

**„VICTOR BABEȘ” UNIVERSITY OF MEDICINE AND PHARMACY
FROM TIMIȘOARA**

FACULTY OF MEDICINE

DEPARTMENT XI: PAEDIATRICS

SALVANTE ENRICA RAFFAELLA GRAZIA



PhD THESIS

**ENHANCING BIOCOMPATIBILITY ASSESSMENTS OF
BIOMATERIALS FOR PAEDIATRIC SURGICAL
APPLICATIONS: INTEGRATING MULTIMODAL
ANGIOGENESIS EVALUATION WITH AI**

- A B S T R A C T -

Scientific Coordinator

PROF. UNIV DR. BOIA EUGEN SORIN, MD PhD

Scientific Co-Coordinator

PROF. UNIV. HABIL. DR. CIMPEAN ANCA MARIA

Timișoara

2024

The present PhD Thesis focuses on biomaterials and their suitability for clinical application and research practice emphasizing the innovation in experimental methodologies and results. The study uses the Chorioallantoic Membrane of Leghorn Hen embryos to evaluate angiogenetic processes as a metric of integration. Various biomaterials were tested on an ethical model of living tissue, with microscopic evaluations enhanced by Artificial Intelligence.

Biomaterials are pivotal in modern medicine, particularly in prostheses, implants, regenerative medicine, and drug delivery systems. They are used to create scaffolds for tissue regeneration, improving treatments for skin burns, bone fractures, heart disease, and neurological disorders. Additionally, biomaterials play a significant role in diagnostic applications through biosensors and diagnostic devices, allowing for early disease detection with high sensitivity and specificity.

The research underscores biomaterials' impact on contemporary medicine by providing innovative solutions to complex medical challenges. The vast realm of biomaterials includes a range from metals and ceramics to biodegradable polymers. The biomaterials are crucial in designing biomedical products like heart valves, joint replacements, and contact lenses.

The study highlights the potential of different types of scaffolds, meshes and dual layers solutions to enhance healthcare outcomes, with ongoing research suggesting a growing impact on the future of medicine.

In summary, this PhD thesis evaluates biomaterials' effectiveness through advanced experimental techniques and highlights their transformative role in medical treatments, diagnostics, and tissue engineering.

Biomaterials enhance, repair, or regenerate biological functions and interact intimately with living tissues. They can be natural (e.g., starch, collagen) or synthetic, and possess diverse mechanical, biological, and chemical properties crucial for medical use. The biomaterials industry has made significant advancements, producing numerous devices and diagnostic products for tissue regeneration and restoring bodily functions. The FDA categorizes over 6000 types of medical gadgets to ensure safety and efficacy, including pacemakers, mechanical heart valves, nerve stimulators, and prosthetic joints. The history of biomaterials dates back to ancient societies, with notable growth post-World War II due to advances in metals, ceramics, and polymers. The term "biomaterials" was broadly defined in 1982, encompassing both inert and active compounds used to treat, augment, or replace tissues, organs, or body functions.

Biomaterials are classified based on their material qualities and medical applications.

The main categories can be summarized as:

Bioceramics: Used in orthopedic surgery and dentistry, examples include aluminum oxide and silicon dioxide. They are biocompatible and non-inflammatory but have a low fracture point. Polymers: Divided into natural (e.g., collagen, chitin) and synthetic (e.g., polyethylene, polypropylene) polymers. Natural polymers are biodegradable, while synthetic ones are versatile and used in prosthetics and implants but can absorb water and proteins, making sterilization challenging. Metals: Include titanium alloys and stainless steel, used in heart valves and joint replacements for their strength and biocompatibility. They face issues like cytotoxicity and corrosion. Liposomes: Lipid bilayer structures used for drug delivery in cancer treatment, vaccinations, and antimicrobial therapy. They protect medicinal agents and enhance cellular uptake. Nanoparticles: Gold and silica nanoparticles are used in disease treatment, enhancing chemotherapy effects and triggering immune responses against cancer.

Collagen is a crucial fibrous protein in the extracellular matrix (ECM) and connective tissue and is vital for tissue stiffness and integrity. The collagen superfamily comprises 28 types of Collagens, each with unique characteristics, making up about 30% of total protein mass of the human body. Collagen chains, containing repeating amino acid motifs (Gly-X-Y), assemble into a right-handed triple helix structure. Proline and hydroxyproline stabilize the

triple helix by forming hydrogen bonds. Collagen types differ in amino acid composition, length, and arrangement of their triple helical domains. Collagen can be categorized into three main groups: fibrillar, fibril-associated, and network-forming collagens. Types I-III make up 80-90% of body collagen. Type I serves as a structural scaffold in musculoskeletal tissue and skin, Type II is found in cartilage, and Type III exists alongside Type I, except in bones and tendons. Type IV forms the main network in the basement membrane, while Type V is crucial for fibrillation of Types I and III. Type VI has a unique beaded filament structure, serving structural and signaling roles. Type IX is associated with tissues containing Type II collagen, and Type XI is primarily found in cartilage. Due to its cell affinity, collagen is widely used in biomedical engineering. It can be naturally degraded by matrix metalloproteinases (MMPs), which play a key role in tissue remodelling. Collagen sources used in biomaterials include xenogeneic or allogeneic origins, with mammalian collagens potentially causing inflammation. Recombinant human Type I collagen (rhCollagen) is an alternative to human collagen but is challenging to produce. Full-length collagen scaffolds provide biochemical and biophysical cues for cells, offering diverse benefits in tissue engineering. Advances in bioprinting technology have enabled the production of diverse scaffold shapes, such as sponges, hydrogels, films, fibers, and meshes. These matrices can encapsulate cells, making them useful in applications like wound dressings, 3D cell cultures, regenerative endodontic therapy, and bone and cartilage substitutes. Techniques to produce collagen sponges include lyophilization, free salt leaching, or solvent casting. Collagen I hydrogels, tunable through variables like pH and temperature, are mainly used in tissue engineering, cosmetic applications, drug delivery systems, intervertebral repair, and wound healing. Understanding collagen gel polymerization is fundamental for creating stable matrices. Collagen gels, useful for cell encapsulation, mimic the natural ECM. Electrospinning collagen solutions produce fibrous mesh scaffolds with high surface area-to-volume ratios and porosity, ideal for wound healing, drug delivery, and skin tissue engineering. Another technique, 3D bioprinting, offers greater geometric and architectural flexibility compared to traditional methods.

Therefore, collagen scaffolds are central to tissue regeneration, replicating the ECM's structural characteristics. Collagen Type I is the most used type in tissue engineering due to its tunable gel-like state modulated by temperature, pH, and ionic strength. Cells cultured on collagen Type I hydrogel exhibit contraction and actuation due to cellular traction. A systematic review by Hameed et al. highlights the differences between 2D and 3D cell cultures: Migration Behavior: 2D cultures exhibit lamellipodia-based migration, while 3D cultures promote amoeboid migration, closely resembling natural tissues. Cell Interaction and Signaling: 2D cultures restrict cell signaling to the ventral surface, whereas 3D cultures enhance cell-to-cell interaction, better representing native tissue environments. Phenotypic Changes: Cells in 2D cultures form stress fibers and upregulate genes like α -Smooth Muscle Actin (α -SMA), leading to myofibroblast-like characteristics. In contrast, cells in 3D cultures show dendritic morphologies, lower α -SMA levels, and reduced stress fiber production. Cell Proliferation: Proliferation rates vary by cell line; for example, rectal and prostate cancer cells proliferate faster in 2D, while breast cancer and kidney cells grow quicker in 3D environments. Drug Testing and Clinical Relevance: 3D models better replicate physiological conditions, showing greater resistance to drugs due to upregulated genes like Bcl2. Overall, studies indicate that 3D culture results align more closely with real-life drug efficacy than 2D models. 3D cell culture models offer significant advantages, including replicating in vivo microenvironments, forming complex tissue-like structures, and promoting enhanced cell-cell interactions. They are essential tools in research fields like cancer biology, drug development, tissue engineering, and regenerative medicine, providing more physiologically relevant data than 2D cultures.

Collagenous Scaffolds, in fact are used in bone lesions and bone regeneration. As a matter of fact, bone regeneration, especially in paediatric patients, requires selecting appropriate materials and scaffolds. Collagen-based scaffolds are favored for several reasons: 1) Biocompatibility: Collagen scaffolds, derived from natural sources, are less likely to provoke immune responses in sensitive paediatric patients. 2) Osteoconductivity: They promote adhesion, migration, and proliferation of bone-forming cells, crucial for growing bones

in children. 3) Biodegradability: Collagen scaffolds degrade over time, eliminating the need for surgical removal. 4) Growth Potential: They support active growth plates, allowing bones to elongate and remodel in children. 5) Reduced Infection Risk: Effective sterilization reduces infection risk. 6) Ease of Manipulation: Collagen scaffolds are easy to shape and use in various surgical techniques, such as cleft palate repair and craniofacial surgeries. 7) Facilitation of Natural Healing: Collagen mimics the body's extracellular matrix, enhancing natural healing mechanisms. In paediatric bone tissue engineering, collagen scaffolds combined with stem cells, like dental pulp stem cells, expedite bone regeneration for congenital anomalies, traumatic injuries, and dental procedures. These scaffolds provide a foundation for stem cell adherence, proliferation, and differentiation into bone-forming cells.

Collagen scaffolds are also promising in paediatric heart surgery, addressing the challenges posed by congenital heart defects. Collagen mimics the extracellular matrix (ECM), essential for creating tissue-engineered vascular grafts (TEVGs) that are biocompatible and resistant to thrombosis and infection. However, synthetic grafts have limited growth capacity, necessitating multiple surgeries for paediatric patients, which poses significant health risks and emotional stress. Therefore, several reasons support cardiothoracic surgical approaches that favor the use of collagen scaffolds. Collagen gels seeded with smooth muscle cells (SMCs) and endothelial cells (ECs) offer potential, though they initially lack sufficient biomechanical properties. Nonetheless, special attention must be considered when using Decellularized Xenogeneic Tissues, since these retain an intact ECM but carry risks of viral infections and compromised mechanical properties. Combining collagen with synthetic TEVGs offers potential solutions, though further investigation and refinement are needed to ensure clinical effectiveness in paediatric cardiovascular surgery.

Tissue engineering holds promise also for congenital urological diseases in children, such as hypospadias, posterior urethral valves, bladder exstrophy, and neurogenic bladder. Current surgical procedures, like urethroplasty and enterocystoplasty, have severe complications and limitations. Tissue engineering aims to create functional tissue mimicking native tissue in structure and function, addressing challenges like biocompatibility, biodegradability, and mechanical similarity to native tissue. For bladder reconstruction, collagen scaffolds support quick tissue regeneration and maintain mechanical strength. They offer a biocompatible, biodegradable platform for addressing congenital disorders like bladder exstrophy and neurogenic bladder. Studies have shown collagen scaffolds promote regeneration of the urothelium, smooth muscle, blood vessels, and nerves. In hypospadias treatment, collagen-based scaffolds derived from decellularized bladder submucosa support cell growth and tissue regeneration, resembling normal urethral architecture without strictures. Silk fibroin-based scaffolds and synthetic polymers also show promise in urethral reconstruction. Overall, collagen-based scaffolds represent a significant advancement in paediatric urology, potentially improving surgical outcomes and reducing complications. Further research is needed to refine these approaches and ensure their effectiveness in clinical practice.

In paediatric gynaecology, collagen-based scaffolds offer significant promise for tissue engineering applications and medical use, due to their biocompatibility, structural support, and biodegradability. The patients suffering from ovarian cancer are treated aggressively, with strategies that can impair ovarian function. For fertility conservation, oocyte harvesting and preservation are standard, but not suitable for pre-pubertal patients. Pre-pubescent girls may benefit from cryopreservation of ovarian cortical tissue, which can be autografted post-treatment to restore fertility. Collagen-based scaffolds support follicle maturation and tissue regeneration, offering a potential solution for preserving ovarian function and fertility without the risk of malignancy. Müllerian Malformations can cause uterine-factor infertility and may require surgical intervention. Endometriosis is a condition involving abnormal growth of endometrial tissue, leading to infertility and pelvic pain. With the help of collagen scaffolds, Tissue Engineering in those pathologies focuses on regenerating uterine tissues using collagen scaffolds combined with materials like Matrigel. The goal is to develop functional

tissue patches that can support embryo implantation and address infertility. Congenital vaginal defects in conditions such as bladder exstrophy and MRKH syndrome often require surgical reconstruction. Collagen scaffolds combined with other materials are used for vaginal reconstruction. Techniques like self-assembly tissue engineering create autologous tissue constructs with native vaginal mucosa characteristics. Techniques can be tailored to paediatric patients even in cases of uterine malformation. Paediatric anatomical sizes and growth patterns are addressed by tissue engineering supported by collagen scaffolds, potentially restoring uterine function as children transition into adulthood. Special considerations are needed for paediatric patients, including age-appropriate materials and monitoring long-term effects on growth and development. Collagen-based scaffolds hold significant potential for addressing gynecological issues and infertility through tissue engineering. While challenges remain, ongoing research and clinical trials are essential for refining these approaches and improving treatment options for women and paediatric patients with reproductive organ pathologies.

Cleft palate-Palatoshizis (CP) is part of a group of orofacial cleft syndromes affecting nourishment, communication, auditory perception, intellectual growth, and social inclusion, often requiring multiple surgeries and lifelong care. These conditions result from a combination of genetic and environmental factors, including maternal smoking, alcohol intake, and vitamin deficiencies. Autologous Bone Grafting is commonly used for palatal cleft repair, and aims to achieve bone continuity and soft tissue closure. Enhance speech and nutrition by ensuring the proper function of the velopharyngeal mechanism are the primary goal of the surgical treatment. Failure to achieve this can lead to velopharyngeal insufficiency (VPI), affecting speech and hearing. Tissue engineering aims to develop osteogenic scaffolds that are customizable, biocompatible, and capable of integrating seamlessly with adjacent tissues. 3D printing allows the production of patient-specific implants. Collagen-based protein scaffolds promote cell adhesion and have been approved by the FDA for bone tissue engineering. Preclinical studies show success in repairing cleft palate bone abnormalities in animal models, but no reports exist for their use in infants with secondary palatal clefts.

Congenital diaphragmatic hernia (CDH) is a serious condition with high morbidity and mortality rates. The severity of cases varies, affecting outcome comparisons. Surgical repair methods depend on defect size, with larger defects requiring patch repairs. Prosthetic patches, although popular, face issues like disruption, hernia recurrences, chest wall deformities, and scoliosis. Prosthetic Patches hold limitations such as lack elasticity and shape memory of normal diaphragmatic tissues and cannot mimic the complex structure of the diaphragm. Vascularization is always a critical challenge, especially for implants in the hemithorax, which have limited contact for integration. The development of skeletal muscle with contractile ability through functional fibrotic structures is the research focus of the last years. Current in vitro models struggle with organized vascular structures. Collagen-based scaffolds have potential for facilitating tissue regeneration and repair in CDH. However, further investigation is needed to address the challenges and ensure effective utilization of collagen-based approaches in this specific application.

This doctoral PhD work evaluates the biocompatibility of various materials, focusing on collagen's role in tissue engineering and regenerative medicine due to its similarity to the extracellular matrix. By implanting collagen, hybrid, and polymeric biomaterials on the chorioallantoic membrane (CAM) of chick embryos, the study assesses how these materials interact with a living vascular system. Vascularization is a key marker for tissue integration, crucial for successful tissue engineering and transplantation. The CAM model is an ethical, sustainable, and cost-effective alternative to mammalian models, providing valuable insights into embryological development and biomaterial integration. This model supports the growth of new blood vessels and the formation of an efficient vascular network, relevant for clinical applications in surgery and tissue regeneration. The study's findings can guide better clinical choices and promote tissue regeneration. Ethical considerations are central, as the CAM model minimizes harm and distress to research subjects. The incorporation of artificial

intelligence (AI) through the IKOSA App enhances the accuracy and efficiency of vascularization assessment, providing precise and reliable analysis. In conclusion, this PhD work aims to advance the understanding of biocompatibility using sustainable models, modern technologies, and ethical research practices, contributing to biomaterials science and regenerative medicine.

This PhD work focuses on evaluating biomaterials' biocompatibility by measuring angiogenesis and their ability to form functional vascular networks. This is particularly important as tissue vascularization remains a significant challenge in tissue engineering.

The methodology involved:

1) The use of the CAM as experimental model. The chorioallantoic membrane (CAM) of chick embryos is an effective model for studying vascular development and the biology of the vascular system. During the first 3-10 days of development, the CAM forms a thin avascular membrane, rapidly developing a robust vascular network. This rapid vascular formation is advantageous for observing developmental pathways and pathological processes, as well as assessing the compatibility of transplanted tissues, cells, or materials. The advantages of the CAM Model are several: -Convenience and Cost-Effectiveness: fertilized eggs are inexpensive, costing 1/100th of a mouse, and easy to obtain and maintain. -Ethical Considerations: The CAM model does not require complex administrative procedures for ethics approval in many European countries, as the chick embryo is not considered a living animal until day 17 of incubation. -Imaging Versatility: The model supports various imaging techniques, including microscopy, MRI, and PET. Its transparent outer layers allow for fluorescence imaging across the visible spectrum. -Reduced Immune Response: The immature immune system of chick embryos reduces unfavorable immune responses, facilitating tissue transplantation. -Pathological Studies: The CAM model is valuable for studying cancer morphology and vascularization, providing insights into oncogenic tissue development and changes in cancer cell morphology. Trichrome staining as well as haematoxylin- eosin staining were used for classical histological examination.

2) The enhancement of the evaluation through the integration of Artificial intelligence. In this research work, two KML Vision products were used: Ikosa Prisma and Ikosa AI. Ikosa Prisma offers specialized applications for microscopy image analysis, including the CAM assay for examining vascular structures on the chorioallantoic membrane of chick embryos and the Network Formation Assay for evaluating endothelial cell networks in in-vitro angiogenesis tests. Ikosa AI provides a user-friendly platform for training AI applications, accessible even to those without technical skills, limits mistakes correlated with repetitive tasks, and gives results in a little amount of time.

3) The use of the quantitative methodology to analyse the angiogenesis. The Ikosa App delivered the results mainly as .xls spread sheets and Images. The .xls spread sheets allow for easy statistical manipulation and exact quantification of the variables.

This doctoral work involves two main experiments. The first experiment **(A)** evaluates the angiogenetic potential of two collagenous scaffolds and a non-collagenous mesh implanted on the CAM of chick embryos. The second experiment **(B)** assesses how vascular network maturity and remodeling are influenced by two different biomaterials: one made purely of collagen and the other a collagenous biomaterial enhanced with glycosaminoglycans. The articles were published in 2024 in two specialty journals. The first article appeared in *In Vivo* (Impact Factor 2.3), and the second article was published in *Biomaterials MDPI* (Impact Factor 4.6).

A) ARTIFICIAL INTELLIGENCE (AI) BASED ANALYSIS OF IN VIVO POLYMERS AND COLLAGEN SCAFFOLDS INDUCING VASCULARIZATION

The main objective of the experiment was to evaluate neoangiogenesis, in response to various scaffolding implants grafted on the chorioallantoic membrane of the chick embryos. This experiment involved the use of two collagen scaffolds, with subsequent comparison of the angiogenetic results with those induced by a non-collagenous mesh. For the investigation, we selected three different biomaterials for implantation on the chorioallantoic membrane (CAM) model of the chicken embryo: a polymeric material and two collagen-based scaffolds. The polymeric material, known as MotifMesh (MOME) is composed of polypropylene and has been commonly used in hernia surgery for some time. However, there are side effects correlated with the use of this mesh, such as inflammation. Instead, collagen-based scaffoldings - Optimaix 2D (OXMD2D) and Optimaix 3D (OXMD3D), manufactured by Matricel GmbH - are chosen for their high collagen content, making them suitable for assessing interactions with human or experimental tissues. The experimental method involved the careful selection of 30 fertilized chicken eggs and their incubation for 72 hours before creating a shell window on each egg to evaluate the viability of the embryo. After dividing the eggs in three groups, we implanted the MotifMesh, Optimaix 2D, and Optimaix 3D fragments on the surface of the CAM using a delicate scratching procedure. The implanted eggs were observed and photographed by microscopy on days 1, 3 and 5. The experiment was completed on day 13. After the experiment was finished, the corresponding membranes and implants were fixed, processed and incorporated into paraffin for histopathological evaluation. The series of sections were coloured and analysed using standard techniques for assessing vascularity. To achieve an accurate mapping of CAM vascularization, we used IKOSA software for automated analysis. The assessment of angiogenesis was performed by stereomicroscopy, histology (hematoxylin/eosin staining and trichrome) and IKOSA/CAM helped to evaluate angiogenesis on stereomicroscopic images. Motif Mesh induced a large inflammatory and necrotic reaction when it was tested on the chorioallantoic membrane and failed to attach enough to the membrane itself. It is possible to state that MotifMesh induced inflammatory-provoked neovasculogenesis. In addition, a fibroblast reaction was discernible in the observational field due to the presence of myofibroblasts. Therefore, we can conclude that MOME is not suitable to be used as a biomaterial. The IKOSA CAM Assay study confirms a clear angiogenic response with several interconnected microvascular structures. This suggests that MOME may neovascularize or stimulate the growth of new blood vessels. However, histological investigations have shown that this was due to the MOME implant, which induced a strong inflammatory reaction.

Vascularization density decreased sharply on the last day of the experiment. This reaction was most likely caused by an increase in stroma cell count, which is likely caused by a myo-fibroblast reaction in MOME compared to normal CAM.

The collagenous scaffolds OXMD 2D and OXMD 3D were analysed. After being implanted on the CAM, the collagen scaffolding did not cause any inflammation. Also in this case, the evaluation involved the use of stereomicroscopy, histology and the IKOSA/CAM approach. The growth of blood vessels around the implant and penetrating the 3D OXMD implant was substantially higher than that of the 2D OXMD implant. Neither the area surrounding the implant nor its interior showed signs of inflammation. The integration of the CAM chorion with the implants OXMD 2D and OXMD 3D was demonstrated both histologically and microscopically. Recruitment of blood vessels in the surrounding area, along with significant vascular response around the implant, showed that the implanted material successfully induced angiogenesis. The collagen fibers of the material showed signs of self-organization, entering both linear and tubular patterns. The results showed that the cells adhere adequately to the 3D framework and that no fibroblast reaction occurred. The presence of red cell-filled tubular structures in the center of the implant has been evidenced by trichrome staining. The 3D-OXMD implant became efficiently vascularized, according to these findings. Assessment of angiogenic status for MOME, 2D OXDM and 3D OXDM was performed also

using IKOSA CAM assay. Artificial intelligence analyzed different quantitative variables for evaluating the impact of angiogenesis. Statistical and computerized analysis confirmed that MOME material is not as suitable as OXMD-2D and OXMD-3D, nor for laboratory settings or clinical practice.

Comparative analysis on days 1 (OBS1), 3 (OBS2) and 5 (OBS5) shows peculiar differences among the three biomaterials used in the present study, confirming the microscopic results.

Statistical analysis of data for three groups of biomaterials and correlation plots for each group shows that no significant correlations have been detected for the increase of new blood vessel development for the non-collagen MOME group ($p\text{-value}=0.200$,) compared to the angiogenic process induced by collagen-based scaffolds. A significant increase of newly formed blood vessels was induced by the OXDM2D scaffold ($p=0.028$,) but the strongest statistically significant angiogenesis induction was done by the OXDM3D scaffold ($p<0.001$).

Collagen and polypropylene-based scaffolds and meshes, commonly used in surgical operations, face controversies due to side effects like inflammation, with polypropylene meshes often causing significant inflammation and collagen-based scaffolds causing less, according to current literature. The angiogenesis and vascularization capabilities of these biomaterials are also debated. Despite their usefulness, collagen and polypropylene scaffolds are rarely tested using the chick embryo CAM model, and few studies utilize AI-driven methodologies for CAM model evaluations. This study compared three scaffolds: one collagen-based and two non-collagenous. The OPTIMAIX 2D collagen scaffold, made of pure porcine collagen, is compatible with multiple cell types and degrades naturally without causing significant inflammation, showing promise in promoting angiogenesis despite its dense structure limiting vascular network expansion. The OPTIMAIX 3D collagen scaffold, produced through a directional solidification process, forms a stable, porous collagen framework that is more resistant to degradation and significantly enhances angiogenesis compared to other materials like DegraPol. Previous studies using advanced imaging techniques corroborate these findings. The IKOSA AI and NFA tools were used to assess angiogenesis, measuring vessel branching points, length, area, and thickness, providing reliable and consistent results. IKOSA CAM assays were validated as a dependable method for evaluating angiogenesis in collagen-based scaffolds on the CAM model. Collagen scaffolds, particularly OPTIMAIX 3D, show substantial potential for enhancing vascularization in tissue engineering. Further research and refinement of these materials and methods are necessary to improve their clinical application and efficacy.

The experiments carried out in our study emphasize the essential role of artificial intelligence (AI) in improving the evaluation of angiogenesis, a hallmark of biocompatibility. AI integration has proven to be essential to facilitate this assessment and can serve as a valuable tool for researchers, in particular with regard to tasks characterized by repetition and complexity, such as the numbering of the branching points of each vessel.

B) GLYCOSAMINOGLYCANS MODULATE THE ANGIOGENIC ABILITY OF TYPE I COLLAGEN-BASED SCAFFOLDS BY ACTING ON VASCULAR NETWORK REMODELING AND MATURATION

The purpose of this experiment was to compare and contrast the neovascularization capacity as well as the ability of vascular remodelling and maturation of Xenoderm and two-layer collagen matrix (DLC), which are two scaffolds made from collagen, the first one containing purely collagen and the second one being enhanced by the presence in its composition of glycosaminoglycans. We compared them in an in vivo setting (in vivo and in ovo), implanting them on the chorioallantoic membrane of the eggs.

The angiogenesis and maturation of the vascular systems are measured by parameters such as the number of vascular loops and the development of vascular networks.

Understanding these issues is essential to evaluate scientific validity and optimize the application of these scaffolds in regenerative medicine. In order to compare the effects of Xenoderm and DLC on chorioallantoic membrane (CAM) vascularization, it was necessary to implant them on the surface of the chick embryo. Stereomicroscopy was used to assess and evaluate vascular formation processes. Using AI through the IKOSA application, we aim to improve the evaluation of the integration of these biomaterials into the living tissue system, investigating their potential to induce angiogenesis and promote the formation of functional vascular networks.

Xenoderm is a dermal substitute manufactured from a collagen-based matrix, obtained from the decellularized pig dermis, cross-linked to ensure the stability of the scaffolding. It finds applications in the clinical environment for the treatment of various skin conditions such as burns, chronic wounds and diabetic ulcers. The dual-layer collagen scaffold (DLC), also known as BIOPAD, comprises two distinct layers. The first layer consists of type I collagen, facilitating cell adhesion and proliferation, while the second layer is composed of glycosaminoglycans (GAGs). In addition to Xenoderm and DLC, the experiment required the use of various materials including 70% concentrated alcohol, ParaPlast sealing tape, 10% buffered formalin solution and paraffin. Microscopic sections were visualized using a stereomicroscope. Preparation of the chorioallantoic membrane test (CAM) started by selecting 60 fertilized chicken eggs, and their cleaning. The eggshells were washed with 70% concentrated alcohol. After an incubation period of 72 hours at 37°C and a humidity of 60%, the egg albumen was extracted from each egg on the fourth day of incubation. The punctures were sealed with ParaPlast. The viability of the embryos and the integrity of the CAM were assessed by cutting a shell window the next day. Xenoderm and DLC grafts were applied to two separate groups of ten eggs each after gently scratching on the CAM surface in a non-invasive manner. After 13 days of incubation, the experiment was successfully concluded. Stereomicroscopy and Ikosa App were applied to the analysis. The IKOSA program was used to conduct an automated study comparing the angiogenic processes of the biomaterials implanted on the CAM. More specifically, the CAM assay and Network Formation Analysis were used for this investigation. Parameters extracted from the CAM assay were used to quantify the quality of the vascularization processes.

The Network Formation Analysis facilitated the examination of vascular loops, and vascular networks. Statistical analysis was performed using XLSTAT and Jamovi, with data exported from the IKOSA application in Excel format. A stereomicroscopic examination was performed and images were captured and analyzed using ImageJ for accurate characterization. Preliminary results show DLC and Xenoderm angiogenic potential by the assessment of branching points and vascular tube morphometry through the IKOSA App. CAM Assay detected the branching points of new blood vessels by pointing them with red dots and automatically counted the branching points number.

The statistical representation of the CAM assay and NFA analysis confirm the microscopical findings. The chosen parameters—vessel total area/region of interest, vessel number of branching points/region of interest, and vessel number of branching points/total area—demonstrate that DLC has a strong capability for organic and sustainable vascular growth. In comparison, while Xenoderm stimulates rapid neoangiogenesis, it does not support long-term vascular functionality. Moreover, the mean vessel thickness value is particularly high for the DLC eggs, indicating that angiogenesis in the DLC-grafted CAMs is efficient, stimulating the formation of strong and patent vessels. These values are consistent with the results reported in the NFA analysis.

The IKOSA network formation assay (IKOSA_NFA) evaluated the remodelling ability of newly formed vascular network (by assessing the vascular loops number and size), but also vascular mean thickness characterizing vessel maturation and stabilization. Significant differences have been found between DLC and Xenoderm. Vascular loop number, size, and density were significantly increased for DLC from day 1 to day 3 and day 5. For a similar period, for Xenoderm, there was a low number of vascular loops.

Inferential statistical analysis were performed. The linear regression analysis of the vascular loops from the eggs implanted with Xenoderm yielded a coefficient of determination (R^2) of 0.949, while the equivalent analysis for the DLC-implanted egg loops displayed a higher R^2 of 0.986. This indicates a stronger correlation between the perimeter and area of the vascular loops in DLC than in Xenoderm, suggesting that the loop perimeter in DLC serves as a more reliable predictor for its area. One possible explanation for this is that DLC may facilitate the formation of more mature and intricate vascular networks in comparison to Xenoderm. Moreover, the greater standard deviation in the area of the loops associated with DLC points to a broader variability in loop size, implying that DLC supports the development of a diverse range of vascular loops, from small to large. Conversely, Xenoderm may primarily encourage the formation of uniformly smaller loops. Consequently, the findings from the linear regression analysis imply that DLC might be more adept at fostering the development of mature and complex vascular networks. This inference is corroborated by the higher coefficient of determination and greater variability (standard deviation) in loop areas observed with DLC.

To account for the discrepancies in the linear regression outcomes between Xenoderm and DLC, we can consider the following:

- Xenoderm may initiate the formation of vascular loops; however, it appears that these loops do not mature or network as efficiently as those formed by DLC, which could account for Xenoderm's lower R^2 value.

- The increased standard deviation in the loop area with DLC suggests it encourages a more extensive array of loop sizes, contributing to the higher variability seen in the analysis.

- DLC might support the development of more elaborate vascular networks, characterized by increased branching and connections (anastomoses), which would also contribute to the higher R^2 value.

These results, in concordance with the inferential data, suggest that DLC may be more successful in stimulating vascular network development and function. This is most likely because GAGs cause blood vessels to mature and stabilize more quickly in the DLC model. This information could be crucial in selecting the appropriate collagen scaffolds for use in tissue engineering of various organs. Due to its high glycosaminoglycan content, dual-layer collagen scaffolds have been shown to be one of the best materials for tissue bioprinting and tissue vascularization. These findings are consistent with previous experimental findings that used DLC as a scaffold for tissue bioprinting. Due to their actions on perivascular smooth muscle cells, GAGs also significantly influence vascular remodeling and maturation in addition to endothelial cell migration, proliferation, and tube formation. On the final day of the experiment, the total tube length for both scaffolds was comparable, but the vessels' mean thickness for DLC was significantly higher than for Xenoderm. This indicates that DLC can cause newly formed blood vessels to mature quickly, most likely as a result of the GAG layer present. A healthy vascular function requires the maturation and remodeling of vessels by the insertion of perivascular smooth muscle cells. The thickness of the newly formed perfused blood arteries in vivo can be evaluated by automated image analysis using the AI-based IKOSA CAM platform, particularly the CAM assay and Network Formation Analysis. Ausprunk provided a description of the location of GAGs throughout the formation of chorioallantoic membrane vessels in chick embryos in 1986. According to the author, sulphated GAGs aid in the stabilization and maturation of CAM vessels. Through activation of angiogenesis, collagen glycosaminoglycan dual scaffold increases the recovery of brain lesions in the mouse model of brain lesions. The same group of researchers found that the collagen glycosaminoglycan dual scaffold appeared to be important in encouraging the growth of peri-endothelial cells, particularly in the region around the lesion. This is consistent with what we found for the mean thickness of the vasculature for a comparable collagen and GAG combination.

In conclusion, with this study, we demonstrated that the incorporation of both type I collagen and glycosaminoglycans on a DLC scaffold can effectively stimulate the process of blood vessel remodeling and angiogenesis. Type I collagen facilitated the early phases of

angiogenesis but had less impact on the subsequent development, maturation, or stabilization of the newly established vascular network. The findings indicate that a combination of glycosaminoglycan and type I collagen is the most effective scaffolding material for tissue creation. This suggests that it has the potential to produce a fully operational vascular network.

This PhD thesis aims to state the following:

1. The study included one scaffold made of a polymer material and four scaffolds made of collagen. The presence of structural heterogeneity in the five different materials resulted in variations in angiogenic processes upon implantation on the chick embryo chorioallantoic membrane.
2. Polymer-based scaffolds caused a misleading false angiogenic response as a result of inflammation when implanted in a micro-environment (the CAM) that is not typically inflammatory. Polymeric-based scaffolds promote the formation of new blood vessels, but enhance the inflammatory property of the material, promoting an inflammatory response and in turn leading to the breakdown of the CAM structure. The inflammation caused by the polymeric-based scaffolds makes it unsuitable to use polymer-based biomaterials in clinical practice, whether for surgical or tissue engineering applications. The inflammatory-induced angiogenesis polymer-based scaffold implants on CAM exhibited a significant remodeling process. However, at the conclusion of the first experiment, it was observed that the vascular network was neither functional nor efficient.
3. In the initial phase of the investigation, we examined another class of matrices known as Oximaids, which are scaffolds made from collagen and typically suitable for cell cultures. In order to conduct the study, we selected two versions of Oximaids: 2D and 3D. The primary distinction between these two types is in the structural composition of the scaffold. Although both scaffolds contain Collagen, they have significantly different constitutions due to being generated through distinct manufacturing procedures. We have demonstrated that variations in the structure of collagen elicit distinct angiogenic responses. The AI-based IKOSA Software conducted a comparative evaluation of Oximaids 2D and 3D and found that OXMD3D is a more suitable collagen matrix for supporting the recruitment and development of new blood vessels. This is because OXMD3D has fine collagen fibers that promote the attachment of endothelial cells and the formation of tubes. Furthermore, the results indicate that the remodeling of newly generated blood vessels is enhanced by the three-dimensional fine fiber arrangement of OXMD3D, as compared to OXMD2D. The implications of our findings could significantly influence both pre-clinical and clinical practice. OXMD3D is a potentially viable framework for tissue engineering of organs that require both rapid vascularization and proper remodeling of the vascular network.
4. The second phase of the current study involved evaluating the angiogenic potential of two additional collagen-based scaffolds. One of these biomaterials (Xenoderm) closely resembles the structure of skin while the other (DLC) is a sponge. These biomaterials are commonly employed in clinical settings respectively for treating burns and promoting hemostasis. Given that both materials possess a comparable form of collagen, it can be inferred that disparities between them, namely in relation to angiogenesis, are caused by the inclusion of GAGs (glycosaminoglycans) in the structure of the Dual-Layer-Collagen sponge. Both collagen-based scaffolds elicit a significant angiogenic response, but the timing and the dynamic of blood vessel formation are influenced by the double-layered structure and the presence of glycosaminoglycans in DLC. This was translated into proper remodeling and maturation processes for DLC compared with Xenoderm.
5. The IKOSA CAM assay and IKOSA network formation assay are two instruments that enhance the accuracy in assessing the angiogenic processes occurring on the CAM surface. The method provides a comprehensive analysis and assessment of the angiogenic response triggered by various types of scaffolds placed and grafted on the CAM surface. These techniques provide us with information not only about the density of newly formed blood

vessels but also about their functional and maturation statuses. The assessment of the dynamic growth of the vascular network stimulated by collagen-based scaffolds enabled us to identify even subtle variations across materials that have a relatively similar structure. Therefore, IKOSA may serve as a valuable digital tool for assessing additional characteristics of biomaterial implants and their capacity to promote neoangiogenesis and vascular maturation.

The present PhD thesis offers different original contributions:

1. This study presents for the first time the de novo assessment of the angiogenic potential and angiogenic characterization of the newly formed blood vessels of four scaffolds made out of collagen (prevalent composition -hybrid- or sole composition) and a polymeric-based scaffold. The evaluation was conducted using a model that involves the chick embryo chorioallantoic membrane ensuring ethical research practice.
2. A multimodal approach was used to study the collagen-based biomaterials-induced angiogenesis. The assessment was performed by using an AI-based analysis program. The analysis included not only the density of the newly formed blood vessels around the implant but also their ability to branch and form networks, therefore both morphology and organic functionality were analyzed. We have demonstrated that the varying structures of different materials significantly influence the architecture and remodeling of the final vascular network, which in turn affects its development.
3. An AI-based software was utilized for the first time to assess the biocompatibility of a scaffold on an experimental model. Classical methods of angiogenesis evaluation cannot provide the same information as digital specimen analysis.