Bachelor's Degree Final Project

Biochemistry and Molecular Biology

ANALYSIS OF THE ANTI-INFLAMMATORY POTENTIAL OF EXTRACELLULAR VESICLES DERIVED FROM MESENCHYMAL STEM CELLS OF SUBCUTANEOUS ADIPOSE TISSUE

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This project is **confidential** and is based on the results obtained in the external practicals carried out in the Inflammatory Bowel Disease research group (IBODI) belonging to the Institut d'Investigació Sanitària Pere Virgili (IISPV), at the Hospital Universitari Joan XXIII in Tarragona (Spain), under the supervision and guidance of Dra. Carolina Serena Perelló and Diandra Monfort Ferré.

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Abbreviations

ABBREVIATION	NAME
EVs	Extracellular vesicles
MSCs	Mesenchymal stem cells
ASCs	Adipose-derived mesenchymal stem cells
SAT	Subcutaneous adipose tissue
CF	Creeping fat
BM-MSCs	Bone marrow-derived mesenchymal stem cells
SVF	Stromal vascular fraction
VEGF	Vascular endothelial growth factor
IBD	Inflammatory bowel disease
CD	Crohn's Disease
PBS	Phosphate-buffered saline
CM	Conditioned medium
qPCR	Quantitative polymerase chain reaction
THP-1	Human monocytic leukemia cell line
BSA	Bovine serum albumin
CRP	C-reactive protein
BMI	Body mass index
LPS	Lipopolysaccharide
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IL10	Interleukin 10
CD163	Cluster of differentiation 163
MRC1	Mannose receptor C-Type 1
FUT4	Fucosyltransferase 4
MPO	Myeloperoxidase
HLA	Human leukocyte antigen
CD11B	Cluster of differentiation 11 β
CD66B	Cluster of differentiation 66 β
TNF	Tumor necrosis factor
IL6	Interleukin 6
IL1B	Interleukin 1β
BAX	Bcl-2-associated X protein
BCL2	B-Cell lymphoma 2

Abstract

Extracellular vesicles (EVs) derived from mesenchymal stem cells (MSCs) from subcutaneous adipose tissue (SAT) offer a promising anti-inflammatory therapy, particularly for inflammatory bowel diseases like Crohn's disease (CD). These EVs, enriched with proteins, lipids, and RNA, possess immunomodulatory and reparative properties. In this study, EVs were isolated from healthy donors and their effects were investigated in creeping fat and intestinal mucosa samples from CD patients.

The results demonstrated that EVs significantly increased the expression of macrophage-specific anti-inflammatory markers (M2) such as *IL10, CD163* and *MRC1* in creeping fat. Similar trends were observed in the intestinal mucosa, although the changes did not reach statistical significance in this tissue. In contrast, when applied to THP-1 cells polarised towards an inflammatory phenotype (M1), EVs did not fully reverse the inflammation to an anti-inflammatory phenotype (M2), likely due to limited cellular uptake in this tumour-derived cell line. Additionally, a sustained anti-inflammatory effect of EVs was observed in creeping fat, with a reduction in pro-inflammatory markers and an increase in anti-inflammatory markers persisting for 72 hours post-treatment.

In conclusion, EVs have promising potential for anti-inflammatory therapies, although further studies are needed to optimise their efficacy and fully understand their mechanisms.

1. Introduction

1.1 Mesenchymal stem cells and their types

Mesenchymal stem cells (MSCs) are a type of non-hematopoietic pluripotent stem cells that grow easily in culture dishes. They possess self-renewal capacity and intrinsic differentiation potentials not previously found in other cells, and they also produce a large amount of useful growth factors and cytokines (Ding et al., 2011; Pittenger et al., 2019; Zhou et al., 2021).

MSCs were first discovered by FriedenStein et al. in the 1960s (Naji et al., 2019; Yu et al., 2023; Zhou et al., 2021). These researchers observed that a subset of rodent bone marrow cells had osteogenic capabilities (Naji et al., 2019). This subset of cells was defined as colony-forming units of fibroblasts or bone marrow stromal cells, which can differentiate into various mesodermal tissues such as muscle, bone, cartilage and fat, as well as cells that form blood vessels, blood cells and the urogenital system (Yu et al., 2023).

Currently, the concept of MSCs implies that there are subsets of stem cells in adult and neonatal tissues that support the homeostasis of other stem cells as well as can provide specialized de *novo* cells of mesodermal lineage (Naji et al., 2019).

The heterogeneity of MSCs is determined by multiple factors including, but not limited to, donors and tissue sources, cell populations, culture conditions, cell isolation techniques, and cryoprotection and thawing protocols (Yin et al., 2019; Zhou et al., 2021). MSCs are adherent cells with a spindle-shaped morphology under standard culture conditions according to the criteria developed by the International Society for Cellular Therapy in 2006 (Naji et al., 2019; Zhou et al., 2021). These cells are characterized by the expression of CD105, CD73, and CD90, but without expression of CD45, CD34, CD14 or CD11b, CD79a, CD19, or HLA-DR. In addition, they have the ability to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro* (Andrzejewska et al., 2020; Naji et al., 2019b; Zhou et al., 2021).

There are many sources of MSCs . These can be extracted from different tissues (**Fig. 1**). The most common and most widely used tissues of adult origin for extracting human MSCs are bone marrow and the stromal vascular fraction of adipose tissue (SVF), thus obtaining bone marrow-derived mesenchymal stem cells (BM-MSCs) and adipose-

derived mesenchymal stem cells (ASC). These human tissues are harvestable as they are considered renewable (bone marrow) or unwanted (adipose) (Pittenger et al., 2019b). MSCs can also be obtained from the human umbilical cord, derived from the placenta and even derived from urine (Yu et al., 2023).

BM-MSCs are a group of heterogeneous cells composed of pluripotent adult stem cells with potential multidifferentiation capacity (Singh et al., 2023). They represent approximately 0,001–0,01% of bone marrow mononuclear cells and express CD73, CD90, and CD105, but do not express CD14, CD45, CD34 or CD11b, CD79α, CD19, or HLA-DR surface molecules on their membrane (Yu et al., 2023)

Human umbilical cord blood MSCs have several characteristics such as a short duplication time, a long survival time, and a high anti-inflammatory capacity. Similarly, placenta-derived MSCs have a higher differentiation potential and self-renewal capacity than other MSCs derived from other tissues (Yu et al., 2023).

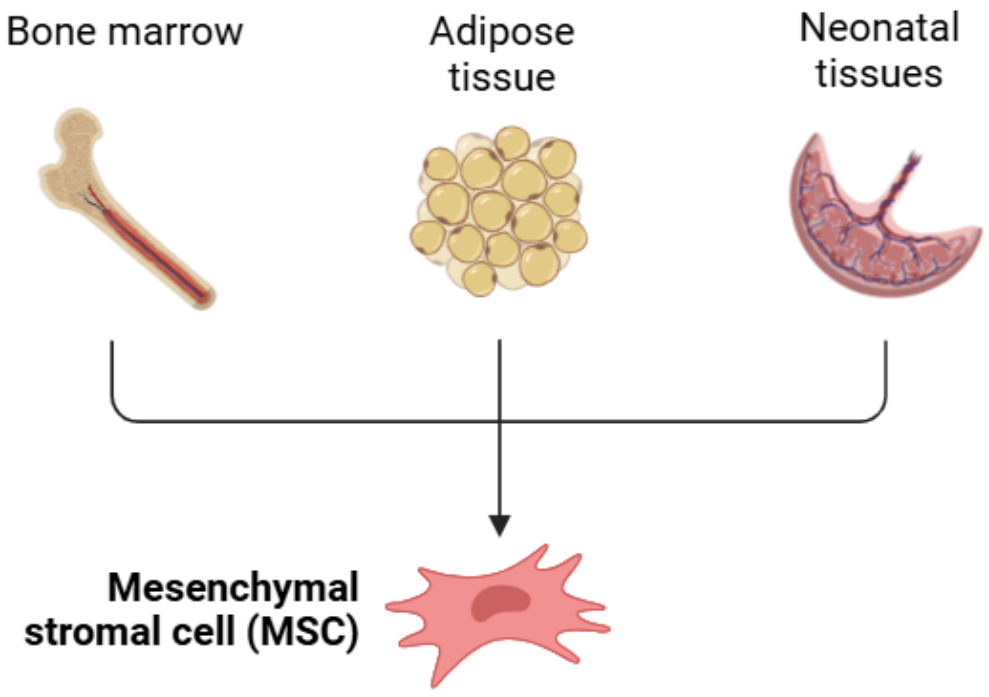

Figure 1. *Sources of Mesenchymal Stromal Cells. Created in BioRender.com.*

1.2 Properties of mesenchymal stem cells

MSCs are cells with very particular characteristics. They have immunomodulatory capacity and paracrine functions, among others.

1.2.1 Immunomodulatory properties

One of the main advantages of MSCs is their immunomodulatory properties. The form of immunomodulation is mediated by cell-cell interactions, cytokines and soluble factors. Depending on the type of stimulation, MSCs perform pro-inflammatory or anti-inflammatory functions in their microenvironment (Mishra et al., 2020). MSCs cultured *in vitro* have the ability to interact and regulate the function of most effector cells involved in primary and acquired immune response processes (Andrzejewska et al., 2019). These cells suppress a wide range of immune cells, including T, B, and natural killer (NK) lymphocytes, and myeloid cells such as monocytes, dendritic cells (DCs), and macrophages, by modulating their activation, proliferation, maturation, cytokine production, cytolytic activity, and antibody production (Naji et al., 2019).

Native MSCs could block the proliferation of de *novo* induced NK cells, although they only partially inhibit the proliferation of already activated NK cells. In addition, they contribute to the reduction of the cytotoxic activity of these cells (Andrzejewska et al., 2019). Under *in vitro* conditions, the presence of proinflammatory cytokines activates a feedback mechanism in MSCs, which increases the production of chemokines. This causes more neutrophils to move towards the site of inflammation where they act mainly by phagocytosis (Mishra et al., 2020). Also, MSCs inhibit the cytotoxic potential of NK lymphocytes and their ability to secrete IFN-γ (Naji et al., 2019).

In the absence of IL6, MSCs promote the proliferation and activation of the M1 macrophage (proinflammatory), favoring its transition from macrophage (M0) to a proinflammatory phenotype (M1). This is due to IFN-γ and IL1, together with the expression of CD40L, a membrane protein. On the other hand, in an anti-inflammatory microenvironment, MSCs induce the transformation of M1 into M2-type cells (anti-inflammatory phenotype). These M2s produce IL10 which inhibits T cell proliferation (Andrzejewska et al., 2019). Furthermore, recent studies have shown that exosomes derived from adipose MSCs stimulate macrophage differentiation towards the M2 phenotype and promote their expression of CCL-18, activating regulatory T cells (Treg) (Mishra et al., 2020).

In vitro studies have demonstrated a direct immunomodulatory effect of MSCs on lymphocytes. During coculture of MSCs with lymphocytes, the differentiation of naive $CD4^+$ T lymphocytes to $Th17^+$ is inhibited and the differentiation into $CD4^+$ $CD25^+$ regulatory T cells is promoted. They also induce anergy in activated T cells and limit the synthesis of immunoglobulins (IgM, IgG, IgA) by B cells, blocking their differentiation into plasma cells. In addition, they reduce the expression of chemokines and receptors in B cells, affecting their migratory capacity (Andrzejewska et al., 2019; Mishra et al., 2020; Naji et al., 2019).

Thus, MSCs have multiple arms for immunosuppression, which therefore validates an assessment of their therapeutic value in various immunological disorders such as hematopoietic stem cell transplantation, solid organ transplantation, Crohn's disease, rheumatoid arthritis, and systemic lupus erythematosus (Naji et al., 2019).

1.2.2 Paracrine properties

MSCs secrete a set of paracrine factors known as secretome. This plays a crucial role in regenerative processes in damaged tissues. The secretome can be divided into two main fractions: the soluble fraction, which includes factors such as cytokines, growth factors, chemokines, microRNAs (miRNAs), lipids and proteins; and the vesicular fraction, which comprises exosomes, microvesicles and apoptotic bodies **(Fig. 2)** (Clua-Ferré et al., 2024). All of them have an impact on the microenvironmental regulation processes (Mishra et al., 2020). Among them, factors such as vascular endothelial growth factor (VEGF) promote angiogenesis, although they can also inhibit it through molecules such as IFN-γ-induced monokine and tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2). Secreted chemokines, such as CCL5, CXCL12 and CCL8, regulate cellular chemotaxis, while growth factors such as HGF, IGF-1 and VEGF have antiapoptotic, antifibrotic and cell proliferation-stimulating effects. The secretome also includes molecules capable of reducing fibrosis during regeneration, such as KGF, HGF, VEGF and Ang-1, and presents antibacterial, antiparasitic and antiviral properties. In addition, certain released factors can influence the proliferation and migration of cancer cells, showing variable effects depending on the type of tumor and the origin of the MSCs (Andrzejewska et al., 2019).

Another broad field that is related to the activity of MSCs is their ability to secrete EVs. Physiologically, they play an important role in regulating biological functions,

homeostasis, and the immune response of the body. Experiments performed using supernatant derived from *in vitro* culture of MSCs showed that the factors contained in their secretome are responsible for a large part of the effects exerted by MSCs during the regeneration of the damaged area including the protection of other cells against apoptosis, the induction of their proliferation, the prevention of excessive tissue fibrosis, the stimulation of the angiogenesis process and immunomodulatory effects, as well as the induction of endogenous stem cell differentiation (Andrzejewska et al., 2019; Kupcova Skalnikova, 2013).

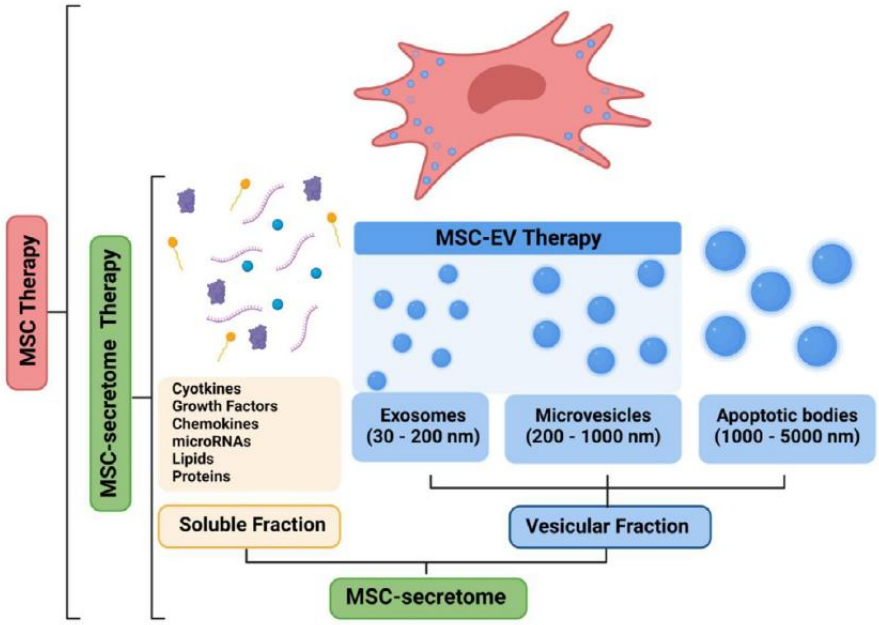

***Figure 2**. Mesenchymal stem cell secretome. Clua-Ferré, L., Clinical and Translational Medicine.2024; 14(11).*

1.3 Adipose tissue and adipose-derived mesenchymal stem cells

Adipose tissue has always been associated with an energy reservoir function, but the discovery of leptin, the first cytokine produced by adipose tissue with systemic repercussions, led to this tissue being reclassified as a dynamic and very complex endocrine organ (Bunnell, 2021). It is mainly composed of extracellular matrix, mature adipocytes and SVF, which includes adipose-derived mesenchymal stem cells (ASCs), preadipocytes, pericytes, endothelial cells, smooth muscle cells, fibroblasts, hematopoietic cells and immune cells (Al-Ghadban & Bunnell, 2020; Trzyna & Banaś-Ząbczyk, 2021).

There are three types of adipose tissue in humans: white adipose tissue (WAT), brown adipose tissue (BAT) and beige adipose tissue ("brite" or "brown/white"). All of them have the capacity to store energy, but BAT and, to a lesser extent, beige adipose tissue, have a thermogenic function since they express Uncoupling Protein 1 (UCP1), a mitochondrial membrane protein (Bunnell, 2021; Trzyna & Banaś-Ząbczyk, 2021). Adipose tissues are obtained from patients by aspirating or removing visceral (VAT) or subcutaneous (SAT) fatty tissue located in the areas of the abdomen, arm, femur or buttocks (Naji et al., 2019; Yu et al., 2023), usually through surgical procedures involving liposuction or lipectomy.

As previously mentioned, adipose tissue represents an important source of MSCs. ASCs are adult, multipotent, self-renewing cells that possess potent immunoregulatory, angiomodulatory, and neuroprotective properties (Al-Ghadban & Bunnell, 2020; Harrell et al., 2022; Trzyna & Banaś-Ząbczyk, 2021). They are a heterogeneous population and for this reason unique surface markers have not yet been described. They notably present CD73, CD90, CD105 and CD36 but possess to a lesser extent CD31, CD45, CD11b, CD106. In addition, these surface markers can also vary depending on the adipose tissue from which the cells are extracted. For example, ASCs from BAT express notably more CD105, HLA-A, B, C and CD-137, but less CD86 and LIN, than WAT (Trzyna & Banaś-Ząbczyk, 2021).

ASCs isolation is simple, has a high yield, and is less invasive than BM-MSCs extraction (Naji et al., 2019a). Furthermore, the obtained ASCs can differentiate into many different lineages including chondrogenesis, osteogenesis, cardiomyocytes, adipogenesis, neurogenic and hepatic differentiation (Trzyna & Banaś-Ząbczyk, 2021; Yu et al., 2023). It is also important to note that the adipose tissue collected for the isolation of ASCs affects their biological properties. Studies have shown that ASCs isolated from SAT demonstrate a higher proliferation rate and differentiation capacity towards adipocytes and chondrocytes, but lower osteogenicity compared to VAT. ASCs from SAT have been shown to be more effective in the treatment of osteoarthritis because they have immunosuppressive capacity to decrease the expression of inflammatory genes (Al-Ghadban & Bunnell, 2020) and it has also been studied that SAT of ASCs operation is a less invasive process (Si et al., 2019). On the other hand, the origin of the ASCs is essential. The age, weight and disease status of the donor can affect the condition and properties of the isolated ASCs. Studies reinforce that, as the donor gets older, the ASCs

have a lower proliferation and differentiation potential and a lower capacity to secrete growth factors. In addition, ASCs obtained from obese donors are characterized by a lower expression of MSCs phenotypic markers, an excessive immune response and, therefore, a lower self-renewal, differentiation potential and a higher capacity for migration, invasion and phagocytosis. In this way, it has also been shown that type 1 and type 2 diabetes mellitus, hypercholesterolemia, hypertension and smoking have negative effects on the pluripotency and self-renewal of the isolated ASCs (Trzyna & Banaś-Ząbczyk, 2021).

The clinical application of ASCs is considered a promising tool for use in regenerative medicine, including the treatment of degenerative, inflammatory and autoimmune diseases. Specifically, it has been successful in the treatment of different conditions such as cardiovascular disease, inflammatory bowel disease, diabetes mellitus, kidney and spinal cord, bone and craniofacial reconstruction, liver cirrhosis, multiple sclerosis, systemic lupus erythematosus and graft-versus-host disease. This makes ASCs a suitable option for use in cell-based therapies (Harrell et al., 2022; Trzyna & Banaś-Ząbczyk, 2021).

1.4 The secretome of adipose tissue-derived mesenchymal stem cells

The secretome of ASCs is a set of paracrine factors that plays a crucial role in tissue repair and regeneration processes. Key components of the secretome include VEGF, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF), monocyte chemoattractant protein 1 (MCP-1), various interleukins, prostaglandin E2 (PGE2), and others (**Fig. 3**). These elements are fundamental to explain many of the therapeutic effects of ASCs (Pinheiro-Machado et al., 2023; Trzyna & Banaś-Ząbczyk, 2021).

The ASCs secretome, as a cell-free therapeutic product, is considered a safer strategy than therapies based on the use of intact cells. This approach eliminates risks associated with traditional cell therapies, such as immune rejection, tumor formation, or excessive cell growth, and addresses ethical concerns related to the use of ASCs (Trzyna & Banaś-Ząbczyk, 2021). In addition, secretome offers cost advantages, as its production and quality control are easier to standardize, ensuring consistent efficacy and quality in therapeutic products (Ratushnyy et al., 2020).

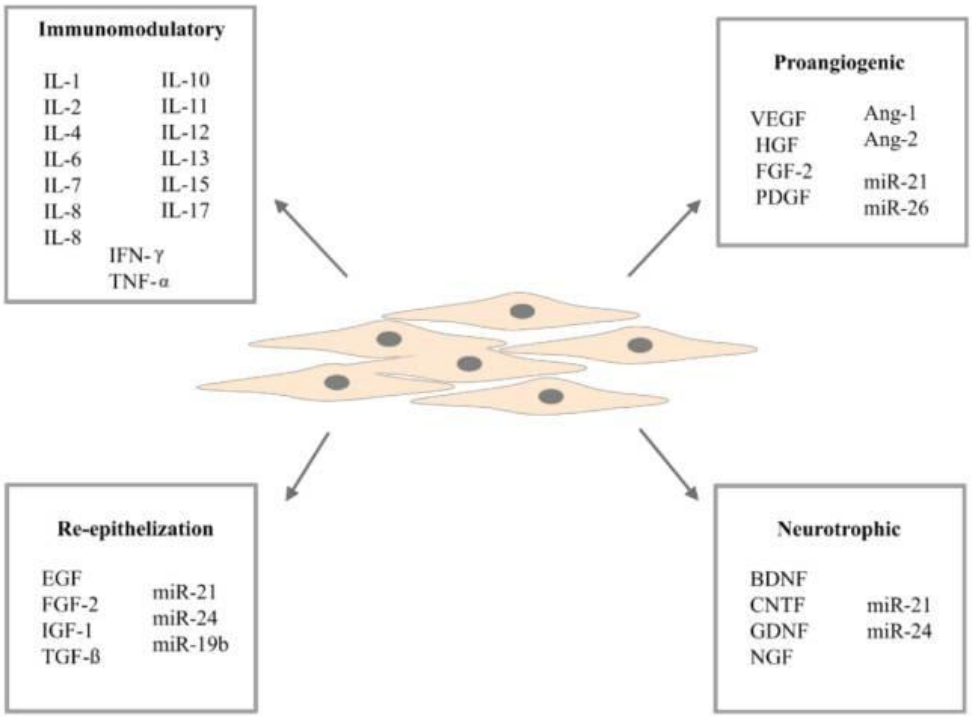

Figure 3. *Key components of the secretome in ASCs that produce therapeutic effects of ASCs. Trzyna A., Biomolecules.2021; 11(6):878.*

The secretome of ASCs is dynamic and adapts to changes in the cellular microenvironment, such as hypoxia or inflammatory stimuli. *In vitro* conditions replicate physiological situations that occur in vivo during regeneration and repair processes, which improves the therapeutic capacity of ASCs (Pinheiro-Machado et al., 2023). Thanks to this, ASCs show greater capacity for differentiation, migration, proliferation and autocrine activity compared to BM-MSCs. Furthermore, ASCs experience senescence later than BM-MSCs, which extends their therapeutic efficacy (Trzyna & Banaś-Ząbczyk, 2021).

Due to limitations in cell therapy, such as the impossibility of indefinitely increasing the dose and frequency of cell application, a promising strategy is to focus on the use of the secretome, with special emphasis on EVs. This approach not only maximizes the therapeutic efficiency of ASCs, but also overcomes the restrictions of using intact cells, offering a safer and more effective method (Trzyna & Banaś-Ząbczyk, 2021).

1.4.1 Extracellular vesicles from adipose tissue-derived mesenchymal stem cells

EVs are bilayer vesicular nanoparticles found throughout the spectrum of body fluids. They are classified into three main categories based on their size and biogenesis:

exosomes, microvesicles, and apoptotic bodies. Exosomes, with diameters ranging from 30 to 200 nm, result from the internalization of extracellular components by the cell through endocytosis (Doyle & Wang, 2019). This process involves engulfment of the cell membrane by invagination, resulting in the formation of endosomes. These endosomes undergo maturation during which the membrane invaginates to produce intraluminal vesicles within the endosome. These intraluminal vesicles contain various internalized molecules, such as antigens, nucleic acids, proteins, and metabolites. Microvesicles, also called ectosomes or microparticles, are EVs between 200 and 1000 nm in diameter that are released into the extracellular environment by direct budding or shedding from the plasma membrane. Finally, apoptotic bodies, which have a larger diameter of 1000–5000 nm, consist of relatively large cell fragments containing organelles, histones, fragmented DNA, and noncoding RNA from apoptotic cells. These vesicles are destined to be removed by phagocytosis (Clua-Ferré et al., 2024; Doyle & Wang, 2019).

ASCs secrete exosomes with a diameter of 30-100 nm. Exosomes contain cytoskeleton, transmembrane proteins, enzymes, lipids and DNA (Trzyna & Banaś-Ząbczyk, 2021). In addition, they carry coding RNA (mRNA) and non-coding RNA (e.g., rRNA, miRNA) in several subtypes but with a strong emphasis on short non-coding RNA. Non-coding RNAs are RNA molecules that cannot be translated into a protein and the functions of many of them are not yet understood, however, transported by exosomes they play a role in cell-mediated communication (Ratushnyy et al., 2020). Interestingly, 1185 proteins were identified in ASC-derived exosomes (ASC-exos) and demonstrated that exosomes are crucial not only for cell-to-cell communication but also for metabolic and cellular processes and the regulation of biological processes (Xing et al., 2020). They also showed that ASC-exos are characterized by exosomal markers such as CD9, CD63, as well as tumour susceptibility gene (TSG) (Trzyna & Banaś-Ząbczyk, 2021).

Exosomes, due to their unique molecular content and specific biological properties, act as modulators with diverse effects on recipient cells. In the case of exosomes derived from ASC, they offer significant therapeutic advantages. Their stability in the human body makes them superior to whole ASCs, which typically die approximately 48 hours after systemic infusion (An et al., 2019). Furthermore, exosomes pose a lower risk of tumorigenicity, making them a safer option. They can be easily produced in large quantities under well-established laboratory protocols and their components are less prone to cellular degradation (An et al., 2019).

A remarkable aspect of exosomes is their ability to influence both neighboring cells and more distant tissues, transporting bioactive cargoes such as proteins, lipids and RNA (Trzyna & Banaś-Ząbczyk, 2021). This makes them a versatile and efficient therapeutic resource.

However, to ensure successful clinical applications, it is crucial to regulate and standardize the content and reproducibility of exosomes. The quantity and function of exosomes depend on the cell donor. For example, ASCs from obese donors and omental fat depots secrete more exosomes than those from lean donors and subcutaneous depots, which is influenced by the metabolic and disease status of the donor. Nevertheless, it is possible to enhance exosome secretion under *in vitro* conditions by using molecular modulators (Trzyna & Banaś-Ząbczyk, 2021).

Exosomal content is not random, it is strictly regulated by post-translational modifications and specific target sequences during its formation. Among the most relevant components are miRNAs. These constitute one of the preferred cargoes of exosomes due to their ability to regulate various cellular processes. In addition, the therapeutic efficiency of exosomes can be enhanced by genetic and chemical modifications, optimizing their content for specific applications. Thanks to these characteristics, exosomes are emerging as ideal vehicles for targeted drug delivery, significantly expanding their potential in regenerative medicine and personalized therapy (Trzyna & Banaś-Ząbczyk, 2021).

1.5 Acute and Prolonged Immune Responses: Neutrophils and Macrophages

The immune system orchestrates a complex defence strategy involving various cell types, among which neutrophils and macrophages play crucial roles in the innate immune response. These cells differ significantly in their timing and function during immune responses.

Neutrophils are the first responders to sites of infection or injury, representing an acute response mechanism. They are polymorphonuclear, granular leukocytes that constitute 50-70% of all circulating leukocytes in humans. Upon activation, neutrophils rapidly migrate to the affected site through the leukocyte adhesion cascade. Their primary functions include degranulation, phagocytosis, production of reactive oxygen species (ROS), and the formation of neutrophil extracellular traps (NETs), which help neutralize pathogens. Although neutrophils are traditionally seen as key players in immediate

antimicrobial defence, they also secrete cytokines and other inflammatory mediators that influence the broader immune response (Rosales, 2020).

In contrast, macrophages are pivotal for a more sustained immune response. They arise from monocytes that differentiate once they migrate into tissues. Macrophages play a dual role: they participate in the innate immune response by phagocytosing pathogens and debris, and they also modulate the adaptive immune response by presenting antigens to T cells. This makes them a critical bridge between the two immune subsystems. Macrophages exhibit a remarkable plasticity, shifting between pro-inflammatory (M1) and anti-inflammatory (M2) states depending on the signals in their environment. M1 macrophages, activated by interferon-γ and lipopolysaccharides (LPS), are involved in the inflammatory response, while M2 macrophages, stimulated by interleukins such as IL-4 and IL-10, contribute to tissue repair and resolution of inflammation. This dynamic adaptation allows macrophages to sustain and regulate the immune response over time (Yang et al., 2023; De Maeyer & Chambers, 2021).

In summary, neutrophils and macrophages serve distinct but complementary roles: neutrophils are critical for rapid, acute responses, while macrophages manage prolonged, adaptive processes, ensuring the immune system’s effectiveness across different stages of immune defence.

1.6 Inflammatory bowel diseases and previous results of extracellular vesicle therapy in Crohn's disease

Inflammatory bowel disease (IBD) includes chronic inflammatory disorders of the gastrointestinal tract. The best known IBD pathologies are ulcerative colitis (UC) and Crohn's disease (CD). UC causes long-lasting inflammation and superficial ulcerative disease in the colon, while CD is characterised by inflammation of the entire gastrointestinal tract, from the mouth to the anus, and there is currently no effective treatment for these diseases (Seyedian et al., 2019). IBD is usually diagnosed between the ages of 20 and 40, but can begin at any age and shows alternating periods of activity and remission. The etiology of IBD may involve the host immune system, genetic variability and environmental factors (Saez et al., 2023).

The idea of this work is a continuation of a project of the Inflammatory Bowel Disease research group (IBODI), investigating whether EVs derived from ASCs could be a promising therapy for IBD.

Previous results from their study show that application of EVs to explants of creeping fat (the adipose tissue that abnormally accumulates in inflamed areas of the digestive tract in CD) and to mucosa for 20h significantly decreases gene expression of proinflammatory cytokines in both tissue types as demonstrated in **Figure 4**.

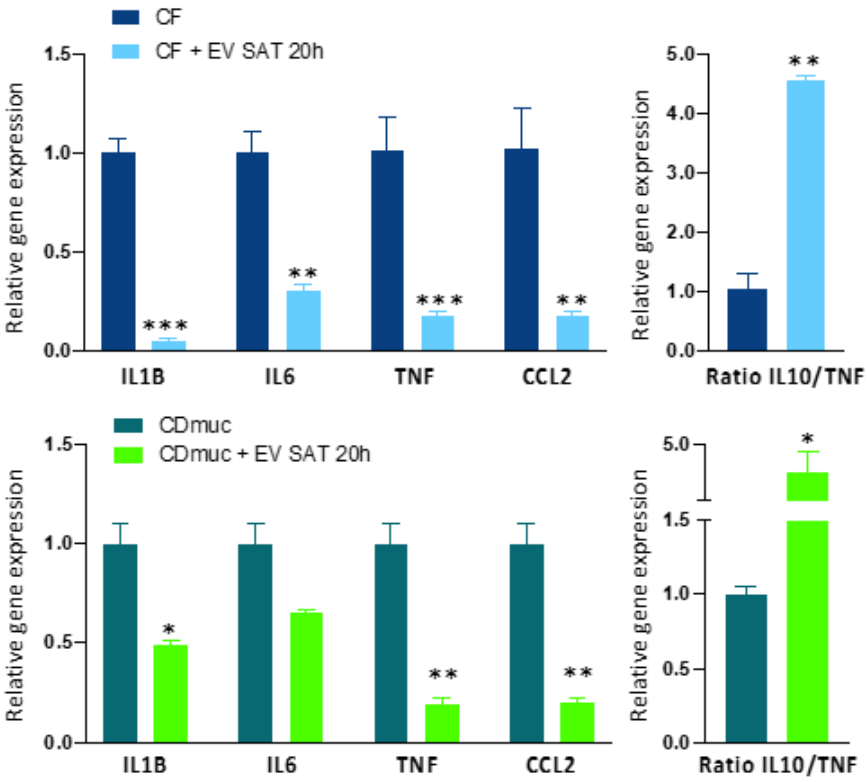

***Figure 4**. EVs isolated from ASCs restore gene expression in CD tissue explants. Proinflammatory cytokine gene expression and IL10/TNF ratio in creeping fat (CF) and mucosal explants from CD subjects treated for 20 hours with EVs at 150 µg/ml (n=4 CD patients) *p<0.05, **p<0.01 and ***p<0.001 versus untreated tissue.*

2. Hypothesis and objectives

2.1 Hypothesis

Extracellular vesicles of mesenchymal stem cells derived from subcutaneous adipose tissue of healthy patients possess significant anti-inflammatory properties and can modulate inflammatory responses in target tissues.

2.2 Objectives

Building upon the established hypothesis, this study aims to:

1. Evaluate whether treatment with EVs from MSCs in creeping fat and intestinal mucosa of CD patients enhances the expression of macrophage-specific anti-inflammatory markers (M2) and modulates gene expression in immune-related cell types, such as neutrophils.
2. Assess the cytotoxicity of EVs in the human monocytic leukemia cell line (THP-1) and investigate whether the anti-inflammatory signalling mechanism of EVs is mediated through macrophages, specifically examining the potential shift from a proinflammatory (M1) to an anti-inflammatory (M2) phenotype in this cell type.
3. Analyze the sustained efficacy of EVs treatment over time in creeping fat, focusing on the temporal dynamics of inflammatory marker expression.

3. Materials and methods

3.1 Patient cohort

EVs were obtained from healthy adipose tissue donors. These donors were healthy individuals undergoing non-acute surgery at several hospitals, including University Hospital Joan XXIII in Tarragona (HJ23), Fundación Jiménez Diaz in Madrid (FJD), Hospital Santa Tecla in Tarragona (HST), Hospital Vall Hebrón in Barcelona (VH) and Hospital La Fe in Valencia (HF). Donor inclusion criteria were: young age (<45 years), body mass index body weight (BMI) < 25 mg/kg^2, and exclusion criteria: smokers or ex-smokers, regular consumers of alcohol; cancer or other chronic pathologies.

On the other hand, the creeping fat and mucosal samples were obtained from six patients with active CD that undergoing surgery for symptomatic complications. All clinical data of the CD patients are shown in **Table 1**.

This study follows the principles established in the Declaration of Helsinki and the Good Clinical Practice guide and is approved by the CEIM of HJ23 (reference CEIM 0145/2023). The personal data of each participant are protected in accordance with LOPD 15/1999 and RD 1720/2007 and the processing of said data will be carried out as established by LOPD 15/1999 and RD 1720/2007.

Table 1. Clinical, anthropometric and demographic characteristics of subjects

	PATIENTS
N	6
Sex (male/female)	6/0
Age	31,5 ± 9,16
BMI	24,73 ± 5,31
Active disease	6/6
Inactive disease	0/6
Smoking status, n (%)	
Current smoker	2 (33,4)
Never-smoker	3 (50)
Ex- smoker	1 (16,6)
Age at diagnosis, n (%)	
A1	3 (50)
A2	2 (33,4)
A3	1(16,6)
Location, n (%)	
L1	0
L2	1 (16,6)
L3	5 (83,4)
Behaviour, n (%)	
B1	4 (66,8)
B1p	0
B2	0
B2p	0
B3	1 (16,6)
B3p	1 (16,6)
CRP (mg/L)	5,73 ± 9,32

Abbreviations: CRP: C-reactive protein; BMI: body mass index.

Age at diagnosis: A1 ≤ 16 years; A2 17-40 years; A3 > 40; Location: L1= Distal ileum; L2= Colon; L3= Ileocolonic; Behaviour: B1 = Non-stenosing, non-fistulizing Crohn's; B1p = Non-stenosing, non-fistulizing Crohn's with perianal disease; B2= Stenosing Crohn's; B2p = Stenosing Crohn's with perianal disease; B3 = Penetrating Crohn's; B3p= Penetrating Crohn's with perianal disease.

3.2 Isolation and characterization of extracellular vesicles from SAT tissue of healthy patients

3.2.1 Ultracentrifuged culture medium

For isolation of EVs, it is important to obtain ultracentrifuged medium. For this, DMEM culture medium supplemented with 20% fetal bovine serum (FBS) (Gibco, Grand Island,

NY, USA) and 1% penicillin/streptomycin (Ab/Ac) (Gibco, Grand Island, NY, USA) should be prepared in a 50 ml falcon tub.

To remove EVs that the medium itself may contain, such as those in FBS, it is ultracentrifuged using a Sorvall WX 100 Ultracentrifuge, Thermo Scientific (Ref: #75000100) at 4ºC for 16 h at 100,000 x g. Once this process is done, the supernatant is transferred to a 50 ml falcon tube. The supernatant obtained is the culture medium without FBS vesicles. Before use, this is filtered using a 0.22 μm filter attached to a syringe to sterilize it. In addition, it must be diluted with DMEM medium to obtain a medium supplemented with 10% FBS.

3.2.2 Cell culture of subcutaneous adipose tissue stem cells.

SAT samples were obtained from donors after a surgical procedure. This tissue was digested with collagenase to obtain SAT tissue stem cell cultures.

ASCs were cultured in DMEM medium supplemented with 10% FBS (Gibco, Grand Island, NY, USA), and 1% Ab/Ac (Gibco, Grand Island, NY, USA) at 37 °C in humidified air containing 5% CO2.

3.2.3 Cell culture to obtain conditioned medium with stem cell vesicles

ASCs were cultured and maintained in a T-75 flask to reach confluence. Cells were washed with 5 ml of phosphate-buffered saline (PBS), and the medium was replaced with 7 ml of ultracentrifuged culture medium for 48 h to collect conditioned medium (CM). At least 25 ml of CM must be collected for column EVs extraction.

If the extraction is carried out in less than 24 h, the CM can be stored in the refrigerator. If the extraction is to be carried out later, the CM must be frozen in cryotubes at -80ºC using a Mr. Froosty of Thermo Scientific™ (Ref: 10110051) to prevent the EVs in the medium from being affected by freezing.

3.2.4 Isolation of extracellular vesicles by Size-Exclusion Chromatography

Isolation of EVs was done by Size-Exclusion Chromatography (SEC).

The column consists of a syringe filled with agarose and sepharose and a valve at the end. The valve serves to control the opening and closing of the column. On the other hand, the function of the agarose and sepharose is to separate the particles according to their size.

In this way, EVs, which are smaller molecules, are eluted before the other components of the MC.

To isolate the EVs on the column, the CM must first be concentrated. For this, the CM is centrifuged at 400 g for 5 minutes to exclude the cells. The supernatant is then transferred to an Amicon filter tube (Amicon Ultra-15 Centrifugal Filter Unit, Milipore. Ref: #UFC910024) pre-moistened with PBS and centrifuged for 30 minutes at 2000g. Once this is done, the CM concentrate that remains on top of the filter is collected for use in the SEC, as this is where the EVs are located. At the same time, before using the column and adding the CM concentrate, the SEC column must first be washed to clean it. For this, 1 ml NaOH, 2 ml TritonX-100, 2 ml distilled water and 2 ml PBS are eluted sequentially.

Once the column and the concentrated CM are ready, the column should be loaded with the sample. A total of 20 fractions of 100µl were collected in different eppendorfs. EVs are generally eluted in fractions 5 to 10. To check this, a Nanodrop analysis (NanoDrop NP-1000 ThermoFisher) is performed at an absorbance of 280nm to confirm that these fractions are enriched with EVs. Eluted fractions should be kept at 4°C for 48h until use or frozen at -80°C with Mr. Froosty.

3.2.5 Characterization of extracellular vesicles by flow cytometry

To characterize the EVs, flow cytometry was performed. This technique allows us to better understand the composition, size, morphology and surface markers of the EVs. Specifically for vesicles, we have looked at the CD63 antibody.

Detecting EVs, due to their microscopic size, can be complicated. For this reason, they can be covalently bound to latex microspheres, which allow the detection of EVs more easily.

First, the microspheres (beads) must be attached to the EVs. To do this, a 1:10 dilution of beads with PBS is made (90 µl PBS + 10 µl beads). Once this is done, we prepare as many tubes as the eluted fractions of EVs we obtain with the SEC and a control to add. 2 µl of the bead dilution and 10 µl of EVs samples are added to each tube. PBS has been used as a control instead of samples. The tubes are left to incubate for 15 minutes so that the microspheres adhere, and then 1 ml of BSB is added. This process is called bead blocking and, in this way, other adhesions with soluble or contaminating proteins to the beads are blocked. The tubes are left in a centrifuge at room temperature for 24 hours.

After this time, immunolabeling is carried out, which is a process that allows the detection and localization of an antigen in a particular site, in our case, the EVs. After removing the tubes from the centrifuge, they are centrifuged at 2000g for 10 minutes and the supernatant is discarded. The pellet in each tube is then resuspended with 75 µl of BCB and the dilutions of the antibody to be used are prepared with BCB. For CD63, the primary antibody needs a dilution of 1:10 and for the secondary antibody it needs a dilution of 1:100. The procedure is continued by adding the dilution of the primary antibody and 50 µl of the sample with beads to a 96-well plate and incubating for 30 minutes at room temperature in the dark. Then, 150 µl of BCB is added per well and the plate is centrifuged at 2000g for 10 minutes to discard the supernatant. Once this is done, the light is turned off and the secondary antibody is added. It is left to incubate for 30 minutes at room temperature and, after this time, 150 µl of BCB is added per well in the dark. Finally, centrifuge again at 2000g for 10 minutes, discard the supernatant to add 100 µl of PBS and analyze the plate in the flow cytometer.

3.2.6 Extracellular vesicles quantification

Colorimetric detection and quantification of EVs was determined with the Micro BCA Protein Assay kit (ThermoFisher).

For use, first prepare bovine serum albumin (BSA) standards by serial dilutions in a known concentration range (0.25 to 200 µg/ml), the EVs samples obtained in the SEC and the working reagent (buffer). The working reagent (WR) is prepared in the dark by mixing the components supplied in the kit in the proportions indicated by the manufacturer.

In each well of a 96-well plate, in the case of preparing the standard, add 150 µL of WR and 150 µL of BSA (in the case of the negative control, add 150 µL of PBS instead of BSA). On the other hand, if the sample is being prepared, 15 µL of sample, 135 µL of PBS and 150 µL of WR are added.

The plate is shaken and incubated at 37 °C for 2 hours in an oven wrapped in aluminium foil to keep out light. Finally, since the reaction generates a purple complex, the absorbance is measured at 562 nm using a Varioskan LUX multimode microplate reader (Ref. VL0000D0) from Thermo Fisher Scientific™.

The protein concentration in the samples is determined by interpolating their absorbance values on a standard curve obtained with the values of the BSA standards.

3.3 Study of anti-inflammatory and immune markers in creeping fat and mucosa of Cronh patients treated with extracellular vesicles

As mentioned above, creeping fat and mucosa were obtained from patients with CD. These tissues were processed in order to apply the EVs (**Fig. 5**) and study their effect on this inflamed tissue by qPCR. The TaqMan™ probes used for qPCR are specified in Supplementary Table 1.

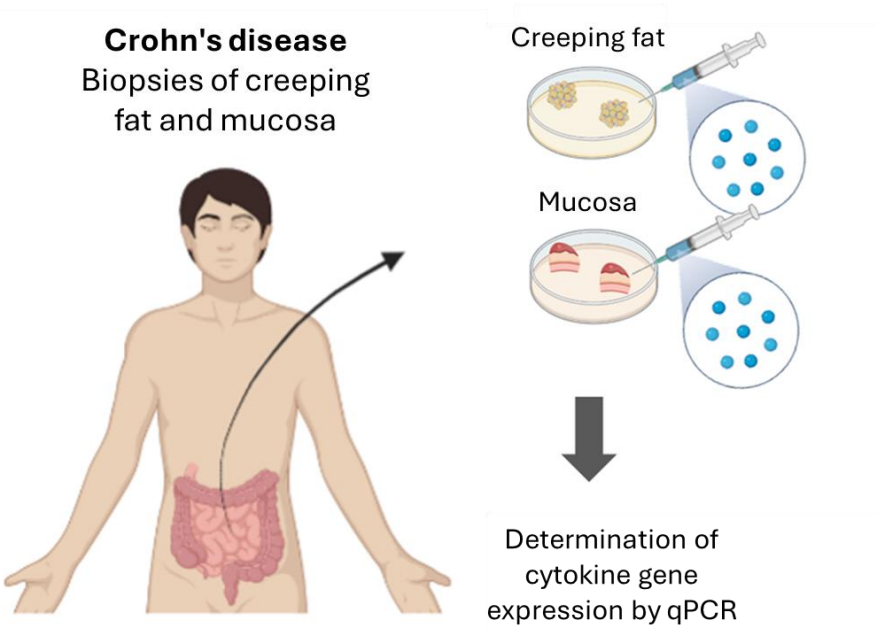

Figure 5*. Creeping fat and mucosa biopsies used for EVs treatment. Created in BioRender.com.*

3.4 Extracellular vesicle viability assay in THP-1 cells

3.4.1 Cell culture

THP-1 were cultured in complete medium RPMI-1640 supplemented with 10% FBS (Gibco, Grand Island, NY, USA), 1% penicillin-streptomycin solution (Gibco, Grand Island, NY, USA) and 0,1% β-mercaptoetanol in 5% CO2 at 37 °C.

Manipulation of the cell lines was carried out in a Class II biological safety cabin.

3.4.2 Cytotoxicity of extracellular vesicles

The cell viability ($1{,}6 \cdot 10^5$ cells per well) was analysed by an CyQUANT™ MTT Cell Proliferation Assay Kit (Ref: V13154) 24h after the incubation with different concentrations of EVs (0µg/ml - 40µg/ml - 80µg/ml - 200µg/ml) in a 96 wells-plate.

After 24h of incubation with the EVs at 37º under 5% CO2 in a humidified atmosphere, 10 µl of MTT was added to each well of cells. The cells were incubated for 4 hours under the same conditions so that viable cells metabolize the MTT to insoluble formazan crystals. Then, 100 µl of DMSO was added and shaken for 15 minutes to dissolve the crystals and turn a purple colour to measure the absorbance (**Fig. 6**). The absorbance was measured with a Varioskan LUX multimode microplate reader (Ref. VL0000D0) from Thermo Fisher Scientific at 540nm.

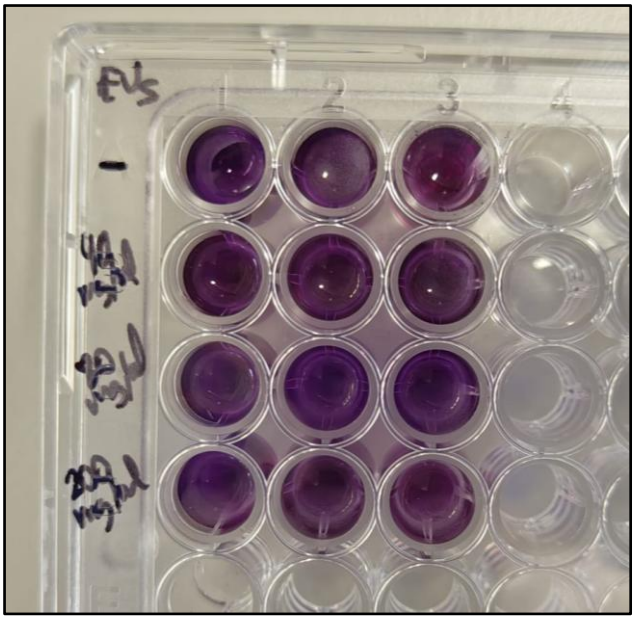

Figure 6*. 96-well plate with MTT assay. The colour change to purple appears after the use of DMSO.*

3.5 Assay of the anti-inflammatory action of extracellular vesicles in THP-1 cells polarised to an inflammatory phenotype

3.5.1 Cell culture

THP-1 cells were cultured in the same conditions as in point 3.4.1.

3.5.2 Polarization of macrophages to an inflammatory phenotype and application of vesicles as an anti-inflammatory action

THP-1 cells were differentiated into macrophages (M0) and then polarized to an inflammatory phenotype (M1). M1 cells were then treated with EVs to test whether the inflammation decreased.

In a 12-well plate, THP-1 cells (8×10^5 cells/pou) were cultured for 48h in RMPI-1640 supplemented with 0.1 µg/mL PMA to differentiate into M0. After this time, THP-1 cells differentiated into M0 were stimulated with LPS (2 µg/ml) for M1 polarization 6h before EVs were applied. Before applying EVs, THP-1 cells were washed with PBS to remove LPS and replaced with RPMI-1640 medium supplemented with 40 µg/mL EVs. After allowing them to act for 24 hours, the gene expression is studied using qPCR.

3.5.3 RNA extraction, cDNA and qPCR

- RNA extraction and quantification

RNA extraction and quantification was performed from THP-1 12-well plates.

To extract RNA, 1 ml of TRIzol™ Reagent (Ref. 15596026) was added to the eppendorf to lyse the cells for 5 minutes. Subsequently, 200 µl of chloroform was added and shaken vigorously for 15 seconds, a colour change being observed. After leaving it for 2-3 minutes at room temperature, the sample was centrifuged at 12000G for 15 min at 4ºC. In another different eppendorf, the upper aqueous phase resulting from the centrifugation was transferred and 500 µl of isopropanol was added. This was homogenized and left at room temperature for 10 minutes. The pellet was then centrifuged at 12000G for 10 min at 4ºC and the supernatant was discarded. The pellet was washed twice, which consisted of adding 1 ml of 75% (v/v) ethanol and centrifuging at 7500G at 4ºC for 5 minutes. The resulting pellet was left to dry. Finally, 30 µl of RNase-free water was added to the RNA pellet (**Fig.7**), homogenized and placed in a Thermoblock at 65ºC for 10 min. After this time, the samples were placed on ice to generate a thermal contrast, then stored at -80ºC.

For RNA quantification in the sample, a µDrop™ Plate (Ref. N12391) from Thermo Fisher Scientific™ was used. 1 µl of RNase-free water, which served as a blank, and 1 µl of each sample were placed on the plate, homogenizing them beforehand. A Varioskan LUX multimode microplate reader (Ref. VL0000D0) from Thermo Fisher Scientific™ showed the absorbance at 260 nm and 280 nm and the RNA concentration (µg/µl) of each sample.

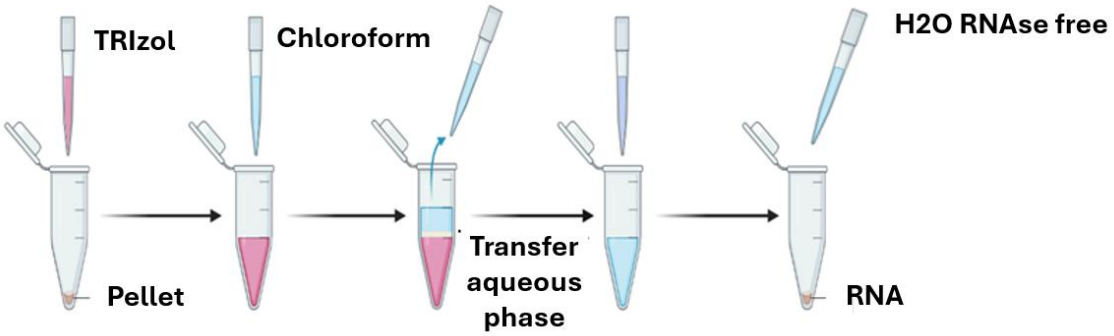

***Figure 7**. RNA extraction process. Created in BioRender.com.*

- cDNA obtainment

To obtain cDNA, 1 µg of RNA is required from each sample. This RNA was subjected to reverse transcription using the High-Capacity cDNA Reverse Transcription Kit with RNAse Inhibitor Kit from Applied Biosystems™ (Ref. 4374966) and the ProFlex 3 x 32 - Well PCR System from Applied Biosystems™ (Ref. 4484073). During the reverse transcription process, the RNA samples were first primed for 10 minutes at 25ºC, then reverse transcription polymerase chain reaction (RT-PCR) was performed for 120 minutes at 37ºC. Finally, the reverse transcriptase was inactivated for 5 minutes at 85ºC.

- qPCR

cDNA samples at a concentration of 50 ng/µl were used for qPCR using the TaqMan™ gene expression assay in 96-well plates from Applied Biosystems™ (Ref. AB0600) to assess the expression level of selected genes. Each plate contained predefined gene assays, which used TaqMan™ probes as detectors obtained from Applied Biosystems™ (Specified in Supplementary Table 1).

The process was carried out on a QuantStudioTM Pro Real-Time PCR System from Applied BiosystemsTM (Ref. A43055). The data obtained from each gene and sample were analysed using the $2^{-\Delta\Delta Ct}$ method, normalizing the expression values to 18S RNA as an endogenous control.

3.6 Study of the action of extracellular vesicles over time in creeping fat

To study the efficacy of EVs over time, the creeping fat of patients with CD was exposed to vesicles for 24 hours. The EVs were then removed, and the plate was washed twice with PBS. This allowed measuring whether, after 72 hours after exposure, the effect of EVs persisted.

Gene expression was studied by qPCR and the TaqMan™ probes used are specified in Supplementary Table 1.

3.7 Statistical analysis

The results were analyzed using GraphPad Prism 8.0. Comparisons between the three and four groups were performed using the ANOVA test and two-by-two comparisons were performed using the T-student test. Values of $p < 0,05$ were considered significant.

4. Results

The results demonstrated that EVs significantly increased the expression of macrophage-specific anti-inflammatory markers (M2) such as IL10, CD163 and MRC1 in creeping fat. Similar trends were observed in the intestinal mucosa, although the changes did not reach statistical significance in this tissue. In contrast, when applied to THP-1 cells polarised towards an inflammatory phenotype (M1), EVs did not fully reverse the inflammation to an anti-inflammatory phenotype (M2), likely due to limited cellular uptake in this tumour-derived cell line. Additionally, a sustained anti-inflammatory effect of EVs was observed in creeping fat, with a reduction in pro-inflammatory markers and an increase in anti-inflammatory markers persisting for 72 hours post-treatment.

5. Discussion

Adipose tissue-derived mesenchymal stem cell EVs unique molecular content and specific biological properties, allowing them to function as modulators with diverse effects on recipient cells (Trzyna & Banaś-Ząbczyk, 2021)

Firstly, thanks to previous results, the effect EVs on proinflammatory markers in creeping fat and mucosa has allowed further investigation underlying mechanisms at play in these tissues. Our results demonstrate a significant increase in the expression of anti-inflammatory markers such as *IL10*, which reflects the effectiveness of EVs in inflamed tissue as in other studies (Kim et al., 2020). In addition, there is a significant increase in the expression of anti-inflammatory macrophage markers such as *CD163* and *MRC1*. With respect to *CD163*, according to the literature, the result obtained is optimal as the expression of this marker is associated with endocytic and phagocytic processes typical of the resolution of inflammation (Adamczyk et al., 2023). On the other hand, increased expression of *MRC1* is characteristic of a phenotype switch to M2 in macrophages (Martínez-Zamora et al., 2024), so the significant increase in the expression of this marker in our results confirms the anti-inflammatory effect of EVs. Similarly, in the mucosa, although similar trends were observed, the results were not significant. This disparity could be due to differences in the immunological microenvironment of the two tissues.

On the other hand, increased gene expression of neutrophil inflammatory markers suggests that EVs treatment may modulate the activity of these innate immune cells, which are typically the first to interact with EVs (Brandel et al., 2022). The significant increase in *MPO* expression in creeping fat points to enhanced production of reactive oxygen species by neutrophils, likely as a result of vesicle clearance and ongoing tissue inflammation. The significant increase in *HLA* gene expression indicates that neutrophils, in response to severe inflammation, may take on antigen-presenting roles. While not statistically significant, the increased expression of *CD11B* and *CD66B* in creeping fat suggests enhanced neutrophil adhesion, activation, and degranulation (Metzemaekers et al., 2023). This points to a heightened presence of neutrophils in the inflamed tissue, which may further exacerbate local inflammation. Similarly, while the gene expression of neutrophil inflammatory markers in the mucosa did not reach statistical significance, the observed trend suggests that a larger sample size may help clarify these results.

Secondly, given the results obtained from the expression of anti-inflammatory genes in creeping fat and mucosa, it was hypothesised that EVs might be processing signals from the macrophage pathway. To assess the potential impact of EVs on cell viability, a viability assay was conducted on THP-1 cells. The literature supported that THP-1 cells maintain excellent viability at a concentration of 20 µg/ml of EVs (Sánchez-López et al., 2022). However, the present study aimed to evaluate the effects of higher EV concentrations. To determine the optimal concentration without compromising cell viability, various doses were tested. The results revealed that high EV concentrations (200 µg/ml) led to a significant reduction in cell viability, which could potentially affect bioavailability and the biological effects of the EVs. In contrast, lower concentrations, such as 40 µg/ml, which was ultimately selected for further experiments, did not negatively impact cell viability.

Once the optimal EVs concentration was known, an attempt was made to test whether macrophages with an inflammatory phenotype (M1) could be polarised to an anti-inflammatory phenotype (M2) using EVs. It can be seen that macrophage polarisation to the M1 phenotype was successful, but EVs treatment failed to reverse inflammation to an M2 phenotype. This result is strange because in other studies with other types of EVs, THP-1 cells do polarise to both M1 and M2 (Kim et al., 2020).

Despite this, a slight increase in the expression of anti-inflammatory markers such as *IL10* can be observed, but levels of pro-inflammatory markers such as *TNF*, *IL6* and *IL1B* remain elevated after treatment. In a study of induced colitis, *TNF* and *IL1B* derived from M1 macrophages induced increased expression of selectins allowing a massive influx of circulating monocytes and lymphocytes into the injured gut leading to increased inflammation and increased expression of these markers (Harrell et al., 2019). There are several reasons for the failure to establish the M2 phenotype, one of them suggesting that the internalisation of EVs in macrophages may have been partial and thus not able to reduce inflammation. Another reason could be that the signalling mechanisms of this immortal cell line are not as efficient as in primary cultures or it could also be due to differences in the composition of the EVs depending on the donor as this could have influenced the results.

Moreover, the results obtained for the study of the effectiveness of the EVs over time in creeping fat show that gene expression of inflammatory markers such as *TNF* and *IL1B*

continues to decrease after 72h. Similarly, the anti-inflammatory markers *CD163* and *MRC1* have a clear tendency to increase over time.

These results indicate that the EVs retain their anti-inflammatory effect in a sustained manner over time, which could represent a therapeutic advantage by reducing the need for repeated applications.

In addition, gene expression of neutrophil inflammatory markers has been obtained. The results obtained show that the expression of markers such as *MPO, CD11B, HLA* and *CD66B* show a temporal pattern after treatment with EVs. At 24 hours, an increase in the expression of these markers is observed, which could reflect an initial inflammatory response. However, at 72 hours, the expression of these same markers decreases, suggesting a progressive resolution of the inflammatory state induced by EVs. This behaviour is consistent with previous studies indicating that EVs may trigger early innate responses before inducing sustained anti-inflammatory effects (Rosales, 2020)

Finally, a progressive decrease in the expression of the pro-apoptotic marker *BAX* and the anti-apoptotic marker *BCL2* was observed over time, which could indicate reduced cell death. However, the increase in the *BAX/BCL2* ratio at 24 hours suggests a transient state where apoptosis might be partially activated before stabilising at 72 hours. This result makes sense when compared to previous studies indicating that EVs can induce early regulatory effects on apoptosis, subsequently adapting to the tissue environment. (Clua-Ferré et al., 2024).

6. Conclusion

EVs derived from ASCs have remarkable anti-inflammatory potential. They have been shown to have the ability to increase expression of anti-inflammatory markers in creeping fat and promising trends in intestinal mucosa. In addition, the anti-inflammatory effect of EVs has been shown to persist for 72h.

Despite the positive results, there are also limitations. For example, EVs have failed to reverse the inflammatory (M1) to anti-inflammatory (M2) phenotype in THP-1 cells. This could be due to the fact that THP-1 cells are an immortalized tumorigenic cell line, which might interfere with the uptake of EVs and limit their effect. This highlights the need for further research to optimise experimental conditions and improve the therapeutic efficacy of EVs.

In conclusion, EVs have promising potential for anti-inflammatory therapies, although further studies are needed to optimise their efficacy and fully understand their mechanisms.

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9. Annex

Supplementary Table 1. TaqMan™ probes used in gene expression analysis by qPCR.

Probes	REFERENCE
18S	Hs99999901_s1
GAPDH	Hs02786624_g1
IL10	Hs00961622_m1
CD163	Hs00174705_m1
MRC1	Gg07156344_m1
FUT4	Hs01106466_s1
MPO	Hs00165162_m1
HLA	Hs00219575_m1
CD11B	Hs00167304_m1
CD66B	Hs00266198_m1
TNF	Hs00174128_m1
IL6	Hs00174131_m1
IL1B	Hs01555410_m1
BAX	Hs00180269_m1
BCL2	Hs00608023_m1