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# **DOCTORAL THESIS**

**Study regarding harvesting and exploring the  
mesenchymal stem cells properties during bioprinting a  
human meniscus structure**

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## **CHAPTER 1: MEDICAL ETHICS ISSUES.**

Patients in the study provided written informed consent, agreeing to surgery and research details, including research duration, expected outcomes, procedures, benefits, risks, data protection, confidentiality, voluntary participation, and opt-out options. Vulnerable populations were excluded from participant selection. Patient data adhered to European and national regulations governing personal medical information. Protocols were established for data collection, processing, and storage, ensuring compliance with legal standards.

The study followed meticulous ethical procedures, starting with approval from the local Ethics Committee and compliance with national, European, and international regulations. Human tissues were acquired during surgical procedures, labeled, and anonymized in accordance with legal requirements. The transfer of samples adhered to strict protocols and legal norms, maintaining the study's integrity.

## **CHAPTER 2: MATERIALS AND METHODS.**

Biological material was collected from four patients hospitalized in the Orthopedics section of the Emergency Military Clinical Hospital "Dr. Victor Popescu" Timisoara. Two patients underwent arthroscopic surgery for anterior cruciate ligament reconstruction, and two underwent knee replacement surgery. Hoffa's fat was harvested during knee arthroplasty. After skin preparation and under spinal anesthesia, a 15 cm incision was made in the middle of the knee. The quadriceps tendon and patellar border were identified, and an arthrotomy was performed. Hoffa's fat, located behind the patellar tendon, was excised to improve access to the knee's external compartment and alleviate potential post-arthroplasty knee pain.

Arthroscopic surgery required precise patient positioning with the lower limbs suspended. Under spinal anesthesia, the knee was accessed through lateral and medial incisions. Specialized techniques, including arthroscopic Hoffa fat harvesting, were employed using forceps or a motorized shaver. Partial Hoffa fat resection was performed when necessary for ligament reconstruction, aided by FDA-approved collection systems like Aquavage. Harvested biological material was carefully transferred to Falcon tubes pre-filled with 15 ml of PBS and a 1% Penicillin-Streptomycin solution. These tubes were placed in a pre-refrigerated container and then transferred to the "Pius Brânzeu" Timișoara County Emergency Clinical Hospital's Immunology department for analysis, ensuring a seamless transition from surgery to scientific evaluation.

In the lab, harvested tissue samples were meticulously handled in sterile conditions. They were placed in sterile 15 cm Petri dishes (Falcon) and washed with a PBS solution containing 2% Pen-Strep within controlled environments. The tissues were then dissected and minced with sterile scalpel blades and scissors. Special Petri dishes were prepared to facilitate mesenchymal cell adhesion, and the fat tissue was transferred to these plates. The Petri dishes were prepared with a growth medium containing human mesenchymal cell (hMSC)-specific components. These dishes were incubated at 37°C with 5% CO<sub>2</sub> for optimal cell growth. Culture medium was regularly replaced every 3-5 days. By day 9, tissue fragments were aspirated, and the culture medium was changed, marking a critical phase in the process. Upon achieving a minimum cell confluence of 80%, trypsinization was performed, followed by centrifugation of the cell suspension. The cells were resuspended in PBS and counted using a hemacytometer. Cultures were maintained at a density of approximately  $3\text{-}5 \times 10^4$  cells per cm<sup>2</sup> and partially frozen, marking the successful culmination of this intricate process.

The culture medium was crucial in differentiating cells into chondrocytes. It contained various substances, including Dexamethasone, Ascorbic acid-2 phosphate, Sodium pyruvate, Proline, Insulin-Transferrin-Selenium, TGF  $\beta$ 1, and 1% Pen-Strep, which facilitated cell differentiation. ChondroDiff medium combined with 1% Pen-Strep was also used to support growth and differentiation. After 24 hours, cell adhesion to Petri dish plastic marked a

significant phase in the experiment. Petri dish 1 continued with MSC-specific culture medium, retaining hMSC identity, while Petri dish 2 underwent a specific differentiation process for later use. The Bioprinting System was a comprehensive technological setup, including hardware like the bioprinter, bioink, and software for scaffold design. Cinema 4D software was used for CAD modeling of a human lateral meniscus, informed by extensive research on meniscus dimensions. The stereolithographic format (.stl) file was transferred to the bioprinter software, ensuring precision. The design was based on empirical research, magnetic resonance analysis, and validation against anatomical data. Average measurements, such as circumference, horn width, and height, were used to ensure anatomical accuracy. This interdisciplinary approach, guided by academic research and medical expertise, highlights the complexity of scaffold design in this advanced medical application.

The INKREDIBLE bioprinter by Cellink in Sweden is a high-precision pneumatic extrusion model with a 10-micron XY axis accuracy and 2.5-micron Z axis accuracy. It offers UV LED crosslinking, 100-micron layer resolution, and handles a wide range of gel viscosities. To maintain sterility, it operates within a Biological Safety Cabinet equipped with a HEPA H14 EN 1822 filter. Cellink bioink combines nanofibrillar cellulose with sodium alginate and is crosslinked using a water and calcium chloride-based agent. This versatile ink adapts to various tissue types and is essential for bioprinting success. The bioprinting process involves sterilization, parameter setting, bioink preparation, and printing. Scaffold size is scaled to 60% of a human meniscus. Controls include acellular structures and test gels. Differentiated chondrocytes from adipose-derived stem cells are incorporated into the meniscus, and mechanical tests assess strength.

The xCELLigence RTCA system, using electrical impedance with microelectrodes, was employed for real-time cell analysis. It assesses cell growth, cytotoxicity, proliferation, differentiation, adhesion, migration, invasion, and ligand-receptor interactions. This system continuously monitors live cells in optimal conditions, providing reliable data throughout the culture. The flow cytometry process involved trypsinization, centrifugation, labeling with multiple antibodies, a 30-minute dark incubation, thorough washing, and resuspension in Cell Wash solution. This method allowed for the detailed investigation of cellular markers and characteristics, demonstrating the rigor of cell preparation for flow cytometry. For immunocytochemistry and immunofluorescence, adherent cells were fixed, labeled with primary antibodies, and incubated. Immunofluorescence was performed in the dark using fluorescent secondary antibodies and DAPI for nuclei staining. Immunocytochemistry involved mouse-produced primary antibodies against specific human markers. Both methods contributed to the detailed analysis of cellular characteristics.

## **CHAPTER 3: RESULTS.**

The experiment started with tissue fragment placement in Petri dishes, and over six days, cells began migrating and adhering to the plastic surface. By the ninth day, a complex network formed, and on day thirteen, cell presence intensified. These observations supported the identification of mesenchymal stem cells derived from adipose tissue, shedding light on their growth patterns. Viability tests via flow cytometry revealed varying cell viability percentages among different passages. Samples 1 and 2 exhibited reduced viability and higher early apoptosis rates. Sample 2 showed significant late apoptosis, rendering it unsuitable for bioprinting. Samples 3 and 4, with higher viability and lower apoptosis rates, emerged as ideal candidates for further bioprinting studies. These findings emphasize the importance of selecting optimal cell material for bioprinting endeavors. Continuous monitoring of cell indices revealed a stabilization period approximately four hours after plating, followed by growth and division within the 4-24 hour window. Comparison between Adipose-Derived Stem Cells (ADSCs) and Bone Marrow Mesenchymal Stem Cells (BM-MSC) demonstrated similar doubling times, highlighting universal cellular dynamics. These results contribute to

understanding stem cell proliferation and offer a reference for researchers working with diverse cell types.

Flow cytometry techniques play a pivotal role in the precise identification and characterization of cell populations, a crucial aspect of our recent investigations into mesenchymal stem cells (MSCs). Specifically, our analyses encompassed MSCs derived from Hoffa adipose tissue (ADSC) and cells directed toward the chondrocyte lineage. This methodological approach involved the assessment of both positive (CD90, CD73, CD29, CD44, CD105, and CD117) and negative (CD106, CD34, CD146, alpha-SMA, E-cadherin, and CD95) markers. Examining the expression of these markers across cell populations from four distinct samples revealed notable differences, indicative of inter-individual variability. This variance underscores the unique attributes of each sample and emphasizes the intricate diversity within MSCs, even when originating from seemingly uniform tissue sources. Inter-individual variability isn't unique to Hoffa adipose tissue-derived stem cells; it is also observed in mesenchymal stem cells from hematogenous bone marrow. These observations underscore that stem cells, regardless of their origin, possess distinct expression profiles that can significantly influence their behavior and therapeutic potential. Despite the inter-individual variability, our results affirm that stem cell-characteristic markers are consistently present, albeit in varying proportions. This emphasizes that while the expression levels of specific markers may differ among samples, the fundamental attributes defining stem cells persist. Thus, variability in marker expression adds complexity to our understanding of these cells rather than negating their inherent stem cell identity.

Our research delved into the unique characteristics of Hoffa adipose tissue-derived stem cells (ASC/ADSC) using various analytical techniques. Firstly, we focused on positive markers CD90, CD29, and CD105, core indicators of stem cell potential. Surprisingly, these markers showed consistent expression across different samples, suggesting uniform stem cell characteristics despite variations in tissue sources. In contrast, CD26 and CD44, related to cell activation and adhesion capacity, displayed significant individual variability, highlighting the complex nature of ASC/ADSC. In the context of chondrocyte markers, our analysis revealed remarkable consistency in the expression of CD90, CD44, CD29, and TGF-beta across diverse samples. Moreover, these markers exhibited similar patterns in comparison to mesenchymal stem cells, implying shared fundamental roles. This uniformity enhances our understanding of chondrocytes and MSCs, particularly in tissue engineering and regenerative medicine applications. We also employed immunocytochemistry to detect specific proteins within the isolated ASC/ADSC cells. Positive staining for CD105 and Vimentin affirmed their presence and contributed to understanding cell identity. Conversely, the absence of VEGF, Ki67, CD117, and cytokeratin revealed distinct characteristics, further delineating the cellular profile. These findings collectively enrich our comprehension of ASC/ADSC, facilitating future research and therapeutic applications by emphasizing the importance of recognizing individual variability in cell properties.

Our study involved bioprinting cellularized menisci, a crucial step in tissue engineering, ensuring the even distribution of cells in the printed structures. This uniformity is essential for replicating the natural meniscus's integrity and function. Moreover, we successfully maintained consistent size and shape characteristics across all printed menisci, mirroring surgically excised human menisci with precision. To achieve accurate measurements, we used a high-precision digital caliper with an error margin of  $\pm 0.015\text{mm}$ . Our measurements encompassed various dimensions, including the average circumference ( $93.2\text{mm} \pm 2.1\text{mm}$ ) and specific measurements for different anatomical regions, reflecting the meniscus's complex geometry and functionality. We also conducted cell viability analysis two days post-printing, revealing that the majority of cells (approximately 40 million out of 50 million) remained within the printed tissue and were viable. Despite some cell loss, this outcome signifies a successful bioprinting process with promising implications for future therapeutic applications. In terms of mechanical strength, compression tests indicated that our bioprinted menisci exhibited unique mechanical properties compared to native tissue. While differences were observed, further exploration of

cell-enriched and non-cell-enriched samples highlighted the role of cells, particularly their secreted extracellular matrix, in modulating mechanical behavior.

## **CHAPTER 4: DISCUSSIONS.**

In tissue engineering, the potential to revolutionize regenerative biomedicine and address the shortage of donor organs is on the horizon. With over 100,000 patients in need of transplants, tissue engineering offers a promising solution to this pressing public health issue. However, creating functional organs from a patient's own cells is a complex process involving various physiological factors, cell cultures, vascular network establishment, innervation, and interaction with adjacent tissues. Three-dimensional printing in the medical field offers a versatile range of applications, from anatomical models for preoperative planning to customized implants, prostheses, and even drug screening tools. In vitro platforms using multidimensional and multicellular interactions enable patient-specific medical solutions. However, while these technologies hold great promise, they also present challenges, such as achieving high cellularization and post-bioprinting cell viability, which necessitate further investigation.

The morphological analysis of adipose-derived mesenchymal stem cells (ADSCs) is essential for their application in regenerative medicine and tissue engineering. These cells demonstrated promising characteristics, including proliferation potential and fibroblast-like morphology, making them valuable for in vitro expansion and potential use in cell-based therapies. Future research should explore the relationship between donor characteristics and ADSC properties, and expand sample diversity to enhance the generalizability of findings. Understanding these cellular aspects is crucial for optimizing ADSCs in tissue engineering and regenerative medicine, promising improved outcomes for patients in need of advanced treatments..

## **CHAPTER 5: CONCLUSIONS.**

In conclusion, this doctoral work has achieved significant milestones with profound implications for regenerative medicine and 3D bioprinting technology. The successful isolation and characterization of ADSC-like mesenchymal stem cells from Hoffa adipose tissue, along with their distinct viability and proliferation patterns, represent a promising source for addressing osteoarticular pathologies. The demonstrated capability of these cells to differentiate into chondrocytes is a critical step forward in cartilage repair applications. This work not only broadens the scope of stem cell therapies but also contributes to the understanding of unique cell sources for tissue engineering.

Furthermore, the creation of a human lateral meniscus using Cinema 4D software and the Inkredible 3D printer marks a pioneering achievement in Romania and signifies a significant technological advancement. While the bioprinted menisci exhibited lower mechanical strength compared to natural counterparts, this discovery provides a foundation for future enhancements in extracellular matrix synthesis and mechanical resilience. Various methods, including the use of pre-differentiated chondrocytes, have been explored, revealing the importance of pre-differentiation in achieving superior cellularization and specific chondrocyte markers.

This comprehensive study not only advances our knowledge of novel stem cell sources and complex 3D tissue structures but also highlights the challenges and opportunities in bioprinting technology. Further research will be essential to address issues such as increasing cellularization and improving post-bioprinting cell viability. Ultimately, the findings of this doctoral work bring us closer to translating these breakthroughs into clinical applications for osteoarticular diseases, offering hope and new possibilities for patients in need of effective treatments.