary function impairment (Mamane et al., 2015; Ratanachina, De Matteis, Cullinan, & Burney, 2020). In particular, studies involving agricultural workers have demonstrated that exposure to organophosphate and carbamate pesticides is associated with compromised lung function (Fareed et al., 2013; Hansen et al., 2021). Furthermore, pesticide flow from treated fields can affect the non-occupationally exposed populations, including children and residents living in close proximity to agricultural areas (Linhart et al., 2019; Teyssere et al., 2021).

1.4.3 ORAL EXPOSURE: THE DOMINANT ROUTE

Oral ingestion represents the primary route of chronic exposure in the entire population, mainly through residues in food, drinking water, and inadvertent hand-to-mouth transfer (National Research Council Committee on Pesticides in the Diets of & Children, 1993). In addition to chronic exposure, acute oral intoxication remains a significant concern in developing countries, where pesticides are stored inappropriately, resulting in accidental ingestion (Nigatu, Bråtveit, & Moen, 2016). Numerous studies have highlighted that dietary intake of pesticide residues through fruits, vegetables, and contaminated water sources may pose substantial risks for endocrine disruption, reproductive toxicity, and carcinogenic issues (Mnif et al., 2011; Sadighara et al., 2024). Biomonitoring studies have consistently detected measurable levels of organophosphates, neonicotinoids, and pyrethroids in human urine and blood samples, even among non-occupationally exposed populations, indicating widespread environmental exposure (Hakme, Poulsen, & Lassen, 2024; Wrobel et al., 2022).

1.4.4 OCULAR EXPOSURE

Although often underestimated, ocular exposure to pesticides can result in acute and chronic adverse effects, including irritation, conjunctivitis, corneal burns, and, in severe cases, retinal damage (Jaga & Dharmani, 2006). This route becomes particularly relevant during manual pesticide application or handling procedures in the absence of adequate protective eyewear. Chemical exposure to the eye can occur through direct splashes, airborne particles, or contact with contaminated hands (Jaga & Dharmani, 2006).

Toxicological evidence indicates that lipophilic pesticide compounds can penetrate the corneal epithelium, reach deeper ocular tissues, and potentially affect the optic nerve and retinal structures (McDonald et al., 2018). Consequently, both occupationally exposed individuals and the general population may be at risk of ocular toxicity following pesticide exposure (Jaga & Dharmani, 2006).

1.4.5 Chronic Low-Dose Exposure and Vulnerable Populations

While acute pesticide poisoning remains a significant issue in low-income settings, chronic low-dose exposure constitutes a more insidious yet substantial global public health challenge. Cumulative exposure through multiple routes has been associated with a wide range of adverse health outcomes, including neurodegenerative diseases (e.g., Parkinson's and Alzheimer's), endocrine and hormonal imbalances, developmental and reproductive disorders, immune dysregulation, and various cancers such as non-Hodgkin lymphoma and leukemia (Chander Shekhar et al., 2024). Mostafalou & Abdollahi (2024) conducted a comprehensive review identifying consistent links between chronic exposure and the incidence of non-communicable diseases, particularly among children, pregnant women, and individuals living in agricultural or rural environment (S. Mostafalou & Abdollahi, 2013; Sara Mostafalou & Abdollahi, 2017).

1.5 THE ISSUE OF THE “COCKTAIL EFFECT”

Pesticides can be harmful to humans not only at high doses, but also at low concentrations or when combined because of the chronic exposure. This phenomenon, commonly referred to as the “cocktail effect”, reflects the potential for combined toxic effects that exceed those predicted based on individual compound toxicity (Laetz et al., 2009). Data from the Human Biomonitoring for Europe (HBM4EU) project (2014–2021) revealed that at least two pesticide residues were detected in 84% of blood and urine samples collected from European participants, reflecting the potential for combined toxic effects that exceed those predicted based on individual compound toxicity (Ottenbros et al., 2023). This issue prompted the EFSA to develop specific methodologies for cumulative risk, moving beyond the traditional single-compound approach; in fact, in 2019, the guidance Scientific Opinion on the update of the guidance on the assessment of the risk for the environment from the exposure to mixtures of pesticides (MIXTOX) promoted the grouping of chemical substances based on shared mechanism of action or common target organ. This has led to the creation of Cumulative Assessment Groups (CAGs), where pesticides with similar toxic effects are evaluated together. The default assumption adopted by EFSA for low-dose assessment is Dose Addition, implemented using tools such as the MCRA (Monte Carlo Risk Assessment) platform, which presumes a simple summation of toxic effects. While Addition assumes that the combined toxic effect is simply the sum of individual chemical doses ($1+1=2$), synergism means the combined effect is greater than expected ($1+1>2$) (Boobis et al., 2008; Sexton & Linder, 2011). Since testing every environmental combination is unfeasible, the unquantified potential for synergism remains the main element of uncertainty in the risk assessment of complex, low-dose chemical cocktails.

1.6 HUMAN GINGIVAL MESENCHYMAL STEM CELLS (HGMSCS) AND HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVECS): THE LINES OF CELLS CHOSEN FOR THE STUDY

Human Gingival Mesenchymal Stem Cells (hGMSCs) and Human Umbilical Vein Endothelial Cells (HUVECs) are the lines of cells chosen to investigate the cytotoxic and molecular outcomes of pesticide exposure.

Among the different MSC populations, Human Gingival Mesenchymal Stem Cells (hGMSCs) are derived from oral connective tissue (Fonticoli et al., 2022), a biological environment continuously exposed to environmental contaminants (Babajko et al., 2020) introduced through contaminated food, drinking water, and/or inhalation of contaminated dust (Hernández et al., 2013; Lozowicka, 2015). Several studies have demonstrated that exposure of MSCs to pesticides induces oxidative stress, DNA damage, mitochondrial dysfunction, and alterations in differentiation capacity. Hochane et al. showed that low-dose exposure to organophosphate pesticides leads to increased reactive oxygen species (ROS) production and senescence-associated phenotypes in human MSCs (Hochane et al., 2017). Similarly, Leveque and colleagues reported that chronic exposure to pesticide mixtures disrupts mitochondrial metabolism and stemness-related gene expression in MSCs (Leveque et al., 2019).

Increasing evidence suggests that exposure to environmental pollutants, including pesticides, may play a role in the onset and progression of oral inflammatory diseases. Epidemiological and experimental reports have linked pesticides exposure with periodontal tissue alterations, dysregulation of host immune responses, and aggravation of chronic inflammatory conditions within the oral cavity (Salazar-Flores et al., 2022a). In particular, oxidative stress and inflammasome activation have been implicated as key factors contributing to the destruction of tooth-supporting periodontal tissues, compromising periodontal tissue homeostasis and tissue regeneration ability (Patil, Dhadse, Salian, & Punse, 2024). This potentially increases the predisposition to periodontitis and subsequently inhibits periodontal tissue healing (Shang, Liu, Zheng, & Zhang, 2023).

Importantly, pesticide-induced oxidative stress in MSCs has been linked to telomere shortening and altered mitochondrial DNA copy number, both hallmarks of cellular aging and impaired regenerative potential (Bártová et al., *Mutation Research*, 2020). Collectively, these findings suggest that long-term exposure to environmental pesticides may compromise tissue repair capacity and contribute to degenerative and inflammatory diseases. Given their human origin, accessibility, high proliferative capacity, and sensitivity to environmental stressors, hGMSCs constitute a robust *in vitro* model for investigating pesticide-related cellular toxicity.

Following systemic absorption, pesticides enter the bloodstream, rendering the vascular endothelium one of the first and most critical targets of exposure (S. Mostafalou & Abdollahi, 2013). Endothelial dysfunction is widely recognized as an early trigger in cardiovascular, metabolic, and inflammatory disorders associated with environmental pollutants (Brook et al., 2010; Deanfield, Halcox, & Rabelink, 2007). Human umbilical vein endothelial cells (HUVECs) are a well-established and extensively validated *in vitro* model for studying endothelial responses to xenobiotics and ecological toxins, including pesticides (Incalza et al., 2018; Jaffe, Nachman, Becker, & Minick, 1973). Experimental evidence indicates that pesticide exposure induces cytotoxicity, oxidative stress, inflammation, and apoptosis in endothelial cells. Moreover, pesticides have been shown to reduce nitric oxide bioavailability and impair angiogenic capacity, key features of endothelial dysfunction and early atherogenesis (González-Curbelo et al., *Environmental Research*, 2017). Chronic endothelial activation by environmental pesticides is also associated with increased expression of adhesion molecules and pro-inflammatory cytokines, contributing to vascular inflammation and altered barrier function (Montanarí et al., 2024; S. Mostafalou & Abdollahi, 2013). Therefore, HUVECs represent a biologically relevant *in vitro* model for investigating the systemic vascular effects of pesticide exposure.

1.6.1 BRIEFING ON MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) were first described by Friedenstein and colleagues in 1966, who identified a population of fibroblast-like cells capable

of adhering to plastic surfaces in vitro and differentiating into bone-forming cells (Friedenstein, Piatetzky, & Petrakova, 1966). These cells were later termed “mesenchymal stem cells” by Caplan et al. in 1991 (Ding, Shyu, & Lin, 2011). According to the criteria established by the International Society for Cellular Therapy, MSCs are defined by three principal characteristics: (i) adherence to plastic under standard culture conditions; (ii) multipotent differentiation capacity toward osteogenic, chondrogenic, and adipogenic lineages; and (iii) a specific immunophenotypic profile characterized by the expression of mesenchymal markers such as CD105, CD90, and CD73, in the absence of hematopoietic and endothelial markers including CD11b, CD19, CD31, CD34, CD45, and HLA-DR (Dominici et al., 2006).

1.6.2 ORAL MESENCHYMAL STEM CELLS (OMSCS)

Among the various sources of MSCs, oral cavity-derived MSCs have attracted increasing attention due to their ease of accessibility, minimal invasiveness during harvesting, high proliferative capacity, and stable genomic profile. Oral MSCs can be isolated from several tissues, including dental pulp, periodontal ligament, apical papilla, dental follicle, and gingival connective tissue (Figure 5). Human gingival mesenchymal stem cells (hGMSCs) represent a particularly promising subpopulation with unique biological properties. These cells exhibit fibroblast-like morphology, robust proliferation, and the ability to differentiate into multiple lineages such as osteocytes, odontoblasts, adipocytes, and myocytes, indicating their multipotent nature (Marconi, Diomedea, Pizzicannella, & Trubiani, 2023; Roato, Chinigò, Genova, Munaron, & Mussano, 2021).



Figure 5: Populations of Oral Stem Cells. DFSCs, dental follicle stem cells; SHED, stem cells from human exfoliated deciduous teeth; SCAP, stem cells from apical papilla; DPSCs, dental pulp stem cells; PDLSCs, periodontal ligament stem cells; GMSCs, gingival mesenchymal stem cells; DSCs, dental stem cells (Y. Li, Duan, Chen, Liu, & Chen, 2022)

1.6.3 HUMAN GINGIVAL MESENCHYMAL STEM CELLS (hGMSCS)

Beyond their differentiation potential, hGMSCs display strong immunomodulatory and anti-inflammatory properties. These cells interact with both innate and adaptive immune systems by suppressing the activation and proliferation of various immune cells, including T cells, B cells, natural killer cells, macrophages, and dendritic cells. Such immunoregulatory effects are mediated through direct cell-to-cell interactions as well as through the secretion of a broad spectrum of bioactive molecules collectively referred to as the secretome (Fonticoli et al., 2022). The hGMSCs secretome includes cytokines, chemokines, growth factors, and extracellular vesicles (EVs), which collectively contribute to tissue repair, immune modulation, and angiogenesis (Ylenia Della Rocca et al., 2022; Silvestro et al., 2020). Moreover, hGMSCs express pattern recognition receptors, such as Toll-like receptors (TLRs), which enable them to recognize pathogen-associated molecular patterns (PAMPs) and respond by modulating immune reactions. TLR signaling has been shown to induce anti-inflammatory mediators release

and promote macrophage polarization toward the M2 phenotype, which is associated with tissue repair and resolution of inflammation (Fonticoli et al., 2022). Clinically and preclinically, hGMSCs and their secretome have demonstrated promising regenerative potential. They have been successfully applied in bone tissue engineering, showing the ability to enhance bone regeneration when combined with biomaterial scaffolds. Similarly, hGMSCs contribute to muscle repair, cutaneous wound healing, and neural tissue regeneration, highlighting their broad therapeutic potential (Diomedea et al., 2018). In summary, hGMSCs represent a readily available, highly proliferative, and immunomodulatory cell population with considerable relevance for regenerative medicine and inflammatory disease research. Their paracrine activity mediated by secreted factors and extracellular vesicles opens avenues for novel cell-free therapeutic strategies, which may improve safety and efficacy profiles in clinical applications.

1.6.4 UTERO-PLACENTAL CIRCULATION

The placenta is a transient yet essential organ for fetal development, originating from both maternal and fetal tissues. Its highly specialized structure enables the efficient exchange of gases, nutrients, hormones, and metabolic waste products between maternal and fetal circulations without direct mixing (Wang & Zhao, 2010).

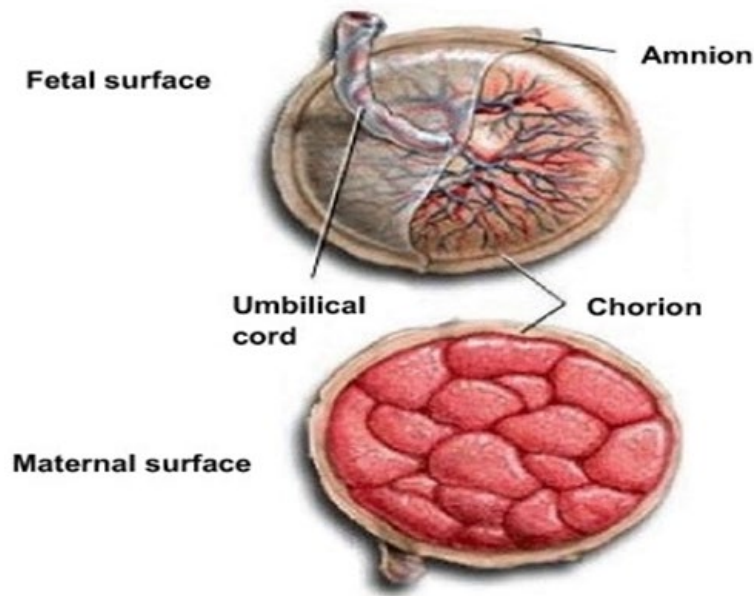


Figure 6: Maternal and fetal surfaces of the placenta (Y. Wang & Zhao, 2010)

Anatomically, the placenta comprises two main components: a maternal portion (the basal decidua) and a fetal portion, consisting of chorionic villi, which constitute the primary functional units (Figure 6). These villi are immersed in maternal blood within the intervillous spaces and contain a dense fetal vascular network derived from the umbilical circulation. Maternal-fetal exchanges occur across a thin barrier composed of trophoblastic and endothelial cells layers (Thornburg & Louey, 2013). Oxygen and nutrients diffuse from maternal to fetal blood, while carbon dioxide and metabolic waste follow the reverse path.

The establishment of the utero-placental circulation begins early in gestation, starting from the third week, with the differentiation of primary chorionic villi into secondary and tertiary villi characterized by mesenchymal cores and fetal capillary formation (Vornic et al., 2024) (Figure 7).

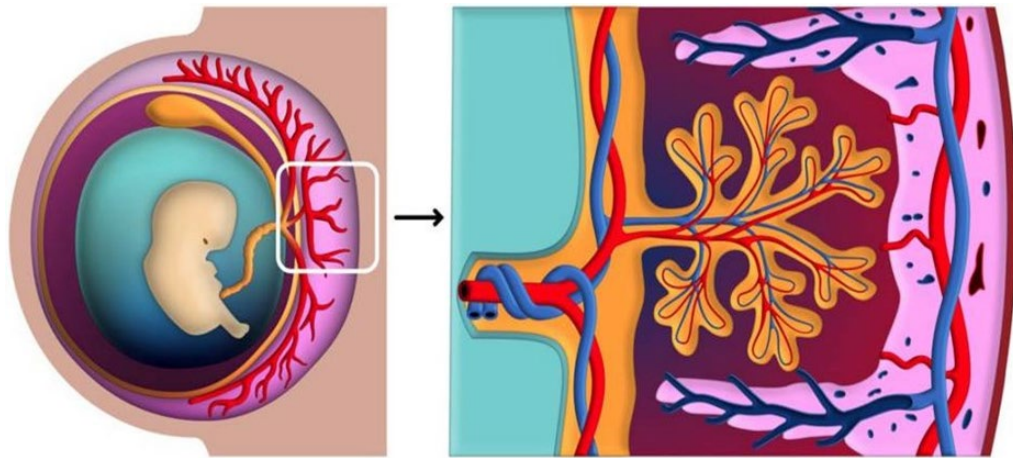


Figure 7: Illustration of the developing placenta and early utero-placental circulation. On the left, an embryo is visible within the amniotic cavity, surrounded by trophoblast and initial placental structures (Vornic et al., 2024).

Concurrently, the spiral arteries undergo extensive remodeling mediated by extravillous trophoblasts invasion, resulting in a reduced vascular resistance and increased placental blood flow (Li, Lorca, & Su, 2018). Endothelial cells within both maternal and fetal compartments play a critical role in angiogenesis, vascular permeability regulation, and immune tolerance at the maternal–fetal interface. Placental endothelium, in particular, regulates vascular permeability, responds to angiogenic signals, and contributes to maintaining an immunologically tolerant environment, which is critical for maternal-fetal coexistence (Li et al., 2018).

The maternal-fetal barrier is primarily composed of trophoblastic and endothelial cells, along with a basal matrix, and exhibits selective permeability. (Wang & Zhao, 2010). Although the maternal–fetal barrier confers selective protection against harmful substances, numerous xenobiotics, including drugs, environmental pollutants, and pesticides, can cross this barrier, particularly when lipophilic or of low molecular weight (Thornburg & Louey, 2013).

Prenatal exposure to such compounds have been associated with adverse developmental outcomes, including congenital anomalies, neurobehavioral

disorders, metabolic dysregulation, or epigenetic alterations. In this context, placental endothelium plays a central role not only as a selective filter but also as an active mediator in the transfer and response to exogenous compounds. This highlights the importance of *in vitro* endothelial models for investigations (Vornic et al., 2024)

1.6.5 HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVECS)

Human umbilical vein endothelial cells (HUVECs) are primary endothelial cells of fetal origin, isolated from the human umbilical vein post-partum (Jaffe et al., 1973). First isolated by Jaffe et al. in 1973, HUVECs have become one of the most widely used *in vitro* models for investigating endothelial biology, vascular function, and disease mechanisms due to their simple isolation, reproducibility, and phenotypic relevance to human vascular endothelium (Jaffe et al., 1973). Endothelial cells line the inner surface of blood vessels and are fundamental for the regulation of vascular tone, angiogenesis, hemostasis, and immune surveillance. Endothelial dysfunction is a central feature of multiple pathologies, including atherosclerosis, thrombosis, hypertension, and inflammatory disorders (Pober & Sessa, 2007). Importantly, HUVECs exhibit expression of endothelial-specific markers, including von Willebrand factor (vWF), platelet/endothelial cell adhesion molecule-1 (PECAM/1/CD31), vascular endothelial-cadherin (VE-cadherin), and endothelial nitric oxide synthase (eNOS), among others (Müller et al., 2002; Nikmanesh, Shi, & Tarbell, 2012). They also display functional behaviors including tube formation, monolayer permeability regulation, and response to shear stress, make HUVECs a reliable proxy for studying human vascular responses *in vitro* (Goncharov et al., 2020). Due to their primary human origin, HUVECs retain important features of endothelial heterogeneity and reactivity to environmental stimuli, including cytokines, growth factors, and toxicants. This makes them particularly suitable for toxicological studies that investigate vascular inflammation, oxidative stress, angiogenesis inhibition, and genotoxicity, all of which are relevant endpoints in the assessment of pesticide-induced vascular toxicity (Cao, 2025; Saquib et al.,

2021). Moreover, HUVECs are responsive to pro-inflammatory cytokines such as TNF- α and IL-1 β , producing increased expression of adhesion molecules (e.g., ICAM-1, VCAM-1, E-selectin) and chemokines (e.g., MCP-1), which are critical in the recruitment of leukocytes to sites of vascular injury (Gogesch et al., 2025; Klein et al., 1995; Makó et al., 2010). This characteristic supports their application in inflammation-related mechanistic studies, particularly in the context of chronic exposure to xenobiotics such as pesticides. Finally, their adaptability to various in vitro platforms—including 2D cultures, flow chambers (for shear stress), co-culture systems (e.g., with smooth muscle cells), and 3D angiogenesis assays—enhances the versatility of HUVECs in preclinical research and chemical safety evaluations (Davies, 1995; Gerhardt et al., 2003; Jaffe et al., 1973; Staton et al., 2004).

1.7 INVESTIGATED PATHWAYS

1.7.1 THE NOD-like Receptor Protein 3 (NLRP3) INFLAMMOSOME PATHWAY

The NOD-like Receptor Protein 3 (NLRP3) inflammasome is a cytosolic multiprotein complex that function as a sensor of cellular stress and a central regulator of innate inflammatory responses (Kelley, Jeltema, Duan, & He, 2019). Upon activation, NLRP3 recruits the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), leading to the activation of caspase-1 (CASP1). Active CASP1 subsequently cleaves the inactive precursors of interleukin-1 β (IL-1 β) and IL-18 into their mature bioactive forms and induces pyroptosis cell death through gasdermin-D cleavage (Huang, Xu, & Zhou, 2021; Zhan, Li, Xu, Xiao, & Bai, 2022). Activation of NLRP3 typically follows a two-signal model. The first, or priming signal, often mediated by NF- κ B activation induces transcriptional upregulation of NLRP3 and pro-IL-1 β . A second activation signal, such as mitochondrial dysfunction, potassium efflux, or lysosomal damage, promotes NLRP3 conformational changes, assembly at mitochondria-associated membranes, ASC oligomerization, and subsequent CASP1 activation, underscoring the pivotal role of mitochondrial oxidative stress in

inflammasome signalling (Bao et al., 2025; Heid et al., 2013). In endothelial cells, including HUVECs, NLRP3 activation leads to CASP1 cleavage, IL-1 β secretion, endothelial barrier dysfunction, and leukocyte recruitment (Figure 8). These events establish a mechanistic link between oxidative stress and vascular inflammation and pathology (Bao et al., 2025). Similarly, in mesenchymal stromal/stem cells, including MSCs, NLRP3 activation has been shown to increase CASP1 activity, shifts differentiation, and impairs regenerative function (L. Wang et al., 2017). Importantly, several pesticides, including the SDHI fungicide boscalid and the strobilurin pyraclostrobin, have been reported to disrupt mitochondrial electron transport, reduce oxygen consumption rate, deplete ATP, impair redox homeostasis, and elevate mitochondrial superoxide in human cells (Carbone et al., 2023). Prolonged IL-1 β release further amplifies inflammation by reinforcing NF- κ B signalling, compromising endothelial integrity, and promoting a senescence-associated secretory phenotype (SASP) in surrounding cells, including MSCs (Chou, Ho, & Hung, 2022; Hsu, Rodriguez-Ortiz, Zumkehr, & Kitazawa, 2021; Romero et al., 2022; Salminen, Kauppinen, & Kaarniranta, 2012). Collectively, these processes link pesticide-induced mitochondrial dysfunction to chronic inflammation, senescence, and tissue dysfunction in both hGMSCs and HUVECs.

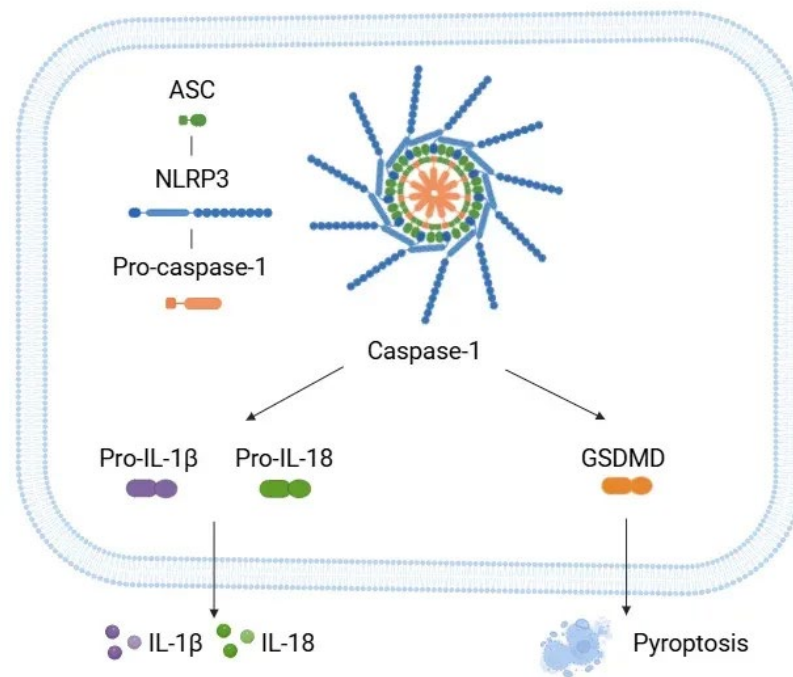


Figure 8: NLRP3 pathway. The NLRP3 inflammasome, composed of NLRP3, ASC, and pro-caspase-1, mediates cleavage of pro-caspase-1 into active caspase-1. Caspase-1 then processes pro-IL-1 β and pro-IL-18 into active cytokines and cleaves GSDMD, inducing pyroptosis (<https://biospective.com/resources/what-is-nlrp3>)

1.7.2 SENESCENCE PATHWAY, TELOMERE LENGTH REDUCTION AND mtDNA COPY NUMBER ALTERATION

Cellular senescence is a multifactorial stress response characterized by stable and irreversible cell-cycle arrest induced by stimuli such as DNA damage, oxidative stress, and telomere dysfunction (Stojanovic et al., 2025). A central regulator of senescence is the p53–p21 pathway (Figure 9). Cellular stress signals lead to stabilization and activation of the tumour suppressor p53, which in turn induces transcription of the cyclin-dependent kinase inhibitor p21 (CDKN1A). p21 inhibits cyclin–CDK complexes required for G1/S transition, thereby enforcing permanent growth arrest (Mijit, Caracciolo, Melillo, Amicarelli, & Giordano, 2020). Both human umbilical vein endothelial cells (HUVECs) and human gingival mesenchymal stem cells

(hGMSCs) display relatively high basal activity of telomerase reverse transcriptase (TERT), which contributes to telomere maintenance and replicative potential (Mazzone et al., 2025; Tomar et al., 2010). However, telomerase activity can be compromised by DNA damage and oxidative stress, often induced by environmental toxins such as pesticides, which impair TERT expression or mitochondrial function (Barnes, Fouquerel, & Opresko, 2019). The resulting telomere shortening or dysfunction generates persistent DNA damage signals that further activate p53, reinforcing p21-mediated senescence (Westin et al., 2011). Beyond its canonical nuclear role, TERT also localizes to mitochondria, where it mitigates ROS production and preserves mitochondrial integrity, thus acting as a protective factor against oxidative stress-induced senescence (Marinaccio et al., 2025).

In addition, telomere attrition has emerged as a fundamental driver of cellular senescence. Telomeres are repetitive DNA sequences at chromosome ends that shorten with each cell division due to the end-replication problem, eventually triggering a persistent DNA damage response and stable cell cycle arrest once a critical length is reached. This telomere-dependent replicative arrest contributes significantly to the aging phenotype and has been associated with organismal lifespan and susceptibility to age-related diseases (Rossiello, Jurk, Passos, & d'Adda di Fagagna, 2022; Zhu, Liu, Ding, Wang, & Geng, 2019).

In parallel, mitochondrial dysfunction has emerged as a hallmark of aging and senescence. Mitochondrial DNA (mtDNA) copy number, reflecting mitochondrial biogenesis and functional capacity, changes in response to aging and cellular stress and has been proposed as a biomarker of metabolic and mitochondrial compromise (Sanchez-Roman et al., 2024; Somasundaram et al., 2024). Alterations in mtDNA copy number have been observed in association with cellular aging and are thought to reflect accumulated oxidative damage and shifts in mitochondrial turnover (Hebert, Lanza, & Nair, 2010).

Increasing evidence suggests that telomere dysfunction can activate p53 and repress key regulators of mitochondrial biogenesis, linking telomere attrition

to compromised mitochondrial homeostasis, while mitochondrial ROS generation can accelerate telomere erosion and contribute to DNA damage accumulation. This bidirectional crosstalk reinforces the concept that telomere shortening, and mitochondrial dysfunction are integrated components of the senescence pathway, rather than isolated phenomena (Sahin & DePinho, 2012). Therefore, simultaneous assessment of telomere length and mtDNA copy number provides a complementary and mechanistically robust framework to investigate the molecular underpinnings of cellular senescence and aging, as well as their implications for organismal health and disease (Lee, Kim, & Lee, 2017). Understanding this interconnected signalling axis is crucial to elucidating how chronic pesticide exposure accelerates cellular aging and dysfunction in key human cell types

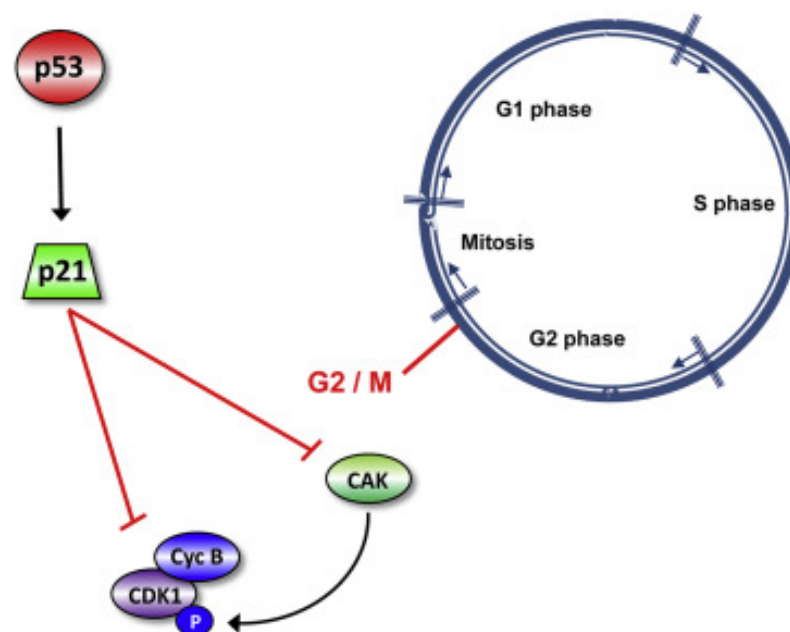


Figure 9: p53/p21 axis in cell cycle arrest (Kulaberoglu, Gundogdu, & Hergovich, 2016)

1.7.3 CROSS-TALK BETWEEN INFLAMMOSOME AND SENESCENCE PATHWAY

Cellular senescence and chronic inflammation are intimately interconnected processes that collectively drive aging and age-related pathology. A defining feature of senescent cells is the senescence-associated secretory phenotype (SASP), which comprises a broad array of pro-inflammatory cytokines, chemokines, growth factors, and matrix remodeling enzymes that can profoundly alter tissue microenvironments and promote chronic, low-grade inflammation (*inflammaging*) (Zhu, Armstrong, Tchkonja, & Kirkland, 2014). SASP factors such as IL-6, IL-1 β , and TNF- α not only reinforce cellular senescence in an autocrine manner but can also induce paracrine senescence in neighboring cells, thereby amplifying inflammatory signaling and tissue dysfunction (Herranz & Gil, 2018; Wu et al., 2022).

Conversely, sustained inflammatory stimuli can drive or exacerbate senescence. Exposure to pro-inflammatory cytokines and oxidative stress enhances DNA damage responses and telomere attrition, accelerates reactive oxygen species (ROS) production, and activates pathways such as NF- κ B that reinforce both the SASP and senescent growth arrest (Correia-Melo, Hewitt, & Passos, 2014). This bidirectional feedback loop between inflammation and senescence establishes a self-perpetuating cycle in which inflammation drives senescence, and senescent cells, through the SASP, maintain and expand inflammatory signaling (X. Li et al., 2023).

At the molecular level, emerging evidence highlights the release of cytosolic DNA species, including mitochondrial DNA (mtDNA) and chromatin fragments, as a mechanistic link between senescence and innate immune signaling (Dou et al., 2025). These cytosolic DNA species activate DNA-sensing pathways (e.g., cGAS–STING), leading to NF- κ B activation and further enhancement of the SASP expression. Through this mechanism, mitochondrial dysfunction, inflammasome activation, and senescence converge to sustain chronic inflammatory states (Dou et al., 2025).

Together, these findings demonstrate that inflammasome activation and senescence are not isolated phenomena but are integrated through multiple

molecular circuits, making their interplay a central driver of aging and chronic disease (Figure 10)

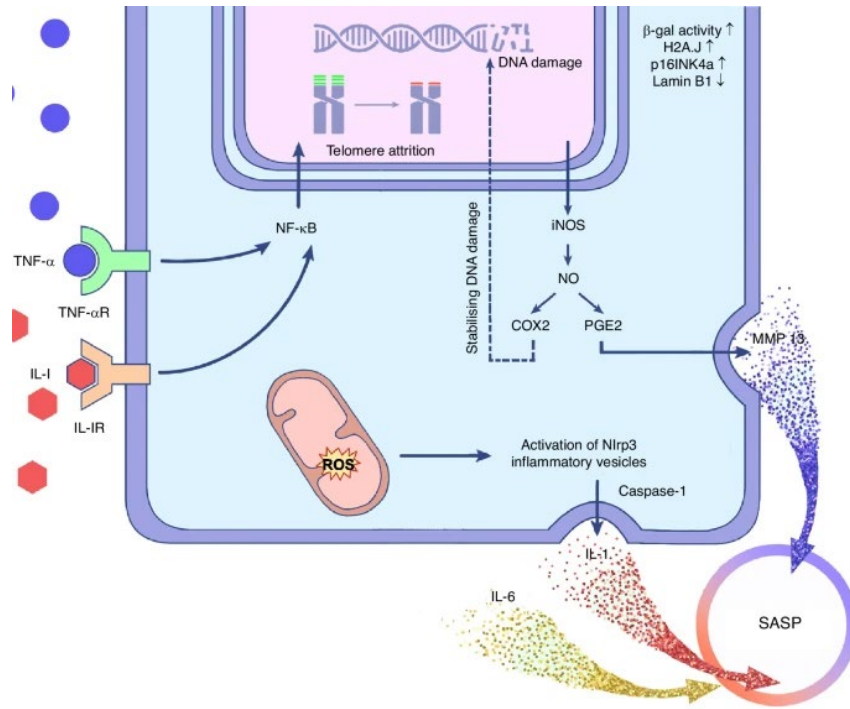


Figure 10: Inflammation and Cellular Senescence Crosstalk. DNA damage and telomere shortening trigger cellular senescence, which is reinforced by inflammatory factors such as IL-6 and IL-8 . These signals increase ROS , activate the NLRP3 inflammasome and caspase-1, and stimulate the production of SASP factors. In parallel, iNOS , PGE2 , and COX2 promote MMP synthesis and stabilize DNA damage, further sustaining the senescent phenotype (Han, Wang, Ding, & Zhang, 2024)

2. MATERIALS AND METHODS

2.1 CELL MODELS

2.1.1 HUMAN GINGIVAL MESENCHYMAL STEM CELLS (hGMSCs)

- **Ethic statement**

An authorization form was signed by the donors registered in the current manuscript. The Medical Ethics Committee formally accepted the study design at the Medical School, “G. D’Annunzio” University, Chieti, Italy (N° 266/17 April 2014). The Department of Innovative Technologies in Medicine and Dentistry and the Laboratory of Stem Cells and Regenerative Medicine are licensed according to the quality standard ISO 9001:2008 (N° 32031/15/S).

- **Isolation and cell culture conditions**

Starting from systemically healthy patients free from oral diseases, gingival tissue biopsy samples were obtained under sterile conditions, transferred to Petri dishes and mechanically fragmented. After washing samples with Phosphate-buffered saline (PBS, Lonza, Basel, Switzerland) with 5% gentamicin to reduce microbial contamination (code, Lonza). The explants were then incubated at 37 °C in a humidified atmosphere containing 5% CO₂ in their specific medium for the growing of the MSCs, TheraPEAK™ MSCGM-CD™ Bullet Kit-chemically defined (MSCGM-CD, Lonza).

The culture medium was refreshed every two days. After approximate two weeks, cells spontaneously migrated from the tissue explants and adhere to the culture surface. When the culture reached 80% confluence, the cells were detached and subcultured for further expansion.

2.1.2 HUVECs

HUVECs (C-003-5C), were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Cells were thawed according to the manufacturer’s instructions and cultured in Human Large Vessel Endothelial Cell Basal Medium (formerly Medium 200) (M200500) (Invitrogen, Carlsbad, CA,

USA) supplemented with Low Serum Growth Supplement (S00310) (Invitrogen, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). Cells at passage 3 were used for all experiments.

2.2 MTS ASSAY

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS) assay was used to measure the metabolic activity of hGMSCs and HUVECs cultured under different conditions, including exposure to pesticides, in a study conducted with the CellTiter 96® Aqueous One Solution Cell Proliferation Assay from Promega, Madison, WI, USA. 3,000 cells were placed in each well of 96-well plates and were grown for 24, 72 h, and 10 days at 37 °C. After each point, 20 µL of MTS dye solution was added to each well and incubated for 3 h at 37 °C. The Synergy™ HT Multi-detection microplate reader from Biotech in Winooski, VT, USA, was used to measure the formazan product, correlating directly with the number of viable cells in culture, by taking absorbance readings at 490 nm. The MTS assay was replicated in three separate trials.

2.3 SCANNING ELECTRON MICROSCOPY (SEM) EVALUATION

Cells were fixed for 1 h at room temperature (RT) in 2.5% glutaraldehyde (EMS, Hatfield, PA, USA) prepared in 0.1 M sodium phosphate buffer (PB; pH 7.2). Samples were post-fixed with 1% aqueous osmium tetroxide for 1 h at 4 °C and washed three times with PB (5 min each). Subsequently, cells were dehydrated through a graded ethanol series (30–100%) and dried using a critical point dryer (CPD 300; Leica Microsystems Inc., Ontario, Canada). Cells cultured on glass were coated with a thin layer of carbon before observation to reduce surface charging. Imaging was carried out using a Regulus 8220 scanning electron microscope (Hitachi, Ltd., Tokyo, Japan) operated at 1 kV.

2.4 IMMUNOFLUORESCENCE INVESTIGATION

To assess protein expression, 10000 cells were seeded onto sterilized glass coverslips (1943-10012A, Bellco Glass, Vineland, NJ, USA). After 10 days of pesticides

treatment, cells were fixed with periodate–lysine–paraformaldehyde (PLP) solution in PB for 30 min at 4 °C. Cells were washed 3 times with PB for 5 min and permeabilized with 0.5% Triton X-100 (9474680K, LKB-Produkter AB, Bromma, Sweden) in PB for 10 min. Non-specific binding was blocked with 5% skim milk in PB for 1 h. Cells were then incubated for 2 h in a humidified environment at RT in the dark with the following primary antibodies diluted in 0.5% skim milk in PB: Primary monoclonal antibodies against human NALP3 (mouse, 1:200, sc-8422, Santa Cruz Biotechnology, Santa Cruz, CA, USA), CASP1 (mouse, 1:200, sc-17810, Santa Cruz Biotechnology), IL-1 β (1:200, sc-32294, Santa Cruz Biotechnology), p21 (rabbit, 1:400, 14-6715-81, ThermoFisher), p53 (rabbit, 1:400, PA5-104741, ThermoFisher) and TERT (rabbit, ZJ4491729A, 1:300, ThermoFisher). After 3 washes of 5 min each in PB, Alexa Fluor 488-conjugated goat anti-mouse (1:400, A11029, ThermoFisher) or goat anti-rabbit (1:400, A11034, Thermofisher) secondary antibodies were applied for 1 h under the same above incubated conditions. Simultaneously, rhodamine-phalloidin (1:150, 2892779, Life Technologies) was added for the duration of this incubation to visualize the actin cytoskeleton. Cell nuclei were stained, with DAPI contained in the mounting medium (Prolong antifade 40,6-diamidino-2-phenyl-indole, dihydrochloride, Molecular Probes, Invitrogen) and the coverslips were mounted on glass slides. Samples were analyzed with a Zeiss Axio Imager M2 Optical Microscope (Carl Zeiss, Jena, Germany), equipped with a 63X oil immersion objective to study the cytoskeleton distribution and the inflammatory markers. Experiments were repeated three times independently.

2.5 RNA ISOLATION AND REAL-TIME (RT-PCR) ANALYSIS

The PureLink RNA Mini Kit, obtained from Ambion, Thermo Fisher Scientific in Milan, Italy, was used to collect total RNA from the samples. To perform reverse transcription into 50 micrograms of cDNA, one microgram of total RNA was required when utilizing M-MLV reverse transcriptase (M1302 Sigma-Aldrich, Saint Louis, MO, USA), following the manufacturer's instructions, which included sequential incubation at 70 °C for 10 min, 37 °C for 50 min, and 90 °C for 10 min. The Mastercycler realplex system from Eppendorf, based in Hamburg, Germany, was used to perform real-time PCR with three separate biological replicates for each

sample. Comparison was made between mRNA expression levels of *NALP3* (Hs.PT.58.39303321), *CASP1* (Hs.PT. 56a.22811633.g), *IL-1 β* (Hs. PT.58.1518186), *CDKN1A* (p21) (Hs.PT. 58.40874346.g), *TP53* (p53) (Hs. PT. 58. 39489752) and *TERT* (Hs. PT. 58. 27489922) in cells cultured without and with pesticides. The *GAPDH* (Hs. PT.39a.22214836) and the *B2M* (Hs.PT.58v.18759587) were used as the endogenous controls to normalize hGMSCs and HUVECs. All the primers were purchased from Integrated DNA Technologies (IDT, Tema Ricerca, Bologna, Italy). The amplification process started with pre-incubation at 95 °C for 3 min, then continued with 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. A melting curve analysis was conducted for each run between the temperatures of 60 °C and 95 °C. Three separate trials were employed.

2.6 ROS

Cells were seeded in a 35 mm imaging dish (μ -Dish, ibidi GmbH, Gräfelfing, Germany) at a density of 85000 cells per dish and exposed to pesticides for 10 days. After treatment, the medium was changed to Normal External Solution (NES) with a composition of 125 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM KH₂PO₄, 5.5 mM glucose, 1 mM CaCl₂, and 20 mM HEPES at a pH of 7.4. Next, the cells were exposed to a culture medium containing 10 μ M of 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA, Thermo Fisher, Waltham, MA, USA) for 30 min at 37 °C, then rinsed with NES. For visualization, only NES was introduced into the plate.

Confocal microscopy (Zeiss LSM800 microscope, Carl Zeiss, Jena, Germany) was used to capture images with consistent acquisition settings using a motorized table named SMC 2009 and the multiple single position acquisition function (Tiles-Advanced setup, carrier 35 mm petri dish) of Zen Blue software (Zen 3.0 SR, Carl Zeiss); the inverted Axio-observer D1 microscope and a W-Plan-Apo 40 $\times$ 1.3 DIC objective (Carl Zeiss, Jena, Germany) has been used for ROS detection using an excitation of 488 nm.

2.7 QUANTIFICATION OF FLUORESCENCE

Protein expression and ROS fluorescence were quantified using ImageJ software (version 8, National Institutes of Health, Bethesda, MD, USA) based on the established protocols (Schneider et al., 2012; Collins, 2007). Regions of interest (ROIs) were selected either manually or using threshold-based automated segmentation, and the mean fluorescence intensity within each ROI was calculated. Background subtraction was applied using a rolling-ball algorithm to correct for non-specific signal and ensure accuracy. To minimize bias, measurements were performed blinded to the sample identity, and multiple images per sample were averaged. The resulting values were normalized relative to the control group to account for inter-experimental variability, allowing quantitative comparison across samples

2.8 STATISTICAL ANALYSIS

Statistical analyses were performed using one-way ANOVA followed by and Tukey's post hoc test (GraphPad 5, San Diego, CA, USA). All differences were considered statistically significant at $p < 0.05$. The comparative $2^{-\Delta\Delta Ct}$ method was employed.

2.9 PESTICIDES DOSE CALCULATION

Boscalid (AST1Y6P006) and Pyraclostrobin (AST1Y6D573) were purchased from Lab Instruments S.R.L. (Castellana Grotte, Italy), while Propamocarb (45638) and Lambda-cyhalothrin (31058) were acquired from Sigma Aldrich (St. Louis, MO, USA). Pesticides concentrations were calculated based on the methodologies described in our previous manuscript (Mazzone et al., 2025). The high Daily Nutritional Intake (hNDI) values were derived from Leveque et al. (2019) and establish the true level of contamination of French citizens from infancy to old age by individual pesticide compounds, based on the method of the European Food Safety Authority (Leveque et al., 2019).

The hNDI calculation followed a process like that of the Theoretical Maximum Daily Intake (TMDI):

$$TMDI = \sum (MRL_i \times F_i)$$

MRL_i represents the maximum residue level in a specific food, and F_i denotes food consumption rates. We included fruits and vegetables in the calculation of the hNDI

because they are the main dietary source of pesticide exposure (Pesticide residues in food: latest figures released). In addition, the World Health Organization (WHO) dietary guidelines recommend a minimum daily consumption of 400 g of fruits and vegetables (Increasing fruit and vegetable consumption to reduce the risk of noncommunicable diseases). Based on this evidence, MRL data were retrieved from the EFSA database, which reports the maximum residue levels of pesticides (https://food.ec.europa.eu/plants/pesticides/eu-pesticides-database_en?utm_source=chatgpt.com). The hNDI values were first converted to mg/kg bw/day, then to g/L, and subsequently to mol/L for in vitro studies (citazione lavori Hochane e Leveque). To estimate the blood concentrations (mg/L and $\mu\text{mol/L}$) to which different organs could theoretically be exposed, complete absorption of the residue concentrations and their distribution in 5 L of blood in a 60 kg individual were assumed. Following those considerations, the hNDI of the tested pesticides are:

- Boscalid: 11,06 μM
- Pyraclostrobim: 1,51 μM
- Propamocarb: 15,64 μM
- Lambda cyhalothrin: 0,19 μM

Pesticide powders were resuspended in 1 mL of DMSO; the final concentration of DMSO was 0,2%. The 0,2% concentration of DMSO was used for control cells.

2.10 EXPERIMENTAL PLAN

- Cells cultured with 0,2% of DMSO (CTRL)
- Cells cultured with Boscalid 11,06 μM (B)
- Cells cultured with Pyraclostrobin 1,51 μM (PY)
- Cells cultured with Propamocarb 15,64 μM (PR)
- Cells cultured with Lambda-cyhalothrin 0,19 μM (LC)
- Cells cultured with Boscalid 11,06 μM + Pyraclostrobin 1,51 μM (BPY)
- Cells cultured with Propamocarb 15,64 μM + Lambda-cyhalothrin 0,19 μM (PRLC)
- Cells cultured with Boscalid 11,06 μM + Pyraclostrobin 1,51 μM + Propamocarb 15,64 μM (BPYPR)

- Cells cultured with Boscalid 11,06 μM + Pyraclostrobin 1,51 μM + Lambda-cyhalothrin 0,19 μM (BPYLC).

3. RESULTS

3.1 MTS

3.1.1 hGMSCs

The MTS assay revealed distinct, time-dependent effects of pesticide exposure on cell metabolic activity at 24 h, 72 h, and 10 days of exposure (Figure 11).

After 24h of exposure, the single pesticides pyraclostrobin (PY) and propamocarb (PR) induced the most pronounced increase in metabolic activity compared with control cells. In contrast, the combined treatment BPYLC resulted in a significant reduction in metabolic activity relative to both control and other experimental conditions. At 72 h, PY remained the treatment that most strongly enhanced metabolic activity, whereas B exhibited the lowest effect among single-pesticide treatments. Interestingly, metabolic activity in cells exposed to the BPYLC combination increased compared to the 24h time point

After 10 days of exposure, B alone induced the highest increase in metabolic activity compared to control cells. Conversely, LC alone and the BPYLC combination led to the lowest metabolic activity, indicating a sustained inhibitory effect over prolonged exposure.

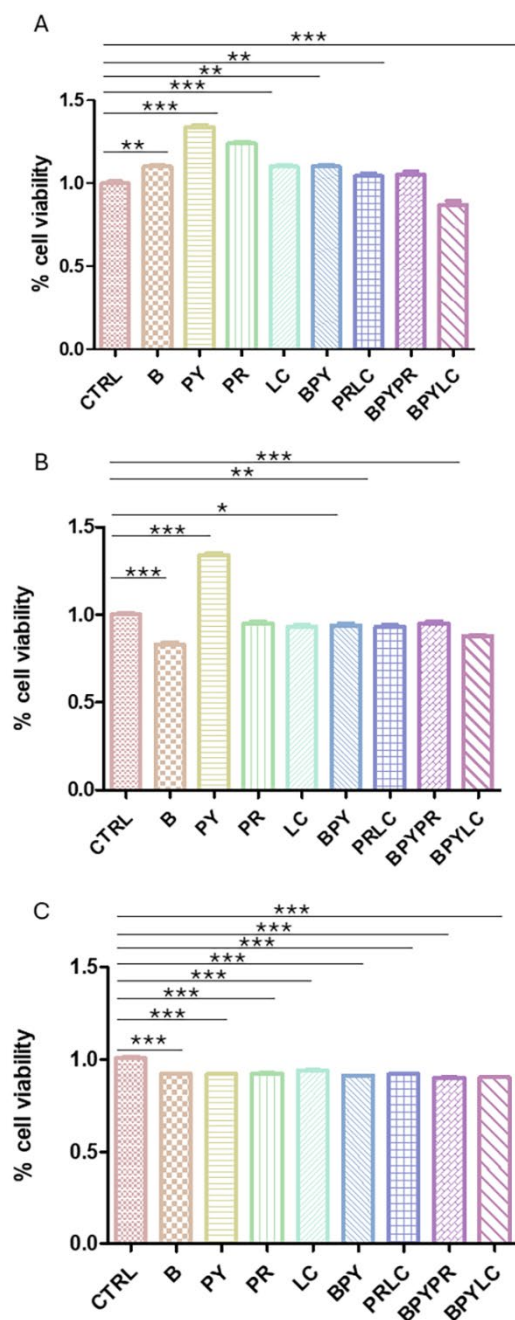


Figure 11: Statistical Analysis of MTS Assay. MTS assay results show cell viability of hGMSCs under control conditions and after exposure to pesticides over different time points (A:24 h, B:72 h, C:10 days). Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison test. Data are expressed as mean $\pm$ standard deviation (n=3). Significant differences compared to control are indicated (*p<0.05, **p<0.01, ***p<0.001). The experiment was conducted in triplicate

3.1.2 HUVECs

The MTS assay demonstrated clear time-dependent variations in the metabolic activity of HUVECs exposed to pesticides compared with control cells (Figure 12).

After 24 h of exposure (Figure 12A), all treated groups exhibited increased metabolic activity compared with controls. At 72 h (Figure 12B), a reduction in metabolic activity became evident in pesticide-treated cells compared to the control. This decrease was further accentuated after 10 days of treatment (Figure 12C), showing reduced metabolic activity. The most pronounced decreases were observed in HUVEC cells exposed to the BPYPR and BPYLC combinations.

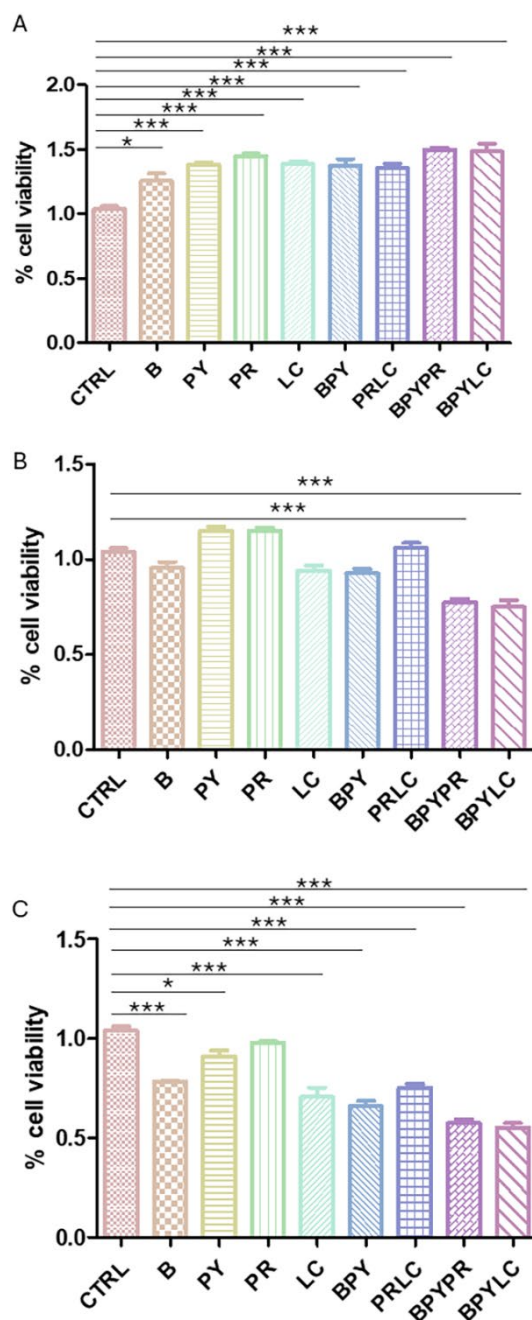


Figure 12: Statistical Analysis of MTS Assay. MTS assay results show cell viability of HUVECs under control conditions and after exposure to pesticides over different time points (A:24 h, B:72 h, C:10 days). Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison test. Data are expressed as mean $\pm$ standard deviation (n=3). Significant differences compared to control are indicated (* p <0.05, ** p <0.01, *** p <0.001). The experiment was conducted in triplicate

3.2 SCANNING ELECTRON MICROSCOPY (SEM) EVALUATION

3.2.1 hGMSCs

SEM analysis (Figure 13) showed that untreated hGMSCs displayed a fibroblast-like morphology, characterized by elongated cytoplasmic extensions and numerous filopodia interacting with neighbouring cells. At the cytoplasmic level, one or more nucleoli were clearly observed in the nuclei.

In contrast, pesticide-exposed hGMSCs exhibited marked morphological alterations. Indeed, treated cells lost the spindle-shaped morphology and appeared smaller and more spherical, showing membrane irregularities. Nuclei and nucleoli were difficult to observe. BPYPR and BPYLC combinations induced the most severe impairment of surface features.

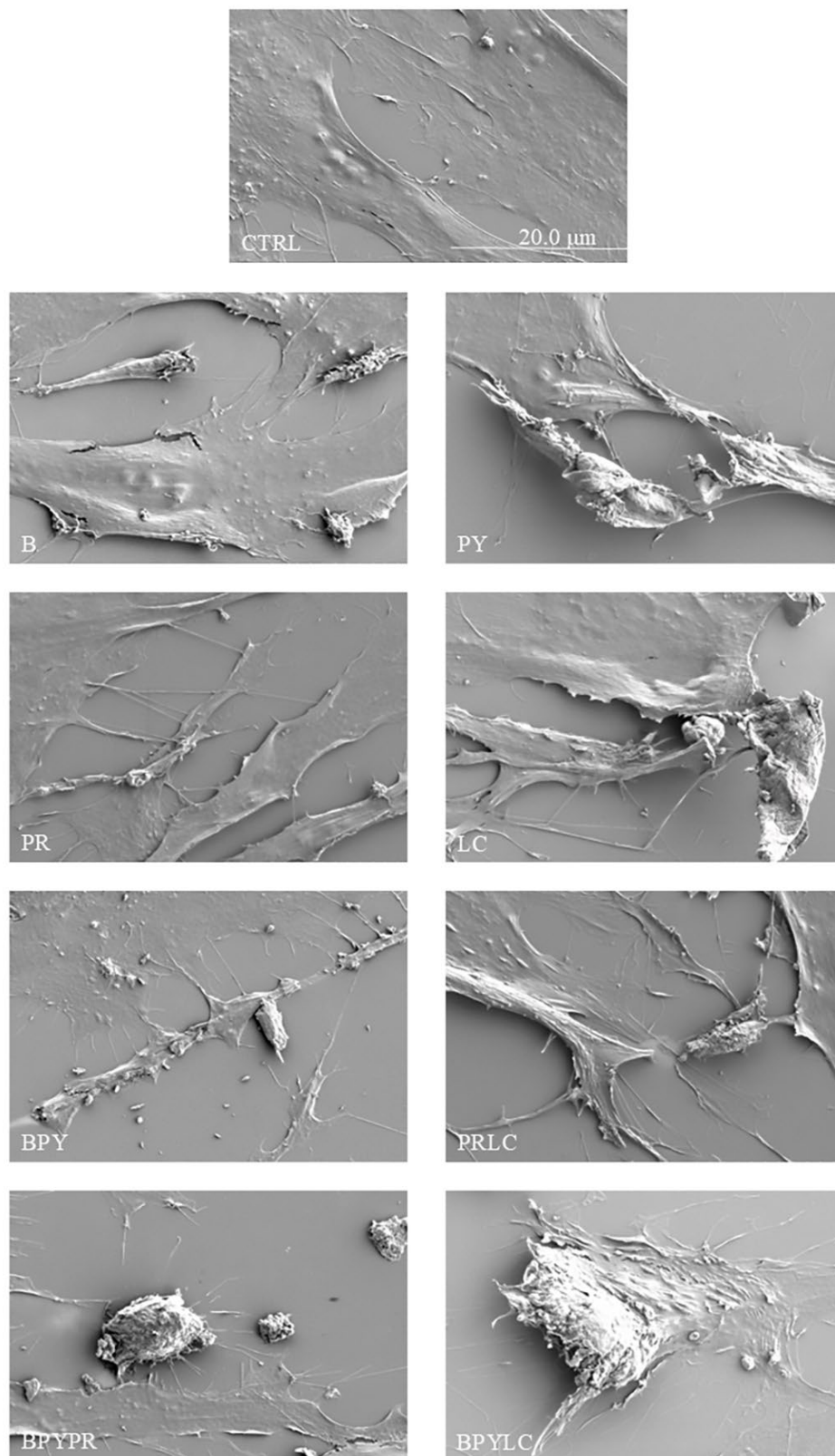


Figure 13: SEM pictures show control cells and treated hGMSCs cells for 10 days.
Scale bar: 20 μm . Control cells were cultured with 0,2% of DMSO

3.2.2 HUVECs

SEM analysis performed after 10 days of treatment revealed morphological differences between control HUVECs and pesticide- treated cells (Figure 14). Control cells exhibited a membrane with a blebbing process, whereas treated HUVECs showed altered cell shape and a marked reduction in membrane blebbing. These changes were particularly evident in cells exposed to the PRLC, BPYPR, and BPYLC combinations, supporting the notion of pesticide-induced cellular stress and dysfunction.

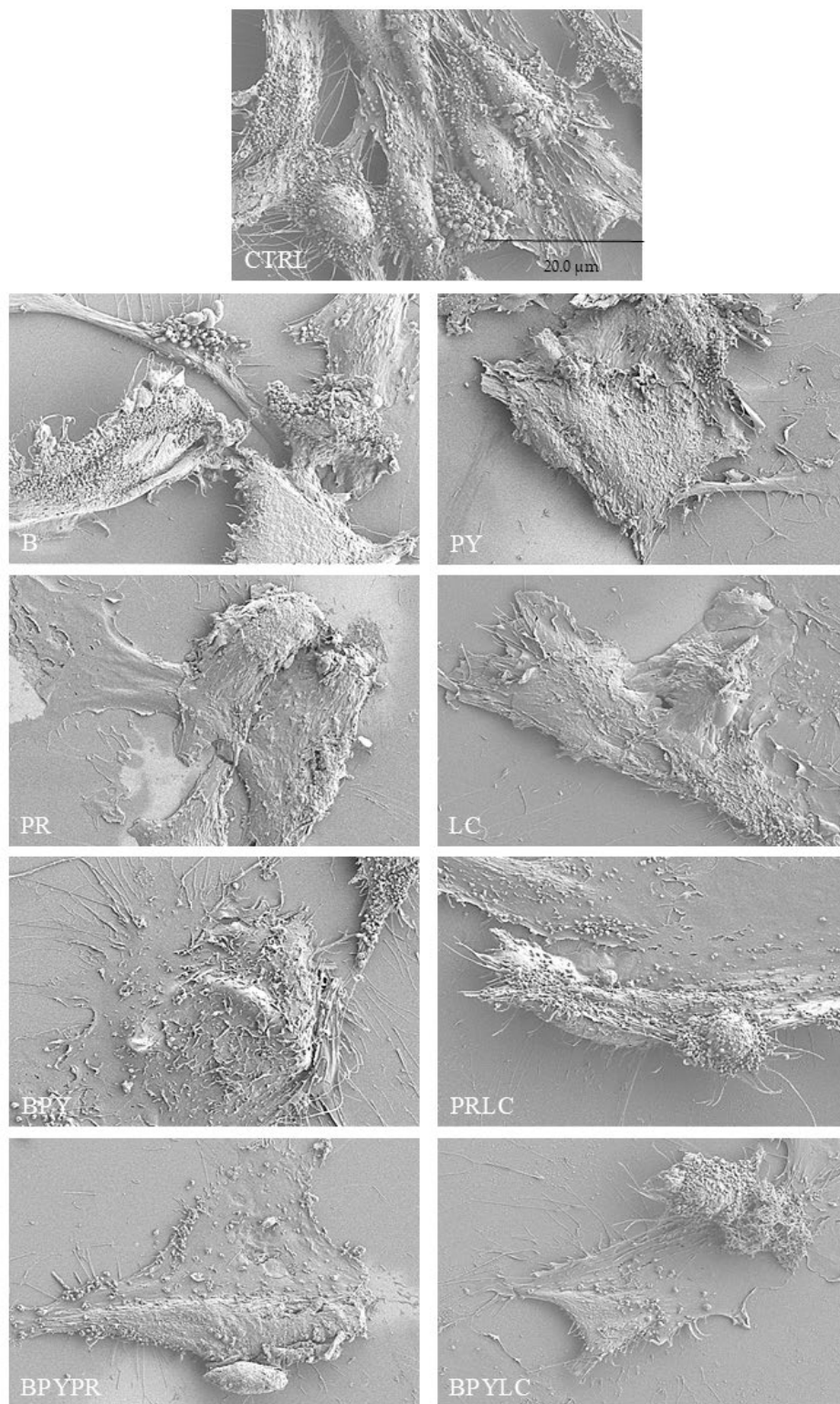


Figure 14: The figure shows SEM pictures in control cells and treated HUVECs cells for 10 days. Scale bar: 20 μm . Control cells were cultured with 0,2% of DMSO

3.3 INPLHAMMOSOME PATHWAY

3.3.1 hGMSCs

3.3.1.1 IMMUNOFLUORESCENCE, PROTEIN QUANTIFICATION AND RT-PCR

The figures 15-20 show immunofluorescence staining, quantitative fluorescence analysis and gene expression levels of NALP3 (Figures 15, 16), CASP1 (Figures 17,18), and IL-1 β (Figures 19, 20) in hGMSCs cultured without and/or with pesticides for 10 days.

Immunofluorescence images demonstrated a clear upregulation of NALP3, CASP1 and IL-1 β in pesticide-treated hGMSCs compared with untreated controls. Among single-pesticide treatments, Boscalid induced the strongest activation of the inflammasome pathway. Overall, pesticide combinations, particularly BPYPR and BPYLC, elicited higher expression levels of all three inflammasome-related markers compared with single treatments and control cells.

Quantitative analyses of fluorescence intensity for NALP3 (Figure 16A), CASP1 (Figure 18A) and IL-1 β (Figure 20A) confirmed the qualitative observations obtained from immunofluorescence imaging. In addition, RT-PCR results (Figure 16B, 18B, 20B) were consistent with protein-level data.

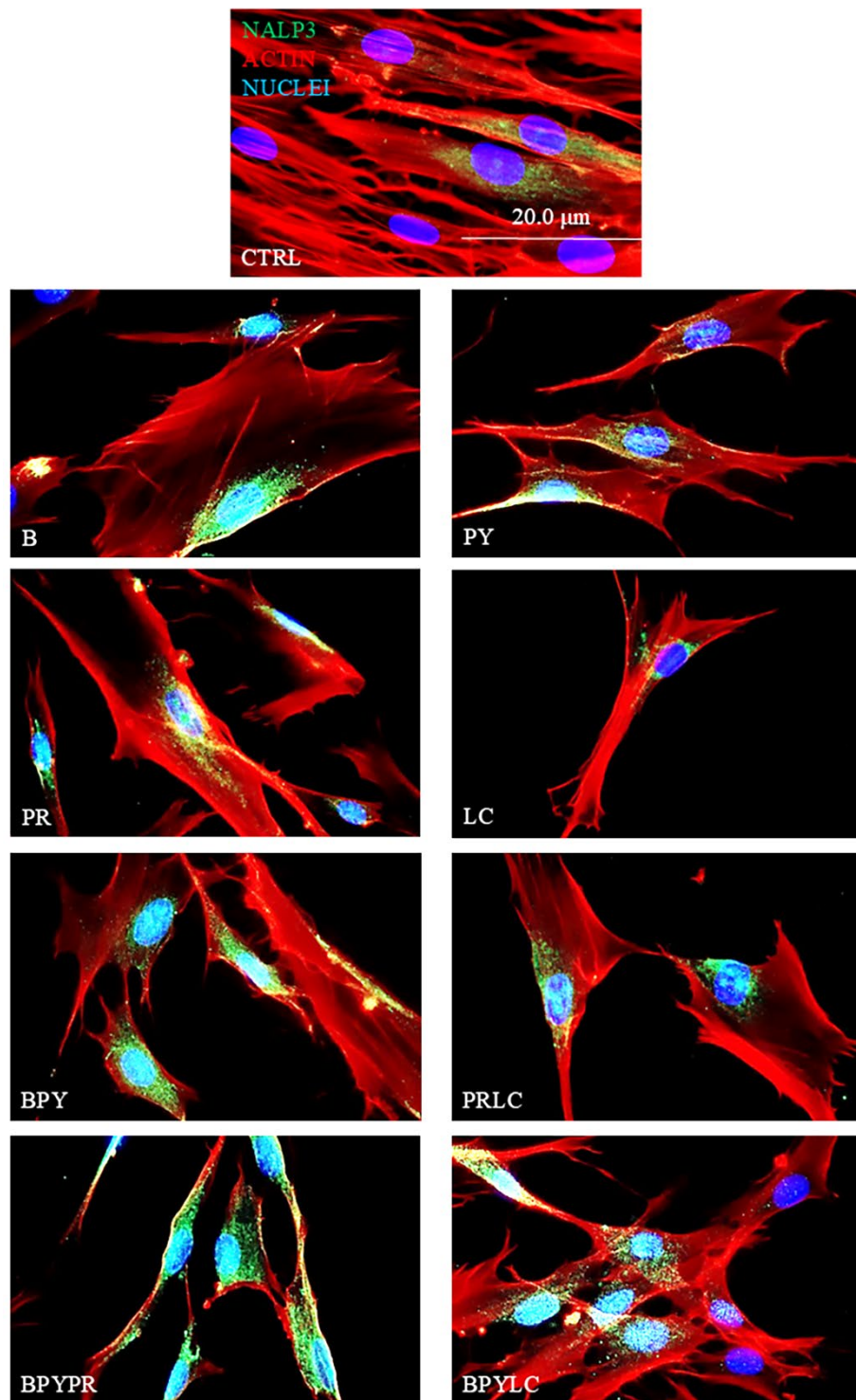


Figure 15: The figure shows Immunofluorescence of the marker NALP3 in hGMSCs control and in hGMSCs treated with pesticides after 10 days. Control cells were cultured with 0,2% of DMSO.

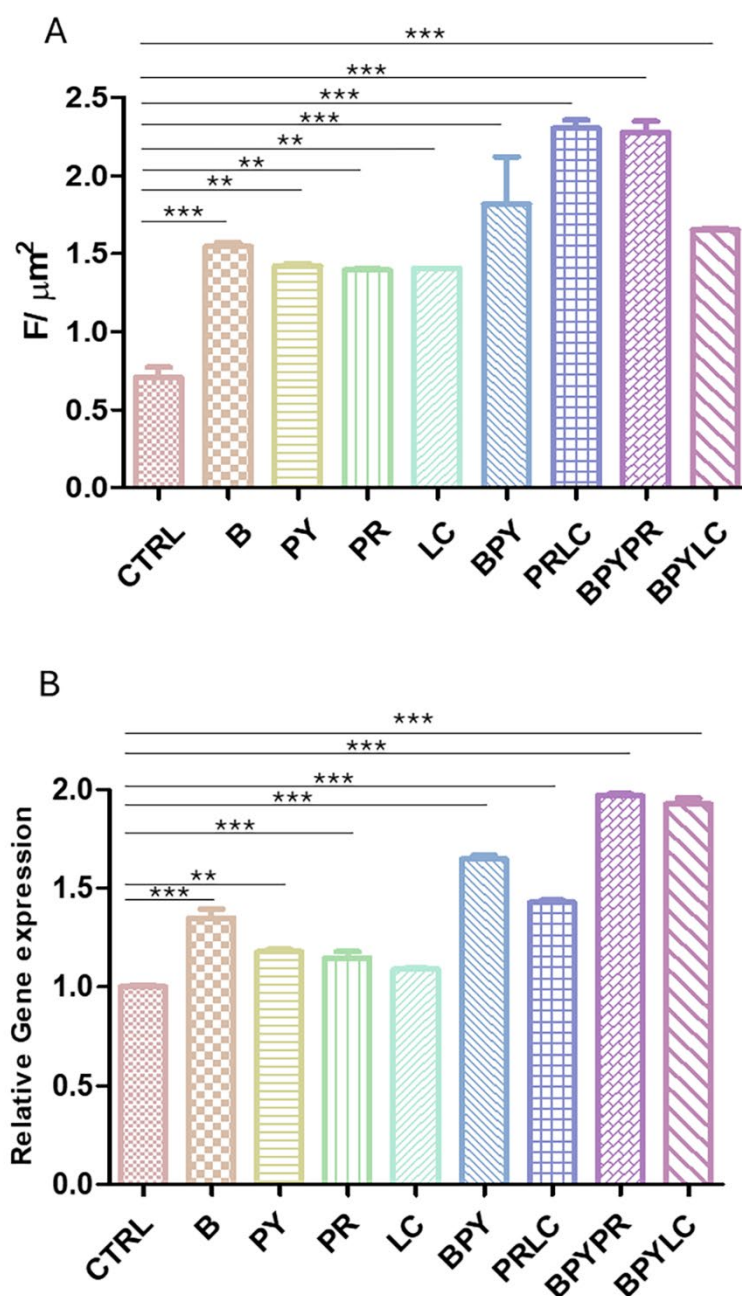


Figure 16: Protein Quantification (A) and RT-PCR analysis (B) of the marker NALP3 in hGMSCs control and in hGMSCs treated with pesticides after 10 days. The bars in the chart show the quantitative assessment of NALP3 fluorescence (A) and mRNA levels (B), reported as mean $\pm$ standard deviation (SD) from three separate experiments. Differences between control and experimental groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Significant differences compared to control are indicated (** $p < 0.01$, *** $p < 0.001$).

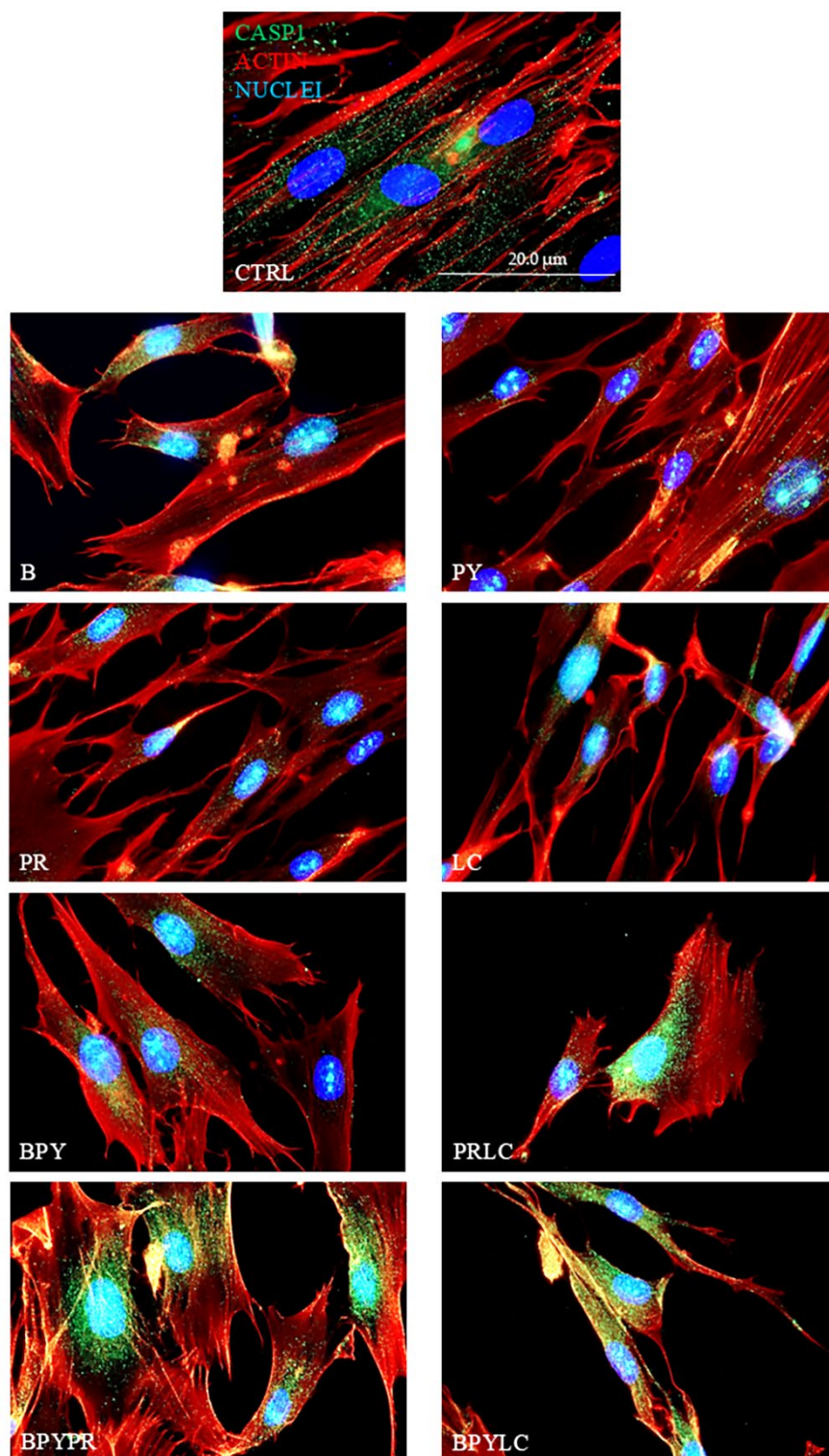


Figure 17: The figure shows Immunofluorescence of the marker CASP1 in hGMSCs control and in hGMSCs treated with pesticides after 10 days. Control cells were cultured with 0,2% of DMSO.

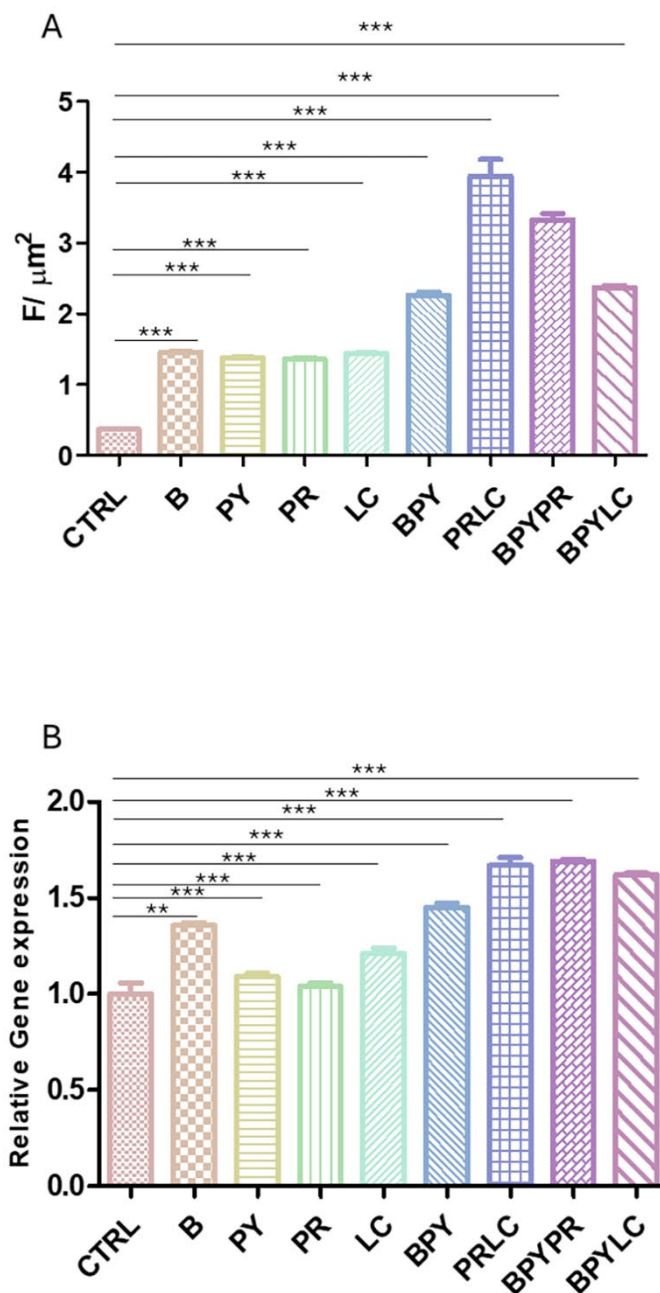


Figure 18: Protein Quantification (A) and RT-PCR analysis (B) of the marker CASP1 in hGMSCs control and in hGMSCs treated with pesticides after 10 days. The bars in the chart show the quantitative assessment of CASP1 fluorescence (A) and mRNA levels (B), reported as mean $\pm$ standard deviation (SD) from three separate experiments. Differences between control and experimental groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Significant differences compared to control are indicated (** $p < 0.01$, *** $p < 0.001$).

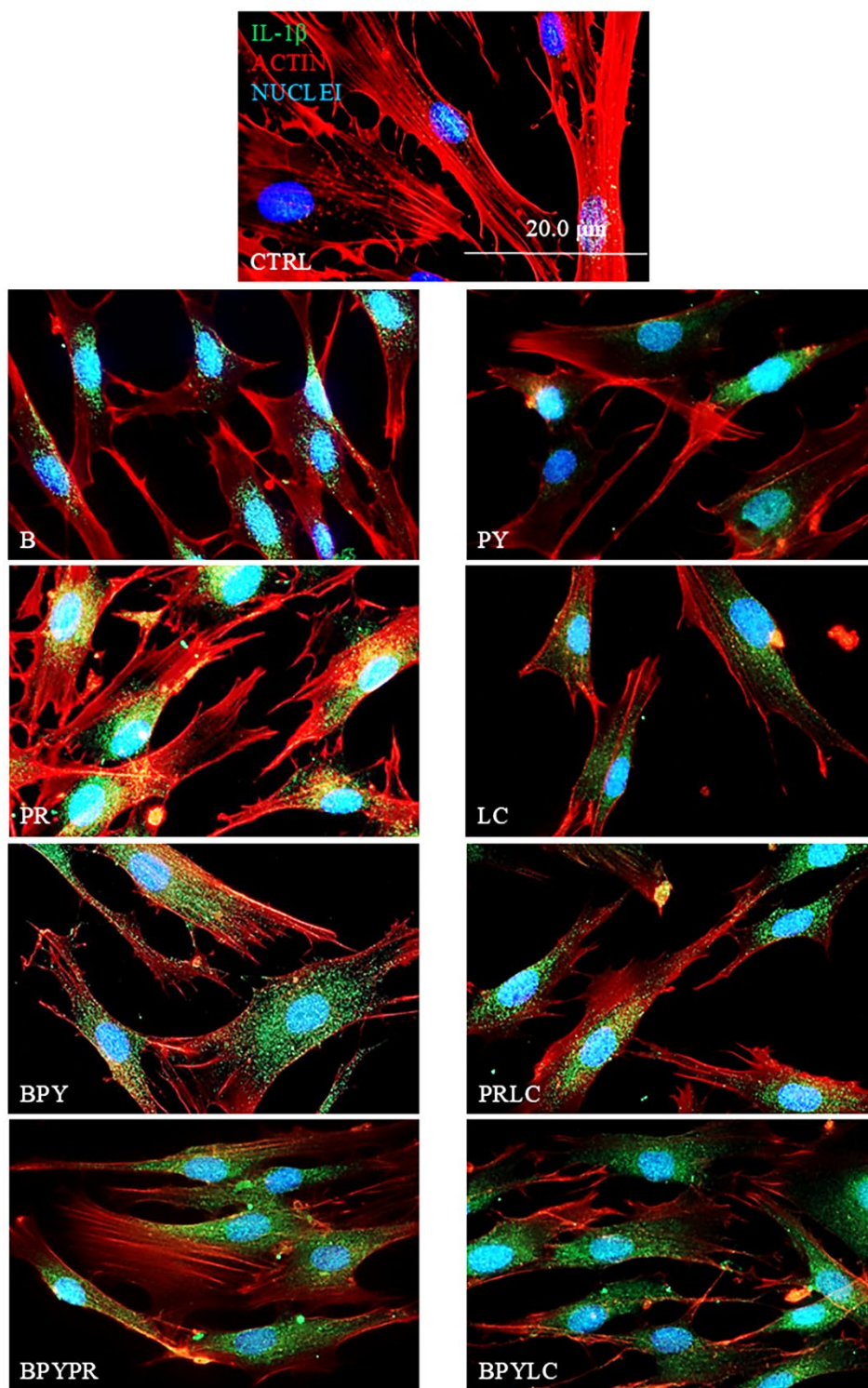


Figure 19: The figure shows Immunofluorescence of the marker IL-1 β in hGMSCs control and in hGMSCs treated with pesticides after 10 days. Control cells were cultured with 0,2% of DMSO.

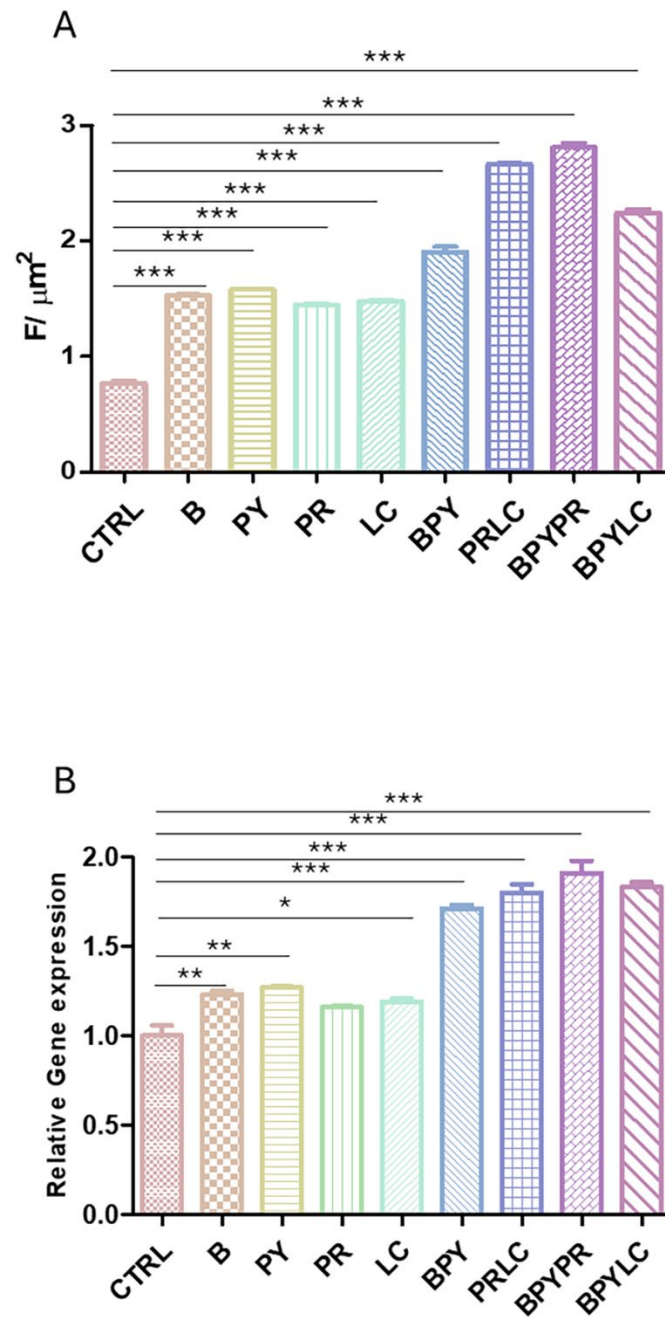


Figure 20: Protein Quantification (A) and RT-PCR analysis (B) of the marker IL-1 β in hGMSCs control and in hGMSCs treated with pesticides after 10 days. The bars in the chart show the quantitative assessment of IL-1 β fluorescence (A) and mRNA levels (B), reported as mean $\pm$ standard deviation (SD) from three separate experiments. Differences between control and experimental groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Significant differences compared to control are indicated (**p<0.01, ***p<0.001)

3.3.1.2 ROS EVALUATION

Figure 21 shows ROS production in hGMSCs, revealing significantly higher levels in pesticide-treated cells compared to control cells. Among the single pesticides, LC induced the highest ROS production, indicating a strong pro-inflammatory and pro-oxidative effect.

The highest ROS levels were observed in cells exposed to the combined treatments BPYPR and BPYLC, which exhibited significantly increased fluorescence compared to both single-pesticide treatments and control hGMSCs. Quantitative analysis of regions of interest (ROIs) confirmed the qualitative findings (Figure 22)

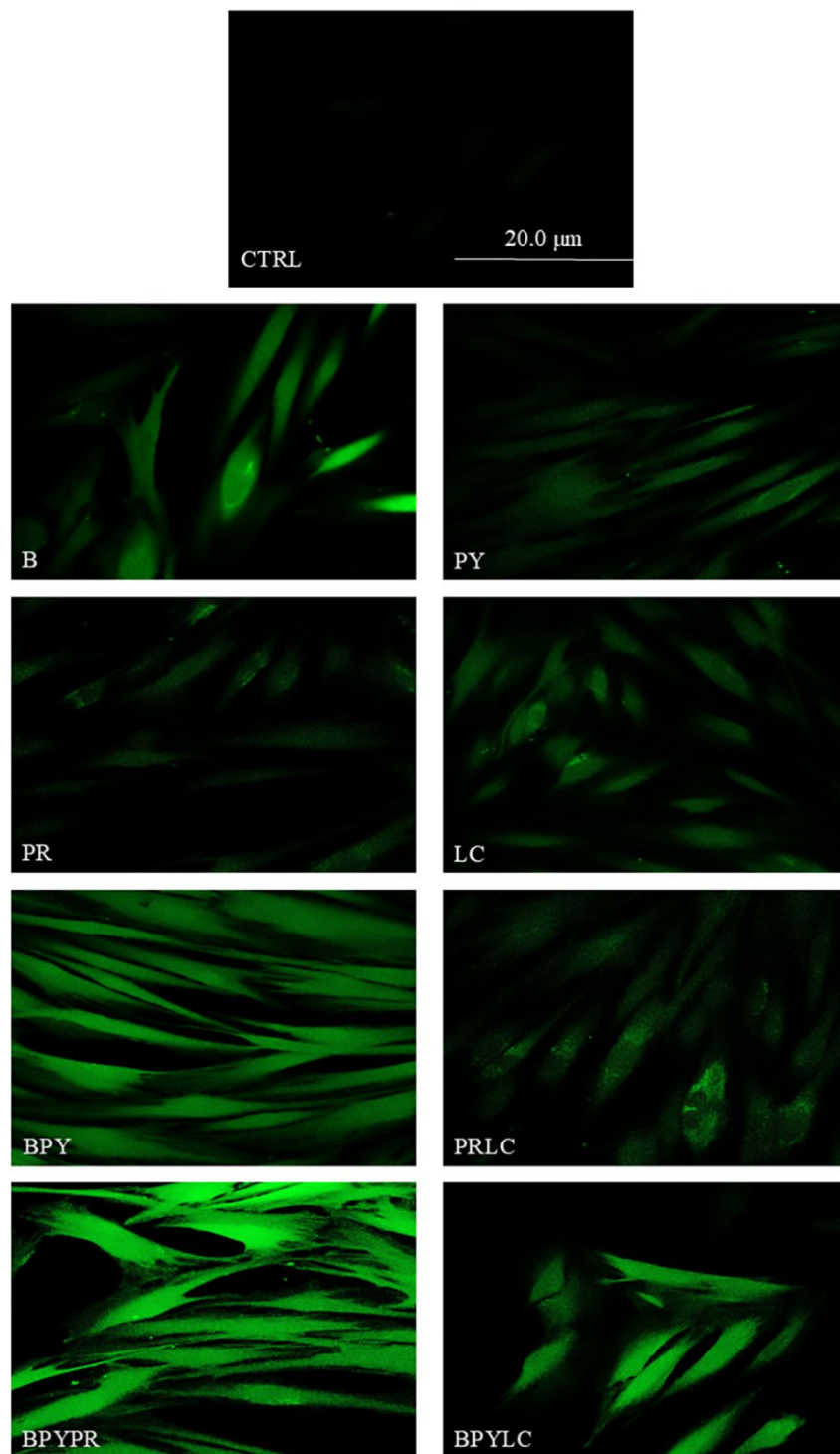


Figure 21: The figure shows ROS measurements in live hGMSCs, loaded with DCFH-DA and acquired by confocal microscopy, cultured alone and with pesticides for 10 days.

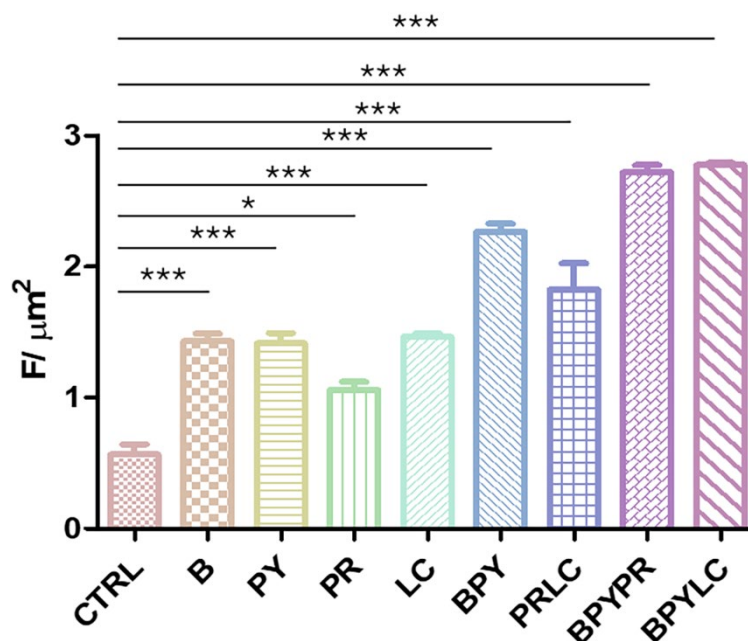


Figure 22: The figure shows the statistical analysis of ROS quantification in hGMSCs control and in hGMSCs treated with pesticides after 10 days determined using one-way ANOVA followed by Tukey's multiple comparison test, expresses data as mean $\pm$ standard deviation (SD) from three independent experiments. Significant differences compared to control are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Control cells were cultured with 0,2% of DMSO.

3.3.2 HUVECs

3.3.2.1 IMMUNOFLUORESCENCE, PROTEIN QUANTIFICATION AND RT-PCR

Figures 23-28 illustrate the immunofluorescence staining, quantitative analysis and gene expression levels of the inflammasome-related markers NALP3, CASP1 and IL-1 β in HUVECs cultured without and/or pesticides for 10 days. The immunofluorescence images evidence the upregulation of NALP3 (Figures 23), CASP1 (Figures 25) and IL-1 β (Figures 27) in treated HUVECs compared to HUVECs cultured alone. Among the single pesticides, B and LC were the most inflammatory; all the combined treatments showed more significant results when compared to the single pesticides and to the control cells, in particular, the combination of the three pesticides BPYPR and BPYLC. The quantification analysis of the immunofluorescence markers' expression, NALP3 (Figures 24A), CASP1 (Figures 26A) and IL-1 β (Figures 28A) confirm the qualitative results observed from the pictures. In addition, the data from RT-PCR (Figure 24B, 26B, 28B) supported the results from the Immunofluorescence analysis.

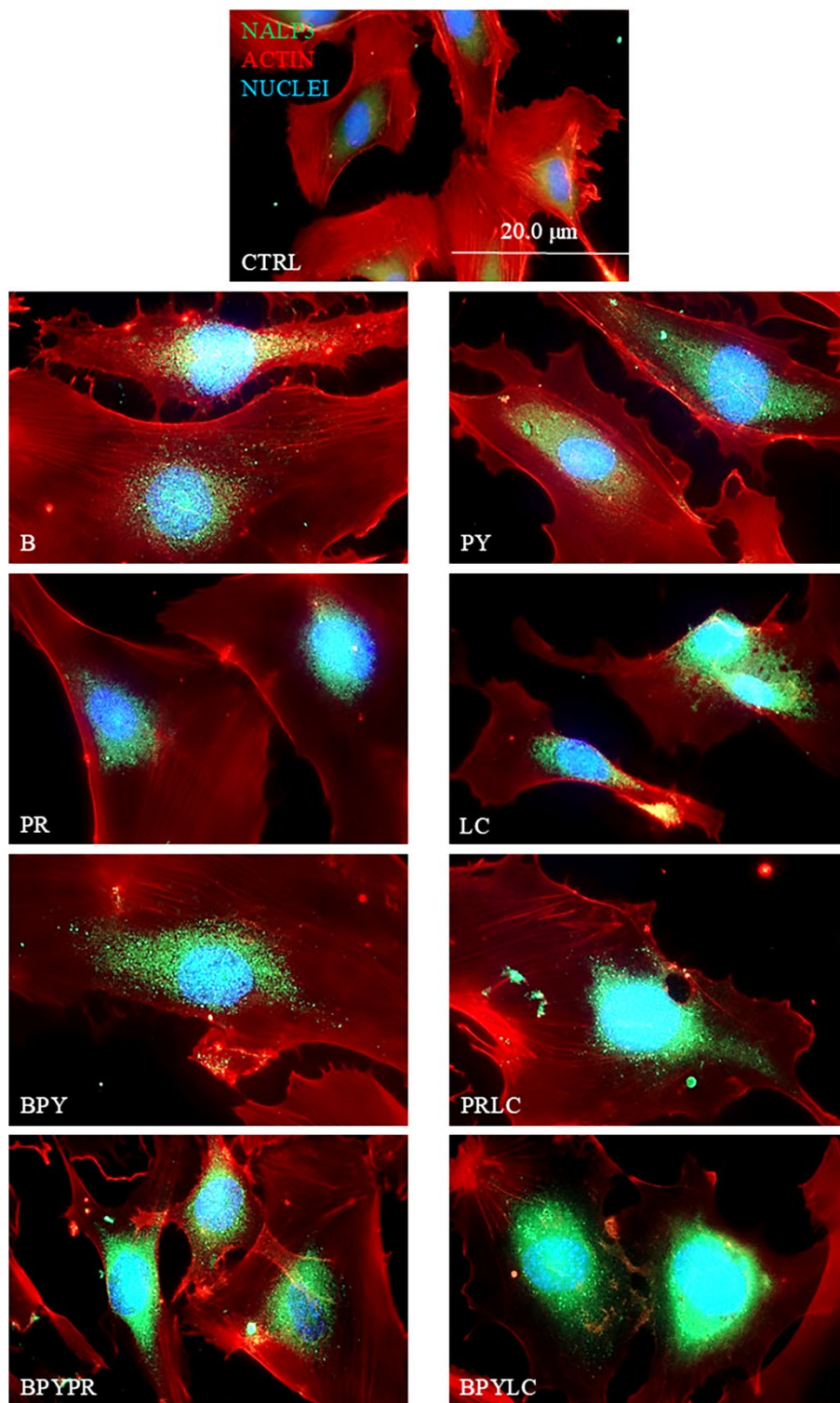


Figure 23: The figure shows Immunofluorescence of the marker NALP3 in HUVECs control and in HUVECs treated with pesticides after 10 days. Control cells were cultured with 0,2% of DMSO.

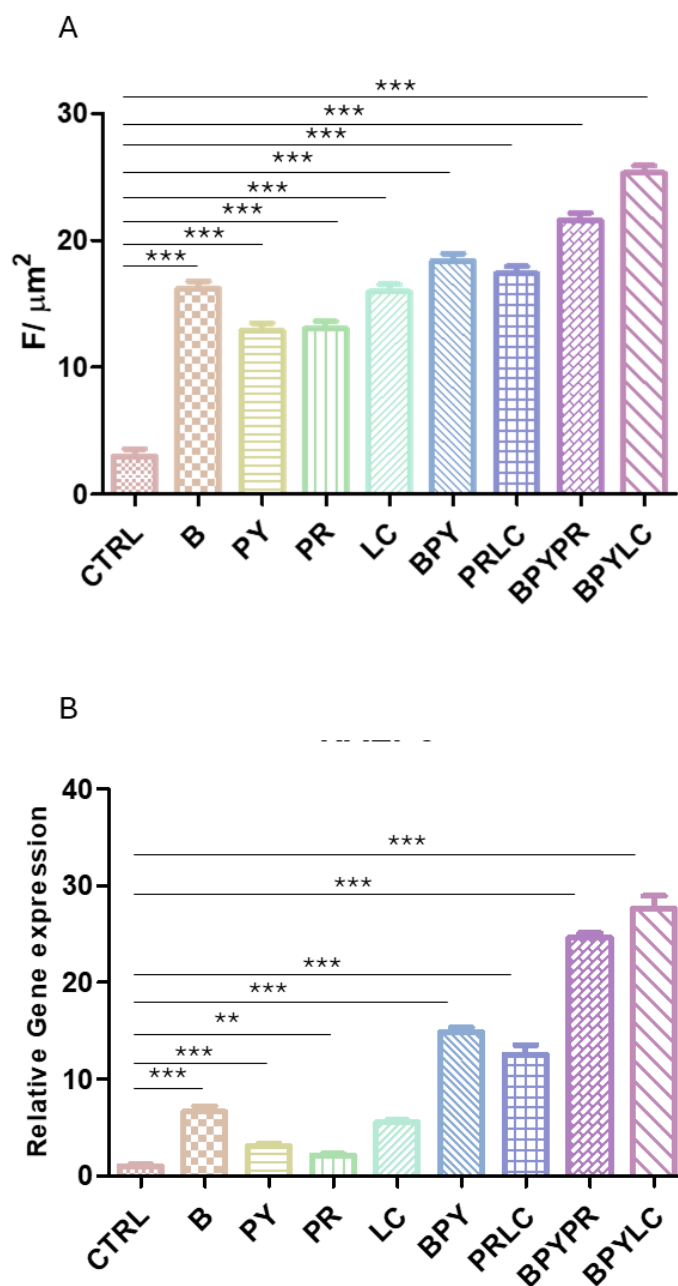


Figure 24: Protein Quantification (A) and RT-PCR analysis (B) of the marker NALP3 in HUVECs control and in HUVECs treated with pesticides after 10 days. The bars in the chart show the quantitative assessment of NLRP3 fluorescence (A) and mRNA levels (B), reported as mean $\pm$ standard deviation (SD) from three separate experiments. Differences between control and experimental groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Significant differences compared to control are indicated (**p<0.01, ***p<0.001).

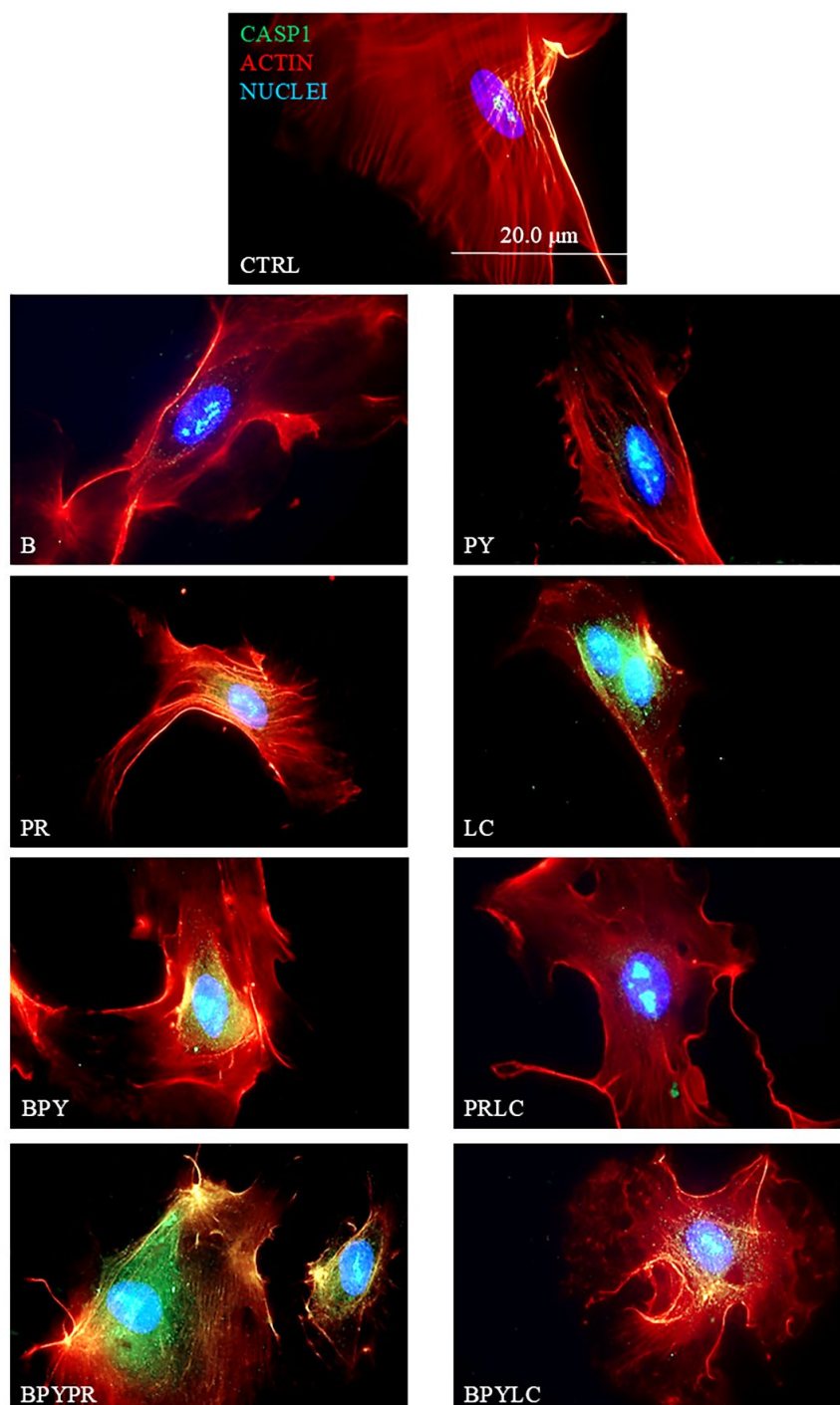


Figure 25: The figure shows Immunofluorescence of the marker CASP1 in HUVECs control and in HUVECs treated with pesticides after 10 days. Control cells were cultured with 0,2% of DMSO.

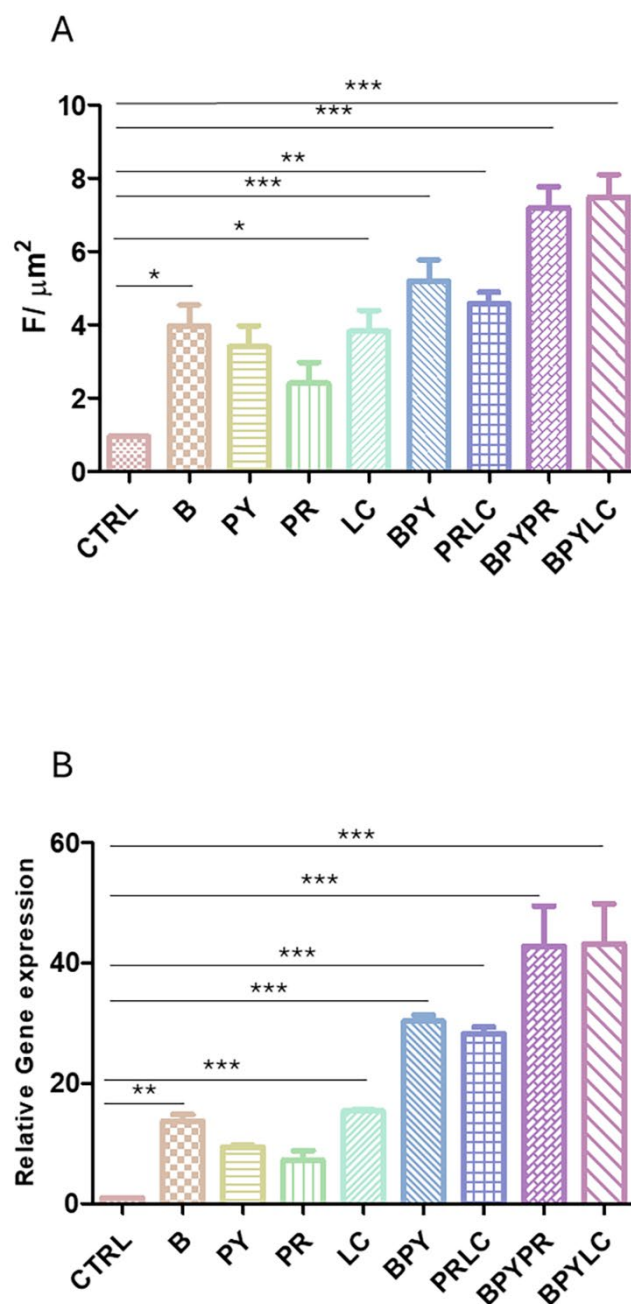


Figure 26: Protein Quantification (A) and RT-PCR analysis (B) of the marker CASP1 in HUVECs control and in HUVECs treated with pesticides after 10 days. The bars in the chart show the quantitative assessment of CASP1 fluorescence (A) and mRNA levels (B), reported as mean $\pm$ standard deviation (SD) from three separate experiments. Differences between control and experimental groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Significant differences compared to control are indicated (* $p < 0.01$, *** $p < 0.001$).

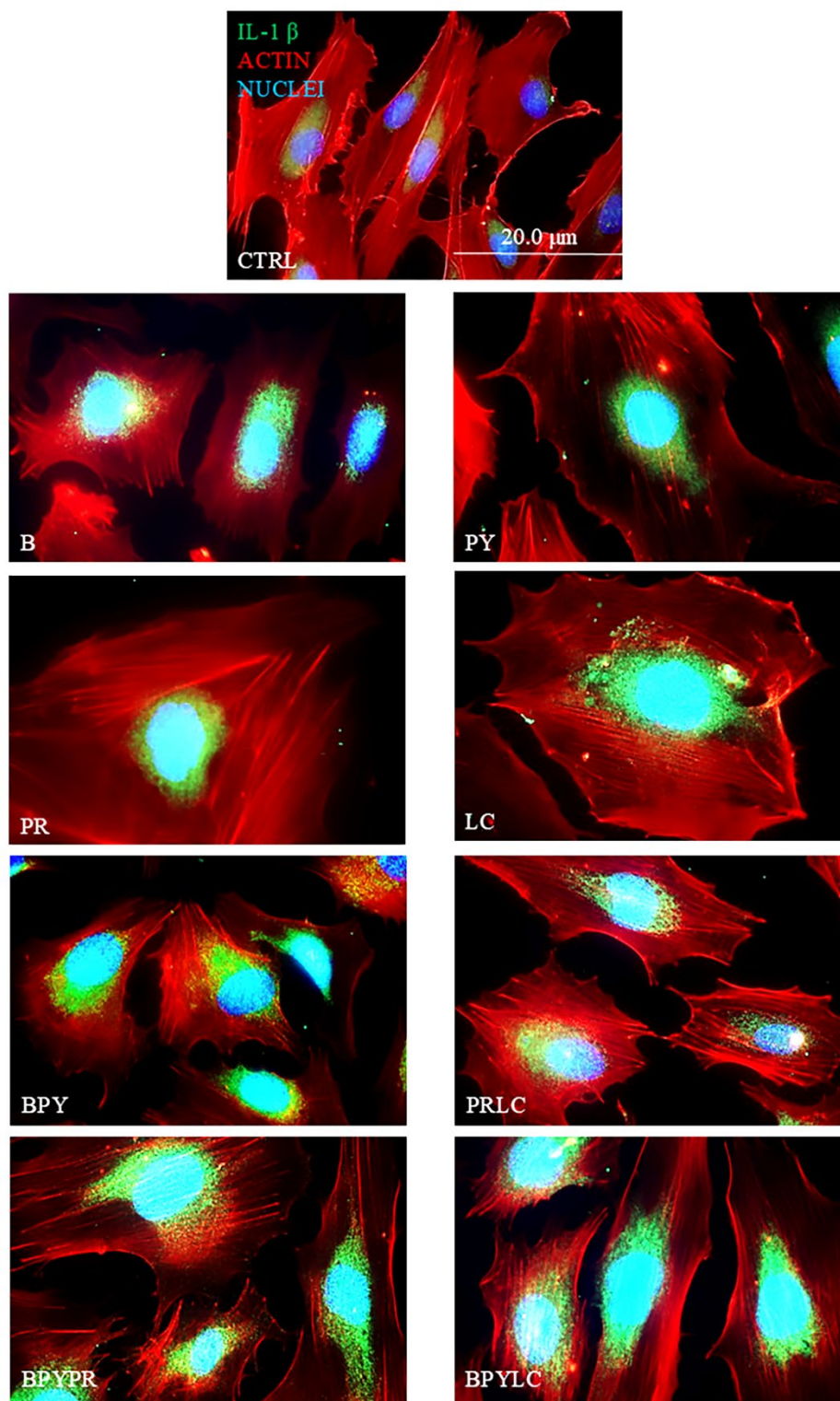


Figure 27: The figure shows Immunofluorescence of the marker IL-1 β in HUVECs control and in HUVECs treated with pesticides after 10 days. Control cells were cultured with 0,2% of DMSO.

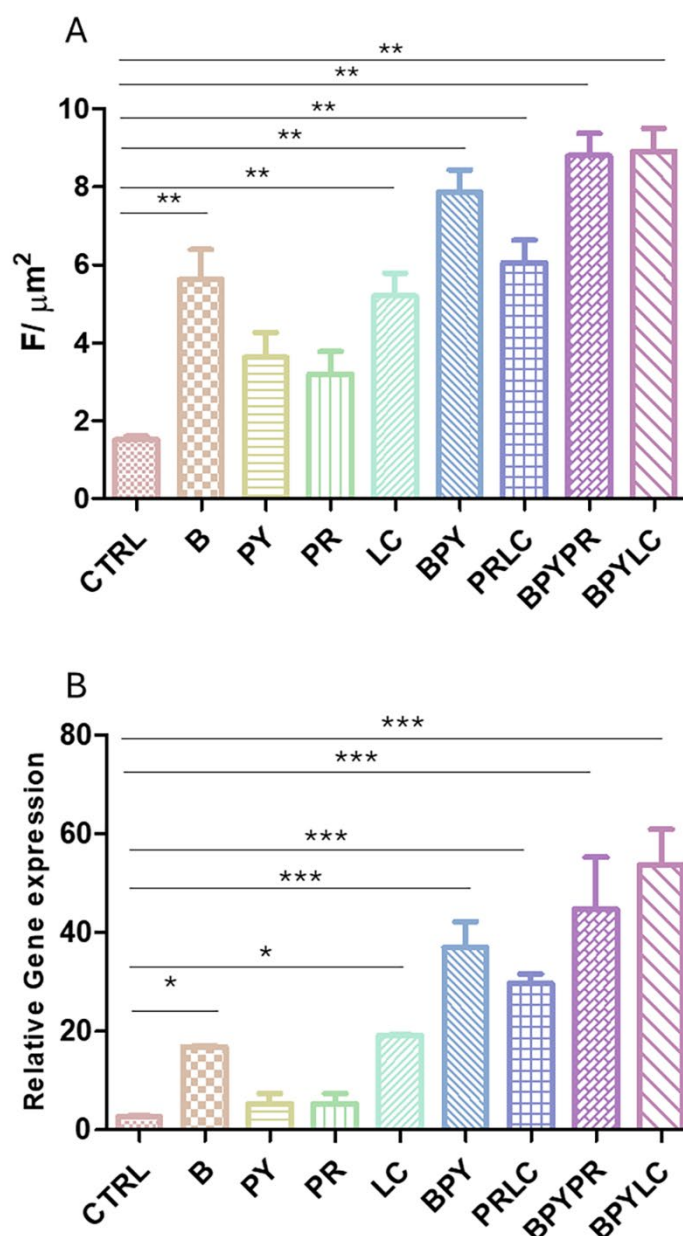


Figure 28: Protein Quantification (A) and RT-PCR analysis (B) of the marker IL-1 β in HUVECs control and in HUVECs treated with pesticides after 10 days. The bars in the chart show the quantitative assessment of IL-1 β fluorescence (A) and mRNA levels (B), reported as mean $\pm$ standard deviation (SD) from three separate experiments. Differences between control and experimental groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Significant differences compared to control are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.3.2.2 ROS EVALUATION

ROS production was assessed using the DCFH-DA probe, which detected an increased fluorescence signal in pesticide-treated HUVECs compared to controls, suggesting heightened oxidative stress. The results followed the same trend as the immunofluorescence data, highlighting the possible link between pesticide exposure and cellular stress responses (Figure 29). In fact, among the single pesticides, B shows the greatest ROS production; combinations of pesticides, in particular the combinations BPYPR and BPYLC, confirmed their stronger activation in inflammation compared to the other pesticides and to control. However, it is noteworthy that the combination of PRLC appeared to induce a relatively lower oxidative stress response compared to other pesticide combinations. ROS results suggest the loop phenomenon between the inflammasome activation and ROS production. Quantification of the ROI areas confirmed the confocal findings (Figure 30).

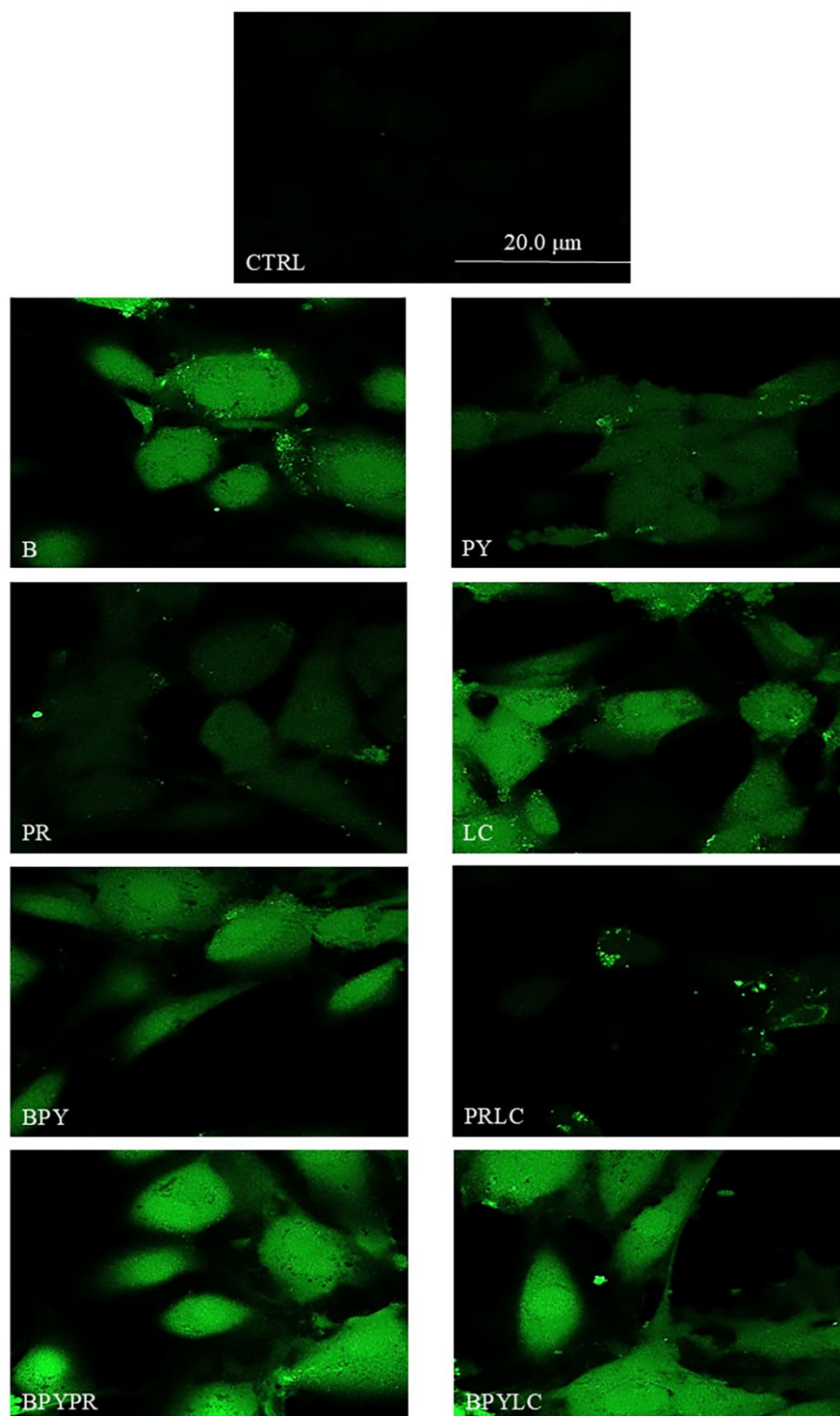


Figure 29: The figure shows ROS measurements in live HUVECs, loaded with DCFH-DA and acquired by confocal microscopy, cultured alone and with pesticides for 10 days.

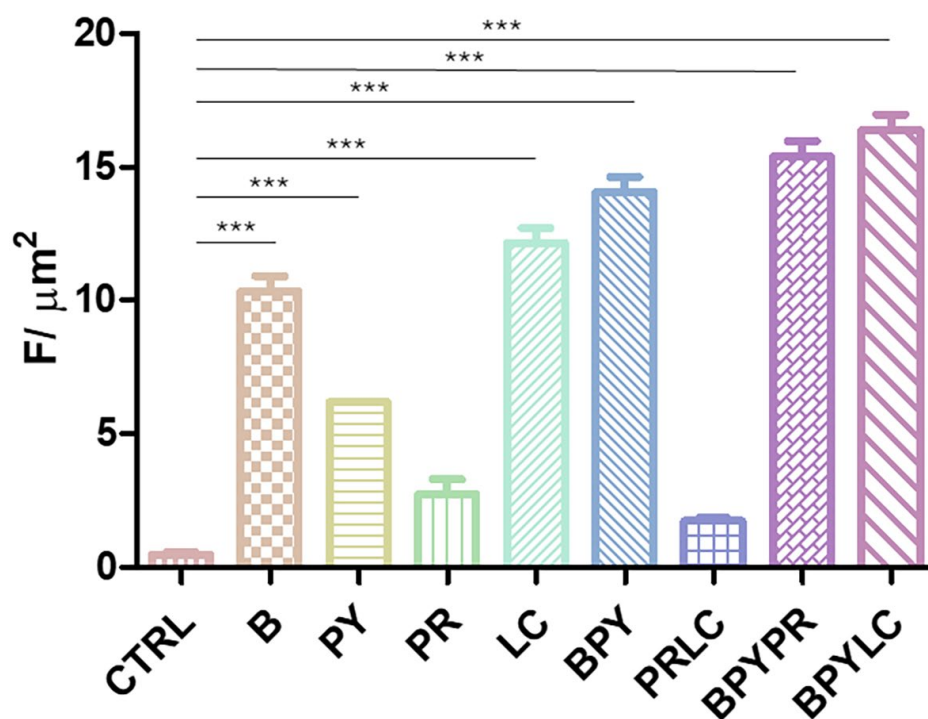


Figure 30: The figure shows the statistical analysis, determined using one-way ANOVA followed by Tukey's multiple comparison test, expresses data as mean $\pm$ standard deviation (SD) from three independent experiments. Significant differences compared to control are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Control cells were cultured with 0,2% of DMSO.

3.4 SENESCENCE PATHWAY, DECREASE OF AVERAGE TELOMERE LENGTH AND VARIATION OF THE MITOCHONDRIAL DNA COPY NUMBER (mtDNA-CN)

3.4.1 hGMSCs

3.4.1.1 IMMUNOFLUORESCENCE, PROTEIN QUANTIFICATION AND RT-PCR

Figures 31-36 illustrate the immunofluorescence staining, quantitative analysis and gene expression levels of the makers p53, p21 and TERT, evaluated through Immunofluorescence, ImageJ software and real-time PCR in hGMSCs cultured without and/or pesticides for 10 days. The immunofluorescence images evidence the upregulation of p53/p21 followed by the downregulation of TERT in treated hGMSCs compared to control hGMSCs. In general, all pesticides caused higher expression of p53 and p21 compared to the control cells. Regarding TERT levels, among the individual pesticides, PY and LC caused the greatest reduction compared with the control cells, as well as with B and PR. Among the pesticide combinations, BPYLC produced the most pronounced decrease. Furthermore, the RT-PCR expression data further corroborated the findings derived from the immunofluorescence analysis.

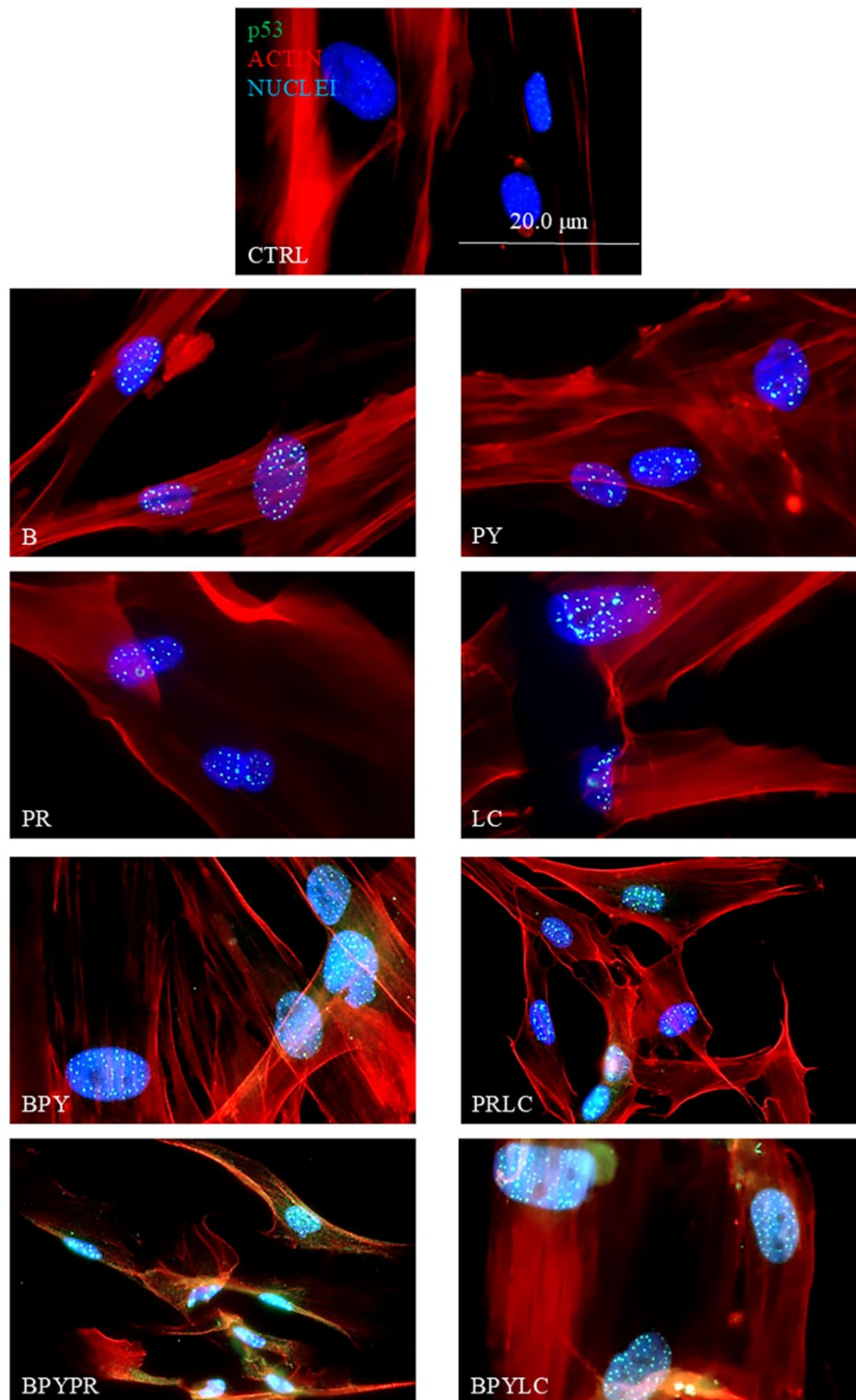


Figure 31: The figure shows Immunofluorescence of the marker p53 in hGMSCs control and in hGMSCs treated with pesticides after 10 days.

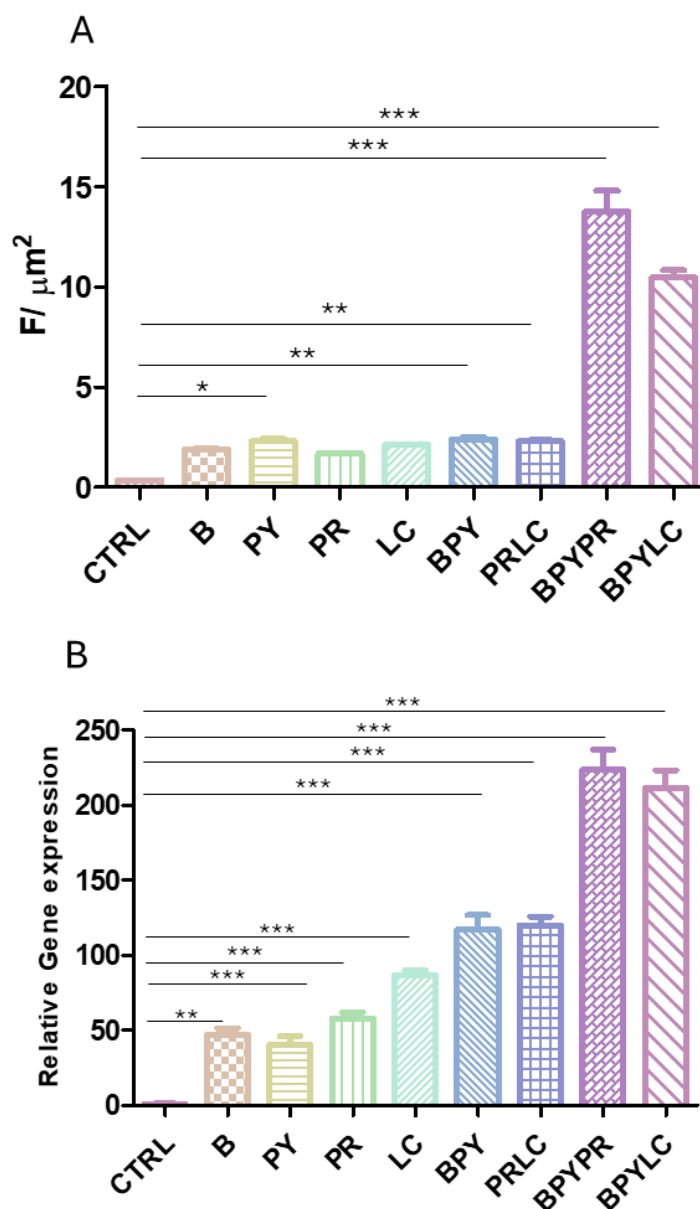


Figure 32: The figure shows Protein Quantification (A) and RT-PCR analysis (B) of the marker p53 in hGMSCs control and in hGMSCs treated with pesticides after 10 days. The bars in the chart show the quantitative assessment of p53 fluorescence (A) and mRNA levels (B), reported as mean $\pm$ standard deviation (SD) from three separate experiments. Differences between control and experimental groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Significant differences compared to control are indicated (** $p < 0.01$, *** $p < 0.001$). Control cells were cultured with 0,2% of DMSO.

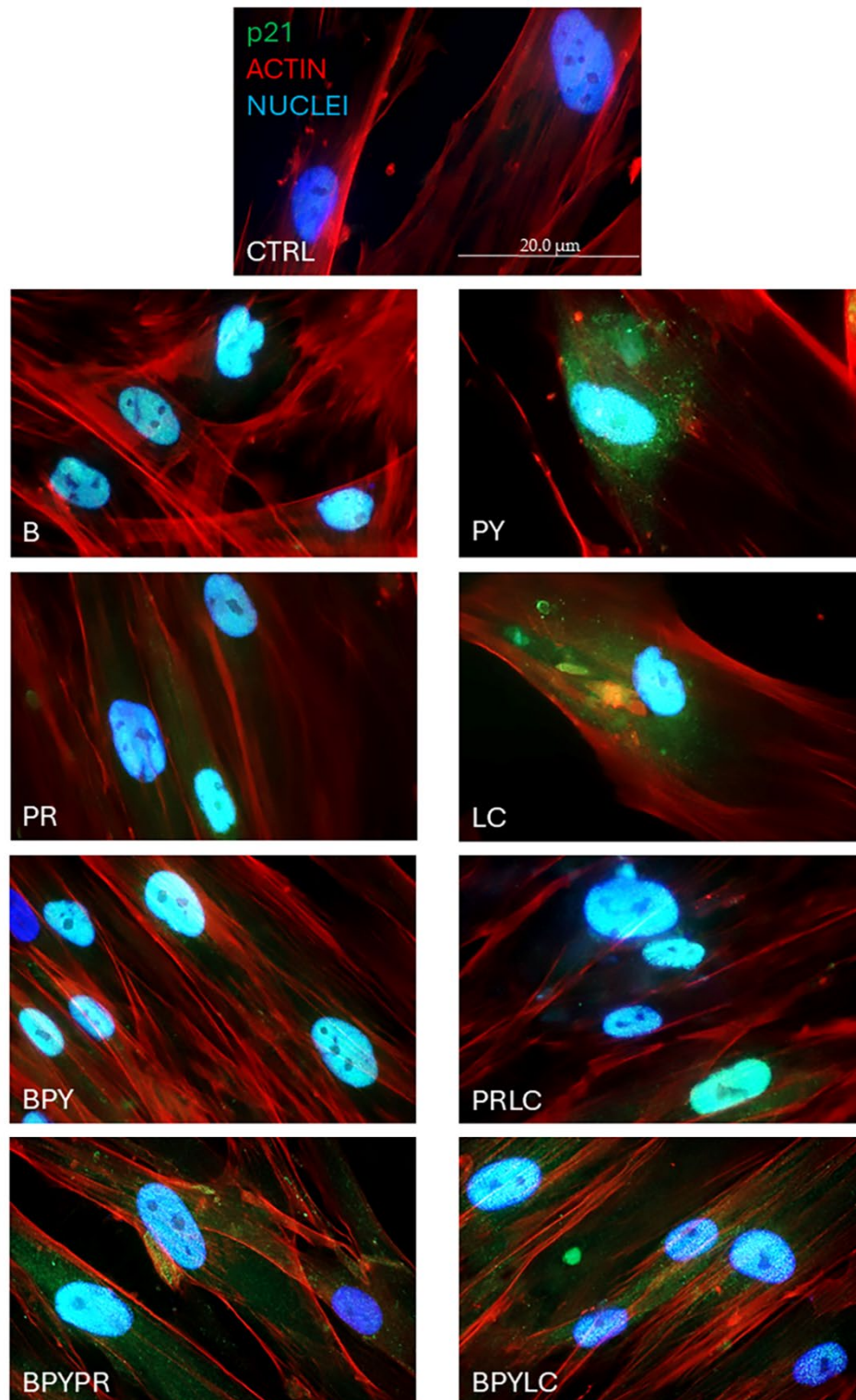


Figure 33: The figure shows Immunofluorescence of the marker p21 in hGMSCs control and in hGMSCs treated with pesticides after 10 days.

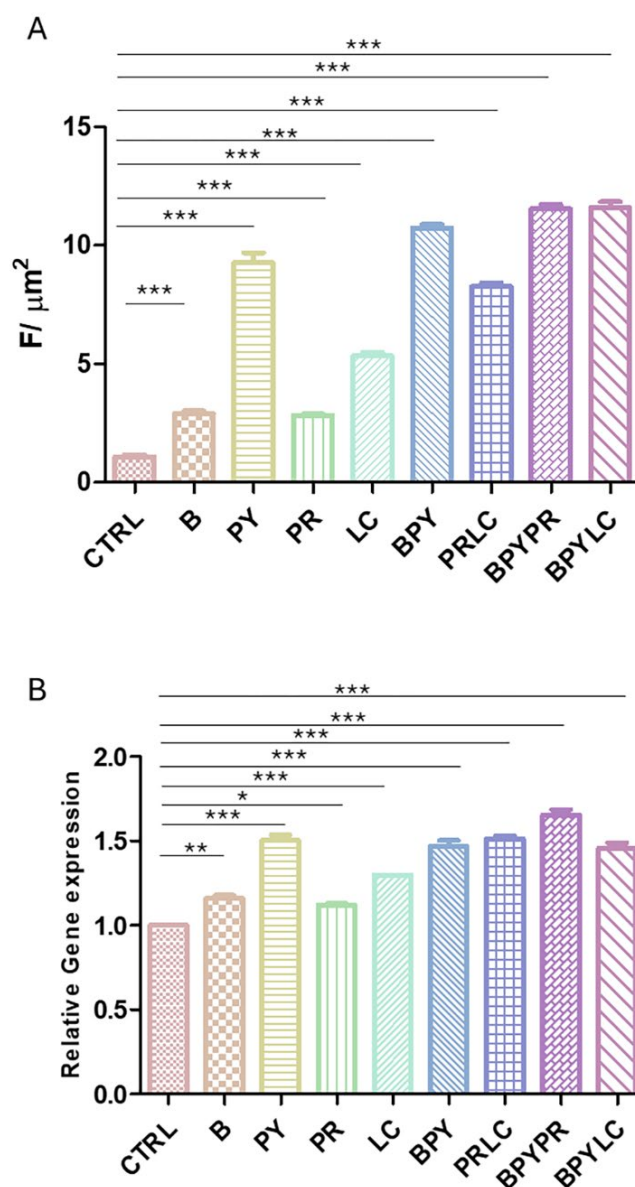


Figure 34: The figure shows Protein Quantification (A) and RT-PCR analysis (B) of the marker p21 in hGMSCs control and in hGMSCs treated with pesticides after 10 days. The bars in the chart show the quantitative assessment of p21 fluorescence (A) and mRNA levels (B), reported as mean $\pm$ standard deviation (SD) from three separate experiments. Differences between control and experimental groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Significant differences compared to control are indicated (** $p < 0.01$, *** $p < 0.001$). Control cells were cultured with 0,2% of DMSO.

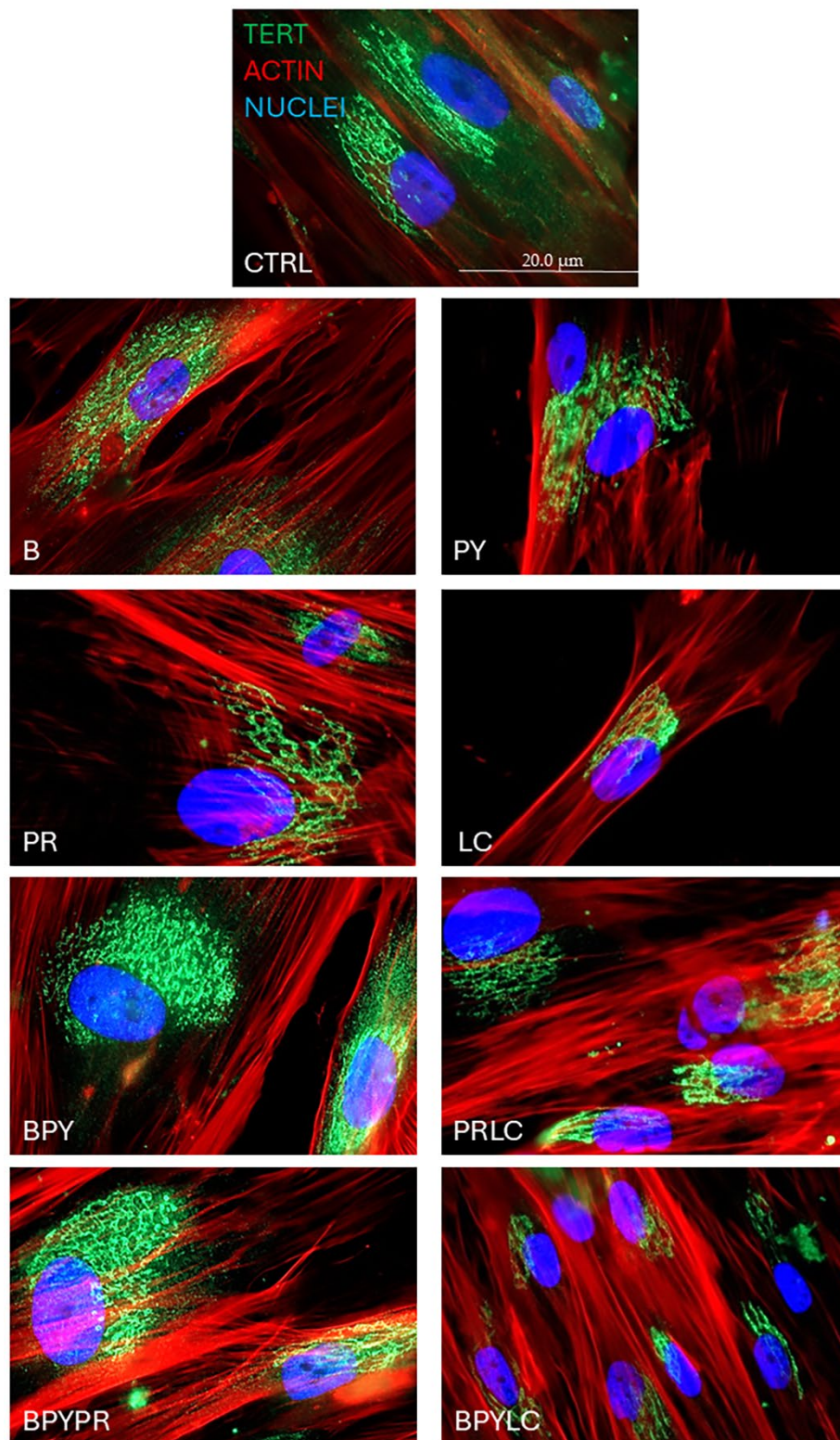


Figure 35: The figure shows Immunofluorescence of the marker TERT in hGMSCs control and in hGMSCs treated with pesticides after 10 days.

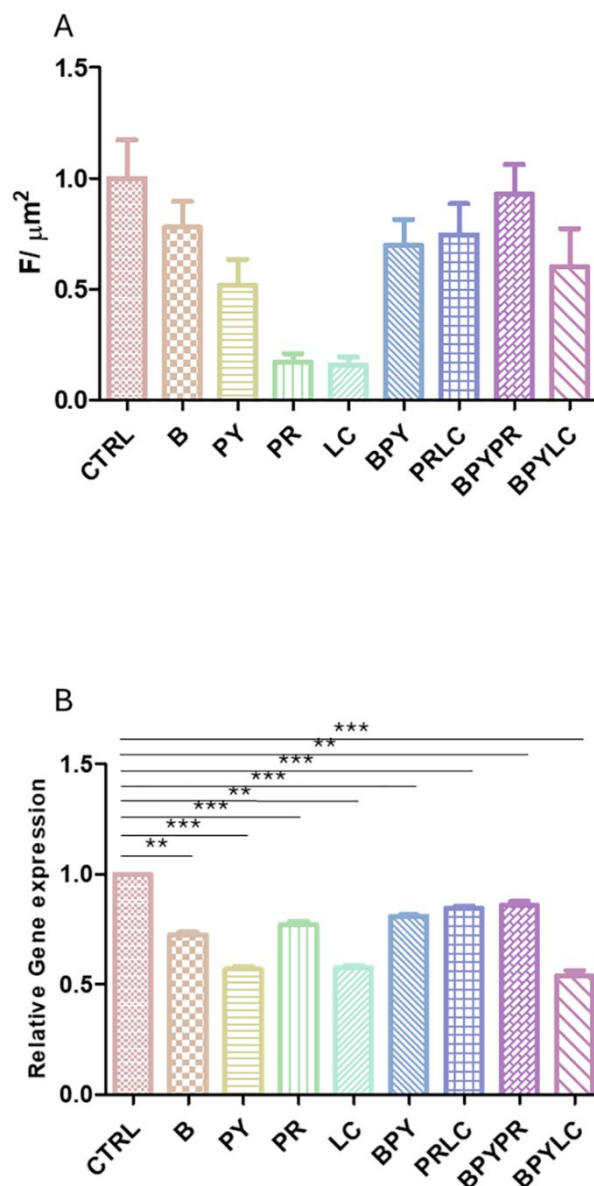


Figure 36: The figure shows Protein Quantification (A) and RT-PCR analysis (B) of the marker TERT in hGMSCs control and in hGMSCs treated with pesticides after 10 days. The bars in the chart show the quantitative assessment of TERT fluorescence (A) and mRNA levels (B), reported as mean $\pm$ standard deviation (SD) from three separate experiments. Differences between control and experimental groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Significant differences compared to control are indicated (** $p < 0.01$, *** $p < 0.001$). Control cells were cultured with 0,2% of DMSO.

3.4.1.2 DECREASE OF AVERAGE TELOMERE LENGTH AND VARIATION OF THE MITOCHONDRIAL DNA COPY NUMBER (mtDNA-CN)

Average TL (Figure 37A) and mtDNA-CN (Figure 37B) were evaluated in the hGMSCs exposed to pesticides compared to control cells. Following statistical analysis, a significantly lower relative TL of the target sample to the reference sample was evidenced in treated hGMSCs compared to the corresponding controls. Among the single pesticides, PY and LC reduce the telomere length, while BPYLC result to be the combination that determines the most significant reduction in telomere length, suggesting a potential correlation between exposure to pesticides and shorter telomere length. In addition, a variation in the mtDNA-CN was further highlighted. More specifically, mtDNA-CN was found to be lower in the hGMSCs treated with B, LC, PY, PR, BPY and PRLC compared to untreated controls, and higher in the hGMSCs treated with BPYPR and BPYLC versus controls.

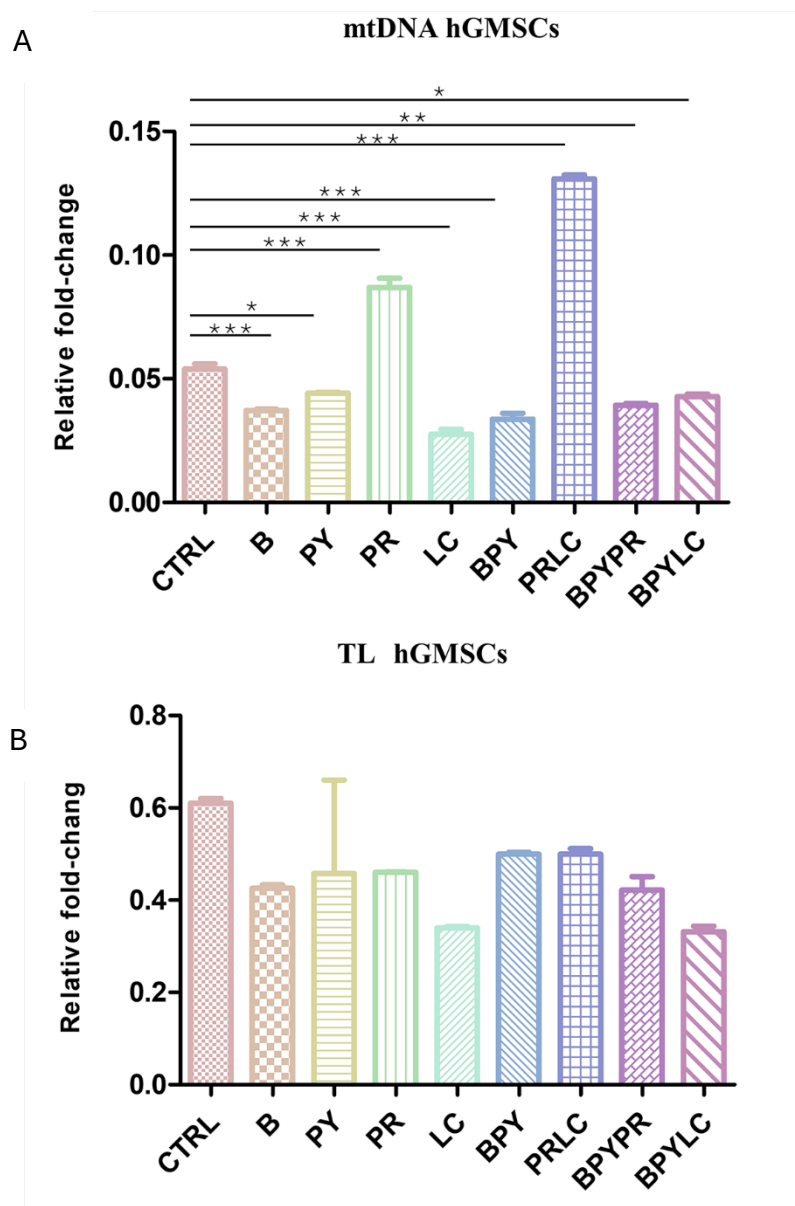


Figure 37: (A) Bar chart with the significant relative fold changes $\pm$ SD indicating the average TL in pesticide-exposed hGMSCs versus the control group after 10 days of treatment. (B) Bar chart with the significant relative fold changes $\pm$ SD evidencing the average mtDNA copy number variation between the hGMSCs exposed to pesticides and the control group after 10 days of treatment. Statistical analysis was performed using the $2^{-\Delta\Delta C_t}$ method (all hypotheses were accepted), considering p-values < 0.05 as significant. The experiment was conducted in triplicate.

3.4.2 HUVECs

3.4.2.1 IMMUNOFLUORESCENCE, PROTEIN QUANTIFICATION AND RT-PCR

Figures 38-43 illustrate the immunofluorescence staining (A), quantitative analysis (B) and gene expression levels (C) of the markers p53/p21/TERT evaluated through Immunofluorescence, ImageJ software and real-time PCR in HUVECs cultured without and/or pesticides for 10 days. The immunofluorescence images evidence the upregulation of p53 and p21, followed by the downregulation of TERT in treated HUVECs compared to control HUVECs. In general, all pesticides caused higher expression of p53 and p21 compared with the control cells. Among all experimental conditions, the BPYPR combination was the strongest inducer of marker expression compared to other pesticides and to CTRL. Moreover, BPYPR was also among the treatments that produced the greatest reduction in TERT levels, together with pesticides B, PR, LC, and the PRLC combination. In addition, the RT-PCR expression data further corroborated the findings derived from the immunofluorescence analysis.

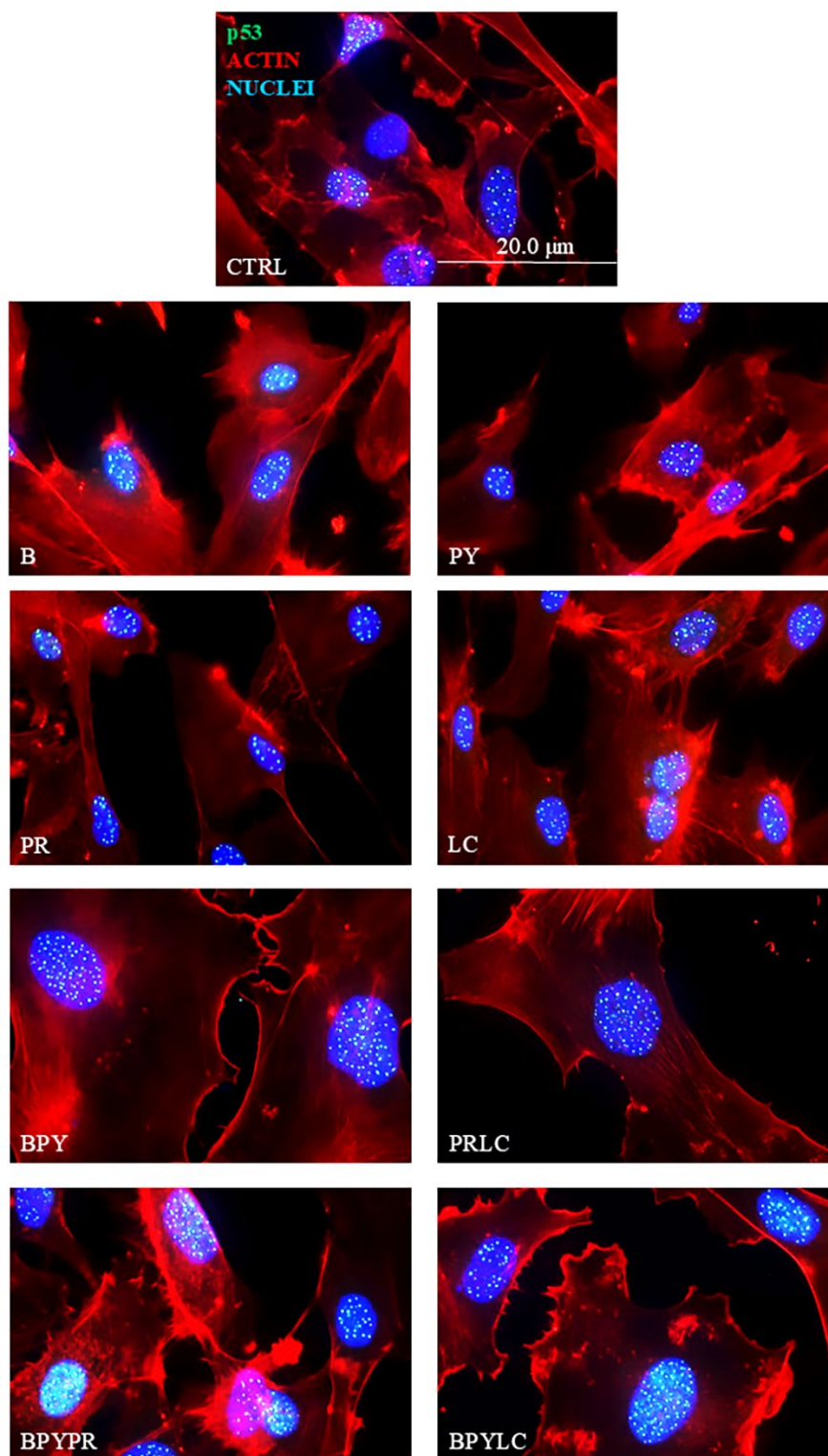


Figure 38: The figure shows Immunofluorescence of the marker p53 in HUVECs control and in HUVECs treated with pesticides after 10 days.

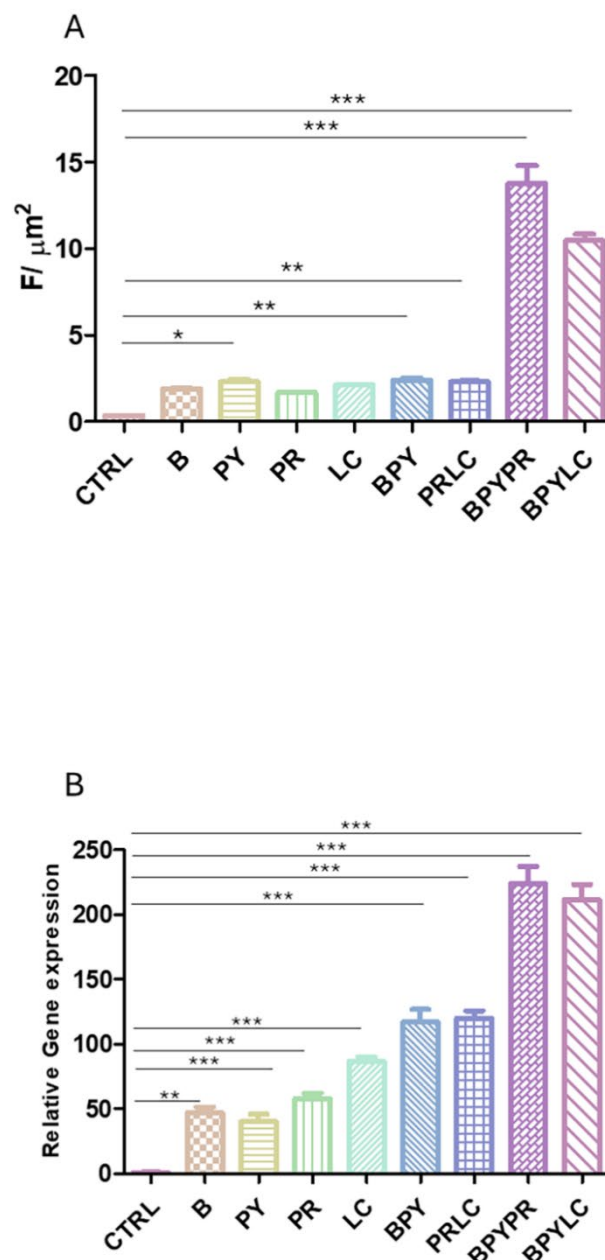


Figure 39: The figure shows Protein Quantification (A) and RT-PCR analysis (B) of the marker p53 in HUVECs control and in HUVECs treated with pesticides after 10 days. The bars in the chart show the quantitative assessment of p53 fluorescence (B) and mRNA levels (C), reported as mean $\pm$ standard deviation (SD) from three separate experiments. Differences between control and experimental groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Significant differences compared to control are indicated (** $p < 0.01$, *** $p < 0.001$). Control cells were cultured with 0,2% of DMSO.

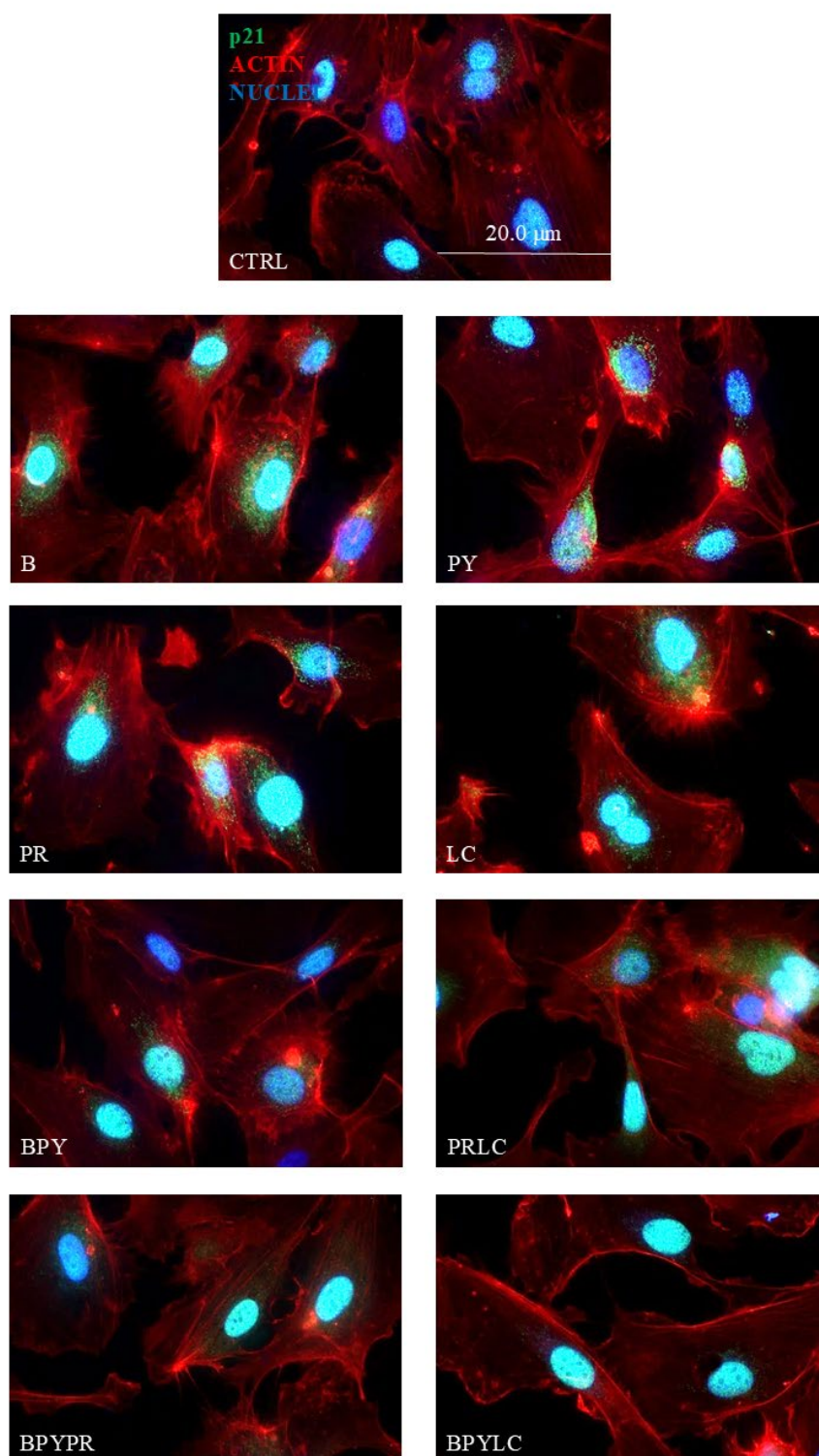


Figure 40: The figure shows Immunofluorescence of the marker p21 in HUVECs control and in HUVECs treated with pesticides after 10 days.

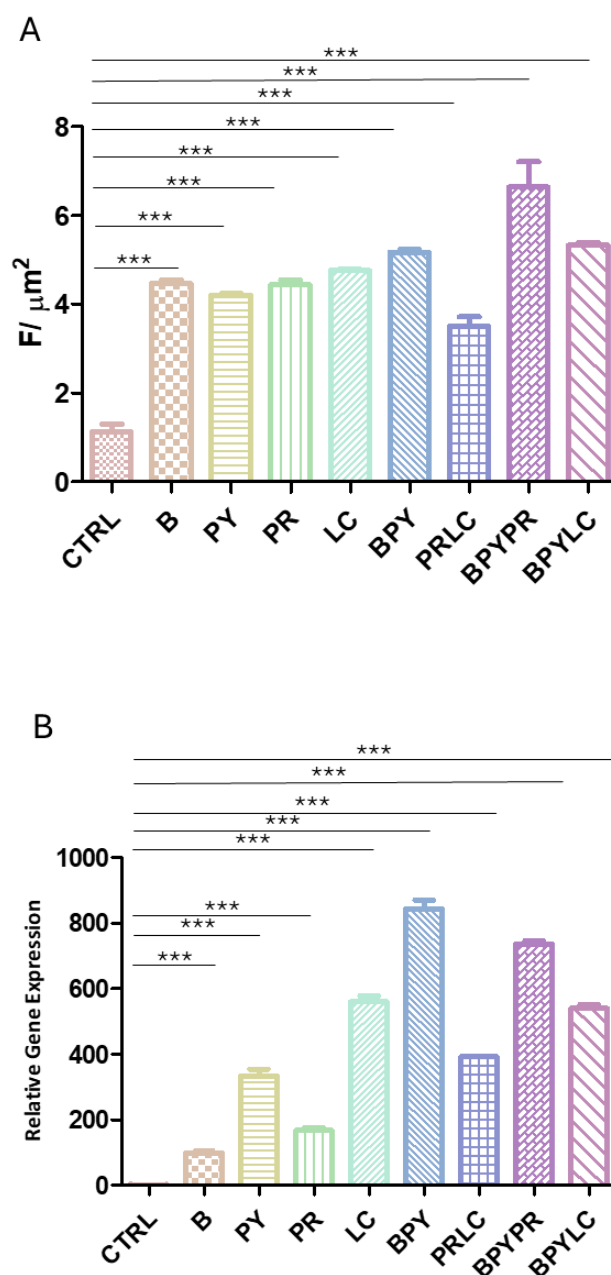


Figure 41: The figure shows Protein Quantification (A) and RT-PCR analysis (B) of the marker p21 in HUVECs control and in HUVECs treated with pesticides after 10 days. The bars in the chart show the quantitative assessment of p21 fluorescence (A) and mRNA levels (B), reported as mean $\pm$ standard deviation (SD) from three separate experiments. Differences between control and experimental groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Significant differences compared to control are indicated (** $p < 0.01$, *** $p < 0.001$). Control cells were cultured with 0,2% of DMSO.

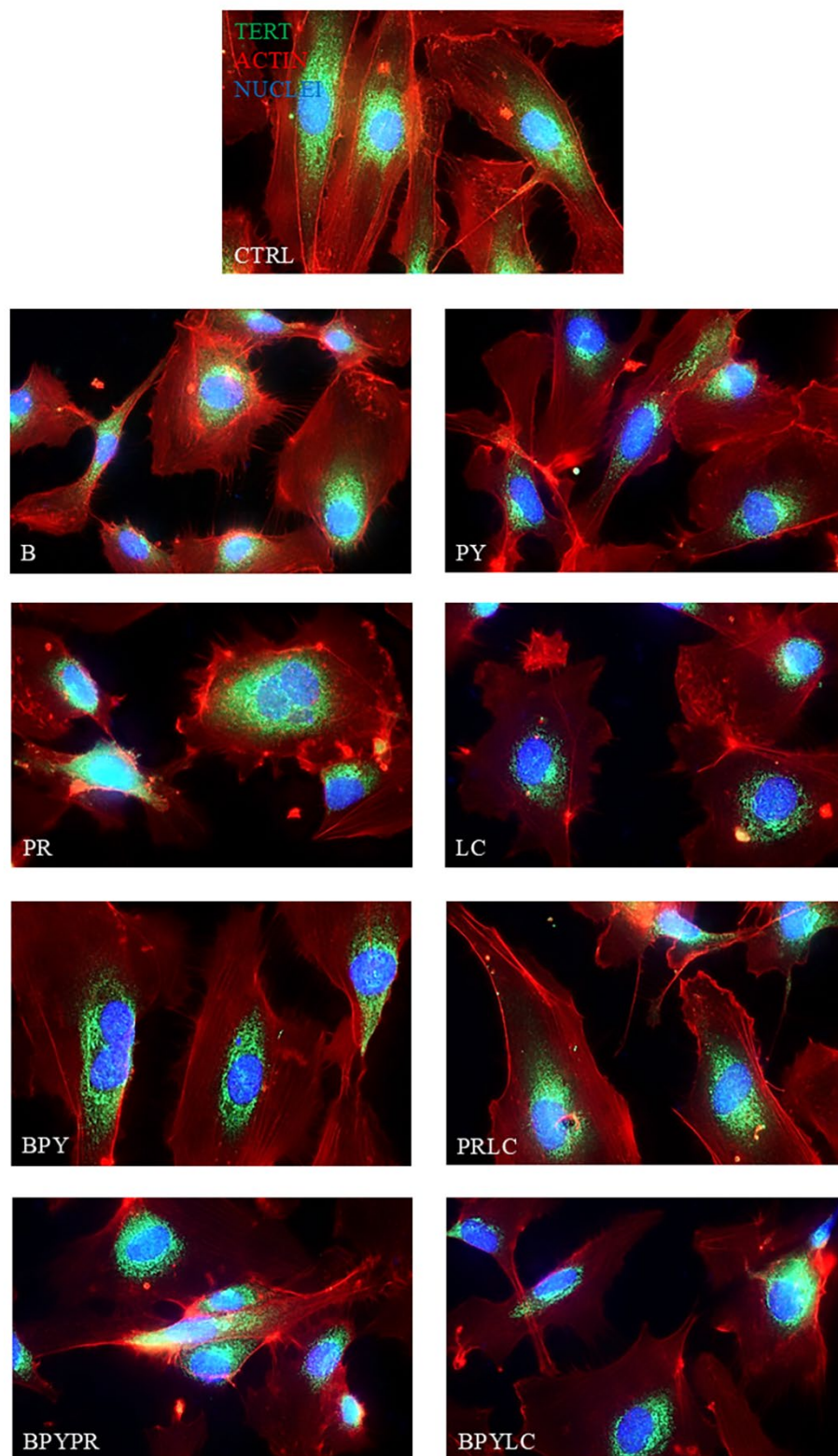


Figure 42: The figure shows Immunofluorescence of the marker TERT in HUVECs control and in HUVECs treated with pesticides after 10 days.

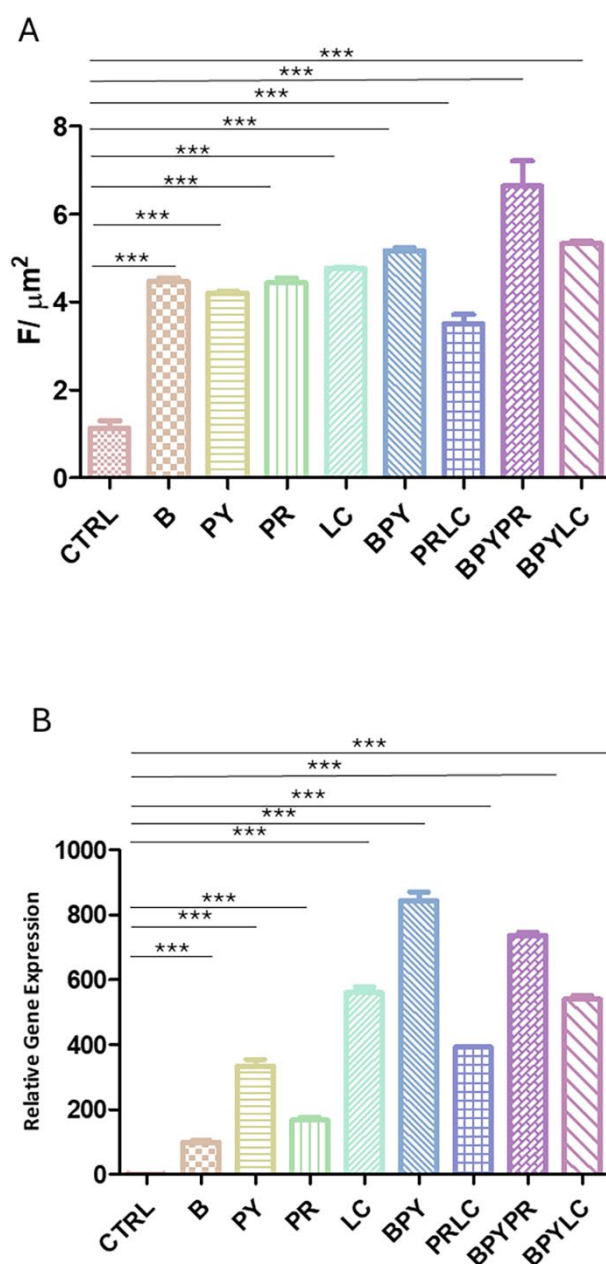


Figure 43: The figure shows Protein Quantification (A) and RT-PCR analysis (B) of the marker TERT in HUVECs control and in HUVECs treated with pesticides after 10 days. The bars in the chart show the quantitative assessment of TERT fluorescence (A) and mRNA levels (B), reported as mean $\pm$ standard deviation (SD) from three separate experiments. Differences between control and experimental groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Significant differences compared to control are indicated (** $p < 0.01$, *** $p < 0.001$). Control cells were cultured with 0,2% of DMSO.

3.4.2.2 DECREASE OF AVERAGE TELOMERE LENGTH AND VARIATION OF THE MITOCHONDRIAL DNA COPY NUMBER (mtDNA-CN)

Average TL (Figure 44A) and mtDNA-CN (Figure 44B) were evaluated in the HUVECs exposed to pesticides compared to non-exposed controls. Following statistical analysis, a significantly lower relative TL of the target sample to the reference sample was evidenced in the pesticide-exposed HUVECs compared to the corresponding control, suggesting a potential correlation between exposure to pesticides and shorter telomere length. In addition, a variation in the mtDNA-CN was further highlighted. More specifically, mtDNA-CN was found to be lower in the HUVECs treated with B, LC, PY, PR, BPY and PRLC compared to untreated controls, and higher in the HUVECs treated with BPYPR and BPYLC versus control.

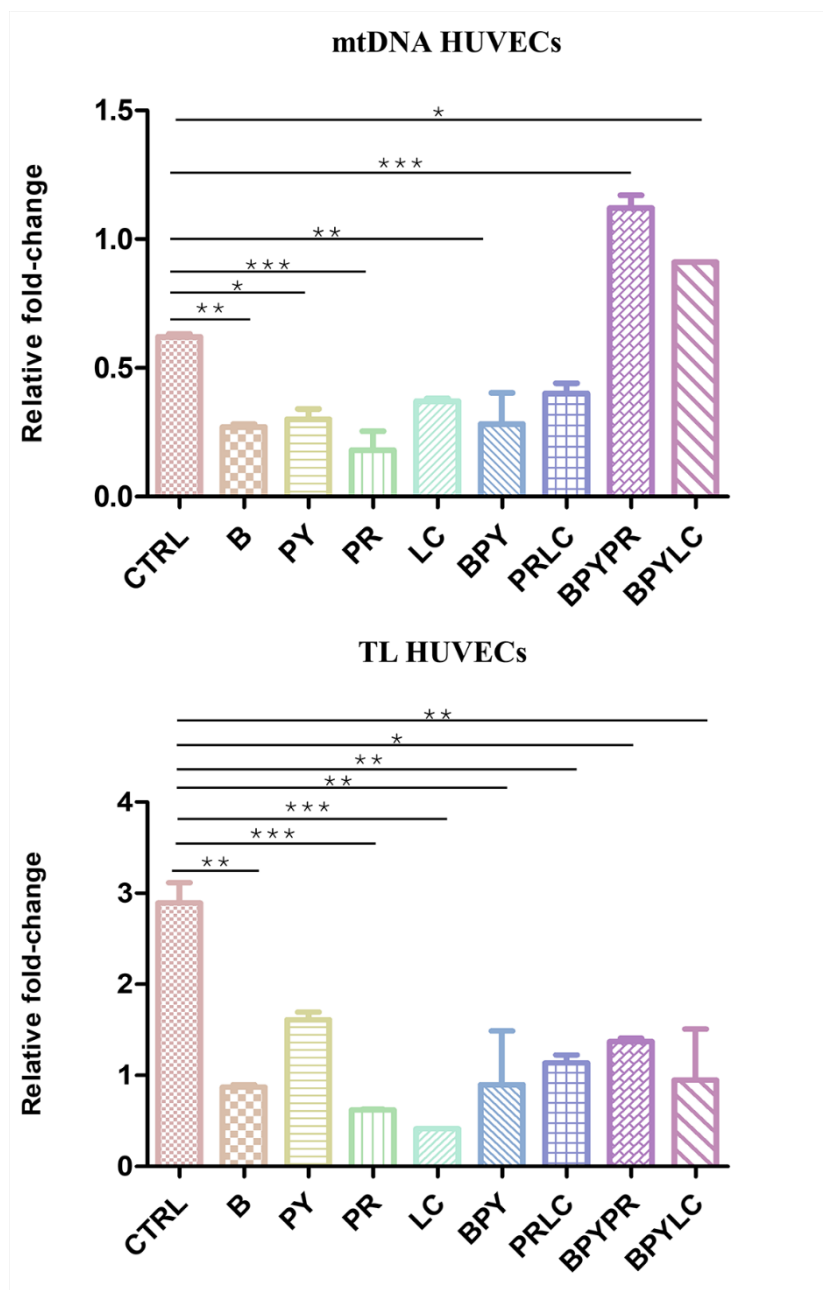


Figure 44: (A) Bar chart with the significant relative fold changes $\pm$ SD indicating the average TL in pesticide-exposed HUVECs versus the control group after 10 days of treatment. (B) Bar chart with the significant relative fold changes $\pm$ SD evidencing the average mtDNA copy number variation between the HUVECs exposed to pesticides and the control group after 10 days of treatment. Statistical analysis was performed using the $2^{-\Delta\Delta C_t}$ method (all hypotheses were accepted), considering p-values < 0.05 as significant. The experiment was conducted in triplicate.

3.5 SUMMARY SCHEMES OF BIOLOGICAL OUTCOMES

3.5.1 SUMMARY SCHEME FOR PESTICIDES-EXPOSED hGMSC

Level of Analysis	Biological Event / Marker	Key Findings	Main Drivers
1. Oxidative Stress	ROS Production	Massive induction of oxidative stress; identified as the primary trigger for cellular damage	LC (single), BPYPR/BPYLC (max)
2. Inflammation	Inflammasome (NLRP3, CASP1, IL-1β)	Significant upregulation of inflammasome components (confirmed via IF and RT-PCR)	B (single), BPYPR/BPYLC (synergy)
3. Cell Cycle Arrest	p53/p21 Axis	Activation of the DNA Damage Response (DDR), acting as molecular "brakes" on the cell cycle	All pesticides vs. Control
4. Genomic Integrity	TERT & Telomere Length (TL)	Potent inhibition of telomerase activity leading to significant telomere shortening	PY/LC (single), BPYLC (max reduction)
5. Organelle Function	mtDNA-CN	Disruption of mitochondrial DNA homeostasis (depletion vs. reactive biogenesis).	B/LC/PY/PR (reduction in copies), BPYLC (increase in copies)

6. Final Phenotype	SEM & MTS (10 days)	Transition to a senescent-like morphology and long-term metabolic exhaustion.	BPYPR and BPYLC (severe damage)
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Table 1: The table summarizes the biological outcomes of the hGMSCs after pesticides exposure of 10 days.

3.5.2 SUMMARY SCHEME FOR PESTICIDES-EXPOSED HUVECs

Level of Analysis	Biological Event / Marker	Key Findings	Main Drivers
1. Oxidative Stress	ROS Production	High ROS levels; evidence of a loop phenomenon between ROS and inflammasome.	B (single), BPYPR/BPYLC (max)
2. Inflammation	Inflammasome (NLRP3, CASP1, IL-1 β)	Strong upregulation at both protein (IF) and mRNA (RT-PCR) levels.	B/LC (single), BPYPR/BPYLC (max)
3. Cell Cycle Arrest	p53 and p21 Axis	Clear upregulation indicating stress-induced growth arrest.	BPYPR (strongest inducer)
4. Genomic Integrity	TERT & Telomere Length (TL)	Significant downregulation of TERT and marked telomere shortening.	B/PR/LC/PRLC and BPYPR
5. Organelle Function	mtDNA-CN	Depletion in single/double treatments; reactive increase in triple mixtures.	BPYPR/BPYLC (upregulation of copies)
6. Final Phenotype	SEM & MTS (10 days)	Loss of membrane blebbing ; transition from early hyper-metabolism to chronic collapse.	BPYPR and BPYLC (severe damage)

Table 2: The table summarizes the biological outcomes of the HUVECs after pesticides exposure of 10 days.

3.5.3 SUMMARY SCHEME: hGMSCs vs HUVECs

Level of Analysis	hGMSCs (Stem Cells)	HUVECs (Endothelial Cells)	Comparative Insight
1. Oxidative Stress	Massive ROS induction (Primary Trigger).	High ROS with a loop phenomenon involving inflammation.	HUVECs show a more complex, self-sustaining cycle of damage.
2. Inflammation	Standard upregulation of NLRP3/CASP1.	Stronger response at both mRNA and protein levels.	Endothelial cells (HUVECs) are more reactive/sensitive to inflammation.
3. Cell Cycle	DNA Damage Response (DDR) activation.	Stress-induced growth arrest.	BPYPR is the dominant driver of arrest in the vascular model.
4. Genome Integrity	Severe TERT inhibition.	Significant shortening & downregulation.	Both lines show "accelerated aging," but drivers vary slightly.
5. Mitochondria	Homeostatic disruption (Depletion).	Reactive biogenesis (Increase) in triple mixtures.	HUVECs show a "last-stand" response (increasing copies) before collapse.
6. Phenotype	Loss of fibroblast-like shape; exhaustion.	Transition from hyper-metabolism to chronic collapse.	HUVECs show a more dynamic metabolic "crash."

Table 3: Summary scheme of the the biological outcomes of hGMSCs compared to HUVECs exposed to pesticides for 10 days.

4 DISCUSSION

The present study aimed to evaluate the molecular and biological effects of pesticide exposure on inflammatory and senescence pathways using an *in vitro* model of hGMSCs and HUVECs. This dual-model approach allowed for a comparative analysis between undifferentiated progenitor cells and terminally differentiated functional cells, addressing two distinct biological targets. As multipotent stem cells, hGMSCs serve as a model for regenerative versatility and long-term repair; investigating this line is crucial for identifying how pesticides may impair stemness maintenance and lineage commitment. Damage at this level suggests a significant risk to the body's future capacity for tissue regeneration and homeostatic balance. Conversely, HUVECs represent the vascular endothelium, which is the primary physiological barrier and the first point of contact for xenobiotics once they enter systemic circulation. By using a differentiated model, we were able to evaluate immediate functional impairment, such as inflammatory response, oxidative stress, and the disruption of endothelial signaling. By integrating these two models, this study moves beyond simple cytotoxicity to demonstrate a systemic biological impact. It proves that pesticide exposure does not simply induce localized damage, but simultaneously compromises both the cellular pool responsible for future healing and the vascular infrastructure essential for current physiological health. This approach provides a more robust validation, ensuring that the observed biological outcomes reflect a generalized systemic risk rather than a cell-specific artifact. Cell viability was investigated by the MTS assay, while morphological alterations were examined by SEM. Activation of the NLRP3 inflammasome pathway was investigated through immunofluorescence, protein quantification, and relative gene expression analyses of NALP3, CASP1 and IL-1 β . In parallel, oxidative stress was evaluated by measuring reactive oxygen species (ROS) production. On the other hand, cellular senescence was assessed through immunofluorescence, protein quantification, and relative gene expression of p53, p21 and TERT, together with the evaluation of telomere length and mitochondrial DNA copy number (mtDNA-CN).

4.1 hGMSCs

Although pesticide exposure is known to alter epithelial cells and the oral microbiota, thereby disrupting oral homeostasis, limited information is available regarding the direct effects of pesticides on oral-derived cells (Salazar-Flores et al., 2022b).

Particularly interesting is the impact of pesticides on the oral cavity, a complex ecosystem, which represents the first physiological line of defense of the human body. Pesticide exposure induces damage in salivary glands, alters oral microbiota, disrupting the homeostasis of the oral environment, favoring pathologies such as caries, periodontal diseases, oral cancer, and odontogenic infection. Moreover, due to the high vascularization of the sublingual and oral mucosa, exposure via oral ingestion may result in systemic absorption since the lipophilic nature of the pesticides (Salazar-Flores et al., 2022a). While pesticide contact is recognized to alter the oral microbiota and the oral mucosal of the epithelial cells by increasing the vulnerability of adherens junctions in the gingival sulcus wall through several systems disrupting the homeostasis of the oral environment, the impact of pesticides on oral cells is still largely unexplored (Salazar-Flores et al., 2022a; Şenel, 2021). Cell junction alteration, allowing passive diffusion of pesticides, can be responsible for morphological modification to the lamina propria, an histological structure with functional significance role for keeping cell populations and for the stem cells that represent the founding core of tissue regeneration (Şenel, 2021).

Gingival mesenchymal stem cells, isolated from gingival tissue, represent an excellent MSC source for regenerative medicine due to their easy accessibility, minimally invasive isolation procedures, high proliferative capacity, and genomic stability during long-term in vitro expansion, making them reliable for large-scale studies and clinical applications. In addition, they showed immunomodulatory and anti-inflammatory features in response to damage caused by toxic substances. Thus, they represent an optimum model for investigating pesticide-induced damage as such exposure may compromise their protective and regenerative functions (Y. Della Rocca et al., 2025; Fonticoli et al., 2022).

Additionally, the high vascularization of the oral and sublingual mucosa allows systemic absorption of pesticides following oral exposure, particularly considering their lipophilic nature. This further supports the relevance of hGMSCs as a model to

study early cellular responses to pesticide exposure (Flannigan et al., 1985; Goswami, Li, & Jasti, 2017; Magomedov, Zeynalov, Suleymanov, Tataeva, & Magomedova, 2022; Mrema et al., 2013).

MTS analysis revealed time and treatment-dependent effects on hGMSC metabolic activity. After 24 h, PY and PR induced the most pronounced increase in metabolic activity, whereas the BPYLC combination resulted in a significant decrease. At 72 h, PY continued to enhance metabolic activity, while B showed the lowest effect compared to the single pesticides. Interestingly, the metabolic activity induced by the BPYLC combination increased over time, suggesting a time-dependent adaptive or stress-related response. After 10 days of exposure, B induced the highest metabolic activity compared to control cells, while LC alone and the BPYLC combination resulted in the lowest values, indicating potential cytotoxic or senescence-related effects following prolonged exposure.

SEM analysis demonstrated marked morphological alterations in pesticide-treated hGMSCs. Control cells displayed a typical fusiform morphology with elongated cytoplasmic extensions and filopodia interacting with neighbouring cells (Mammana et al., 2019). The alteration of these features resulted particularly in hGMSCs treated with a mixture of pesticides compared to hGMSCs treated with single pesticides and control cells, suggesting the synergic effects of pesticides.

Immunofluorescence analysis showed that hGMSCs treated with pesticides for 10 days increased the expression of inflammatory mediators NALP3, CASP1 and IL-1 β when compared to cells cultured alone. Notably, among single pesticides, B induced the highest inflammatory response. Regarding the combination, BPYPR and BPYLC demonstrated to be the most potent combination when compared to the other pesticides and control cells. Quantitative ROI analysis and gene expression data corroborated these findings; in fact, levels of mRNA were higher in treated cells compared to control hGMSCs, highlighting the synergistic pro-inflammatory effects of pesticide mixtures.

Consistency, ROS production was evaluated to further confirm the activation of inflammation in treated hGMSCs compared to the control cells. In fact, our results evidenced that ROS production was higher in cells treated with pesticides compared to CTRL. In detail, LC was found to be the most inflammatory pesticide among the

single pesticides. The highest ROS production can be observed with the combinations BPYPR and BPYLC when compared to the other experimental points and with control cells. The observed results suggest that pesticide exposure may trigger the activation of the inflammatory pathway, which, when combined, highlights their synergic effect.

The immunofluorescence results regarding the senescence showed that exposure to pesticides determined an increase in the expression of the markers p53 and p21 compared to non-exposed cells; in parallel, the expression of the TERT was significantly decreased in treated cells when compared to untreated cells. In particular, the combination BPYPR and BPYLC led to the highest expression of p53/p21, in parallel with the lowest expression of TERT, compared to the other pesticides and control hGMSCs. Real-Time PCR analysis supports the findings achieved by Immunofluorescence; in fact, also regarding the relative gene expression, the combinations of BPYPR and BPYLC still led to the highest upregulation in the senescence genes when compared to the other experimental points.

In parallel, the reduction of telomere length was investigated: all pesticide treatments resulted in significantly shorter telomeres compared to control cells. Among the single pesticides, PY and LC determined the shortest telomere length, while among the combinations, BPYPR and BPYLC led to the greatest decrease in the telomere length. Reduction in telomeres indicates an activation of cellular senescence and dysfunction, since telomere is a marker widely used for cellular aging (Sanders & Newman, 2013). In addition, mtDNA copy number variation was employed. The results highlighted that the pesticide PR alone and in combination with LC determined a significant increase in mtDNA levels compared to CTRL and to other pesticides, while the other experimental points caused a significant decrease. Decrease of mtDNA-CN can affect mitochondrial function, as in the case of extensive oxidative stress, which exceeds the mitochondrial capacity to make up for oxidative damage and reduce mtDNA content. An increase, on the other hand, may aim to compensate for mtDNA damage and mitochondrial dysfunction (Liu et al., 2003; Y. C. Wang et al., 2011).

4.2 HUVECs

Pesticides are well known to induce oxidative stress, telomere shortening, and various health issues, including endothelial dysfunction (Liu et al., 2003). Endothelial tissue lines the blood vessels and is a key regulator of vascular homeostasis, including nitric oxide production, vascular permeability, and blood flow control (Rubanyi, 1993). These functions can be compromised during aging processes, which directly contribute to vascular dysfunction observed in aging and cardiovascular diseases (Hwang, Kim, Herman, Gorospe, & Lee, 2022). HUVECs are derived from the human umbilical vein and represent a robust and widely used *in vitro* model for endothelial tissue due to their preserved functional and molecular characteristics. They closely mimic adult vascular endothelial properties, including the expression of specific markers such as von Willebrand factor, PECAM-1, and vascular endothelial cadherin (McCloskey et al., 2022; Müller et al., 2002). Furthermore, their ability to respond to oxidative stress, inflammatory stimuli, and changes in vascular permeability make them particularly suitable for studying the cellular responses to pesticide exposure (Cao et al., 2017).

MTS analysis showed that after 24 h of exposure, treated cells exhibited an increased metabolic activity compared to the control. By 72 h, cellular metabolic activity began to decrease in treated cells when compared to the control, being more evident after 10 days of treatment in all treated groups, particularly in HUVECs exposed to the BPYPR and BPYLC combinations. Based on these findings, subsequent assays were performed using a 10-day pesticide exposure protocol. Our results are consistent with previous reports demonstrating that organochlorine pesticides induce reactive oxygen species (ROS) generation, upregulate NADPH-oxidase, reduce nitric oxide (NO) bioavailability, and lead to endothelial dysfunction. (Ghosh et al., 2017).

SEM analysis revealed alterations in pesticide-treated HUVECs, including the loss of physiological morphology and the presence of membrane blebbing, further supporting the occurrence of pesticide-induced cellular stress and dysfunction. Moreover, changes in the cellular blebbing have been observed between control and treated cells that showed a loss in that cellular phenomenon observed in the combinations PRLC, BPYPR, and BPYLC. Such alterations may significantly affect communication between the endothelium and the other tissues. In fact, being the “endothelium”, the

single cell-lining tissue formed by the endothelial cells in the blood and lymphatic vessels, it plays a critical role in regulating the exchange of oxygen and nutrients between the vessel contents and underlying tissues.

Regarding the inflammasome activation, the biological outcomes showed the upregulation of the mRNA of the relative gene expressions of the markers NALP3, CASP1 and IL-1 β in treated HUVECs compared to control cells. Among the single pesticides, B and LC were the most inflammatory compared to control cells; on the other hand, all the combined treatments showed an increase in the gene levels when compared to the other experimental points. In particular, the combination of the three pesticides BPYPR and BPYLC revealed the highest mRNA levels compared to the other experimental points. Fluorescence microscopy revealed that the presence of NALP3, CASP1, and IL-1 β proteins was significantly increased in HUVECs with pesticides when compared to untreated HUVECs, following the results obtained from the RT-PCR; in fact, the combined pesticide treatment still elicited the greatest increase in their expression. Quantitative analysis was obtained through ROI measurements. All these findings suggest the additive or synergistic effects induced by the pesticide mixture.

In parallel, ROS production, assessed by the DCFH-DA probe, detected an increased fluorescence signal in pesticide-treated HUVECs compared to controls, indicating heightened oxidative stress. The results followed the same trend as the immunofluorescence data, reinforcing the link between pesticide exposure and cellular stress responses. However, it is noteworthy that the PRLC combination appeared to induce a relatively lower oxidative stress response compared to other pesticide combinations. This finding is in line with the previous results discussed about the hGMSCs' ROS production.

The immunofluorescence results regarding the senescence pathway showed an increase in p53 and p21 expression in HUVECs exposed to pesticides compared to control cells; on the other hand, a reduction in the TERT marker was observed in treated cells when compared to untreated cells. Those results suggest the correlation between upregulation of p53/p21 markers and the downregulation of TERT expression, suggesting that pesticide exposure may trigger the activation of the senescence pathway in HUVECs. Real-Time PCR analysis supports the findings

achieved by Immunofluorescence; in fact, HUVECs treated with combinations of BPYPR and BPYLC led to the highest upregulation in the senescence genes when compared to control cells.

In parallel, the investigation of the telomere reduction revealed that all pesticide treatments resulted in significantly shorter telomeres compared to control cells, suggesting pesticide exposure accelerates telomere attrition in endothelial cells. Telomere length is a crucial marker of cellular aging and replicative potential; thus, shorter telomeres indicate an increase in cellular senescence and dysfunction. (Sanders & Newman, 2013).

Dysfunction of the mitochondrial network was evaluated through analysis of mtDNA copy number. Figure 33 revealed that the combinations BPYPR and BPYLC led to a significant increase in mtDNA levels compared to CTRL, while the other pesticides caused a significant decrease. Decrease of mtDNA-CN can affect mitochondrial function, as in the case of extensive oxidative stress, which exceeds the mitochondrial capacity to make up for oxidative damage and reduce mtDNA content. An increase, on the other hand, may aim to compensate for mtDNA damage and mitochondrial dysfunction (Liu et al., 2003; Y. C. Wang et al., 2011).

5 CONCLUSION

Despite the inherent limitations of *in vitro* models, this study demonstrates that pesticide exposure activates inflammasome and senescence pathways in both hGMSCs and HUVECs, showing distinct cellular behaviors. Indeed, our findings reveal that the response to these toxins is strictly cell-specific: while hGMSCs experience a 'silent' impairment of their regenerative potential—marked by TERT inhibition and metabolic exhaustion—HUVECs exhibit a more aggressive, acute collapse. Pesticides mixtures consistently exerted stronger effects than individual compounds, supporting the concept of a synergistic “cocktail effect”. The findings highlight a close interplay between oxidative stress, inflammation, and cellular senescence.

Understanding the molecular crosstalk between these pathways may contribute to the development of preventive and therapeutic strategies targeting age-related diseases and improve regenerative medicine approaches. Future studies will aim to validate these findings *in vivo* and to investigate whether antioxidant or protective molecules can mitigate or reverse pesticide-induced cellular damage.

Overall, this thesis provides novel insights into the cellular mechanisms linking environmental toxins to stem cell dysfunction and vascular pathology, offering a foundation for future translational research.

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