EDUCATIONAL SERIES

# Autologous & Tissue Specific Human Exosomes

## A non-synthetic clinical intervention for hair follicle regeneration

ISHRO ETS ITALIA
SBARRO HEALTH RESEARCH ORG.
International Academy of Regenerative and Precision Medicine

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# About Us

At Sbarro Health Research Organization (SHRO) we specialize in research aimed at finding cures for cancer, cardiovascular and other diseases by identifying their underlying molecular mechanisms. We are engaged in exploring regenerative technologies to provide safer & effective options to Health care practioners.

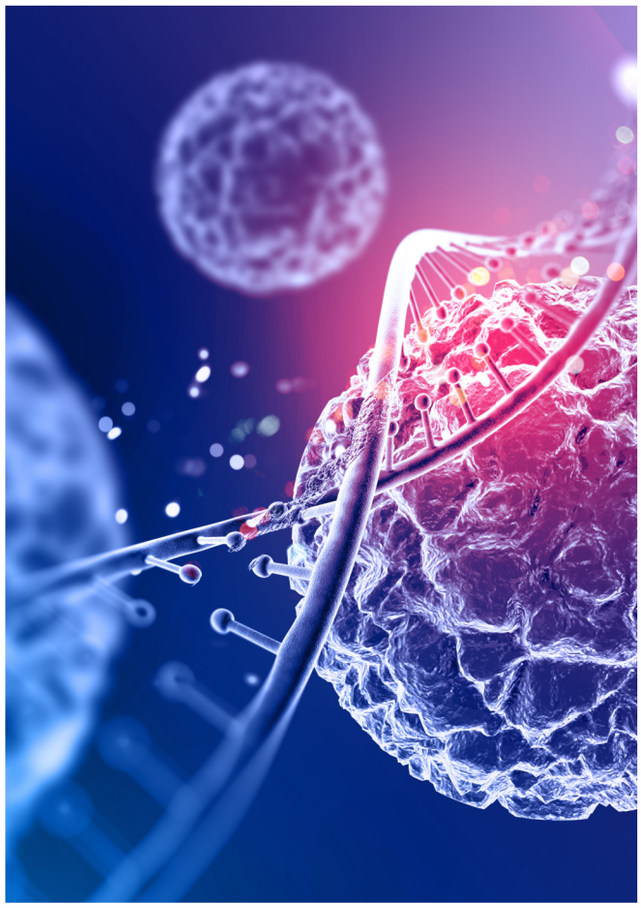
SHRO is dedicated to supporting scientific research aimed at finding cures for cancer, cardiovascular, and other diseases by identifying their underlying molecular mechanisms. SHRO includes the Sbarro Institute for Cancer Research and Molecular Medicine located at Temple University in Philadelphia, PA, and an SHRO affiliated laboratory located at the University of Siena in Siena, Italy. Over 200 SHRO molecular biologists, geneticists, physicists, and chemists work to develop new methods to understand, diagnose, and cure disease.

Sbarro Health Research Organization Italia (SHRO Italia) established in Candiolo, in the province of Turin, is a massive 12,000 square meters facility. This facility has been coordinated by the Sbarro Health Research Organization (SHRO) and other biotech companies.

In addition to giving young researchers a place to call home, SHRO Italia hopes to also attract many talented people & this new entity is a great Italian reality taking shape.

# Introduction

## Paracrine communication the bridge for hair growth



Mesenchymal and epithelial cells interact to drive the development of hair follicles (HF) and hair growth. This interaction is critical for the regulation of hair cell migration and proliferation in adults. Two important regulators of HF development and growth are dermal papilla cells (DPCs) and outer root sheath cells (ORSCs). DPCs are a particular kind of specialized mesenchymal cell that have been shown to influence ORSC proliferation and migration (1),

Recent data (2) indicates that DPCs primarily use a paracrine mechanism to carry out their regulatory role for HF growth. DPCs release a variety of factors to promote the proliferation and differentiation of follicular epithelium and regulate the interactions between mesenchymal and epithelial tissues, such as epidermal growth factor, transforming growth factor-β, and keratinocyte growth factor.

> *Hair follicle (HF) homeostasis is regulated by various signaling pathways. Disruption of such homeostasis leads to HF disorders, such as alopecia, pigment loss, and hair aging.*

The extracellular vesicles (EV) also known as exosomes act as a medium for this paracrine communication(3). According to a recent study, the diameter of DPC-exosomes is roughly 105 nm (4). In mice, DPC-exosome injection postponed catagen and quickened the onset of HF anagen. DPC-exosome treatment increased the migration and proliferation of ORSCs in vitro and induced the expression of Shh and β-catenin(1,3,4).

A recent systemic review (5) identified 16 studies (15 preclinical & 1 clinical) showing varying degrees of efficacy using exosomes derived from sources including adipose-derived stem cells (ADSCs) and dermal papilla cells (DPCs). Applications of exosomes isolated from ADSCs (ADSC-Exo) and DPCs have shown early promising results in preclinical studies corroborated by results obtained from different model systems. The study highlighted the need to warrant and define mechanisms of action, efficacy, and important safety concerns(5).

# Autologous versus Allogenic Exosomes

## US FDA does not approve the use of lab-grown exosome products

**Consumer Alert on Regenerative Medicine Products Including Stem Cells and Exosomes**

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Date: July 22, 2020

If you were hurt or had a bad side effect following treatment with anything that was supposed to be a regenerative medicine product, including, for example, stem cell products and exosome products, we encourage you to report it to the FDA's MedWatch Adverse Event Reporting program. Additional information for patients on reporting adverse events for these products can be found here.

The market is flooded with formulations of exosomes obtained from diverse sources. The main distinctions between the exosomes produced by these manufacturers are the exosome concentration and the exosome source (10, 18).

Currently, placental stem cells, adipocytes, and platelets are the most widely used non-autologous sources of exosomes, which are derived from human donors and utilized in most aesthetic medical procedures (11 ).

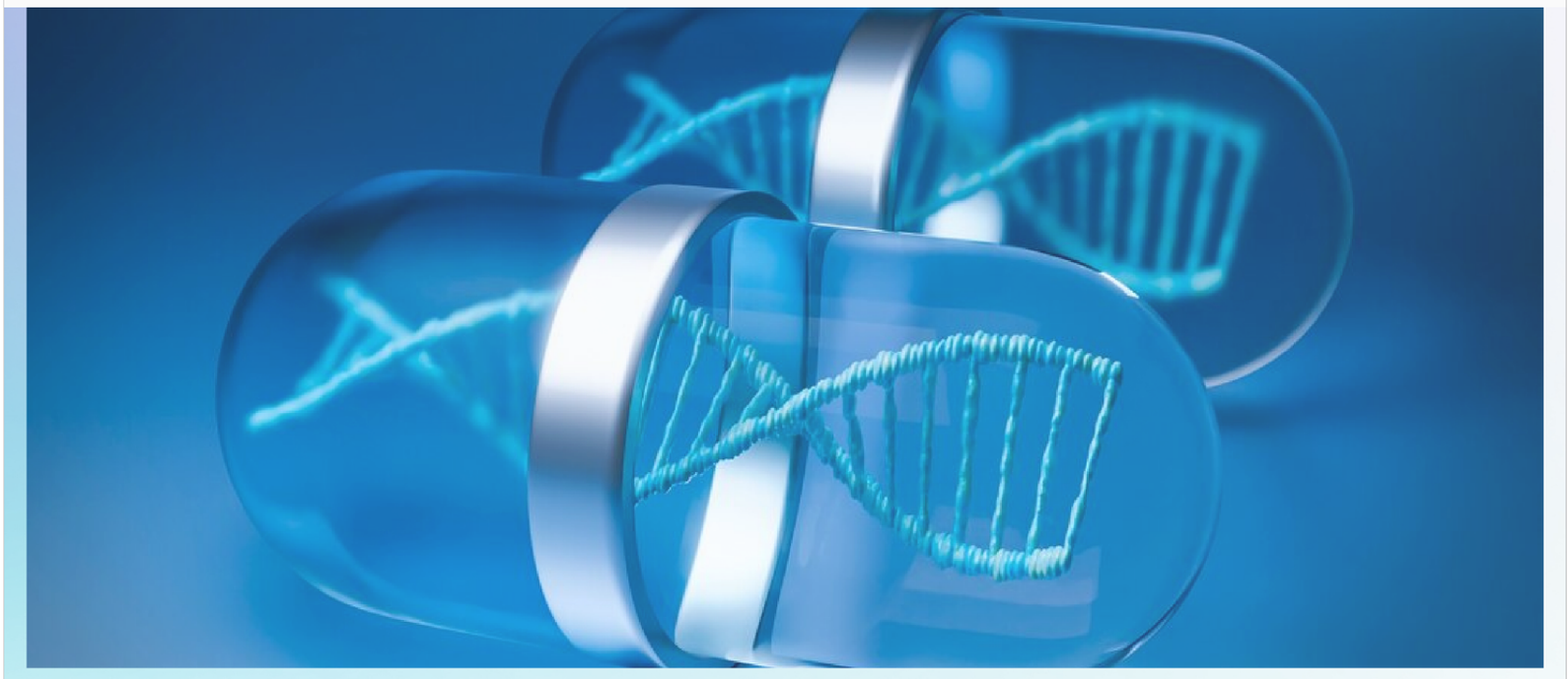
Studies (12) have proven that allogeneic exosomes can induce T cell proinflammatory allogeneic immune response in vitro and in vivo, due to different donors. In addition, the response & from allogenic exosomes depends on the heterogeneity and donor age, sex, body mass index, drug use, race, and other factors (13).

> *Since the use of exosomes derived from human donors is prohibited in many nations now manufacturers are synthesizing exosomes from plants and animals.(19)*

Studies have demonstrated that only live cells or tissues can be used to produce or extract effective exosomes (14) Such formulation of exosomes must either be kept refrigerated and then reconstituted before use, or they must be stored in a specific deep freezer and defrosted beforehand. Any kind of product formulated with autologous & bio-viable exosomes will not have long shelf stability and will have a short lifespan which makes them extremely expensive & difficult to store (15).

Despite an increasing surge of exosome use throughout the aesthetic arena, a paucity of published exosome-based literature exists, and no exosome-based products are currently USA FDA-approved (16). On the other hand, Autologous Micrografting Technology (AMT®) by Rigenera® is a well-established clinical intervention used by physicians to reverse the miniaturization of hair follicles. This technology is supported by more than 80 publications and is the only autologous, homologous, tissue-specific regenerative technique currently available for physicians.

# The Big Question is; the Production & Formulation of Exosomes.



Several technical challenges hinder the widespread use of exosomes The unique heterogeneity and other characteristics of exosomes deter their engineering production(6). The application of exosomes in medical aesthetics is inseparable from their large-scale production and preservation (7).

Engineering processes including separation, purification, and preservation methods have a greater impact on the yield and active components of exosomes (7).

Due to inadequate manufacturing conditions, cases of severe infections were reported some years ago, despite the therapeutic product being marketed as "cell-free" and claimed to be less likely to cause tumorigenesis and infections than stem cell therapy ( 7,8 ).

Adherence to good manufacturing practice (GMP) is paramount for minimizing risks of contamination in the cell-culture system, such as microbes including Mycoplasma, as well as intracellular contents that can trigger the host immune response.

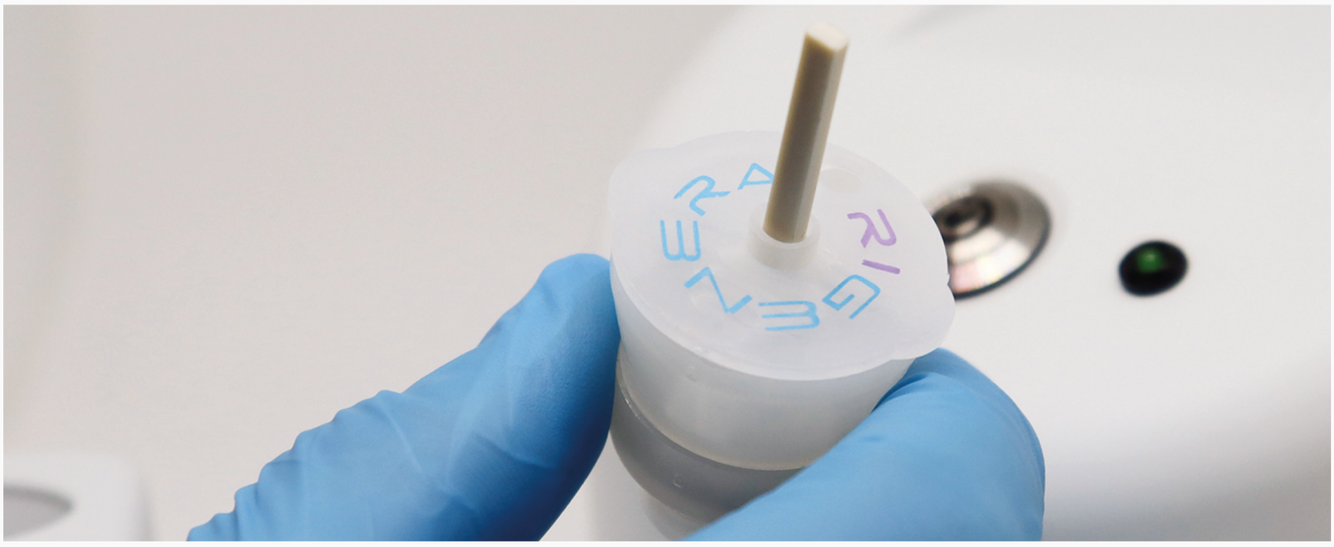
**Major limitations Of EV based therapies**

- *Scalable production of EVs*
- *Uniformity in EV production*
- *Cargo and mechanism of action*
- *Dosage regime*
- *Safety*

To influence biological activity, exosomes must be recognized by surface receptors, fuse to the target cell membrane, and be ingested through endocytosis by the target cell (9).

# Autologous Micrografting

AMT® is the most advanced, innovative, simple, safe, and effective regenerative method, capable of obtaining Autologous Micrografts using the Rigenera® System.




The AMT® technology developed by Rigenera® is an innovative clinical approach to obtain in one surgical time autologous micrografts able to promote derma and bone regeneration. This technology allows the mechanical disaggregation of small tissue samples previously harvested from the patient. Lastly, the device also performs selective filtration, collecting only the cells and tissue particles smaller than 80µm. At the end of the procedure, which lasts only 2 min, the micrografts are collected from the reservoir located at the bottom of the Rigeneracons.

The regenerative role of micrografts was also reported in several in vitro studies, and more important, displayed high positivity to mesenchymal stem cell markers such as CD73, CD90, and CD105. Therefore, the obtained autologous micrografts were identified as progenitor-cell enriched micrografts capable of restoring paracrine communication with the help of cytokines & tissue-specific exosomes (17).

> A recent study was undertaken with University of Ferrara & University of Barcelona to quantify & screen the exosomes present in the micrograft suspension obtained by using the Rigenera® System.

# Study Conducted at Ferrara University

## The study aims to characterize the population of extracellular nanovesicles isolated from mechanically disaggregated skin biopsies using Rigeneracons AS

Rigeneracons devices enable the mechanical disaggregation of tissue biopsies and the collection of the disaggregated product.

Biopsies from cranio-facial (FC) and abdominal (AD) areas were examined in this study. From each disaggregation, a suspension of the disaggregated product was obtained from which extracellular nanovesicles were subsequently isolated.

These extracellular nanovesicles were quantitatively characterized through protein analysis and qualitatively through NTA analysis, providing information about the diameter and distribution of vesicles. Additionally, cytometric analysis was employed for the analysis of surface markers. Activity analysis was conducted through gene expression analysis of markers related to the tissue regeneration process, such as growth factors and metalloproteinases, on inflamed in vitro human dermal fibroblasts (HDF).

Rigeneracons AS

The data provided by the analysis of extracellular nanovesicles isolated in this study confirm that the Rigeneracons AS device is capable of isolating exosomes with dimensions of approximately 150 nanometers, displaying specific surface markers for exosome characterization. These results affirm the presence of dimensional characteristics and surface markers compatible with those reported in the literature.

The characterization of vesicles revealed the presence of populations of extracellular nanovesicles with an average diameter ranging from 95.9 nm to 119.5 nm and a concentration in the range of in the range of $10^8$-$10^{10}$ particles/ml. With a biological sample of 2.5mm, the suspension had a concentration of 8-9 Billion particles/ ML.

This concentration was increased up to 55 billions* when the number of biopsies was increased to three 2.5mm each (Figure 1). The populations of extracellular nanovesicles tested positive for the presence of exosome-specific surface markers such as CD81, CD63 and CD9 that as well established in the literature are exosomes-specific. (Figure 2). *depending on the protocol applied and patient variables

The disaggregation of skin biopsies using Rigeneracons AS devices allows obtaining a suspension containing cutaneous-derived exosomes. These exosomes have demonstrated the ability to modulate the gene expression of inflamed human dermal fibroblasts, influencing specific genes involved in tissue regeneration mechanisms, matrix remodeling, angiogenesis, and cell proliferation such as collagen A3, FGF-2, MMP-9, and IL-10 (Figure 2).

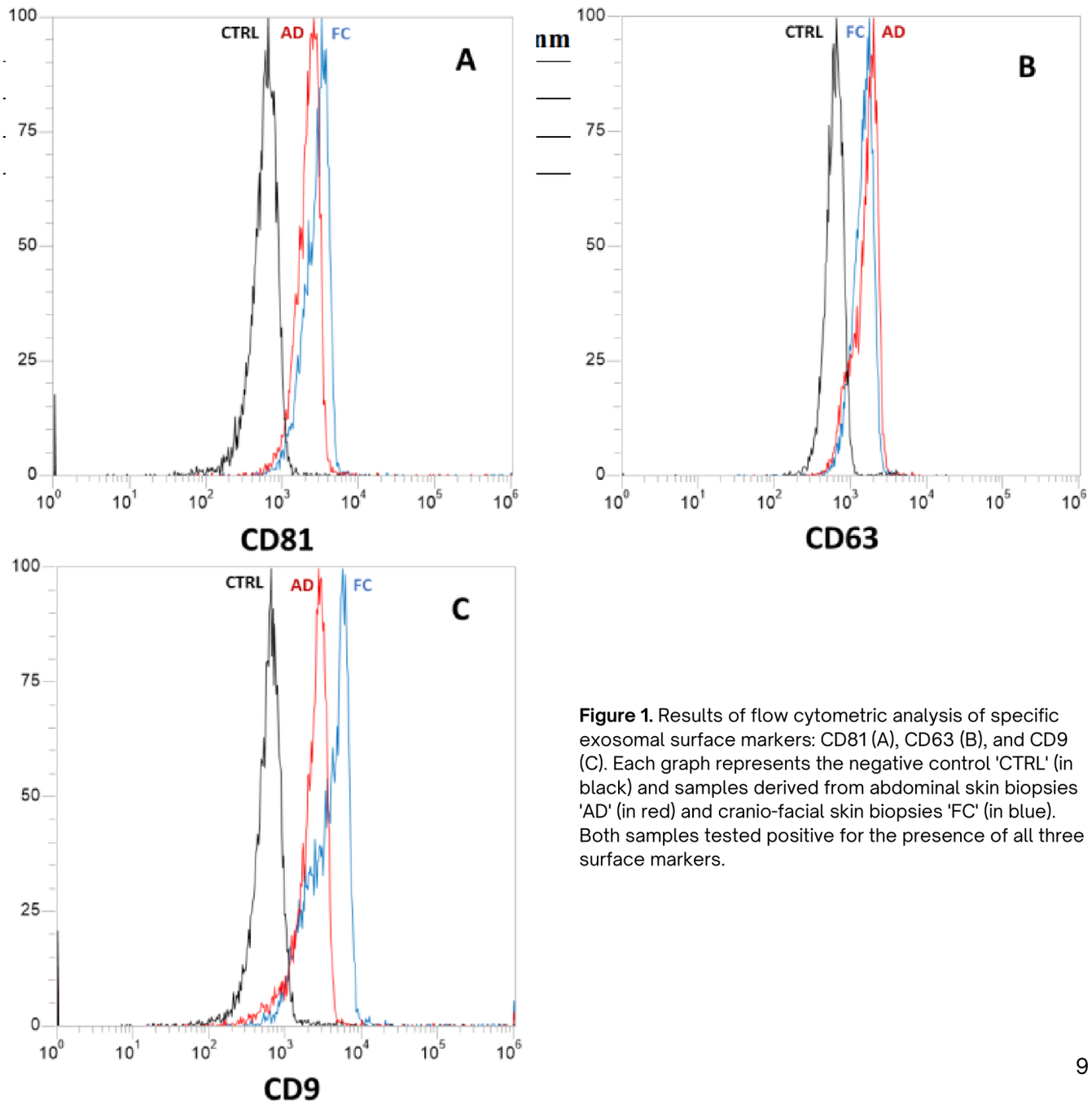



**Figure 1.** Results of flow cytometric analysis of specific exosomal surface markers: CD81 (A), CD63 (B), and CD9 (C). Each graph represents the negative control 'CTRL' (in black) and samples derived from abdominal skin biopsies 'AD' (in red) and cranio-facial skin biopsies 'FC' (in blue). Both samples tested positive for the presence of all three surface markers.

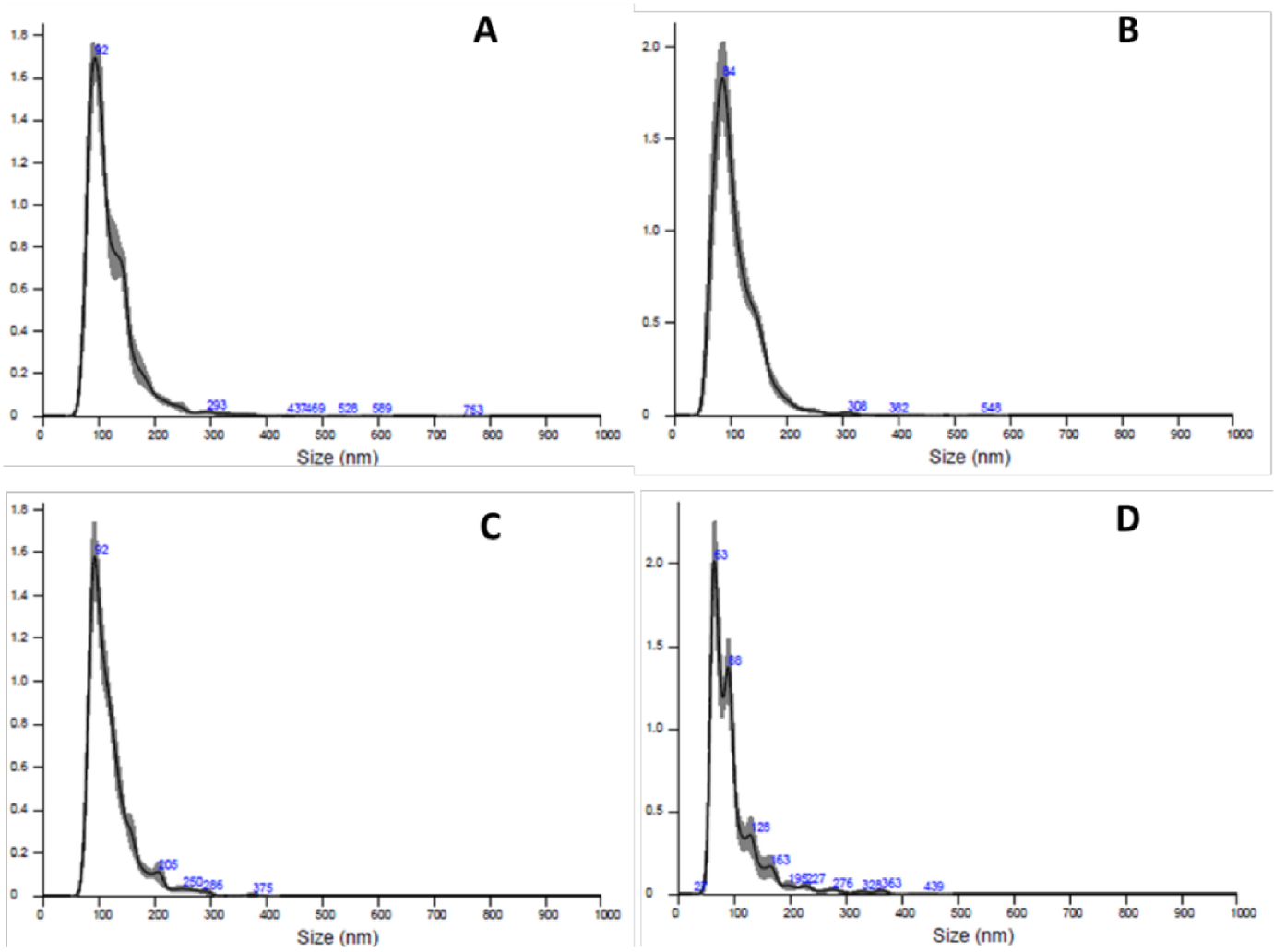



**Figure 2.** Results of the quantitative and dimensional analysis of exosome populations isolated from skin samples carried out by Nanoparticle Tracking Analysis (NTA).

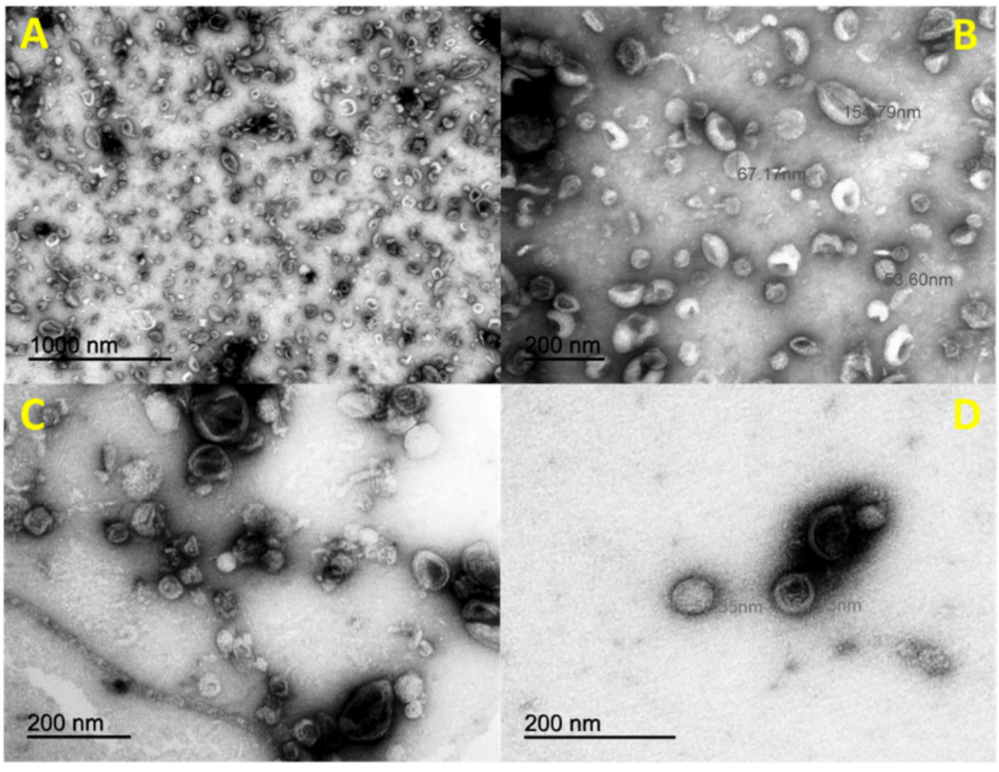



**Figure 3.** Transmission electron microscopy (TEM) images obtained at different magnifications of the microvesicles purified from an AMT solution.

The presence of exosomes in the micrograft suspension obtained by using the Rigenera ® System was also confirmed by a report from the **University of Barcelona**

# Conclusion

In conclusion, the data obtained so far identify the ability of skin tissue-derived exosomes obtained by using the Rigenera® system can influence the gene expression of inflamed human fibroblasts, with the potential to facilitate tissue regeneration.

The existence of autologous & tissue-specific exosomes in the suspension prepared by using 3 biopsies of 2.5mm each explains the paracrine communication-induced restoration & regeneration of hair follicles.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University of Ferrara


# STUDY REPORT

Characterization of exosomes
from skin treated with the 'RIGENERACONS'

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| Device | ID | Biopsy | Donor |
|---|---|---|---|
| Rigeneracons AS | AD1 | 3 punches of 3 mm from abdominal skin biopsy | Gender F Age 87 |
| Rigeneracons AS | FC1 | 2 punches of 3 mm from cranio-facial skin biopsy | Gender M Age 38 |
| Rigeneracons AS | FC2 | 1 punches of 3 mm from cranio-facial skin biopsy | Gender M Age 46 |
| Rigeneracons AS | FC3 | 1 punches of 3 mm from cranio-facial skin biopsy | Gender M Age 26 |

| Device | ID | Biopsy | Donor |
|---|---|---|---|
| Rigeneracons TS | FC3 | 3 punches of 3 mm from abdominal skin biopsy | Gender M Age 61 |
| Rigeneracons TS | FC4 | 2 punches of 3 mm from cranio-facial skin biopsy | Gender M Age 45 |
| Rigeneracons TS | FC5 | 1 punches of 3 mm from cranio-facial skin biopsy | Gender M Age 23 |

**Table 1.** The table lists the processed samples and the devices used.

### 2.2 Isolation of Extracellular Nanovesicles

The obtained disaggregated material was centrifuged at 1200 rpm for 4 minutes to sediment cellular debris. After centrifugation, the supernatant was collected and filtered by passing it through a 0.22µm filter. Vesicles were isolated from the filtrate through ultrafiltration.

### 2.3 Quantitative and Dimensional Characterization

The vesicle population was analyzed in quantitative and dimensional terms using Nanoparticles Tracking Analysis (NTA) with NanoSight (Malvern Panalytical, Malvern, United Kingdom). This analysis provided information on the average size and concentration of vesicle populations.

### 2.4 Characterization of Surface Markers

The presence of extracellular nanovesicles, such as exosomes, within the obtained vesicle suspension was confirmed through flow cytometric analysis, identifying the presence of specific exosomal surface markers such as CD81, CD63, and CD9[1]. Flow cytometric analysis was performed using the Attune NxT Acoustic Focusing Cytometer (Life Technologies, Carlsbad, CA, USA), and the data were analyzed with Attune NxT version 2.5 software (Life Technologies).

### 2.5 Culture and Treatment of Human Dermal Fibroblasts

The human dermal fibroblasts (ATTC, Manassas, Virginia) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Euroclone, Milan, Italy) supplemented with 10% Fetal Bovine Serum (Euroclone) and 2% Penicillin-Streptomycin (Sigma) at 37°C, 5% CO2 in a humidified environment until reaching confluence. The experiment involved seeding $0.05*10^6$ cells/well in a 24-well plate. After 48 hours of seeding, an inflammatory treatment was administered; the fibroblasts were incubated for 24 hours with inflammatory DMEM medium (Euroclone) supplemented with 10ng/ml TNFα (Merck, Darmstadt, Germany). At the end of the inflammation period, the medium was replaced with fresh culture medium. The cells were then treated with the previously isolated nanovesicle suspensions and incubated for an additional 24 hours.

### 2.6. Extraction and Purification of Cellular RNA

At the end of the incubation period, the cells were lysed, and total RNA was extracted using Total RNA Purification Kits (Norgen Biotek Corp, Thorold, Ontario, Canada), following the protocol provided by the manufacturer.

### 2.7 Gene Expression Analysis

The RNA samples were quantified using NanoDrop (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Reverse transcription was performed using the SensiFAST cDNA Synthesis Kit (Meridian Bioscience, Cincinnati, Ohio, United States), and gene expression analysis was carried out using Rotor-Gene Q (Qiagen, Hilden, Germany) and SensiFAST™ SYBR® No-ROX Kit (Meridian Bioscience) following the manufacturer's protocol. The data were processed using Q-Rex Software (Qiagen).



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## 3. Results

### 3.1. Characterization of Extracellular Nanovesicles

All samples were processed, and the vesicles were analyzed.

Samples AD1, FC1, FC2, FC3 processed with AS:

The characterization of vesicles revealed the presence of populations of extracellular nanovesicles with an average diameter ranging from 95.9 nm to 119.5 nm and a concentration in the range of $10^8$-$10^{10}$ particles/ml (Table 2, Figure 1). The populations of extracellular nanovesicles tested positive for the presence of exosome-specific surface markers (Figure 2).

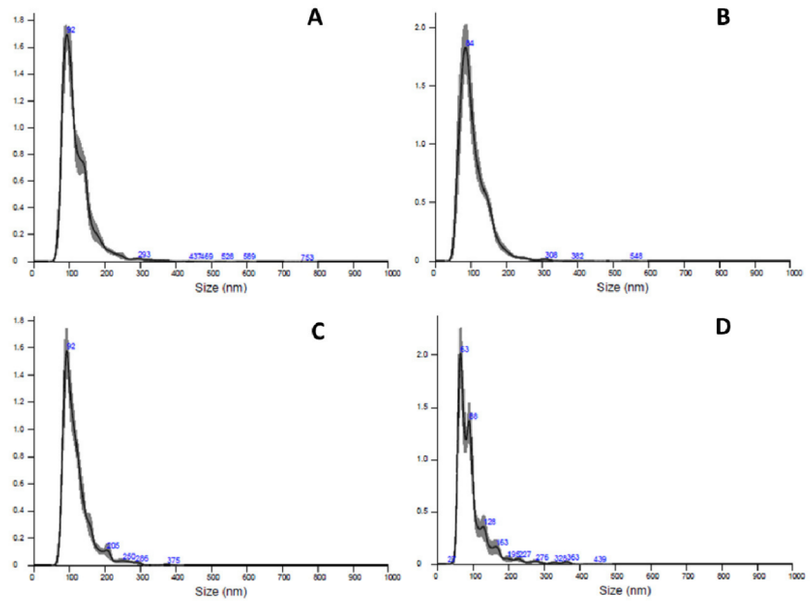


**Figura 1** e **Tabella 2.** Risultati dell'analisi quantitativa e dimensionale delle popolazioni di esosomi isolate dai campioni cutanei effettuata mediante Nanoparticle Tracking Analysis (NTA). **(A)** campione AD1, **(B)** campione FC, **(C)** campione FC2, **(D)** campione FC3.

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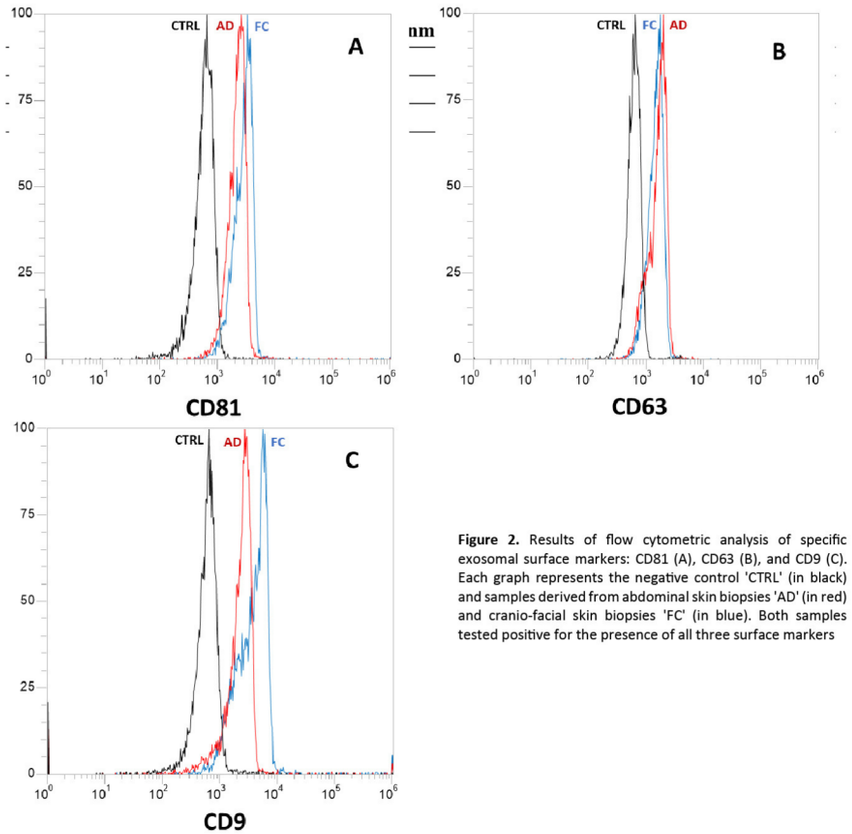


**Figure 2.** Results of flow cytometric analysis of specific exosomal surface markers: CD81 (A), CD63 (B), and CD9 (C). Each graph represents the negative control 'CTRL' (in black) and samples derived from abdominal skin biopsies 'AD' (in red) and cranio-facial skin biopsies 'FC' (in blue). Both samples tested positive for the presence of all three surface markers

Samples FC4, FC5, FC6 processed with Rigeneracons TS did not yield a sufficient quantity of exosomes for analysis. The received samples were, in fact, of sizes and quantities incompatible (too few) with those required by the device (received 1 punch instead of the 5 needed for processing).

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### 3.2. Gene Expression Analysis

The exosomes isolated from AS1, FC1, FC2, FC3 samples treated with Rigeneracons AS were used to treat inflamed human fibroblasts. The obtained data were collected and normalized to the untreated inflamed control. Gene expression analysis revealed a differential expression of genes involved in wound healing and regeneration among the cell populations treated with vesicles compared to the control, such as IL10, FGF2, VEGFA, TGFB1, IL1B, TNF, MMP1, MMP9, COL1A1, COL3A1 (Table 3, Figure 3).

| Gene | | Fold Change | |
|---|---|---|---|
| | | AD | FC1 |
| TNF | Tumor Necrosis Factor | 1,08 | 1,08 |
| IL1B | Interleukin 1 Beta | 1,95 | 2,69 |
| COL1A1 | Collagen 1 A III | 0,71 | 0,65 |
| COL3A1 | Collagen 3 A I | 2,34 | 3,39 |
| FGF2 | Fibroblast Growth Factor 2 | 5,92 | 8,91 |
| VEGFA | Vascular Endothelial Growth Factor | 1,98 | 2,68 |
| IL10 | Interleukin 10 | 1,97 | 6,70 |
| TGFB1 | Transforming Growth Factor Beta 1 | 0,75 | 0,74 |
| MMP1 | Matrix Metalloproteinase 1 | 1,89 | 2,21 |
| MMP9 | Matrix Metalloproteinase 9 | 2,93 | 7,19 |

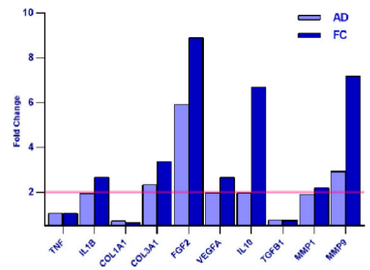

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**Table 3** and **Figure 3.** Result of the differential gene expression analysis performed by RT-qPCR. The graph depicts the AD and FC samples - as indicated in the legend - on the x-axis and the differential expression indicated as the Fold Change value on the y-axis. A Fold Change value ≥ 2 indicates an increase in the expression of the examined gene in the sample compared to the control (highlighted in yellow), while a value < 2 indicates that no difference in expression was detected between the two conditions.

The difference in gene expression is defined as the Fold Change, a value ≥ 2 indicates an increase in the expression of the examined gene in the sample compared to the control, a value < 2 indicates that no difference in expression was detected between the two conditions.

**4. Discussion and Conclusion**

Exosomes are extracellular nanovesicles of significant interest in regenerative medicine. These vesicles are constitutively secreted by various cell types, and their content and biological functions are closely linked to the biological state of the progenitor cell. Characterized by dimensions ranging from 30 to 200 nm in diameter and the presence of specific surface markers such as CD81, CD63, and CD9[1], these nanovesicles play a crucial role in cellular communication.

The data provided by the analysis of extracellular nanovesicles isolated in this study confirm that the Rigeneracons AS device is capable of isolating exosomes with dimensions of approximately 150 nanometers, displaying specific surface markers for exosome characterization. These results affirm the presence of dimensional characteristics and surface markers compatible with those reported in the literature (Table 2, Figure 1, Figure 2).

The disaggregation of skin biopsies using Rigeneracons AS devices allows obtaining a suspension containing cutaneous-derived exosomes. These exosomes have demonstrated the ability to modulate the gene expression of inflamed human dermal fibroblasts, influencing specific genes involved in tissue regeneration mechanisms, matrix remodeling, angiogenesis, and cell proliferation (Figure 3, Table 3).

The analysis does not indicate a significant difference in the effect of exosomes derived from abdominal and cranio-facial biopsies, except for the expression of the anti-inflammatory IL10, which



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is higher in facial samples. However, we do not consider this difference significant, considering the patient's age (87) in the abdominal sample, much higher than the average age of donors for facial region samples (average age 40).

It has been identified that treatment with exosomes isolated from cranio-facial tissue biopsies leads to an increase in the synthesis of IL10 (an inhibitor of pro-inflammatory cytokine synthesis[2]), FGF2 (a factor stimulating cellular proliferation mechanisms and tissue repair[3]), VEGFA[4] (a factor promoting angiogenesis in wound healing), MMP9[5] (involved in angiogenesis, wound closure, and matrix remodeling), COL3A1 (a protein involved in the structural integrity of many tissues), IL1B (an inflammation mediator), and MMP1[6] (involved in extracellular matrix remodeling). No significant differential expression was observed for TGFB1 (an inhibitor of cytokine secretion), TNFa (a pro-inflammatory factor), and COL1A1 (a structural protein present in many tissues)[7].

In conclusion, the data obtained so far identify the ability of skin tissue-derived exosomes to influence the gene expression of inflamed human fibroblasts, with the potential to facilitate tissue regeneration.

The limited amount of tissue processed for samples FC2 and FC3 did not allow the identification of significant gene expression variations, and therefore, they are not included in this report. Based on the same consideration, data from subsequently obtained samples (FC4 M 61a, FC5 M 45a, and FC6 M 23a) are not presented in this report.

UNIVERSITAT DE BARCELONA

# STUDY REPORT

## Detection of exosomes in the AMT ® suspension obtained with the Rigenera® System

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**Client:** Regenera Activa Worldwide.

**Report:** 493

## DETECTION OF EXOSOMES IN AMT EXTRACT

**Objective:**

Isolation and observation of exosomes from an AMT solution obtained on the Rigeneracons 7945 AS device.

**Experimental procedure:**

1. **AMT extract**

The AMT extract consists of a human skin biopsy homogenate obtained in saline with the Rigeneracons 7945 AS device (see **Figure 1**).

The samples (biopsies) are placed on the grid of the device, then 0.9% saline solution is added and mechanically disaggregated using the Sicurdrill mechanical disruptor. The AMT solution, which includes the starting tissue components, is then recovered.

**Figure 1.** Rigeneracons 7945 AS Device.

2. **Isolation of exosomes.**

The isolation of exosomes is performed using fresh AMT solution by means of differential ultracentrifugation technique, which consists of a sequence of centrifugations applied to the sample with increasing centrifugal forces (g) (300xg, 2000xg, 10,000xg, 100,000xg) to remove larger particles and ultimately precipitate the exosomes. With this technique, the most of the exosomes that are isolated remain intact.

3. **Characterization of the isolated exosomes.**

The isolated and precipitated exosomes, are resuspended in phosphate buffer for characterisation by transmission electron microscopy. The sample is loaded onto 200 mesh copper grids with Formvar and carbon coating. For observation, negative contrast staining with uranyl acetate is performed. The samples are visualised in a 100kV JEOL JEM 1010 transmission electron



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microscope, especially indicated for studies of biological samples. The microscope is equipped with an Orius CCD digital camera (Gatan Inc.) to acquire images of the samples.

**Results:**

Images of electron microscopy slides, obtained at different magnifications, show vesicular structures with a lipid bilayer membrane, ranging in size from 50 to 150 nm and compatible with exosomes (**Figure 2**).

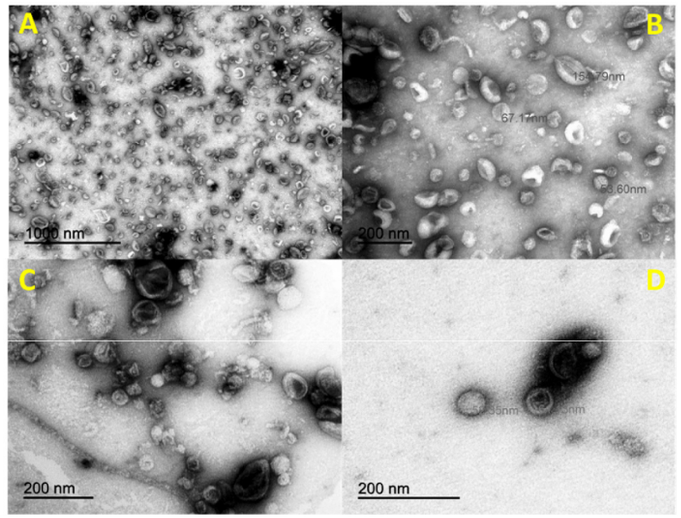


**Figure 2.-** Transmission electron microscopy (TEM) images obtained at different magnifications of the microvesicles purified from an AMT solution.

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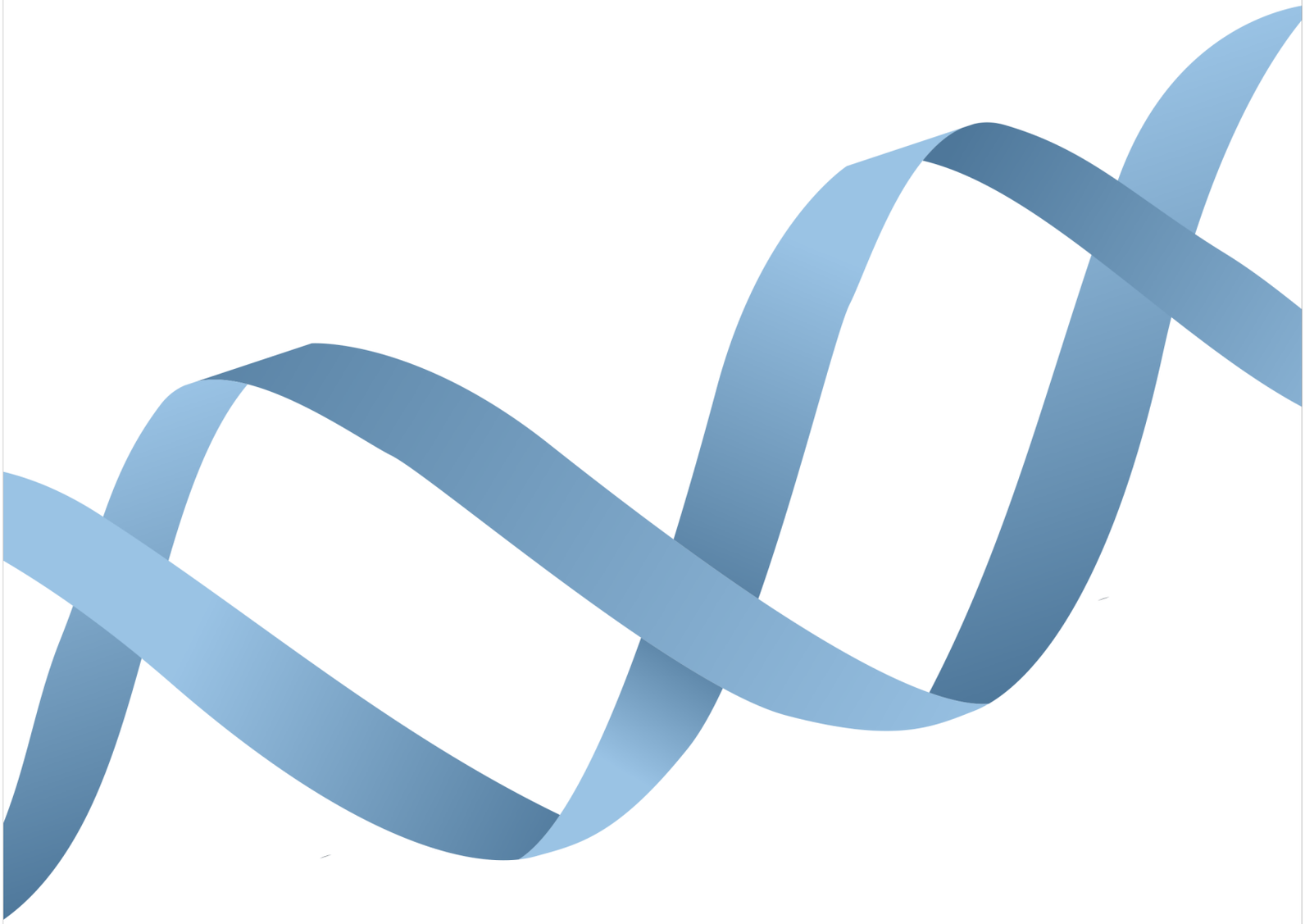
Barcelona, the 14th of February of 2024

Dr. David Ricart i Jané
Project Manager
CEREMET

**CEREMET has a management system certified according to ISO 9001 by SGS ICS.**



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