# "CAROL DAVILA" UNIVERSITY OF MEDICINE AND PHARMACY, BUCHAREST
# DOCTORAL SCHOOL
# MEDICINE

***Optimization of the osteochondral regeneration process of the knee, through the use of new composite materials based on collagen, hydroxyapatite and keratin***

## DOCTORAL THESIS SUMMARY

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# DOCTORAL THESIS SUMMARY

# 1. Purpose and objectives of the study

**1. <u>The fundamental research problem:</u>**

Osteochondral defects of the knee, which simultaneously involve the articular cartilage and subchondral bone, constitute a complex challenge in orthopedics and sports medicine, having a significant impact on joint functionality and quality of life of patients(1,2). These lesions can develop as a result of trauma, degenerative processes (such as osteoarthritis) or congenital malformations and can rapidly progress to severe joint degeneration, especially in weight-bearing joints(3,4). The lack of effective and clinically translatable biomaterial solutions imposes the need for new regenerative strategies aimed at synergistic restoration of cartilage and subchondral bone(5,6).

**2. Research hypothesis:**

It is assumed that the development of innovative biomimetic composites based on collagen (COL), hydroxyapatite (HA) and keratin (CHER/K) may lead to the obtaining of materials capable of efficiently supporting the three-dimensional regeneration of the osteochondral unit(7,8). By combining the osteoconductive properties of hydroxyapatite, the structural and immunomodulatory capacity of keratin, and the biocompatibility of collagen, the central hypothesis is that these composites can stimulate cell differentiation, tissue integration and efficient osteo-cartilaginous regeneration(9,10).

**3. Research objectives:**

The research objectives aimed to obtain biomimetic composites based on collagen, hydroxyapatite and keratin (COL:HA:K), using reproducible synthesis methods by chemical crosslinking and lyophilization, adapted to the processing of natural materials. The resulting composites were subjected to rigorous physicochemical characterization by scanning electron microscopy (SEM), FTIR and Raman spectroscopy, as well as by absorption and biodegradability tests, to evaluate their stability and behavior in biological environments. In the in vitro stage, the biocompatibility and osteoinductive capacity were tested using human mesenchymal stem cells (MSC)(11,12), by cell viability tests (XTT) and immunofluorescent staining for specific markers. Based on the results, the most promising formulations were selected for in vivo testing on an animal model (Wistar rat), in order to validate the biological

performance under physiological conditions. Postoperative monitoring included imaging assessments (radiographs, micro-CT)(13), histological analyses and serological determinations of cytokines (IL-6, IL-8, IL-10, TNF-α)(14) and osteogenic markers (ALP, CRP)(15), to analyze tissue integration and immunological profile of the tested composites.
- Identification of an optimal formulation (e.g. F6) with superior regenerative and immunological properties, which would offer potential for clinical translation in the treatment of osteochondral defects.

**4. Research methodology:**

The research was structured in three major stages, following a progressive and integrative approach, from the synthesis of materials to their in vivo testing.

**Stage I - Synthesis and characterization of composites**

The biomimetic formulations were obtained by chemical crosslinking with glutaraldehyde, followed by lyophilization, to generate porous, stable structures suitable for tissue regeneration. The physicochemical characterization included:

 Scanning electron microscopy (SEM), used to analyze the morphology and porosity of the structure;

 FTIR and Raman spectroscopy, to identify the chemical composition and molecular interactions between components;

 Functional tests regarding water absorption, stability in enzymatic media (collagenase) and biodegradability of the scaffolds.

**Stage II - In vitro studies**

The biocompatibility of the composites was tested using human mesenchymal stem cells (MSC), evaluated by:

 XTT test, to determine cell viability;

 Eosin-Hoechst and immunofluorescence staining, highlighting the specific markers vimentin (cytoskeleton) and fibronectin (extracellular matrix);

 Analysis of cell adhesion and proliferation, monitored by examining the behavior of cells on the surface of the scaffolds.

**Stage III - In vivo studies (animal model)**

Two preclinical experiments were conducted on Wistar rats to evaluate the regenerative functionality of the selected composites (F1, F2, F5, F6). The stage included:

 Bilateral femoral implantation and monitoring of tissue integration;

 Imaging monitoring through standardized radiographs and micro-CT for three-dimensional morphological analysis of bone regeneration;

 Histological and immunohistochemical analyses, for qualitative evaluation of regeneration;

 Serological analyses at 30 and 60 days post-implantation (IL-6, IL-8, IL-10, TNF-α, CRP, ALP), for characterization of the inflammatory and osteogenic response;

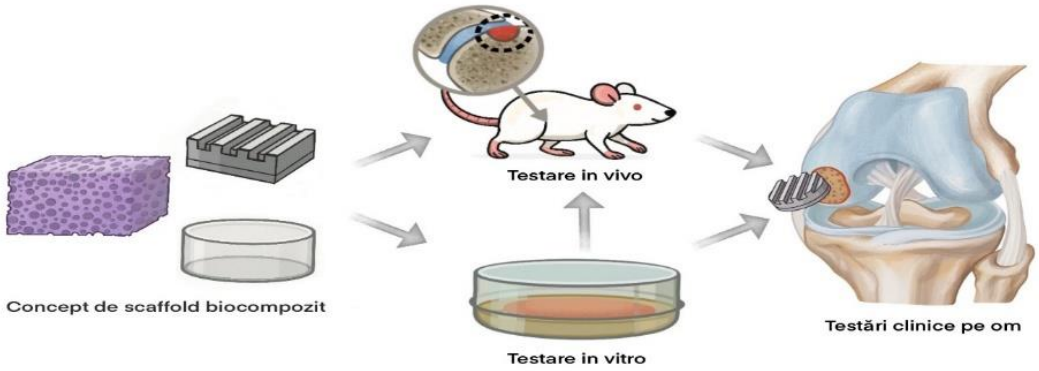
 Hemocompatibility testing, performed according to the ASTM F756-00 standard.

The data obtained from the physicochemical characterization, in vitro testing and in vivo evaluations were statistically correlated to validate the best performing formulation – F6. A strong correlation was identified between the histological scores and the mean pixel intensity in the radiographs (Pearson coefficient r = 0.94), confirming the methodological validity and translational potential of the F6 composite. The figure below illustrates the conceptual synthesis of the stages carried out in the study, which highlights the translational approach from the development of the composite scaffold to the in vitro, in vivo testing and the targeted clinical applications.

In the documentation stage carried out within the general part of the thesis, we followed a systematic and critical analysis of the specialized literature on osteochondral defects of the knee - a complex pathology and still insufficiently resolved from a therapeutic point of view. By studying recent and relevant bibliographic sources, including articles from international journals of orthopedics, bioengineering and regenerative medicine, we have deepened the aspects related to the anatomy and physiology of the osteo-cartilaginous unit, as well as the evolution of regenerative treatments, from cellular techniques to composite biomaterials. In this context, we identified the need for a new approach, which combines osteoconductive properties, structural biocompatibility and an efficient control of the local inflammatory response.

Thus, against the background of a rigorous documentation on the potential of natural materials, we noted that, although it is less used in osteochondral regeneration, keratin presents important valences through its immunomodulatory, proangiogenic and tissue organization supporting capacity. Consequently, the choice to better exploit the potential of keratin within biomimetic composites represented an approach with solid scientific justification and innovative character, opening promising perspectives for the development of new therapeutic solutions.

This documentation stage was essential for defining the theme, formulating the hypothesis and subsequently designing the experiments, significantly contributing to the integrated understanding of osteochondral issues and emerging regenerative solutions.




## 2. Thesis chapters

First, in the documentation stage carried out within the General Part of the thesis, I pursued a critical, systematic and extensive analysis of specialized bibliographic sources regarding the etiopathogenesis, diagnosis and current therapeutic options in the treatment of osteochondral defects of the knee. By studying recent and relevant specialized literature, including articles published in renowned international journals in the fields of orthopedics, bioengineering and regenerative medicine, I delved into aspects related to the anatomy and physiology of the osteo-cartilaginous unit, as well as the current state of regenerative treatments - from cellular techniques to the use of composite biomaterials.

I identified the potential of a new biomimetic composite, which would combine osteoconductive properties, structural osteoinductivity and an efficient control of the local inflammatory response. The choice to better capitalize on the potential of keratin in this context emerged as a scientifically justified and innovative approach, supported by the data and conclusions from the specialized literature. This initial stage was important for defining the theme, formulating the hypothesis, and subsequently designing the experiments.

# I. SPECIAL PART - PERSONAL CONTRIBUTIONS

## 1. Development of New Composite Materials Based on Collagen, Hydroxyapatite and Keratin

This chapter describes the development, characterization and in vitro validation of novel biocomposites based on collagen, keratin and hydroxyapatite (COL:K:HA), intended for osteochondral regeneration. Against the backdrop of recent advances in tissue engineering and nanotechnology, the proposed materials were designed to offer superior biomechanical and biological properties, responding to the regenerative needs of osteo-cartilaginous defects. In this work, the terms ‘composites’, ‘biocomposites’ and ‘scaffolds’ refer to the same three-dimensional structures with applicability in osteochondral regeneration.

The biocomposites were obtained by chemical cross-linking with glutaraldehyde and lyophilization, and the physicochemical characterization included morphological analysis (SEM), water absorption, enzymatic stability, FT-IR and Raman spectroscopy. SEM and morphometric analyses demonstrated an optimal porous structure (50–200 μm) for scaffold applicability, with efficient dispersion of components. Formulation F6, containing 1% collagen, 0.5% keratin and 1% hydroxyapatite, was distinguished by an optimal balance between stability, porosity and physicochemical integration of the phases.

In vitro biological testing, using human mesenchymal stem cells (MSC), revealed increased viability and good cell colonization for formulations F5 and F6, accompanied by the expression of fibronectin and vimentin, markers of a solid regenerative activity. These results indicate the potential of the developed composites to be used in orthopedic regenerative medicine, especially for the reconstruction of osteochondral defects. The conclusions support the superiority of formulation F6 and the synergistic role of the three components in stimulating tissue regeneration.

The overall conclusions of the study highlight the promising potential of collagen, hydroxyapatite and keratin-based composites in optimizing the osteo-cartilaginous regeneration process. At the same time, they emphasize the importance of continuing research efforts in the field of personalized biomaterials and the development of innovative therapeutic strategies capable of accelerating healing, reducing postoperative risks and contributing to reducing the costs associated with modern orthopedic treatments.

## 2. In vivo evaluation of composites obtained from collagen, hydroxyapatite and keratin, on the experimental animal model

Chapter II of the paper presents the in vivo evaluation of biocomposites based on collagen (COL), hydroxyapatite (HA) and keratin (K) using the preclinical animal model with Wistar rats. The main aim of this study was to test the biological performances of four biomaterial compositions: F1 (simple collagen – control), F2 (collagen + keratin), F5 (collagen + hydroxyapatite) and F6 (collagen + hydroxyapatite + keratin), in the context of osteochondral defect regeneration.

The introduction highlights the major challenges in the treatment of osteo-cartilage lesions and the importance of developing biomimetic biomaterials capable of inducing bone and cartilage regeneration. Collagen, hydroxyapatite and keratin have already been investigated in vitro for their osteoinductive and biocompatible properties. Combining these three components in a composite represents an innovative approach for osteo-cartilage substitution.

In terms of methodology, the study involved 32 male Wistar rats, each animal receiving the implant in a single femur, while the contralateral femur was kept as an internal control. The implants were evaluated 30 and 60 days after surgery by radiological and histological analyses. The compositions were selected based on in vitro performance, and the surgical interventions followed rigorous anesthesia, implantation and postoperative care protocols. The results revealed significant differences between the compositions. Body weight monitoring showed good systemic tolerance for all formulations, without significant weight loss or mortality. Groups F5 and F6 showed a sustained increase in body weight, suggesting a favorable interaction between the biomaterials and the organism.Analizele radiologice au utilizat parametri cantitativi precum media intensității pixelilor, deviația standard, asimetria (skewness) și kurtoza, obținuți cu software-ul ImageJ. Compoziția F6 s-was characterized by a homogeneous distribution of bone density, a low standard deviation and high kurtosis values, indicating an organized and advanced bone regeneration. Group F5 performed well but inferiorly compared to F6, while F1 and F2 showed limited bone regeneration.

Histological evaluation confirmed these differences. Group F6 showed thick and well-organized trabeculae, high cell density, extensive vascularization and excellent integration with the host tissue, while residual inflammation was minimal. F5 showed moderate

regeneration with well-formed trabeculae and adequate vascularization. F2 showed abundant collagen deposition and partial mineralization, and F1 performed the worst, with limited bone formation.

The correlations between the imaging and histological data were significant, with a Pearson coefficient $r = 0.94$ between the mean pixel intensity and the total histological scores. This suggests that radiological analysis can be a reliable tool for the semiquantitative assessment of bone regeneration in vivo.

The discussions highlighted the superiority of the F6 composition, which combines the benefits of hydroxyapatite (osteoconduction) with those of keratin (biocompatibility, cellular organization). This formulation demonstrated the best performance in bone regeneration and integration into the host tissue. F2 and F5 had intermediate results, and F1, as a control formulation, showed a limited capacity to induce regeneration.

In conclusion, the study demonstrated that the integration of hydroxyapatite and keratin in a collagen matrix leads to the obtaining of a composite biomaterial (F6) with superior regenerative performances. The radiological and histological results were convergent and support further research on the F5 and F6 compositions, in larger animal models and with more advanced evaluation methods, such as microCT and biomechanical tests. The F6 formulation thus emerges as a promising candidate for clinical applications in the treatment of osteochondral defects.

# 3. Convergent immunological, histological and in vivo imaging evaluation of composites

Chapter III presents a detailed in vivo evaluation of three biomaterial composites – F1 (plain collagen), F5 (collagen + hydroxyapatite) and F6 (collagen + hydroxyapatite + keratin) - using a preclinical model on Wistar rats, aiming to investigate their capacity to regenerate osteochondral defects. The selection of these formulations is based on the conclusions of the in vivo study in Chapter II, where four compositions were initially analyzed, and F1, F5 and F6 stood out for their superior performance. Their choice for in-depth analysis reflects a scientifically grounded decision, with direct relevance for the development of effective and clinically applicable biomaterials. The introduction of this chapter supports the need for innovative regenerative approaches, emphasizing the role of collagen as a structural support, of hydroxyapatite for osteoconductivity and of keratin for its immunomodulatory and proangiogenic effects.

The study was conducted on 15 Wistar rats, organized into three experimental groups. The evaluation was performed over a 60-day period and included preoperative tests (hemolysis, FTIR and Raman spectroscopy), body weight monitoring, serological analyses (cytokines, CRP, ALP), radiographs, micro-CT scans and histological examinations.

The results showed that all composites were well tolerated systemically, without significant weight loss or adverse effects. F6 demonstrated the best biocompatibility and integration profile, highlighting a steady increase in body weight concomitant with increased alkaline phosphatase levels, indicating enhanced osteogenic activity. Radiographs and digital imaging analysis showed more advanced mineralization and a more uniform bone distribution in the F6 group, while micro-CT confirmed a denser and more organized trabecular architecture.

Histologically, composite F6 showed high cell density, extensive collagen deposition, advanced mineralization and intense vascularization, with minimal residual inflammation. Histological scores and radar graphs confirmed the superiority of this composite over F1 and F5. Raman and FTIR spectroscopy indicated the chemical stability of the materials and a favorable interaction with the biological environment.

The cytokine profile of composite F6 was characterized by significant decreases in IL-6, IL-8 and TNF-α, concomitantly with an increase in IL-10, highlighting an anti-

inflammatory immune response favorable to tissue regeneration. Low levels of CRP and increased ALP activity reinforced this conclusion.

The correlations between radiological, histological and micro-CT parameters were strongly positive, suggesting coherence between the evaluation methods. The F6 composite was highlighted as having the greatest translational potential for clinical applications in the reconstruction of osteochondral defects, by effectively combining structural support, biological integration capacity and immune response modulation.

# Conclusions and personal contributions

In conclusion, the study supports continued research on the F6 composite in higher-order animal models, biomechanical validation, and expansion of applications in orthopedic regenerative medicine.

**(i) The effectiveness of the materials developed in the study**

The work aimed at the development and testing of innovative biocomposites based on collagen, hydroxyapatite (HA) and keratin, with biomimetic potential for the restoration of the osteo-cartilaginous unit. These materials, obtained by homogenization, chemical crosslinking and lyophilization, demonstrated a stable porous structure, compatible with cellular infiltration and nutrient diffusion. In vitro and in vivo testing showed a superior osteoconductive and osteoinductive capacity in the case of collagen-hydroxyapatite-keratin composites, confirming a synergistic effect favorable to bone and cartilage regeneration. Thus, the work contributes to the development of orthopedic biomaterials with real clinical potential.

**(ii) In vivo evaluation of COL:HA:K biocomposites on preclinical animal model**

The evaluation of the compositions F1, F2, F5 and F6 in the Wistar rat model indicated the superiority of the F6 formulation in terms of osseo-cartilaginous regeneration and implant integration. The association of the three components favored osseointegration and reduced the inflammatory response. The results emphasize the importance of the appropriate selection of the biomaterial and the implantation technique in the success of osteochondral reconstruction.

**(iii) The role of COL:HA:K composites in regeneration and biocompatibility**

COL:HA:K composites have been shown to be effective in stimulating osteoblastic and chondroblastic differentiation, with a porous structure favorable to regeneration. Keratin contributes through immunomodulatory and proangiogenic properties, collagen supports cell adhesion, and hydroxyapatite provides osteoconductivity. This combination provides robust biological support, accelerating healing and reducing the risk of complications.

**(iv) Importance of the study and research perspectives**

The study provides a solid scientific basis for the use of COL:HA:K composites as advanced regenerative solutions. They can be applied in orthopaedics, dental implantology and orthoprosthetics. Future research should aim at the integration of active therapeutic agents and the use of technologies such as 3D printing for implant customization. Clinical validation, structure optimization and the establishment of standardized protocols are essential for the transition to clinical practice. Collaboration between researchers, clinicians and industry is crucial for the implementation of these innovative solutions for the benefit of the patient.

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