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COMPUTATIONAL METHODS TO SIMULATE ANGIOGENESIS

ANA CATARINA COUTO SOARES GUERRA

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Computational Methods To Simulate Angiogenesis

Ana Catarina Couto Soares Guerra



Doctoral Program in Biomedical Engineering

Supervisor: Professor Doutor Renato Manuel Natal Jorge

Co-Supervisor: Professor Doutor Jorge Américo Oliveira Pinto Belinha

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Abstract

Angiogenesis is the formation of new blood vessels from pre-existent ones and it is crucial for the success of wound healing. Sprouting angiogenesis is regulated by several biological factors; however, the vascular endothelial growth factor (VEGF) is the most preponderant pro-angiogenic compound. Given the complexity of angiogenesis, computational modelling can be extremely useful in this field since it allows to study this process in different scales and uses reproducible, more economic and less time-consuming methodologies, comparatively to the laboratory ones. Accordingly, this thesis aims to analyse the pertinence of using a meshless method approach to simulate angiogenesis and to validate the numerical results using an *in vivo* angiogenesis assay.

In the proposed 2D model, the endothelial cells migrate according to a diffusion-reaction equation for the VEGF and the Radial Point Interpolation Method (RPIM) was used to solve its governing equations. An *in vivo* angiogenesis assay was used to obtain some important parameters to model the branching process. The numerical results regarding the capillary network pattern and the angiogenic response were quantitatively and qualitative compared with the experimental ones in order to validate the computational model and to analyse its predictive capacity. Moreover, since mechanical loads can affect endothelial cell behaviour, the effect of compressive loading in angiogenesis was studied.

The obtained results demonstrated that the RPIM formulation developed could efficiently simulate the angiogenic process modulated by the VEGF diffusion. In all the performed simulations, endothelial cells migrated accordingly to the chemotaxis effect and following the VEGF gradient. Regarding the capillary network morphology obtained *in silico* and *in vivo*, some parameters were compared – total branching number, total vessel length and branching angle – and similar results were obtained (*p-value* higher than 0.05). Moreover, the capillary network pattern was compared between the *in vivo* and the *in silico* methodologies using the difference between the total capillary volume fractions and a good agreement was

obtained with values between 10% and 15%. In order to analyse the predictive capacity of the developed model, the angiogenic response for different VEGF concentrations was analysed. The numerical results showed that VEGF promotes angiogenesis, increasing the total vessel number in 25% for VEGF 50ng (vs. control, $p < 0.01$) and in 75% for VEGF 100ng (vs. control, $p < 0.01$) as well as the vessel length in 18% for VEGF 50ng (vs. control, $p < 0.01$) and in 44% for VEGF 100ng (vs. control, $p < 0.01$). These quantitative results were compared with the experimental ones and it was demonstrated that they were very similar. Additionally, it was found that the effect of compressive loading in the branching process was well simulated. The numerical results showed that all the compressive loadings tested increased the vascular network length and the number of branches. Among all the compressive loadings tested (5%, 10% and 30% strain), 5% strain loading was the most effective one, presenting a 33% increase in the vascular network length and a 68% increase in the number of branches.

Therefore, in this thesis the capillary network from an *in vivo* assay was simulated, for the first time, with a realist structure and morphology. The computational model was validated using *in vivo* data and the predictive capacity of the developed model was addressed. The combination between computational models and experimental assays performed in this thesis will contribute, in the future, for the development of robust and accurate angiogenesis models capable of increasing the knowledge in this field as well as to reduce the time and cost of experimental methodologies and the number of animals used in science.

Resumo

A angiogénese é a formação de novos vasos sanguíneos a partir dos já existentes e é um processo fundamental na cicatrização de feridas. A angiogénese é regulada por diversos fatores biológicos; no entanto, o fator de crescimento do endotelial vascular (VEGF) é o composto pró-angiogénico mais preponderante. Considerando a complexidade da angiogénese, os modelos computacionais podem ser extremamente úteis nesta área, pois permitem estudar o processo angiogénico em diferentes escalas e utilizando metodologias reprodutíveis, mais económicas e menos demoradas, comparativamente às laboratoriais. Assim, esta tese tem como objetivo analisar a pertinência da utilização do método sem malha para simular a angiogénese e validar os resultados numéricos utilizando um ensaio de angiogénese *in vivo*.

No modelo 2D proposto, as células endoteliais migraram de acordo com uma equação de reação-difusão para o VEGF e a utilização do *Radial Point Interpolation Method* (RPIM) permitiu resolver as equações obtidas. Para adquirir alguns parâmetros importantes para modelar o processo de ramificação foi utilizado um ensaio de angiogénese *in vivo*. De forma a validar o modelo computacional e analisar a sua capacidade preditiva, os resultados numéricos relativos ao padrão da rede capilar e à resposta angiogénica foram comparados de forma quantitativa e qualitativa com os experimentais. Para além disso, como as cargas mecânicas podem afetar o comportamento das células endoteliais, estudou-se o efeito da carga compressiva na angiogénese.

Os resultados obtidos demonstraram que a formulação RPIM desenvolvida permite simular com eficiência o processo de angiogénese modulado pela difusão do VEGF. Em todas as simulações realizadas, as células endoteliais migraram de acordo com o efeito da quimiotaxia e seguindo o gradiente de VEGF. Considerando a morfologia da rede capilar obtida *in silico* e *in vivo*, compararam-se os parâmetros número total de ramificações, comprimento total do vaso e ângulo de ramificação e obtiveram-se resultados semelhantes (valor de p superior a 0,05). Adicionalmente, comparou-se o padrão da rede capilar entre as metodologias *in vivo* e *in silico*

usando a diferença entre as frações de volume capilar total e obteve-se uma boa concordância, com valores entre 10% e 15%. Para avaliar a capacidade preditiva do modelo desenvolvido, analisou-se a resposta angiogénica para diferentes concentrações de VEGF. Os resultados numéricos mostraram que o VEGF promove a angiogénese, aumentando o número total de vasos em 25% para o VEGF 50ng (vs. controlo, $p < 0,01$) e em 75% para o VEGF 100ng (vs. controlo, $p < 0.01$), assim como o comprimento dos vasos em 18% para o VEGF 50ng (vs. controlo, $p < 0.01$) e em 44% para o VEGF 100ng (vs. controlo, $p < 0.01$). Compararam-se os resultados quantitativos com os experimentais e foi demonstrado que estes eram muito semelhantes. Verificou-se também que o efeito da carga compressiva no processo de ramificação foi bem simulado. Os resultados numéricos mostraram que todas as cargas compressivas testadas aumentaram o comprimento da rede vascular e o número de ramificações. Considerando todas as cargas compressivas testadas (5%, 10% e 30% de deformação), a carga de 5% foi a mais efetiva, apresentando um aumento de 33% no comprimento da rede vascular e de 68% no número de ramos.

Deste modo, nesta tese simulou-se pela primeira vez e com uma estrutura e morfologia realista a rede capilar presente num ensaio *in vivo*. O modelo computacional foi validado utilizando dados *in vivo* e a capacidade preditiva do modelo desenvolvido foi estudada. A conjugação de modelos computacionais e ensaios experimentais realizada nesta tese contribuirá, no futuro, para o desenvolvimento de modelos de angiogénese mais robustos e precisos, capazes de aumentar o conhecimento nesta área, bem como reduzir o tempo e custo de metodologias experimentais, bem como o número de animais usados na ciência.

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Abbreviations

CAM	Chick Chorioallantoic Membrane
EC	Endothelial Cell
ECM	Extracellular Matrix
FEM	Finite Element Method
RPIM	Radial Point Interpolation Method
VEGF	Vascular Endothelial Growth Factor

List of Figures and Tables

Figure 1.1: Angiogenesis process.....	1
Figure 1.2: Timeline of the most relevant continuum, cell-based and hybrid models of wound healing angiogenesis.	4
Figure 2.1: Schematic representation of the angiogenesis steps.	11
Figure 3.1: Tip cell migration profile. (a) VEGF diffusion gradient (VEGF concentration in g mm^{-3}); (b) and (c) Simulation results from individual tip cell migration in different time steps, respectively, with no random motion and with random motion, using a regular nodal discretization mesh.	58
Figure 3.2: Tip cell migration profile in direction of two wounds placed at different location. (a) VEGF diffusion gradient (VEGF concentration in g mm^{-3}); (b) Final simulation results for the three tip cell migration with no random motion; (c) Final simulation results for the three tip cell migration with random motion.....	58
Figure 3.3: Capillary network. (a) VEGF diffusion gradient (VEGF concentration in g mm^{-3}); (b) Final simulation results for cell migration from individual sprouting tip (left), from two sprouting tips (middle) and from two parent vessels (right), using a regular nodal mesh.	87
Figure 3.4: Capillary network profile obtained in simulations (1 st column) and in CAM images (2 nd column) for some of the performed examples and the corresponding difference between the total capillary volume fractions (3 rd column). Images sized: $5 \times 5 \text{ mm}^2$	88
Figure 3.5: <i>In vivo</i> and <i>in silico</i> quantitative analysis of angiogenesis. Quantification of total vessel number and total vessel length for each treatment from CAM processed images (<i>in vivo</i>) and	

from simulation results (*in silico*) ($n_{\text{Control}} = n_{\text{VEGF 50ng}} = n_{\text{VEGF 100ng}} = 6$, One-way ANOVA, pairwise comparison Tukey's post-hoc test, *: $p < 0.05$, #: $p < 0.01$)..... 114

Figure 3.6: Capillary network profile obtained in CAM images (1st column) and in simulations (2nd column) for control, VEGF 50ng and VEGF 100ng and the corresponding difference between the total capillary volume fractions (3rd column). In all the images the biomaterial is located in the lower left corner. Images sized: 5 × 5 mm²..... 114

Figure 3.7: Final simulation results obtained for unloaded and under 5%, 10% and 30% strain loading, for a VEGF release region located in the superior boundary (1st row), a circular VEGF release region (2nd row) and a VEGF release region located in the middle (3rd row), using a regular nodal discretization mesh. The arrows mark the initial sprouting cells location. Images sized: 5 × 5 mm². 144

Figure 3.8: Quantification of the number of branches **(a)** and the total vessel length **(b)** for unloaded and under 5%, 10% and 30% strain loading, for the three different initial geometry domains tested: VEGF release region in the superior boundary (1st row), circular VEGF release region (2nd row) and VEGF release region in the middle (3rd row). All the results were normalized to the unloaded situation. The experimental data was obtained in [38]. 145

Table 3.1: Capillary network parameters analysed with both methodologies under study (*in silico* and *in vivo*). 88

Contents

Abstract	i
Resumo.....	iii
Acknowledgements.....	v
Abbreviations	vii
List of Figures and Tables	ix

Chapter I – Structural Overview

1.1. Introduction	1
1.2. Wound Healing Angiogenesis Models	2
1.3. Motivation and Aim	5
1.4. Thesis Outline	6

Chapter II – Theoretical Background

2.1. Introductory Remarks.....	9
2.2. Contribution I: Modelling skin wound healing angiogenesis: A review	11
2.2.1. Highlights of Contribution I	11
2.2.2. Contribution I.....	13

Chapter III – Original Studies

3.1. Introductory Remark	55
3.2. Contribution II: A preliminary study of endothelial cell migration during angiogenesis using a meshless method approach	57
3.2.1. Highlights of Contribution II	57
3.2.2. Contribution II.....	61
3.3. Contribution III: Sprouting Angiogenesis: A Numerical Approach with Experimental Validation.....	87

3.3.1.	Highlights of Contribution III	87
3.3.2.	Contribution III.....	89
3.4.	Contribution IV: Simulation of the Process of Angiogenesis: Quantification and Assessment of Vascular Patterning in the Chicken Chorioallantoic Membrane ..	113
3.4.1.	Highlights of Contribution IV	113
3.4.2.	Contribution IV	117
3.5.	Contribution V: Using a meshless method to assess the effect of mechanical loading in angiogenesis	143
3.5.1.	Highlights of Contribution V	143
3.5.2.	Contribution V	147
 Chapter IV – Thesis Considerations		
4.1.	General Discussion.....	171
4.2.	Conclusions and Future Work.....	175
References.....		177
Appendix I – Meshless Methods		181
Appendix II – Compilation Authorization		191

Chapter I

Structural Overview

1.1. Introduction

Angiogenesis is the process by which new blood vessels grow from the pre-existent vasculature, allowing the growth of the vascular system (Figure 1.1). This process is indispensable to support and to maintain cell and tissue viability, since every cell in the body needs to be within a small distance of 100 – 200 μm from a capillary because the diffusion of oxygen, nutrients and waste products does not occur at higher distances [1]. Angiogenesis is observed during embryogenesis and in adult life, in particular situations such as wound healing, tissue remodelling and pregnancy. Angiogenesis is also up-regulated in some diseases such as cancer, psoriasis and atherosclerosis [2]. In this thesis, angiogenesis during the wound healing process was studied.



Figure 1.1: Angiogenesis process.

Wound healing is a natural process that takes place when the human skin is damaged. Given the high frequency of skin damage during human life, skin wounds are an important health

and economic concern. Depending on their healing time, wounds can be defined in acute or chronic [3]. Acute wounds normally heal over a short period of time, normally a few weeks, without needing extra care. However, chronic wounds require a long period of several months to heal and usually are associated with other pathologies, such as diabetes and obesity, requiring additional health care [4].

After skin is damaged, wound healing takes place in order to restore tissue homeostasis. Wound healing is usually divided into four phases: haemostasis, inflammation, proliferation and tissue remodelling [5]. Briefly, in haemostasis, a fibrin clot is formed allowing to prevent blood loss. The inflammation phase is characterized by tissue swelling and it allows to control the bleeding and to prevent the infection. During proliferation, new tissues and blood vessels are formed in order to rebuild the wound area. Finally, in tissue remodelling phase, the granulation tissue, formed in the previous phases, matures into scar and tissue's tensile strength increases. Accordingly, the vascular network is re-established through angiogenesis what allows the adequate delivery of oxygen and nutrients to the new tissue. In fact, a poor blood supply to the wound is probably the single most important factor adversely affecting the wound healing process [6].

Angiogenesis is regulated by different mediators. However, the vascular endothelial growth factor (VEGF) is considered to be one of the most important proangiogenic factors during wound healing [7]. Indeed, the effect of VEGF in wound healing is well established. Several *in vivo* studies have already shown that increasing the VEGF concentration in the wound site accelerate the healing time. In contrast, animals that have reduced levels of VEGF tend to heal more slowly [8]. Although mechanical cues also have a role in angiogenesis regulation, its effect is less explored. Nevertheless, it has already been demonstrated that stretch, shear stress and pressure modulate endothelial cell processes such as proliferation and migration, signal transduction, gene expression and extracellular matrix remodelling [9-12]. In Chapter 2, the skin wound healing process is described in detail, emphasizing all the steps of the wound healing angiogenesis and its regulation.

1.2. Wound Healing Angiogenesis Models

Wound healing angiogenesis is a very complex process, and despite all the experimental studies already conducted to fully understand this process [13-15] there are some aspects that remain unclear. In order to overcome these gaps, in the last decades computational modelling

of angiogenesis has been under focus. This approach allows studying angiogenesis in different scales, using reproducible, more economic and less time-consuming methodologies, comparatively to the laboratory ones. Accordingly, to develop a computational model, first it is necessary to create a mathematical formulation capable to address the angiogenic process. Since the mathematical formulation (the set of equations that established the interactions between the model's components) is not analytical tractable, it is necessary to convert the mathematical model into the computational model, which is formed by the algorithmic solution of these equations. This methodology, named discretization, will allow to simulate angiogenesis in different degrees of complexity. In the field of wound healing angiogenesis there are three kinds of models: continuum models, cell-based models and hybrid models [16, 17].

Continuum models [18-22], by application of a partial differential equations system, are extremely effective to analyse, at the tissue scale, the behaviour of different cell populations and chemical concentrations of oxygen and growth factors. However, these models do not consider the individual detail of the tissue constituents, such as cells, and the capillary formation due to angiogenesis is often simulated as changes in vascular density [18, 20]. In contrast, cell-based models [23-26] analyse the capillary network, allowing to determine the effect of oxygen and growth factors in its morphology and structure. With these models, it is possible to study the proliferation and migration processes [23], the individual endothelial cell behaviour and morphology [24, 25] as well as the blood vessel length [23, 26]. Nevertheless, cell-based models present some disadvantages since the information acquired at tissue scale is limited and they are computationally more expensive. Therefore, hybrid models were developed in order to merge the advantages that continuum and cell-based models present individually. These models [27-30] allow to couple the interaction between distinct spatiotemporal scales, addressing the vessel network and cell behaviour modulated by oxygen and growth factors. Using hybrid models it is possible to study branching and anastomosis processes [27, 28, 30], to analyse individual cell behaviour, such as proliferation, differentiation and migration [29], and to analyse the morphology and structure of the capillary networks [27, 28]. However, hybrid models are more complex than other models, being less explored. Figure 1.2 shows a summary of some models previously published. In Chapter 2, a careful revision of the wound healing angiogenesis models described in the literature is presented.

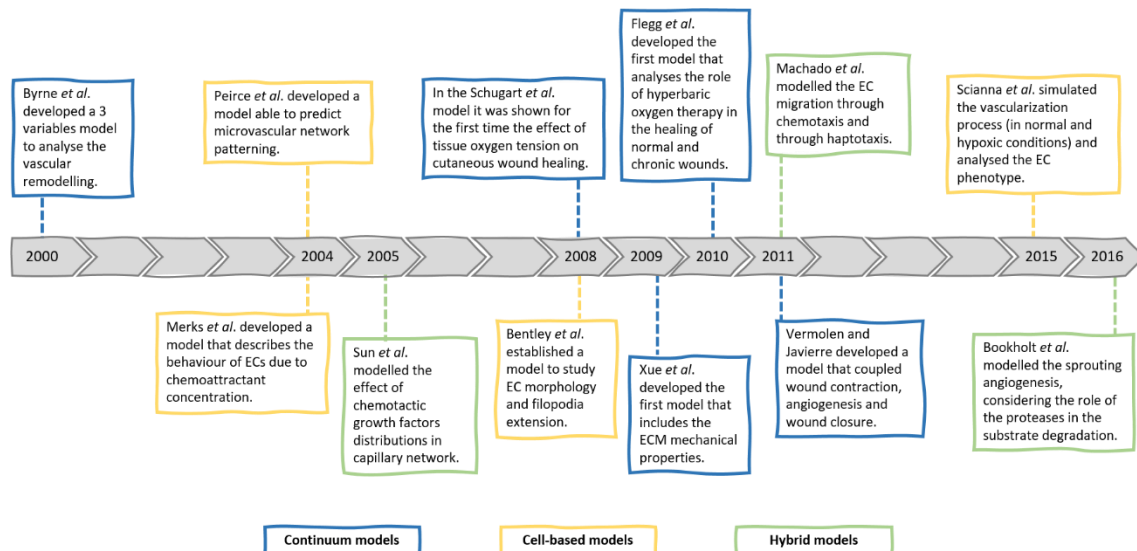


Figure 1.2: Timeline of the most relevant continuum, cell-based and hybrid models of wound healing angiogenesis.

Every wound healing angiogenesis model described in the literature presents some limitations. Biological processes are quite complex and modulated by several multiplayers, being impossible to capture the entire situation in just one computational model. Most of the developed models analysed the effect of growth factors in angiogenesis. However, continuum models do not mimic the individual behaviour of endothelial cells, neither the capillary network pattern nor fundamental processes of angiogenesis such as the branching and anastomosis. Furthermore, the effect of mechanical cues in individual endothelial cell behaviour and capillary network is less explored since the existing models only addressed the mechanical effect between the endothelial cells and the extracellular matrix (ECM) or the fibroblasts mechanical contraction in wound contraction [31]. To establish a computational model it is necessary to insert some parameters in the algorithm. Given the complexity of biological processes, it is usually necessary to use a huge number of estimated parameters. In order to develop models as close as possible to reality, it is necessary that computational modelling and biological experimentation work together. That way, it is possible to develop models in which the capillary network will be modelled using experimental data and the numerical results will be validated using *in vivo* angiogenesis assays, allowing to reproduce the *in vivo* angiogenesis.

1.3. Motivation and Aim

Skin wounds cause pain and discomfort and they are an unguarded region for pathogens to enter in the body. In modern societies, the occurrence of wounds and its treatment is a relevant economic, clinical and social topic. In 2018, a retrospective study conducted by Medicare identified that about 8.2 million people had at least one type of wound or related infection [32]. Furthermore, cost projections from Medicare for all wounds ranged between \$28.1 billion and \$96.8 billion. These projections include costs related to infection management, among which surgical wounds and diabetic ulcers were the most expensive to treat [32]. Chronic wounds may have different aetiologies but all of them represent a burden to the health care system. Moreover, chronic wounds are predicted to increase as the population ages and the incidence of comorbidities that affect the wound healing process such as diabetes, cardiac disease and obesity increase. All these pathologies are characterized by a poor tissue vascularization that impairs the wound healing process. Accordingly, it is necessary to understand and to address new strategies to promote wound healing angiogenesis.

In silico models, a type of computational model formulated to study biological research carried out entirely in a computer, are very useful to understand the dynamics of biological systems in different scales by using different approaches. To simulate angiogenesis, advanced discretization techniques, regardless being mesh dependent or meshless, are the most applied ones. Meshless methods have some advantages such as being able to analyse structures with complex geometries, such as soft tissues; to use high-order continuous weight functions to construct the test functions, which allows to achieve smoother variables fields; to analyse large deformations problems and moving discontinuities; and, where mesh refinement is required, to insert locally a higher number of nodes in order to obtain accurate local solutions, without any extra computational cost [33, 34]. Moreover, meshless methods provide the analysis of any phenomenon that is governed by a differential equation [33] as the endothelial cell migration due to chemotaxis. *In silico* models help researchers to study angiogenesis using less time-consuming, reproducible and cheaper methodologies. However, these models require the proper feedback from experimental assays in order to validate the computational model and to determine how close the model is to reality.

Accordingly, in this thesis, a meshless-model to study the angiogenic process in the context of wound healing was presented. Furthermore, the numerical results were compared with an *in vivo* angiogenesis assay.

Based on the previously discussed, the aims of the present thesis are:

- To review the literature in what regards the wound healing angiogenesis models;
- To analyse the pertinence of using a meshless method to simulate angiogenesis;
- To simulate angiogenesis using different domain geometries in order to mimic several hypothetic biological situations;
- To analyse the effect of VEGF diffusion and concentration in angiogenesis;
- To analyse the effect of compressive loading in angiogenesis;
- To validate the numerical results using experimental data;
- To contribute to a better knowledge related with the angiogenic process.

1.4. Thesis Outline

The structure of this thesis is based on the published or submitted papers in international journals and it is organized in four main chapters.

In **Chapter I** the thesis subject is introduced and it is divided into four sections. In Section 1.1. the angiogenesis process and its importance in wound healing is briefly described. In Section 1.2. the relevance of using *in silico* models to study angiogenesis is addressed and a review of some of the most relevant models in this field is presented. In Section 1.3. the motivation and the objectives of this work are presented. Finally, in Section 1.4. the thesis outline with a brief explanation of its structure is provided.

In **Chapters II** (Review Paper) and **III** (Original Papers) the papers written during the project are presented. Therefore, in these chapters, the obtained results are described in great detail. These chapters were designed to develop a computational model able to simulate angiogenesis, comprised one review paper and four original studies, each one with different aims. The sequence of the contributions is organized as follows:

Chapter II, Section 2.2.	Contribution I
Title	<u>Modelling skin wound healing angiogenesis: A review</u>
Authors	Guerra A, Belinha J, Natal Jorge RM
Published in	Journal of Theoretical Biology, 2018, 459: 1-17
DOI	https://doi.org/10.1016/j.jtbi.2018.09.020

Chapter III, Section 3.2.		Contribution II
	Title	<u>A preliminary study of endothelial cell migration during angiogenesis using a meshless method approach</u>
	Authors	Guerra A, Belinha J, Natal Jorge RM
	Published in	International Journal for Numerical Methods in Biomedical Engineering, 2020, 36(11): e3393, 2020
	DOI	https://doi.org/10.1002/cnm.3393
Chapter III, Section 3.3.		Contribution III
	Title	<u>Sprouting Angiogenesis: A Numerical Approach with Experimental Validation</u>
	Authors	Guerra A, Belinha J, Mangir N, MacNeil S, Natal Jorge RM
	Published in	Annals of Biomedical Engineering, 2021, 49(2): 871-884
	DOI	https://doi.org/10.1007/s10439-020-02622-w
Chapter III, Section 3.4.		Contribution IV
	Title	<u>Simulation of the process of angiogenesis: Quantification and assessment of vascular patterning in the chicken chorioallantoic membrane</u>
	Authors	Guerra A, Belinha J, Mangir N, MacNeil S, Natal Jorge RM
	Published in	Computers in Biology and Medicine, 2021, 136: 104647
	DOI	https://doi.org/10.1016/j.combiomed.2021.104647
Chapter III, Section 3.5.		Contribution V
	Title	<u>Using a Meshless Method to Assess the Effect of Mechanical Loading in Angiogenesis</u>
	Authors	Guerra A, Belinha J, Natal Jorge RM
	Published in	Submitted to an International Journal

Chapter IV displays the final considerations and it is divided into two sections. Section 4.1. presents an integrated discussion of the obtained results and the thesis' main contributions. Section 4.2. summarizes the main conclusions and possible future research.

Chapter II

Theoretical Background

2.1. Introductory Remarks

As previously mentioned, this thesis was conducted in order to improve the knowledge in the field of wound healing angiogenesis and to develop a computational model able to address this complex process. In this section, the first publication (2.2.2. Contribution I) is presented. In this review paper, the continuum, cell-based and hybrid mathematical models of wound healing angiogenesis are carefully reviewed in order to identify possible improvements. Moreover, the relevance of angiogenesis in wound healing and the variables that guide this regenerative phenomenon, a brief overview of the anatomy and structure of the human skin and a detailed description of the wound healing angiogenesis process are provided.

In this chapter, the theoretical background is presented as a full review paper and the main highlights are presented as an extended abstract.

2.2. Contribution I: Modelling skin wound healing angiogenesis: A review

2.2.1. Highlights of Contribution I

In silico models are more economical and less time-consuming approaches comparatively to experimental methodologies and can help to test new strategies to promote/optimize angiogenesis during wound healing.

Therefore, the main contents of this publication include:

- I. Brief overview of the anatomy and structure of the human skin;
- II. Concise description of the skin wound healing process. This process is usually divided in four phases: haemostasis, inflammation, proliferation and tissue remodelling. During the proliferative phase the vascular network is restored due to angiogenesis. Angiogenesis is an indispensable process during wound healing since a deficiency in vessel formation leads to a delay in wound healing and promotes the development of chronic wounds;
- III. Summary of angiogenesis steps during wound healing (Figure 2.1) and its regulatory species;

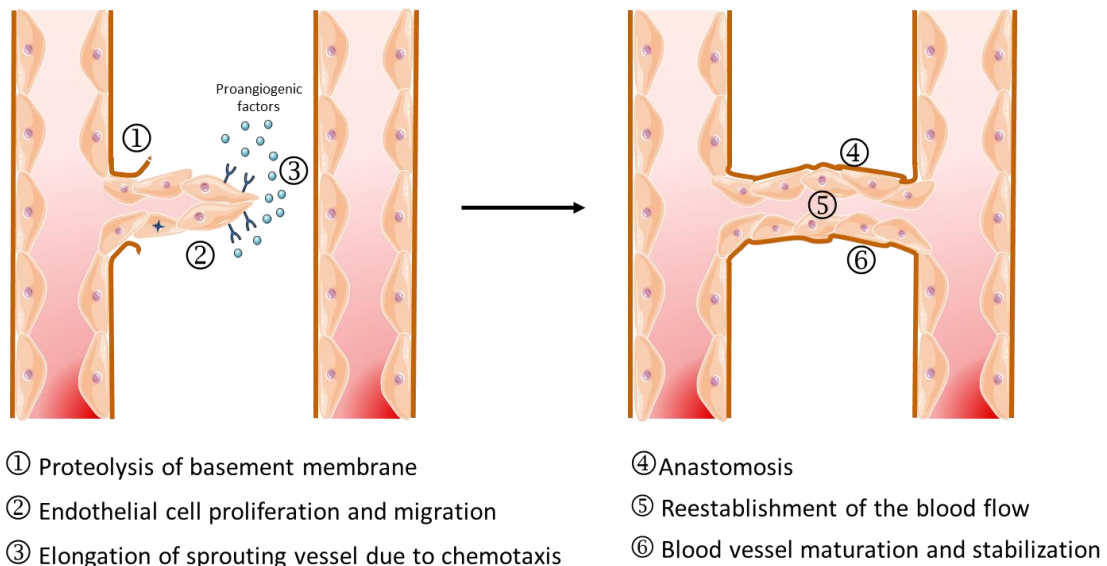


Figure 2.1: Schematic representation of the angiogenesis steps.

- IV. Comprehensive revision of the mathematical and computational models of wound healing angiogenesis described in the literature. Usually these models are divided in continuum, cell based and hybrid approaches. Accordingly, the most relevant models were discussed considering: the scale of interest addressed in the model (molecular, cellular or tissue), the biological processes captured in the model and the chemical and cellular species involved. Moreover, the advantages and disadvantages of using each kind of modelling approach were also analysed;
- V. Discussion of the simplifications, assumptions and decisions that have to be done in order to develop a mathematical model of wound healing angiogenesis;
- VI. Conclusion highlighted that the current models are able to capture the wound healing angiogenesis as a whole. However, some specific steps such as vascular network remodelling and the ECM components are less explored.

The complete document can be found in the next sub-chapter.

2.2.2. Contribution I

Modelling skin wound healing angiogenesis: A review

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Modelling skin wound healing angiogenesis: A review

Ana Guerra^a, Jorge Belinha^b and Renato Natal Jorge^a

^aINEGI, Faculty of Engineering, University of Porto, Porto, Portugal.

^bSchool of Engineering, Polytechnic of Porto, Porto, Portugal.

ABSTRACT

The occurrence of wounds is a main health concern in Western society due to their high frequency and treatment cost. During wound healing, the formation of a functional blood vessel network through angiogenesis is an essential process. Angiogenesis allows the reestablishment of the normal blood flow, the sufficient exchange of oxygen and nutrients and the removal of metabolic waste, necessary for cell proliferation and viability. Mathematical and computational models provide new tools to improve the healing process. In fact, over the last thirty years, *in silico* models have been continuously formulated to describe the effect of several biological and mechanical factors in angiogenesis during wound healing. Additionally, with different levels of complexity, these models allow coupling the human skin structure, to distinct cell types and growth factors, to study extracellular matrix composition and to understand its deformation. This paper discusses how *in silico* models, which are more economical and less time-consuming comparatively to laboratory methodologies, can help test new strategies to promote/optimize angiogenesis. The continuum, cell-based and hybrid mathematical models of wound healing angiogenesis are reviewed in the present paper, in order to identify possible improvements. Accordingly, the development of higher dimension models incorporating multiscale analysis at molecular, cellular and tissue level remains a challenge that future models should consider.

KEYWORDS

Angiogenesis modulation;
Computational modelling;
Human skin.

1. INTRODUCTION

Skin wounds are an important health concern since they are very frequent during human life. Wounds in the skin can be a consequence of damage in traumatic accidents, the result of surgery incisions, may result from long periods of immobility and are also associated to other pathologies, such as obesity and diabetes. Wound treatment represents high economic and social costs in modern societies. It is estimated that the United States Health Care spends approximately 25 billion dollars every year for the treatment of wounds (Sen et al., 2009). In the United Kingdom, the National Health Service estimated an annual range of £2.3-3.1 billion for the treatment of chronic wounds (Posnett and Franks, 2008). Thus, it is mandatory to understand the wound healing process in order to address new and more effective therapies.

Angiogenesis consists in the formation of new blood vessels from pre-existing ones and it is an indispensable process during wound healing. Several *in vitro* (Herman and Leung, 2009; Staton et al., 2009) and *in vivo* (Chen et al., 2013; Wong et al., 2011) studies have been carried out in order to fully describe this biological process during wound healing. However, angiogenesis is very complex, and includes many mediators and regulatory factors (Carmeliet and Jain, 2011). Due to differences in biology between human and other species, as well as distinct experimental designs, the studies already performed present some disadvantages that have to be taken into consideration. Consequently, *in silico* models have been under focus as they allow modelling angiogenesis with different and more economic approaches. In fact, several mathematical models have been developed in order to describe biochemical and biomechanical mediators of wound healing angiogenesis at molecular, cellular and tissue scale (Bentley et al., 2008; Javierre et al., 2008; Maggelakis, 2003; Matsuya et al., 2016; Merks et al., 2004; Olsen et al., 1997; Pettet et al., 1996a; Pillay et al., 2017; Scianna et al., 2015; Sun et al., 2005).

The aim of this paper is to review the continuum, cell-based and hybrid mathematical models of wound healing angiogenesis in order to identify possible improvements. Therefore, scientific databases such as “PubMed” and “Google Scholar” were reviewed for the words: “skin”, “wound healing”, “angiogenesis” and “mathematical models”. First, in order to understand the mechanism and the variables that guide this regenerative phenomenon, we will provide a brief overview of the anatomy and structure of the human skin and of the wound healing angiogenesis process. Then, we will describe the most relevant continuum, cell-based and hybrid models focusing in the modelling strategies and different mediators. In the end, a comparison between the distinct approaches to model wound healing angiogenesis and potential improvements for future models will be discussed.

2. HUMAN SKIN

Human skin (Figure 1) is an important physical barrier between the body and the external environment (Proksch et al., 2008) being the largest adult organ, with approximately 2 m² in area and around 2.5 mm of thickness.

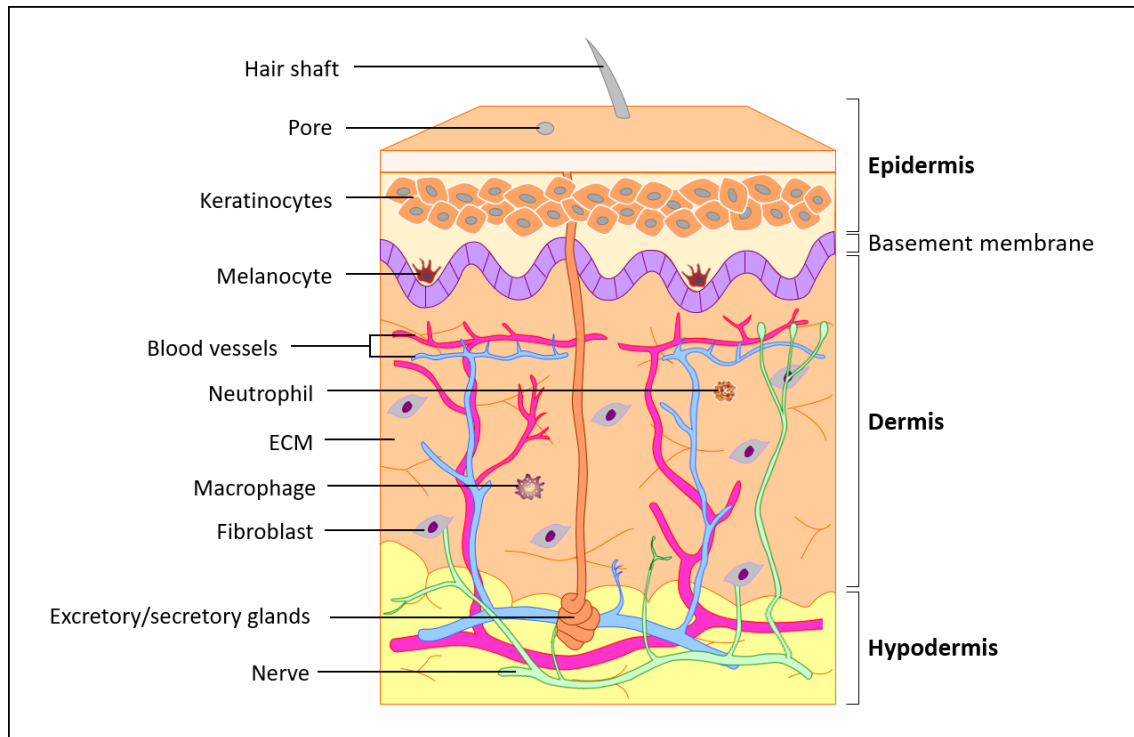


Figure 1: Schematic representation of human skin and underlying structures.

This tissue is composed of two layers: epidermis and dermis. The epidermis is the upper layer and it is very thin. For this reason, its contribution to the mechanical behaviour of the skin is minimum. The epidermis is mainly composed of keratinocytes but presents other cell populations such as melanocytes, Langerhans cells and Merkel cells (Javier and Ackerman, 2001). The dermis, the layer below the epidermis, is approximately 2 mm of thick and provides the structural and the nutritional support for this tissue. It is mainly composed by extracellular matrix (ECM), produced by fibroblasts (Supp and Boyce, 2005) but also contains blood and lymphatic vessels, nerves, excretory and secretory glands, keratinizing structures, sensory nerve receptors and immune cells. In fact, the dermis acts as a mucopolysaccharide gel in which collagen fibres deliver strength and toughness, elastin maintains normal elasticity and flexibility and proteoglycans provide viscosity and hydration. The dense collagen and elastin network of dermis is responsible for the mechanical properties of the skin (Reihsner et al., 1995). Some argue that a third layer can be considered, the hypodermis, which would be mainly composed

of fat connective tissue that connects the dermis with the skeletal components (Cooper, 2002). Although the principal functions of the human skin is to protect the body against mechanical, chemical and pathogenic damage and to prevent loss of water and of extracellular fluid (Wertz, 2013), this tissue possesses other important functions, such as body temperature regulation, sensory perception, absorption of some substances, immunological response (Pasparakis et al., 2014) and synthesis of hormones (Zouboulis, 2009).

The maintenance of the correct interconnection between all the cells populations, ECM components, regulatory mechanisms and vascular system in the skin is essential to the correct function of this tissue and to body homeostasis.

3. SKIN WOUND HEALING

Under particular situations, the human skin may experience damage. In fact, skin wounds are considerably common during human life and an important issue in health care. Depending on their healing time, wounds can be divided in acute or chronic (Dreifke et al., 2015). Acute wounds can have different causes, such as surgical incision, thermal, abrasion or laceration. In people with no serious health conditions these wounds generally heal over a short period of time. Conversely, in chronic wounds the healing fails and the repair process cannot successfully form an anatomic and functional tissue. These wounds present a delay in the healing process and they are usually associated with other pathologies, such as diabetes mellitus and obesity.

After the skin damage, wound healing takes place in order to repair the damage. Wound healing is usually divided in four phases: haemostasis, inflammation, proliferation and tissue remodelling (Singer and Clark, 1999). Briefly, in haemostasis, platelets and fibrin fibres promote coagulation by forming a clot that reduces the blood loss. Besides coagulation, platelets also release the platelet derived growth factor (PDGF) that stimulates cellular activity allowing the bleeding to stop. In the following phase, inflammation, the wound is cleaned from foreign particles, bacteria and dead tissue. Neutrophils, the first population of cells that arrives to the wound area, remove pathogens. Then, macrophages migrate into the wound and continue the cleaning process. These inflammatory cells release chemical signals that establish gradients, allowing other cell populations such as keratinocytes, fibroblasts and endothelial cells (Tranquillo and Lauffenburger, 1987) to be attracted. The following proliferative phase involves new granulation tissue formation, reepithelization and the restoration of the vascular network. Growth factors stimulate fibroblasts that will proliferate and synthesize collagen, the main component of ECM and which restores the desired mechanical properties of the healed skin

(Chiquet et al., 2009). Growth factors also stimulate keratinocytes to proliferate, thus allowing the reepithelization (Simpson et al., 2011). In this phase, the vascular network is also re-established through angiogenesis. This process is very important since it provides nutrients and an oxygen supply to the new tissue (Li et al., 2005). Due to its importance, angiogenesis will be presented with detailed in section 3.1. When the wound is closed, the remodelling phase begins. This phase is characterized by the reorganization and contraction of the ECM, which decreases the dimension of the scar on the skin. In the remodelling phase, collagen fibres type-III, that have been quickly produced by fibroblasts are gradually replaced by a much stronger type-I collagen, which increases the ultimate tensile strength of the skin. The remodelling phase may continue for months or even years, however the healed area never regains the full ultimate tensile strength of unwounded skin (Blais et al., 2013).

For the correct skin wound healing this orderly sequence of healing events have to occur. If any step of the healing sequence is altered, pathologic responses may take place leading to hypertrophic scars (fibrosis) or chronic non-healing wounds. Chronic wounds frequently presents a delay in the inflammatory or proliferative phases (Enoch et al., 2006). It is well known that effective wound healing is dependent on sufficient oxygen supply, functional vascular network and diffusion distance. Furthermore, economic and social impact of wounds in our society is very high and there is still a lack of wound healing, non-invasive methodologies capable to fully describe this biological process in humans. Mathematical and computational models are becoming an interest approach within the scientific community because they are a more economical and less time consuming methodology, allowing these new strategies to be tested. Thus, it is mandatory to fully understand the wound healing mechanism in order to address new strategies to improve the healing process. Consequently, it is necessary to develop new/enhanced mathematical models capable to mimic wound healing. Nevertheless, testing new therapies using non-invasive methods remains a challenge.

As already mentioned, angiogenesis is an indispensable process during wound healing. In fact, a deficiency in vessel formation is one of the main factors in the delay of wound healing and plays a major role in the development of chronic wounds.

3.1. Angiogenesis in wound healing

Angiogenesis is a physiological process essential during wound healing since it provides the reestablishment of the normal blood flow and consequently the sufficient exchange of

oxygen and nutrients and the removal of metabolic wastes (Carmeliet and Jain, 2011). A defect in the regulation of the blood vessels growth can cause dehiscence and ulceration.

In healthy tissues, blood vessels are maintained in a quiescent state. Mature vessels are surrounded by a basement membrane, made up mainly of collagen type-IV and laminin coated with smooth muscle cells and pericytes that promote endothelial cell survival and allow for vessel stability (Carmeliet, 2003). In situations such as wound healing, quiescent vessels are exposed to proangiogenic factors and adjacent endothelial cells become stalk cells that start to proliferate and to migrate in the direction of the tip cell, resulting in elongation of the sprouting vessel (Carmeliet and Jain, 2011). Afterwards, blood vessels density increases and vascular sprouts will fuse with neighbouring sprouts vessels, in a process called anastomosis that allows re-establishing the blood flow. Then, the unperfused vessels will regress by apoptotic processes. Finally, the vasculature returns to a quiescent state, the basement membrane is restored and the new blood vessel will be coated by smooth muscle cells and by pericytes that stabilize the vessel (Carmeliet and Jain, 2011) (Figure 2). If the tissue wound healing was correctly performed, the number of vessels normalizes and returns to a level close to the one observed in uninjured skin (Yamashita et al., 2014).

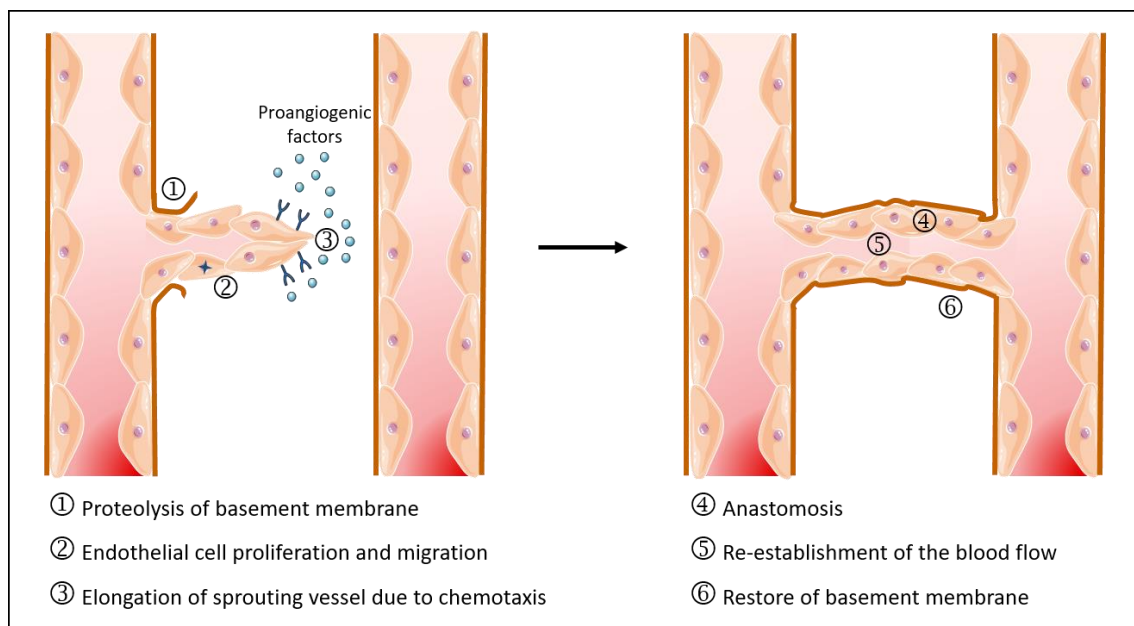


Figure 2: Schematic representation of the angiogenic process. In the presence of pro-angiogenic stimulus the basement membrane detach (1) and the endothelial cells proliferate and migrate (2), allowing the elongation of the sprouting vessel due to chemotaxis (3). Then, sprouting vessels fuse with neighbouring sprouts vessels (4), allowing the re-establishing of the blood flow (5). Finally, the basement membrane is restore and the new formed vessel is stabilize (6).

Angiogenesis is precisely regulated by genetic programs and it is strongly modulated by several chemical factors that allow the activation of cellular related pathways. The main regulator of angiogenesis is the vascular endothelial growth factor (VEGF) family, which includes VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). These soluble factors bind specifically to the tyrosine kinases receptor (VEGFR1 and VEGFR2) on the cell surface and to non-tyrosine kinase receptors of the neuropilin (NRP-1 and NRP-2), which function as co-receptor for VEGFRs. Each soluble factor binds specifically to its receptor, which will allow the activation of cellular pathways downstream. Within this family of proteins, the VEGF-A, or simply VEGF, is the most important proangiogenic factor and stimulates endothelial cells to proliferate, to migrate, to differentiate and to survive, allowing new blood vessel formation. Indeed, the binding of VEGF to VEGFR2 is the best known pathway for stimulating endothelial cells proliferation and migration and the increasing in vascular permeability (Koch and Claesson-Welsh, 2012). The effects of these pathways are regulated via downstream signalling including the MEK-MAPK pathway (proliferation and migration), the PI3K-Akt pathway (survival) and the Src-eNOS pathway (permeability) (Koch and Claesson-Welsh, 2012; Moens et al., 2014). Besides the effect of VEGF, the angiogenic process is stimulated by other growth factors, such as basic fibroblast growth factor (bFGF), interleukin-8, platelet derived growth factor (PDGF), transforming growth factor β (TGF- β), hypoxia-inducible factor (HIF) and PlGF (Bao et al., 2009; Penn et al., 2012).

Cutaneous wounds present a high level of VEGF, which is produced in response to injury by multiple cells types, including keratinocytes, macrophages and fibroblasts (Brown et al., 1992; Willenborg et al., 2012). Hypoxia causes activation of the transcription factor HIF, resulting in the transcription of the VEGF gene (Elson et al., 2000). This is one of the reasons that explains the VEGF increase during wound healing.

In the literature, there are some contradictory results about angiogenic regulation and the promotion of wound healing. Some studies highlight that inhibiting angiogenesis does not have significant effects in wound healing progress (Lange-Asschenfeldt et al., 2001; Roman et al., 2002). Other authors argue that blocking angiogenesis significantly delays wound repair (Rossiter et al., 2004; Streit et al., 2000). One reason for these contradictory results could be explained by the fact that in many of these studies, angiogenesis was analysed by counting the number of blood vessels or the blood vessels density and did not evaluate the functionality of the vessels. In fact, many blood vessels formed during wound healing are not perfused (Bluff et al., 2006), thus the decrease in blood vessels density by the selectively elimination of nonperfused blood vessels may not have a significant effect in wound healing. It is also necessary to take into account that wounds angiogenesis normally correlates with the inflammatory process, given that inflammatory cells release abundant proangiogenic factors (Koh and

DiPietro, 2011). Fetal skin wounds (Wilgus, 2007) and oral mucosa wounds (Szpaderska et al., 2003) present reduced scar formation when compared to normal healing in adult skin wound. Some authors correlated this observation with the lower levels of angiogenesis that fetal skin wounds and oral mucosa wounds show. They also exhibited reduced inflammation, reduced capillary growth and a more quick maturation of capillary network which may justify improvement in the healing process (DiPietro, 2016). Accordingly, a number of existent studies point that, apart from the increase in capillary density, it is also necessary that these blood vessels are functional and well perfused in order to establish the correct blood flow and to allow the delivery of oxygen and nutrients that sustain the metabolism of the cells in the wound area. It has already been demonstrated that the reduction on VEGF activity by using neutralizing antibodies or small molecules that block VEGF signalling or conditional genetic deletion of VEGF delays the wound healing process (Rossiter et al., 2004).

As previously discussed, angiogenesis is a considerably complex process, being difficult to establish the effect of changing one biological factor, such as oxygen, nutrients or growth factor concentration, and its influence in wound healing. Consequently, mathematical models capable of reproducing biological systems are powerful tools that can be used to understand the complexity of biological systems, their regulatory processes and thus allowing to study the effect of different variables in these complex systems (Galle et al., 2005; Morel et al., 2001).

4. MATHEMATICAL AND COMPUTATIONAL MODELS

Computational modelling is a powerful tool that provides better knowledge about biological systems, since it allows virtual simulation of the interactions between biochemical and biomechanical effects and to better understand the effect of these biological factors in skin wound healing as a whole (Kitano, 2002).

The first step when simulating a biological process is to create a mathematical model suitable to address the biological feature under study. However, because of the mathematical model (the set of equations) is not analytically tractable, so it is necessary to convert it into a computational model, composed by the algorithmic solution of these equations. This methodology, known as discretization, will allow to simulate the biological phenomena in various degrees of complexity. In the context of wound healing angiogenesis, there are two mathematical approaches available: continuum models and cell-based models (Anderson et al., 2007; Buganza-Tepole and Kuhl, 2013). However, this classification is not fully adopted by all the researchers in this area. Some authors describe the cell-based models as discrete models

(Buganza-Tepole and Kuhl, 2013). Continuum models are often used to modulate concentrations of chemical mediators, such as VEGF and TGF- β , and of mechanical factors, such as forces and deformation. Cell-based models are frequently used to modulate cell populations allowing researchers to study the behaviour of individual cells, such as fibroblasts and macrophages. Yet, the scale of interest and the underlying mathematical formulation will determine which modelling approach is more appropriate. Generally, one can say that components at molecular or cellular level can be modulated using cell-based models and, at tissue, organ or system levels the continuum models are a better approach.

In continuum models, the evolution of different fields is frequently governed, in space and time, through partial differential equations. These equations establish: (i) how a field interact with other fields through coupling terms; (ii) how a field evolves in time; and (iii) how a field evolves in space over the domain of interest. These kind of models are good approaches to study spatial variations of chemical concentrations and mechanical strains or stress (Buganza-Tepole and Kuhl, 2013). In cell-based models, the behaviour of each entity can be governed by one or more differential equations. These equations establish: (i) how a characteristic variable of a single entity interacts with other variable through coupling terms and (ii) how a characteristic variable evolves in time (Buganza-Tepole and Kuhl, 2013).

Concerning the numerical discretization process, in continuum models, the partial differential equations can be discretize both in time and space. Time is frequently discretize into small intervals using the finite difference method. Space may be discretized by several distinct methodologies: the domain of interest may be represented in a regular mesh using a finite difference method or a finite volume method (Thackham et al., 2009). Additionally, arbitrarily shaped domains, such as particular parts of the body, may be discretize using advanced discretization techniques (finite element method or meshless methods). When time and space are discretized, a system of algebraic equations is obtained and the resolution of these equations characterizes the evolution of all continuum fields in space and time. Cell-based models are conceptually simpler than continuum models but because each entity needs to be represented through its own equation they are computationally more expensive (Buganza-Tepole and Kuhl, 2013).

In order to select the best modelling approach, first, it is necessary to identify which angiogenic phase will be studied. In addition to the scale of the analysis (molecular, cellular or tissue level), it is important to carefully define the natural and essential boundaries of the problem (numeric simplifications). Thus, the most relevant approaches about modelling wound healing angiogenesis are discussed below.

4.1. Modelling wound healing angiogenesis

Several experimental studies have been performed in order to understand in depth and explain the angiogenic process (Carmeliet and Jain, 2011; Ferrara, 2000; Flamme et al., 1997). Modulating angiogenesis stimulated the interest of the scientific community since this process is essential in wound healing. Due to the complexity of angiogenesis, there is still a lack of experimental studies and some aspects of this process are not fully described. Moreover, costs involved are high and time consuming to study angiogenesis using only laboratory-based biological techniques. Thus, *in silico* studies have started to be under focus since they enable angiogenesis to be modulated with different approaches.

In the last 30 years, several modelling strategies for wound healing angiogenesis have been developed. It is important to take into account that if it is intended to model the behaviour of a reduced number of cells, the wound may be better described using a cell-based model, in which individual entities are considered. Wounds are characterized by the interaction between several chemical factors and cell population, which make the continuum models a better approach. Recently, the scientific community has started to investigate hybrid models, combining the microscale analysis (cell level) with the macroscale analysis (tissue level). Hybrid models allow explicitly describe the behaviour of cells at the microscale and, simultaneously, predict the variation of the species' concentration due to reaction and diffusion (oxygen, glucose, VEGF, drugs, etc.) at a macroscale.

Wound healing and tumour growth can be modelled through similar mathematical approaches (Chaplain and Byrne, 1996). Therefore, several studies that model angiogenesis in solid tumours (Byrne and Chaplain, 1996; Holmes and Zachary, 2005; Levine et al., 2000; Levine et al., 2001; Levine et al., 2002; Plank et al., 2004) contributed to develop mathematical models for wound healing angiogenesis and *vice versa*. Because tumour angiogenesis is not the scope of this review, these models will not be addressed. The first model of angiogenesis was proposed, in 1985, by Balding and McElwain (Balding and McElwain, 1985). The authors established a model that simulated the interaction between blood vessel density, capillary tip density and tumour angiogenic factor concentration in a 1D framework, referred as a “snail-trail” model. Although this model has been developed to study tumour-induced angiogenesis, it supported the subsequent mathematical models of wound healing angiogenesis. Then, in 1990, Sherratt and Murray (Sherratt and Murray, 1990) developed the first mathematical model of wound healing. Although this model did not include the angiogenic process, it introduces several aspects about cell migration, proliferation and death that the upcoming models have adopted.

In the following subsections, the most relevant approaches in modelling angiogenesis during wound healing, considering each model (continuum, cell-based and hybrid), will be presented.

4.1.1. Continuum models

In the field of angiogenesis, continuum models (Table 1) are extensively used to describe, at tissue scale, the behaviour of cell populations and chemical concentration, using a system of partial differential equations (PDEs).

Pettet and co-workers developed the first two models of wound healing angiogenesis. For the first time, using a 1D framework model (Pettet et al., 1996a), Pettet *et al.* modelled the interactions between six chemical and cell species: capillary tips, capillary sprouts, fibroblast, chemoattractants, oxygen and ECM. In their 3-species model (Pettet et al., 1996b) the authors simplified the mechanism and described the behaviour of capillary-tip, macrophage-derived chemoattractants and new blood vessels formation during the tissue repair process. The authors proposed a model that includes a system of partial differential equations to describe the evolution of the dependent variables in space and time: capillary-tip density (ρ_c), chemoattractant concentration (C_c) and blood vessel density (ρ_{bv}). Accordingly, the capillary tip density (ρ_c) was modelled with:

$$\frac{\partial \rho_c}{\partial t} = \underbrace{\mu_c \frac{\partial^2 \rho_{ct}}{\partial x^2}}_{\text{random motility}} - \underbrace{\chi_c \frac{\partial}{\partial x} \left(\rho_c \frac{\partial C_c}{\partial x} \right)}_{\text{chemotaxis}} + \underbrace{\lambda_1 C_c \rho_{bv}}_{\text{budding}} - \underbrace{\lambda_2 \rho_c}_{\text{decay}} - \underbrace{\lambda_0 \rho_c^2}_{\text{anastomosis}} \quad (1)$$

Being μ_c the assumed constant of capillary-tip random motility coefficient and χ_c the chemotaxis coefficient. The model assumes that tip production or budding is proportional to the product of the attractant concentration and the vessel density, with the constant of proportionality λ_1 . For simplicity, it was assumed that natural death and tip-to-tip anastomosis constitute the dominant contributions and tip-to-branch anastomosis was neglected. Natural death was modelled by a linear decay of tips, with rate λ_2 , while tip-to-tip anastomosis was modelled using a term proportional to the square of the tip density, with rate λ_0 . The chemoattractant concentration (C_c) was modelled by means of:

$$\frac{\partial C_c}{\partial t} = D_a \frac{\partial^2 C_c}{\partial x^2} - \frac{\lambda_3}{2} \left(\underbrace{1 + \tanh \left(\frac{b \rho_{bv}^{char} - \rho_{bv}}{\lambda_{apr}} \right)}_{\text{production}} \right) - \underbrace{\lambda_4 C_c}_{\text{decay}} - \underbrace{\lambda_5 C_c \rho_{bv}}_{\text{removal}} \quad (2)$$

In which D_a is the attractant diffusion coefficient, $b\rho_{bv_{char}}$ is the typical (characteristic) blood vessel density and $\lambda_{a_{pr}}$ determines the rate at which attractant production falls with increasing vessel density. The model assumes that attractant production occurs at the maximum rate λ_3 . In addition, it was supposed that natural decay and removal by the local vascular network were the dominant mechanisms for attractant loss and that these processes occurs at rates λ_4 and λ_5 , respectively. Finally, the evolution of blood vessel density (ρ_{bv}) was governed by the equation:

$$\frac{\partial \rho_{bv}}{\partial t} = \underbrace{\mu_{bv} \frac{\partial}{\partial x} \left(\rho_c \frac{\partial \rho_{bv}}{\partial x} \right)}_{\text{random motility}} - \underbrace{\left(\mu_c \frac{\partial \rho_c}{\partial x} - \chi_c \rho_c \frac{\partial C_c}{\partial x} \right)}_{\text{snail trail}} \quad (3)$$

where μ_{bv} is the constant blood vessel random motility coefficient, being similar in magnitude with μ_c . With this approach the authors successfully modelled the propagation of wound healing angiogenesis and several other authors used this model as a base for the upcoming studies. Byrne *et al.* (Byrne et al., 2000) developed a simpler model, with the same variables as the model of Pettet *et al.* (Pettet et al., 1996b): capillary tip density, concentration of angiogenic factors and blood vessel density. This model also included the effect of vascular remodelling. The authors compared the results obtained for wound surface area in the numerical simulations with independent clinical measurement of normal and chronic wounds and they found good qualitative agreement in both cases. Maggelakis (Maggelakis, 2003) developed a model slightly different from the previously ones, based on diffusion equations. The model is capable of describing the motion of oxygen through the wound space, using a diffusion equation:

$$\frac{\partial C_{O_2}}{\partial t} = D_0 \nabla^2 C_{O_2} + F(n) - G(C_{O_2}), \quad 0 \leq x \leq R \quad (4)$$

where C_{O_2} is the oxygen concentration, D_0 is the oxygen diffusion coefficient, $F(n)$ represents the supply of oxygen to the damaged tissue by the capillaries and $\nabla = \partial/\partial x$. The consumption of oxygen by the macrophage-derived growth factors (MDGFs) was described by a sink term, which is taken to be $G(C_{O_2}) = \lambda_0 (C_0/C_\theta)$, where λ_0 represents the oxygen consumption rate, and C_θ represents the critical level of oxygen concentration below which macrophages appear at the wound site. The model also included the production, depletion and absorption of macrophage derived growth factors (MDGFs) and the growth of capillary density. The numerical simulation showed that there is a negative feedback mechanism that regulated the tissue wound healing. Thus, an increase in the amount of feedback results in higher levels of oxygen, which decrease the production of MDGFs. However, in the areas where oxygen concentration is low,

the concentration of MDGFs increase and, consequently, there is a higher capillary density. These results suggest that the production of MDGFs and consequently the capillary growth is affected by the amount of negative feedback present. These numerical results were consistent with experimental data (Bennett and Schultz, 1993; Davidson and Broadley, 1991; Stokes et al., 1990). Schugart *et al.* (Schugart et al., 2008) established the first mathematical model that studies the effect of tissue oxygen tension on cutaneous wound healing. As the previous models, this also included a system of nonlinear PDEs that described the interaction in space and time between several biological factors but also incorporated more variables, such as capillary sprouts, inflammatory cells (macrophages and neutrophils), fibroblasts, ECM and chemoattractants (VEGF and other growth factors). The results obtained showed that extremely hypoxia is adverse for vascular growth and for the healing process. Instead, hyperoxia promotes angiogenesis and wound healing. These results were in accordance with experimental literature data (Gibson et al., 1997; Liao and Johnson, 2007; Sheikh et al., 2005; Sheikh et al., 2000). Flegg *et al.* (Flegg et al., 2010) extended the works of Pettet *et al.* (Pettet et al., 1996b) and Schugart *et al.* (Schugart et al., 2008) and developed a mathematical model that studies, for the first time, the role of hyperbaric oxygen therapy (HBOT) in the healing of normal and chronic wounds. Their model described the interactions in space and time of biological factors, such as the concentration of oxygen and chemoattractant, and the densities of capillary tip, blood vessel, fibroblast and ECM. The authors verified that HBOT did not improve the treatment of wounds presenting low growth factor production, low chemotactic response or excessive ECM deposition. In wounds that did not heal due to excessive oxygen-consuming bacteria, it was demonstrated that intermittent HBOT can accelerate the healing of these wounds. Nevertheless, HBOT sessions should be continued until complete healing is achieved. Thus, the results obtained suggest that HBOT has a reduced benefit for the treatment of normal wounds. Xue *et al.* (Xue et al., 2009) extended the work of Schugart *et al.* (Schugart et al., 2008) and established the first model that incorporates mechanical properties of the ECM in the study of ischemic dermal wounds. The authors described the ECM by its density, ρ_{ECM} , and velocity, $\mathbf{v}_{ECM}$, using the equation:

$$\frac{\partial \rho_{ECM}}{\partial t} + \frac{\partial}{\partial x}(\rho_{ECM} \mathbf{v}_{ECM}) = G \rho_{ECM}(\rho_f, C_{O_2}, C_{PDGF}) \quad (5)$$

where $G \rho_{ECM}(\rho_f, C_{O_2}, C_{PDGF})$ is the growth and decay term due to collagen secretion by fibroblasts and degradation by matrix metalloproteinase (MMPs). This term depends directly on the density of fibroblasts, ρ_f , and the concentrations of oxygen, C_{O_2} , and PDGF, C_{PDGF} . Hence, similarly with previous models, this model combined biological and chemical factors, such as

concentration of oxygen, VEGF and PDGF, densities of macrophages, fibroblasts, capillary tips and capillary sprouts. In this model, ECM is assumed to be a viscoelastic material with growth. The results obtained suggested that ischemic conditions limit the recruitment of macrophages which may delay wound closure. The numerical results were in good agreement with experimental data, such as the radius of normal and ischemic wounds and also the macrophage abundance at the wound edge (Roy et al., 2009).

Contrary to the models previously mentioned, Olsen *et al.* (Olsen et al., 1997) and Gaffney *et al.* (Gaffney et al., 2002) described wound healing angiogenesis without considering chemical factors regulation. Olsen *et al.* (Olsen et al., 1997) established a continuum model that addressed the interactions between endothelial cells and ECM involved in wound angiogenesis for the first time. The model was composed by two coupled partial differential equations, for endothelial cells and ECM densities. The authors extended the model to two space dimensions and the results showed that angiogenesis occurs because the endothelial cells and ECM moving inside from both the wound margins and the wound base. Gaffney *et al.* (Gaffney et al., 2002) established a model to study the angiogenic response related to the motility and the budding of capillary tips that affect the velocity of wounding. This model differs from the others because it focuses only on endothelial cells and capillary tips, allowing the understanding of the capillary tip migration.

Javierre *et al.* (Javierre et al., 2008) combined the models of wound closure due to Arnold and Adam (Arnold and Adam, 1999) and the model of angiogenesis due to Maggelakis (Maggelakis, 2003) to obtain a new one that couples the two processes. This model has the same variables that the model of Maggelakis (oxygen and MDGFs concentrations and capillary density) and incorporates the epidermal growth factor (EGF) concentration, that regulates cell mitosis and motility allowing wound closure. The two-dimensional model was solved by the finite element method and an adaptive mesh algorithm was implemented because of the discontinuous production of EGF. This model allowed the studying of the effect of oxygen concentration on the rate of wound closure. Vermolen and Javierre (Vermolen and Javierre, 2011) developed a 2D model that coupled wound contraction, angiogenesis and wound closure for the first time. The model's PDEs were solved using the finite-element method. The temporal evolution of fibroblasts, ECM, VEGF, capillary profiles, oxygen, EGF and epidermal cell density were modelled in a dermal wound gap. The numerical simulations showed that the re-epithelialization progress occurs in a different way in early and later stages of wound closure. In the early stages, wound closure is characterized by keratinocyte migration from the undamaged epidermis into the wounded epidermis. In the later stages, the keratinocytes move from the basal membrane, between the epidermis and dermis, directed to the top surface of the

epidermis. These results were in accordance with the ones reported in clinical experiments (Escamez et al., 2004; Laplante et al., 2001). Valero *et al.* (Valero et al., 2013) extended the work of Javierre *et al.* (Javierre et al., 2008) and incorporated the effect of fibroblasts contraction, coupling angiogenesis and wound contraction. The authors presented a model that includes biological (capillaries and fibroblasts), chemical (concentration of oxygen and MDGF) and mechanical factors (cell traction forces and ECM deformation) that regulate skin wound healing. The stress of one fibroblast cell per unit of ECM, p_{cell} , that corresponds to the mechanical stimulus which in turn regulates the forces exerted by the cells, was considered. In the study, p_{cell} was a piecewise linear function depending on the tissue volumetric strain, θ , and modelled as:

$$p_{cell}(\theta) = \begin{cases} K_{pas}\theta & \theta < \theta_1 \\ \frac{K_{act}p_{max}}{K_{act}\theta_1 - p_{max}}(\theta_1 - \theta) + K_{pas}\theta & \theta_1 \leq \theta \leq \theta^* \\ \frac{K_{act}p_{max}}{K_{act}\theta_2 - p_{max}}(\theta - \theta_2) + K_{pas}\theta & \theta^* < \theta \leq \theta_2 \\ K_{pas}\theta & \theta > \theta_2 \end{cases} \quad (6)$$

where θ^* is defined as p_{max}/K_{act} and θ_1 and θ_2 are the compression and tension strain limits, respectively. The maximal contractile force that the actomyosin machinery can exert is defined as p_{max} , and K_{act} and K_{pas} is the stiffness moduli of the active and passive components of the cell, respectively, whereas the skin was modelled as a hyperelastic material and the authors argue that it was a more appropriate approach due to the tension/deformation that skin undergoes during wound healing. The model included two types of wound of the same size and different shapes (circular and elliptical). The numerical simulation demonstrated that elongated wounds had faster vascularization. Nevertheless, the contraction experienced by both wounds was similar. In the literature, not much experimental data seems to be available to validate computational results. The existent studies were mainly performed using animal models, frequently rats and pigs. Nevertheless, the contraction curve obtained in the Valero et al. simulations were qualitatively in accordance with the one obtained for the *in vivo* animal models (Gross et al., 1995; McGrath and Simon, 1983). Furthermore, the macrophage density curve of non-ischemic wounds reported experimentally (Roy et al., 2009) is also qualitatively equal to the one obtained in the simulation.

As presented above, continuum models are particularly useful to describe angiogenesis during wound healing. However, the prediction of the structure of vascular network of these models are limited.

4.1.2. Cell-based models

Several cell-based models were developed in order to overlap the biological features that continuum models do not allow to estimate. Cell-based models (Table 2) are a better approach to determine the effect of nutrients, oxygen and growth factors in the morphology of such network in two- or three-dimensional frameworks. These cell-based models include cellular automata and cellular Potts models. Cellular automata models consist in particles that occupy some or all sites of a regular lattice (Deutsch and Dormann, 2005). Each particle is characterized by one or more state variables, by a set of rules that describe the evolution of their state and position as well as that of neighbouring particles. The change of state of the particles and their movement depend on their current state and that of their neighbouring particles. At each step, each cell will move to a neighbouring site according to its corresponding instantaneous velocity. In biological applications, cells are treated as point-like objects with an internal state but no spatial structure. Thus, in these models the shape of the cells during migration is not accounted for. Eukaryotic cells normally move by remodelling their cytoskeleton and consequently change their shapes. Thus, shape change can influence the cell migration pattern and this characteristic needs to be taken into account in modelling. Consequently, cellular Potts models that describe cell volume and shape more realistic started to be a research focus in biological cell mathematical modelling. These models allowed to modulate cell growth, proliferation, motility, adhesion, apoptosis, differentiation and polarity (Merks and Glazier, 2005). Cellular Potts models describe the individual cell behaviour and the interactions with the ECM, determined by energy considerations.

The first stochastic model that described angiogenesis by ordinary differential equations was developed by Stokes and Lauffenburger (Stokes and Lauffenburger, 1991). The model was based in particular characteristics of the microvessel endothelial cells such as cell speed and chemotactic responsiveness that allowed the description of the microvessel endothelial cells migration and the vessel branching. The results obtained showed that microvessel endothelial cells motility and chemotaxis are essential in the angiogenic process, affecting the vascular network structure. In the following paragraphs, we summarize the most pertinent cell-based models for wound healing angiogenesis. Matsuya *et al.* (Matsuya et al., 2016) used a discrete dynamic model for angiogenesis and verified that the deterministic interaction between endothelial cells can lead to cell-mixing behaviour, elongation and bifurcation. The motion of endothelial cells in a branch was modelled as:

$$\mathbf{x}_n^{t+1} = \mathbf{x}_n^t + \mathbf{v}_n^t \quad (7.1)$$

$$\mathbf{v}_n^{t+1} - \mathbf{v}_n^t = -\gamma \mathbf{v}_n^t + \sum_{k \neq n} F(\mathbf{x}_n^t - \mathbf{x}_k^t) \quad (7.2)$$

where n refers to endothelial cell, $\mathbf{x}_n^t \geq 0$ is the position of the endothelial cell measured from the origin of time step, γ denotes the coefficient of conflict, F denotes the two body interaction and $\mathbf{v}$ is the velocity of the endothelial cells. However, the model did not take into account anastomosis, chemotaxis and remodelling. Peirce *et al.* (Peirce et al., 2004) developed a cellular automata model to predict microvascular network patterning. The model could predict the cell behaviour in response to epigenetic stimuli and molecular signals. Their work allowed for the first time to study the living multicellular systems growth, the cell behaviour adaptation and the epigenetic engineering of vascular tissues. The increase in vascular density obtained in the cellular automata model were similar to the one obtained in rodent model after exogenous VEGF treatment. Furthermore, the numerical method predicted a vessel maturation pattern consistent with those measured experimentally. The network assembly, the vascular pattern and the vascular remodelling predicted by the computational model were independently verified by experimental observations. Bentley *et al.* (Bentley et al., 2008) established a hierarchical agent-based model to investigate how angiogenic sprouting is modulated, in tip cells, by notch signalling due to different concentrations of VEGF and filopodia dynamics. The numerical simulation demonstrated that VEGF concentration, VEGF gradients and filopodia extension determine the robustness of tip/stalk morphology. In order to validate the predictions of the mathematical model, experimental tests were performed. The predictions were: levels of DII4/VEGF-2 oscillate in synchrony along a vessel in high VEGF environments; the VEGF gradient increases tip cell selection rates.

Some authors combined the cellular Potts model, which modulates cell behaviour, with PDEs, that describe chemical concentrations. Merks *et al.* (Merks et al., 2004) developed a 2D framework combining a cellular Potts model, that described the behaviour of endothelial cells, with a PDEs model, for chemoattractant concentration. Accordingly, the authors implemented a preferential motion for the cells along gradients of the chemoattractant c defined as:

$$H' = H - \sum_i \chi_c \frac{c(\mathbf{x}, t)}{s c(\mathbf{x}, t) + 1} (1 - \delta_{ni, nj}) \quad (8)$$

where H is the Hamiltonian (defined as $H = \sum_{i,j} J_{ni,nj} (1 - \delta_{ni,nj}) + \beta \sum_i (a_{ni} - A_{ni})^2$), n_i represents the index of a single biological cell, $J_{ni,nj}$, represents the characteristic bond energy between two lattice sites (n_i, n_j) of unlike index, $\delta_{ni,nj} = 0$, being δ the Kronecker delta ($\delta_{i,j} = \{1, i = j; 0, i \neq j\}$), χ_c is the strength of the chemotactic response and the saturation s sets the Michaelis-Menten constant of the chemotactic response. The volumetric constrain on a

biological cell n_i is imposed by $(a_{ni} - A_{ni})$ and the resistance to compression is defined by β . The effect of cell elongation in the vascular pattern was also analysed by the addition of a cell-length constraint to the Hamiltonian:

$$H'' = H' + \lambda_L(l - L)^2 \quad (9)$$

where l is the length of the cell along its longest axis, L is its target length, and λ_L is the strength of the length constraint. The results obtained allowed us to study intercellular adhesion and cell morphology and showed that endothelial cell adhesion is essential for the formation of stable vessels. The authors validated their model by comparing its numerical results with time-lapse video-microscopy experiments of endothelial cell culture under different physiological and pathological conditions similar to the ones used in the simulations (Dougherty and Lotufo, 2003). Scianna *et al.* (Scianna et al., 2015) also established a cellular Potts model to simulate the vascularization process and the subsequent reoxygenation of a hypoxic tissue. The model included reaction–diffusion equations that describe the diffusion, decay, uptake and production of VEGF and the diffusion and consumption of oxygen. In this model, endothelial cells, upon VEGF stimulus and according to delta-notch pathway, turn from a quiescent phenotype into a stalk or a tip one. In their iterative model, the domain is discretized by a regular lattice, $\Omega \subseteq \mathbb{R}^2$, defined by identical close grid sites, which are identified by their centre $\mathbf{x} \in \mathbb{R}^2$. Thus, the behaviour of tip cells, at each time step t , is described with a Boltzmann-like probability function between a source site, $\mathbf{x}_s$, and a target site, $\mathbf{x}_t$:

$$\begin{aligned} P(n(\mathbf{x}_s) \rightarrow n(\mathbf{x}_t))(t) = \\ = \begin{cases} \tanh\left(T_{\Sigma_{\mathbf{x}(\mathbf{x}_s)}(t)}\right) \min\left\{1, \exp\left(\frac{-\Delta H + w_{pol}}{T_{\Sigma_{\mathbf{x}(\mathbf{x}_s)}(t)}}\right)\right\}, & \text{if } \tau(\Sigma_{\mathbf{x}(\mathbf{x}_s)}) = T; \\ \tanh\left(T_{\Sigma_{\mathbf{x}(\mathbf{x}_s)}(t)}\right) \min\left\{1, \exp\left(\frac{-\Delta H}{T_{\Sigma_{\mathbf{x}(\mathbf{x}_s)}(t)}}\right)\right\}, & \text{if } \tau(\Sigma_{\mathbf{x}(\mathbf{x}_s)}) = \{Q, S\}; \\ \tanh\left(T_{\Sigma_{\mathbf{x}(\mathbf{x}_t)}(t)}\right) \min\left\{1, \exp\left(\frac{-\Delta H}{T_{\Sigma_{\mathbf{x}(\mathbf{x}_t)}(t)}}\right)\right\}, & \text{if } n(\mathbf{x}_s) = 0; \end{cases} \quad (10) \end{aligned}$$

where ΔH is the net difference of the Hamiltonian due to the proposed change of domain configuration, w_{pol} describes the directional persistent movement of polarized cells and $T_{\Sigma_{n(\mathbf{x})}}(t) \in \mathbb{R}^+$, for $\mathbf{x} \in \{\mathbf{x}_s, \mathbf{x}_t\}$, is a Boltzmann temperature. The oxygenated state of the site $n(\mathbf{x})$ is defined by $\tau(\Sigma_{n(\mathbf{x}_s)})$, for which Q, S and T represent the three types of vascular cells: quiescent, stalk and tip, respectively. The vascular network obtained in the simulations manifests a good degree of realism. The contribution of this model was the introduction of the interaction between endothelial cells and extracellular matrix, which characterized the host

tissue. van Oers *et al.* (van Oers et al., 2014) developed a cellular Potts model to study the motility of the endothelial cells, and combined them with the finite element method to study cell deformation. This approach allowed us to study the mechanical communication between endothelial cells and ECM components and also the collective cell behaviour that the formation of vessels network and sprouting. For that, the model was based on the following assumptions: endothelial cells can exert mechanical strains in the substrate (cell traction), they perceive a stiffening of the substrate along the strain orientation (strain stiffening) and they extend preferentially on stiffer substrate (durotaxis). The numerical simulation results were in accordance with *in vitro* observations of bovine aortic endothelial cells culture in acrylamide gels (Reinhart-King et al., 2008) and also with experimental observations of fibroblasts culture on compliant substrates (Takakuda and Miyairi, 1996).

As presented, cell-based models are very useful to describe the individual endothelial cell behaviour. However, the information obtained with these models at tissue scale is more limited and they are computationally more expensive. In order to couple the advantages of each kind of model (continuum and cell-based) and overlap its drawbacks, several authors developed hybrid models.

4.1.3. Hybrid models

Hybrid models (Table 3) were developed in order to achieve more reliable mathematical/computational models. These models allowed to simultaneously predict the cell behaviour and the evolution of species' concentration along distinct time and space scales. Sun *et al.* (Sun et al., 2005) developed a nonlinearly system of partial and ordinary differential equations for modelling angiogenesis. In this model, the space was discretized using cell centre finite difference method and the time was discretized using the backward Euler method. The ECM was considered heterogenic and anisotropic. With this numerical simulation the authors aimed to study the effect of chemotactic growth factors distributions in capillary network. The set $S(t)$ indicates at time t the collection of all existent sprout tips, $s_i \in S$, with $i \in \mathbb{N}$. Anastomosis and branching were described as sudden events. Anastomosis was modelled as:

$$S(t^+) = A(S(t^-)) \quad (11)$$

where anastomosis operator $A(\cdot)$ is defined as:

$$\begin{aligned}
A(S(t^-)) &= S(t^-) \setminus \{s : s \in S(t^-) \text{ and } n(x_s, y_s, t) = 1\} \\
&\quad \setminus \left\{ \begin{aligned} &s_1, s_2 : s_1 \in S(t^-), s_2 \in S(t^-), s_1 \neq s_2, \mathbf{p}_{s_1} = \mathbf{p}_{s_2} \\ &\text{and } \frac{d\mathbf{p}_{s_1}}{dt} \cdot \frac{d\mathbf{p}_{s_2}}{dt} < 0 \end{aligned} \right\} \\
&\quad \setminus \left\{ \begin{aligned} &s_1 : s_1 \in S(t^-), s_2 \in S(t^-), s_1 \neq s_2, \mathbf{p}_{s_1} = \mathbf{p}_{s_2} \\ &\text{and } \frac{d\mathbf{p}_{s_1}}{dt} \cdot \frac{d\mathbf{p}_{s_2}}{dt} \geq 0 \end{aligned} \right\}
\end{aligned} \tag{12}$$

where $\mathbf{p}_s$ denotes the tip position vector $\mathbf{p}_s = \{x_s, y_s\}$. for tip s . In the anastomosis operator, the second term in the right-hand side of the equation represents the tip-to-sprout anastomosis. The third and the fourth terms describe the tip-to-tip anastomoses. The branching mechanism were described as:

$$S(t^+) = B(S(t^-)) \tag{13}$$

After branching, one sprout tip becomes two tips. The two new tips formed from the existent tip, s , are defined as $b_1(s)$ and $b_2(s)$. Thus, the branching operator $B(\cdot)$ can be defined as:

$$B(S(t^-)) = \{b_1(s), b_2(s) : s \in B_0(S(t^-))\} \cup S(t^-) \setminus B_0(S(t^-)) \tag{14}$$

and:

$$B_0(S(t^-)) = \{s \in S(t^-) : \text{age}(s) \geq \tau_a \text{ and } |\nabla \mathbf{u}_n(\mathbf{p}_s(t), t) \cdot \mathbf{u}_n(\mathbf{p}_s(t), t)^\perp| \geq \tau_u\} \tag{15}$$

$S(t^-)$ is the set of sprout tips at time $t - \varepsilon$ (ε is an arbitrarily small positive number), $B_0(S(t^-))$ is the set of sprout tips at time $t - \varepsilon$ that will undergo branching at time t , and $S(t^+) = B(S(t^-))$ is the set of sprout tips at time $t + \varepsilon$ immediately subsequent to the sprout branching at time t has performed. The sprout extension was modelled as a continuous-in-time process as:

$$\frac{\partial \mathbf{p}(x_i, t)}{\partial t} = \mathbf{u}_p(x_i, t) \quad \text{with} \quad \mathbf{p}(x_i, t) = \mathbf{x}_i(t), \forall x_i(t) \in S(t) \tag{16.1}$$

$$\mathbf{u}_p(\mathbf{x}, t) = \mathbf{f}_p(\mathbf{u}_n(\mathbf{x}, t)) \tag{16.2}$$

$$\mathbf{u}_n(\mathbf{x}, t) = \frac{\mathbf{u}(\mathbf{x}, t)}{|\mathbf{u}(\mathbf{x}, t)|} \tag{16.3}$$

$$\mathbf{u}(\mathbf{x}, t) = \mathbf{K} \nabla c \tag{16.4}$$

where $\mathbf{u}_p$ is the sprout extension velocity vector, $\mathbf{u}_n$ is the normalized velocity vector, $\mathbf{u}$ is the auxiliary sprout extension velocity vector, $\mathbf{f}_p(\mathbf{u}_n(\mathbf{x}, t))$ is the velocity modulator based on the proliferation rate and $\mathbf{K}$ is the conductivity of the extracellular matrix for the movement/extension of capillary cells. The computational simulations created capillary

networks with a very realistic structure and morphology, when compared to data described in *in vitro* studies. The simulations also showed that areas with low capillary densities demonstrated high growth factor concentration. Lower growth factor concentration was obtained in areas with higher capillary network, due to growth factor consuming by endothelial cells. The replacement of the traditional endothelial cell density by the capillary indicator function permits to model the capillary network precisely at fine scales. Machado *et al.* (Machado et al., 2011) established a hybrid discrete-PDE formulation to model the endothelial cells migration through chemotaxis, in response to VEGF gradients, and through haptotaxis, in response to gradients in the ECM. In fact, endothelial cell produces matrix degrading enzymes that degrade the ECM, allowing the haptotaxis response. The authors modelled the migration of individual sprout endothelial cell by the equation:

$$\frac{\partial \rho_e}{\partial t} = \mu_e \nabla^2 \rho_e - \nabla \cdot \left(\frac{\chi_c}{1 + \xi C_{VEGF}} \cdot \rho_e \nabla C_{VEGF} \right) - \chi_h \nabla \cdot (\rho_e \nabla C_{ECM}) \quad (17)$$

where ρ_e represents the endothelial cell density per unit of area, μ_e is the endothelial cell random motility coefficient, χ_c is the endothelial cell chemotaxis coefficient, ξ is the endothelial cell chemotactic receptor saturation factor, C_{VEGF} represents the VEGF concentration, χ_h is the endothelial cell haptotaxis coefficient and C_{ECM} is the ECM concentration. The particular migration direction of each sprout tip was determined by:

$$\frac{\partial C_{VEGF}}{\partial t} = -\lambda_{VEGF} q_i C_{VEGF} \quad (18.1)$$

$$\frac{\partial C_{ECM}}{\partial t} = \lambda_{Epr} q_i - \lambda_{Ede} \rho_m C_{ECM} \quad (18.2)$$

$$\frac{\partial \rho_m}{\partial t} = \lambda_{Mpr} q_i + \lambda_{Mdi} \nabla^2 \rho_m - \lambda_{Mdc} \rho_m \quad (18.3)$$

where ρ_m is the MMPs density and q_i a Boolean value (1 or 0) indicating the presence or absence of a tip cell at a given position. λ_{VEGF} , λ_{Epr} , λ_{Ede} , λ_{Mpr} , λ_{Mdi} and λ_{Mdc} are rate constants for: VEGF uptake by endothelial cell, ECM production, ECM degradation, MMPs production, MMPs diffusion and MMPs decay, respectively. The study also included an *in vivo* wound healing angiogenesis assay based on confocal imaging of a dorsal skin-fold window chamber preparation of mouse previously perfused with fluorescein isothiocyanate-dextran. The results obtained in the computational simulation for wound areas, vessel densities and vessel junction densities were in excellent agreement, temporally and spatial with the results obtained in *in vivo* experiment. This study was the first that directly compared between morphological data obtained to a mathematical model and data obtained during experimental wound healing assay. Bookholt *et al.* (Bookholt et al., 2016) studied the sprouting angiogenesis in a 3D mesh taking

into account the role of the proteases, secreted by endothelial cells, in the substrate degradation. The model combined a cell-based model to study cell motility with diffusion-reaction equations to model VEGF, protein delta-like ligand 4, MMPs and urokinase plasminogen activator concentrations. With this model, the authors aimed to study the effect of local chemical conditions in the differentiation of endothelial cells into tip and stalk cells. Moreover, an *in vitro* assay that analysed the effect of different concentrations of VEGF in sprouting angiogenesis in dermal endothelial cells was also performed. The results obtained both in numerical simulation and in experimental assay demonstrated that the area of sprouted perimeter was of the same order of magnitude. Furthermore, the morphology of the sprout was similar in the two approaches. Pillay *et al.* (Pillay et al., 2017) developed a 2D cellular automata model of angiogenesis in the corneal assay, based on the “snail-trail” process. The authors used PDEs for the spatiotemporal evolution of the tip cells and endothelial cell densities. With this approach they derived the PDEs model to a 1D continuum model that represents the 2D angiogenesis in the corneal assay. The simulation showed that the vessel formation due to tip cells movement can be modelled as a source term of tip cells on the macroscale. It was also demonstrated that anastomosis imposes cell occupancy and density restrictions on the discrete model and continuum model, respectively.

The distinct numerical approaches used to model wound healing angiogenesis described in this work present relevant differences amongst themselves related to their complexity. The model’s complexity is related to the biological feature that we want to study and is translated in the model dimension, in the number of angiogenic factors and cell types considered and in the biological level of interest (molecular, cellular or tissue). It is important to take into account that each model presents its limitations. Several mathematical models were developed in 1D or 2D, however biological processes occurs at 3D. Frequently, all the chemical species and growth factors that modulate angiogenesis are combined into one specie. The mechanical behaviour of the skin, the ECM composition and collagen and fibrin fibres orientation are frequently neglected. The interaction between different cell types that participate in wound healing angiogenesis, such as endothelial cell, mural cells, inflammatory cell, fibroblasts and others ECM components are often neglected. In the following section some limitations, biological features and parameters analysed in the different models described in this work will be discussed

5. DISCUSSION

The wound healing angiogenesis is not yet fully understood. *In vivo* and *in vitro* models that address this biological process have already been established. Nevertheless, the development of models capable to simulate wound healing angiogenesis as close to reality as possible, remain a demanding challenge. Physiologic variables that affect wound healing, such as temperature, pH levels, circulation, local oxygen tension and adequate nutrient supply, had already been manipulated in *in vitro* cell culture. This methodology also allows the study of cell proliferation, migration and apoptosis. However, studies based on cell cultures have some disadvantages because they lack the ECM composition, the immune cells activity and the cell-cell and cell-ECM interactions. Thus, more complex studies that included animal models have been developed, usually rodent animals. Nevertheless, in rodent animals the wound healing process is quite different and occurs mainly due to contraction (Ansell et al., 2012). For these reasons, some authors suggest the use of large animals such as porcine (Roy et al., 2009) because the skin structure of these animals is closer to human (Xie et al., 2007). Working with these animals requires large expenses for animal acquisition and housing and there is a lack of molecular tools to perform sample analyses. Thus, the use of large animals for the study of wound healing angiogenesis may be one of low practice applicability. Consequently, *in vitro* cell culture and *in vivo* experiments previously performed do not fully describe the wound healing angiogenesis process. Additionally, these methodologies are expensive, time consuming and to work with animals involves ethical reflections that need to be taken into account. For these reasons, mathematical models became a focus of interest. In fact, mathematical models are a non-invasive procedure capable to present different levels of complexity, allowing to couple human skin structure, distinct cell types, different growth factors, ECM composition and deformation.

For the development of a mathematical model of wound healing angiogenesis, some simplifications, assumptions and decisions have to be considered. The first assumption concerns the spatial scale. It is necessary to decide whether the behaviour will be described at the level of molecules, cells, tissue or across multiple scales. The wound geometry is also an important aspect to consider. The shape of the wound could be circular, elliptical, rectangular or irregular and this parameter may influence the timescale and other aspects of the healing process. In fact, Valero and co-workers (Valero et al., 2013) simulated the time of vascularization and the contraction of two wounds with different shapes, circular and elliptical. The authors verified that the circular wound centre manifested a more severe hypoxia and consequently an increase in MDGFs production. Conversely, the elliptical wound presented a faster fibroblasts invasion of

the wound and thus higher contraction stress. However, the wound contraction was similar in both wounds shape. The spatial dimension of the wound is also an aspect to ponder. Frequently, it is considered that wounds are much longer than wide or deep, and in these situations 1D models are a proper approach. In fact, several studies modelled angiogenesis in a 1D framework (Byrne et al., 2000; Flegg et al., 2010; Gaffney et al., 2002; Maggelakis, 2003; Olsen et al., 1997; Pettet et al., 1996b; Schugart et al., 2008; Xue et al., 2009). These models are conceptually simpler, numerical tractable and present less computational costs. However, if dealing with wounds with a larger surface extent, deep into the dermis is what is intended, then it is necessary to add more complexity to the model and developed it in two dimensions. These kind of models provide additional insights useful for studying more realistic geometries, similar to those visualized in real wounds and, for this reason, some authors choose this geometry (Javierre et al., 2008; Machado et al., 2011; Matsuya et al., 2016; Peirce et al., 2004; Sun et al., 2005; Valero et al., 2013; Vermolen and Javierre, 2011). The 3D models (Bentley et al., 2008; Bookholt et al., 2016) simulate skin structure and cell-ECM interactions closer to reality and allow an accurate representation of the involved phenomena. These models are analytically more complex and represent a high computational cost. For these reasons, in the literature, 3D models that simulate wound healing angiogenesis are still scarce. Nevertheless, in order to understand how the increase in blood vessel density regulates wound closure on realistic wound geometries it will be necessary to develop higher dimensional models. For example, the model of Merks *et al.* (Merks et al., 2004) could be extended to 3D by replacing the reference cell area, A_{ni} , by a reference volume, V_{ni} , and the cell area, a_{ni} , by the cell volume, v_{ni} , in the Hamiltonian of Equation 8. Mathematical models can be solved with fine resolution in 2D and 3D, using complex geometries obtained from clinical data. These models may allow to test new therapies for the treatment of acute, chronic or ischemic wounds and to reduce the use of expensive and time-consuming experimental methodologies.

After deciding the wound geometry, it is necessary to decide the number of chemical molecules or cells per unit of volume in the wound. If cell densities and chemical concentration are to be considered, continuous models are a good option. Continuum models are very useful to describe the early steps of angiogenesis, when the motion of endothelial cells is regulated by chemical gradients. These models are based on partial differential equations are largely used due to their simplicity and to the availability of ready-to-use numerical solvers. Thus, several authors used this strategy to model angiogenesis (Byrne et al., 2000; Flegg et al., 2010; Gaffney et al., 2002; Javierre et al., 2008; Maggelakis, 2003; Olsen et al., 1997; Pettet et al., 1996a; Pettet et al., 1996b; Schugart et al., 2008; Valero et al., 2013; Vermolen and Javierre, 2011; Xue et al., 2009). Notwithstanding, these models need to be extended, becoming more complex, in order

to describe all the steps of the process, such as cell adhesion to ECM, vessel maturation and mechanical interaction between endothelial cell and ECM. Wound healing angiogenesis usually takes place on a very small spatial scale and for this reason cannot be fully described by deterministic partial differential equations based models. Cell-based models (Bentley et al., 2008; Matsuya et al., 2016; Merks et al., 2004; Peirce et al., 2004; Scianna et al., 2015; Stokes and Lauffenburger, 1991; van Oers et al., 2014) capable of describing stochastically the structure and morphology of capillary networks (concerning individual endothelial cell behaviour) are required. Cell-based models will allow the study of biochemical and biomechanical mechanisms that regulate cell behaviour during wound healing. The cell-based models provide useful information about individual endothelial cell behaviour during vascular network formation and stabilization, the morphological evolution of endothelial cells and its adhesion. Nevertheless, a limitation of these models is that they may not provide useful information about specific/localized processes, such as vessel branching, anastomosis, pruning and vessel remodelling. Cell-based models do not provide the description of the biological process as a whole and they are computationally expensive. Consequently, it is necessary that the upcoming models combine continuum and discrete descriptions. Hybrid models (Bookholt et al., 2016; Machado et al., 2011; Pillay et al., 2017; Sun et al., 2005), which combine the interactions between distinct spatiotemporal scales, possess unique characteristics that allow explaining the behaviour of vessel network in response to chemical gradients and the substrate degradation. Besides, hybrid models permit to predict individual cell behaviour, such as endothelial cell proliferation, differentiation and migration and cell-cell or cell-ECM interaction. Due to their complexity until now they were less explored.

Another crucial aspect is to decide how many interacting species the model will include. Accordingly, authors developed mathematical models with different levels of complexity. Olsen *et al.* (Olsen et al., 1997) and Gaffney *et al.* (Gaffney et al., 2002) modelled angiogenesis considering only endothelial cells and blood vessels. Several other authors (Bookholt et al., 2016; Peirce et al., 2004; Schugart et al., 2008; Xue et al., 2009) developed more complex models, in which they included chemical species such as oxygen, VEGF, MDGFs, EGF, PDGF, TGF- β , different types of cells such as capillary tip, capillary sprout, fibroblasts, inflammatory cells, smooth muscle cell and also ECM. This number of variants can be changed substantially depending on the scope of the model. In general, most of the studies were focussed in the endothelial cells and do not consider the inflammatory cells, required for the establishment of chemical gradients that allow to attract different cell populations, neither smooth muscle cells or pericytes, required to stabilize the new blood vessels formed. Vessel maturation is an important step in angiogenesis that allows the stability of vascular network and for this reason should be included

in the upcoming mathematical models. Nevertheless, a model with a larger number of variants may be difficult to implement or to be efficient and for this reason the majority of the proposed models were simplified at their genesis. Therefore, it is necessary to establish the most completed model as possible but without redundant species.

The mechanical properties of human skin are an important aspect that should be considered in mathematical models. These properties depend on the skin anatomical localization, orientation, depth and age. In fact, over time, skin becomes less elastic and loses its recovery capacity. During wound healing, the mechanical properties of the surrounding skin are also important since they influence the correct process. Skin presents a non-linear behaviour and may be characterized as a viscoelastic or hyperelastic material. Traditionally, human skin has been assumed as a viscoelastic material (Javierre et al., 2009; Manoussaki, 2003; Xue et al., 2009). This kind of approach captures the time-dependent properties of the skin. Nonetheless, there are some limitations. In fact, *in vivo* and *in vitro* studies have shown that modelling skin behaviour as a hyperelastic material is a better option. Indeed, hyperelastic models provides a more accurate approximation of the mechanical state of the tissue, allows for the incorporation of fibre remodelling in the model and permits the study of skin's healthy behaviour. Valero *et al.* (Valero et al., 2013) developed the first wound healing model that considers skin as a hyperelastic material and coupled it with wound contraction. However, the authors considered the hyperelastic skin behaviour as a function of the matrix density instead of skin composition, which would be a more realistic option.

Most of the wound healing angiogenesis studies considered the skin as an isotropic matrix (Machado et al., 2011; Scianna et al., 2015; Valero et al., 2013; van Oers et al., 2014). Still, anisotropic skin behaviour is clearly observed in experimental studies. The use of skin anisotropy on the mechanical model improved the accuracy of healing kinetics in the simulation. Some authors also highlighted that the influence of anisotropy will be more important in the remodelling phase, in which fibres are formed into the wound (Valero et al., 2015). Groves *et al.* (Groves et al., 2013) performed *in vitro* human tensile tests and found that the skin presents hyperplastic and anisotropic behaviour. Anisotropy means that the resistance of the ECM for the sprout extension might be stronger in some directions and weaker in others. This mimics the wound healing process because tissues often present a layered orientation. Consequently, the inclusion of material anisotropy provides the model with an additional realistic degree.

Wound contraction plays an important role in delayed ischemic wound healing due to reduced fibroblasts activity. In fact, it has been demonstrated that ischemic wounds manifested a delay in wound closure. This may be due to the fact that they present a decrease in the number of myofibroblasts and also a decrease in collagen type-I mRNA (Alizadeh et al., 2007). *In vivo*

studies demonstrated that the application of exogenous mechanical loading to mouse incisions results in increased wound fibrosis, similar to human hypertrophic scars (Tatlidede et al., 2009a; Tatlidede et al., 2009b). In fact, these studies replicated the repetitive cycles of ischemia followed by reperfusion associated with compression, a characteristic of human pressure ulcers leading to skin injury. Yet, these experimental findings were never included in mathematical models.

As discussed above, the approach chose by each author allows the study of angiogenesis analysing different biological features. Several authors peruse angiogenesis considering different chemical species' concentrations (oxygen, VEGF, MDGFs) and cells densities (endothelial cells and inflammatory cells) in the wound area, over time. Other authors develop models which are able to mimic the capillary network and study specific angiogenic processes such as anastomosis and branching. Table 4 summarizes the biological features analysed in each model in order to perform an overview that can be helpful when considering modelling wound healing angiogenesis. Some of these models can be used to test new techniques able to improve and better understand the wound healing process, to evaluate and compare other possible treatments and drug delivery mechanisms and their simulation results can be used to justify the reason of unsuccessful wounds (Maggelakis, 2003; Flegg et al., 2010; Machado et al., 2011).

Experimental studies had shown that several factors such as oxygen and growth factors concentration, nutrients supply and pH levels, affect wound healing. The effect of oxygen and growth factors in wound healing angiogenesis has already been modelled in several studies (Flegg et al., 2010; Javierre et al., 2008; Maggelakis, 2003; Schugart et al., 2008; Scianna et al., 2015; Valero et al., 2013; Vermolen and Javierre, 2011). The effect of nutrients in wound healing angiogenesis has been less explored. Wound healing is an extremely demanding process since it is necessary to increase cell proliferation, protein synthesis and enzyme activity. Hence, several macronutrients and micronutrients are necessary in this biological process. Proteins are required for synthesis of collagen and the production of fibroblasts (Harris and Fraser, 2004). Fatty acids are components of cell membranes and the substrate for eicosanoids synthesis, which promotes the inflammatory response (Shingel et al., 2008). Some vitamins and minerals also promote wound healing. In fact, vitamin A is required for epithelial and bone tissue development, cellular differentiation and immune system function, collagen cross-linkage increase and wound breaking strength (Greenwald et al., 1990). Vitamin C increases collagen production and stability, neutrophil function and angiogenesis (Gross, 2000). Zinc is essential for DNA synthesis, cell division and protein synthesis, all of which are fundamental steps in tissue regeneration and repair (Prasad, 1995). Iron is a cofactor of enzymes that participate in collagen synthesis and also of the haemoglobin, and therefore very important in the transport of oxygen

to the wound area (Mazzotta, 1994). Perthame *et al.* (Perthame et al., 2014) included in their mathematical continuum model the nutrients density, allowing the study of nutrients supply from blood vessels in the growth of solid tumours. These research works demonstrated the relevancy of including nutrients density laws into mathematical models of wound healing angiogenesis. The effect of pH levels in the regulation of wound healing is also an important physiological aspect but it has been less explored. The literature shows that, up to now, only two works modelled the effect of pH in wound healing (Callejón et al., 2012; Sirkka et al., 2016). The authors modelled the interaction between wound healing electrophysiology and changes of pH levels (Callejón et al., 2012), and studied the spatial pH-distribution in a wound (Sirkka et al., 2016). Experimental studies showed that the wound healing process is associated with a spatiotemporal change in pH in the wounded area (Schneider et al., 2007) and that the decrease of pH levels promotes new blood vessel formation. Furthermore, skin acts as physical barrier for many pathogens for which pH is optimum in more alkaline solutions (Schneider et al., 2007; Thomas Hess, 2011). Thus, the effect of an acidic environment in the promoting of angiogenesis should be included in the mathematical models of wound healing angiogenesis.

6. CONCLUSION

In the last three decades, computational mathematical modelling has been under focus in the scientific community due to its possibility to mimic biological processes and to test, using non-invasive procedures, new therapies. With this review, it is intended to provide a global view of the current approaches to model wound healing angiogenesis, pointing out the advantages and disadvantages of using different mathematical models.

The current models are able to apprehend the wound healing angiogenesis as a whole, however, some specific steps such as vessel maturation and reperfusion are less explored. The vascular network remodelling and the involvement of ECM components should also be included in the upcoming models in order to visualize the angiogenic process at the tissue level.

It is important to take into account that *in silico* models require the proper feedback from experimental *in vitro* and *in vivo* studies. These experimental works help validate the computational models and also to determine how close the wound healing model is to reality. Nevertheless, computational models are usually focused in human wounds and the experimental studies are developed in animal models, typically rodent. Rodents exhibited some differences in wound the healing process that were extensively discussed along this work. Thus, it is only possible to obtain a tendency of wound healing and the biological factors analysed

should be adjusted in order to be used in human models. Additionally, mathematical models require several parameters such as diffusion coefficients, cells densities, production and decay rates. Ideally, all the parameters required in the model should be obtained in direct *in vivo* measurements. Alas, this is not always possible due to the lack of technical support or due to ethical issues. Accordingly, many parameters used in the model are estimated and this is an important subject in the scope of wound healing modelling.

The development of wound healing angiogenesis models with higher dimension and incorporating multiscale analysis at molecular, cellular and tissue level, remain a challenge. Thus, there is still much to research and improve.

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Table 1: Continuum mathematical models, based on partial differential equations, of wound healing angiogenesis on multiple biological scales.

Authors	Dimension	Scale of interest exploring in the model			Experimental validation
		Molecular	Cellular	Tissue	
Pettet et al. (1996b)	1D	• MDGFs concentration	• Capillary tip density	• Capillary formation • Blood vessel density	
Byrne et al. (2000)	1D	• Proangiogenic factors concentration	• Capillary tip density	• Blood vessel density • Vascular remodelling	• Qualitative agreement when compared to surface area of normal and ischemic wounds
Maggelakis (2003)	1D	• Oxygen concentration • MDGFs concentration	• Capillary tip density	• Capillary growth	• Numerical results consistent with experimental data (Bennett and Schultz, 1993; Davidson and Broadley, 1991; Stokes et al., 1990)
Schugart et al. (2008)	1D	• Oxygen concentration • Chemoattractant concentration	• Capillary tip density • Capillary sprout density • Macrophages density • Neutrophils density • Fibroblasts density • ECM density	• Capillary growth	• Numerical results consistent with experimental data (Gibson et al., 1997; Liao and Johnson, 2007; Sheikh et al., 2005; Sheikh et al., 2000)
Flegg et al. (2010)	1D	• Oxygen concentration • Chemoattractant concentration	• Capillary tip density • Fibroblast density • ECM density	• Blood vessel density • Wound area • Oxygen concentration at wound edge	
Xue et al. (2009)	1D	• Oxygen concentration • VEGF concentration • PDGF concentration	• Capillary tip density • Capillary sprout density • Macrophages density • Fibroblasts density • ECM density • ECM velocity	• Chemokine concentrations at wound area • Cell densities at wound area	• Numerical results consistent with experimental data: radius of normal and ischemic wounds and macrophage abundance at the wound edge (Roy et al., 2009)
Olsen et al. (1997)	1D/2D		• Endothelial cell density • ECM density • Endothelial cell - ECM interaction		
Gaffney et al. (2002)	1D		• Capillary tip motility • Capillary tip budding • Capillary tip migration		
Javierre et al. (2008)	2D	• Oxygen concentration • MDGFs concentration • EGF concentration	• Capillary density	• Capillary formation • Wound closure • Oxygen concentration at wound area	

Vermolen and Javierre (2011)	2D	• Oxygen concentration • VEGF concentration • EGF concentration	• Fibroblasts density • Capillary density • Epidermal cell density • ECM profile	• Wound closure • Chemokine concentrations • Cell densities in wound	• Numerical results consistent with clinical experiments (Escamez et al., 2004; Laplante et al., 2001)
Valero et al. (2013)	2D	• Oxygen concentration • MDGFs concentration	• Capillary density • Fibroblasts density • ECM deformation • Cell traction forces	• Chemokine concentrations in wound • Cell densities in wound • Wound area	• Contraction curve obtained in the simulation was qualitatively similar to experimental data (Gross et al., 1995; McGrath and Simon, 1983) • Macrophage density curve obtained in the simulation was qualitatively equal to experimental data (Roy et al., 2009)

ECM, extracellular matrix; EGF, epidermal growth factor; MDGF, macrophage-derived growth factor; PDGF, platelet derived growth factor; VEGF, vascular endothelial growth factor.

Table 2: Cell-based models of wound healing angiogenesis on multiple biological scales.

Authors	Dimension	Scale of interest exploring in the model			Experimental validation
		Molecular	Cellular	Tissue	
Stokes and Lauffenburger (1991)	2D	• Angiogenic factors	• Microvessel endothelial cell motility • Microvessel chemotactic responsiveness	• Vascular network structure • Branching	
Matsuya et al. (2004)	2D		• Endothelial cell interaction • Cell-mixing phenomenon	• Vessel elongation • Vessel bifurcation	
Peirce et al. (2004)	2D	• VEGF diffusion • PDGF release • TGF- β release	• Endothelial cell proliferation and migration • Smooth muscle cell proliferation and migration • Interstitial precursor cell proliferation and migration • Perivascular cell differentiation • Cell-cell contact	• Microvascular network remodelling • Vessel length	• Predicted network assembly, vascular pattern and vascular remodelling was verified via direct experimental observations
Bentley et al. (2008)	3D	• VEGF gradients • Notch1 levels • DII4/Notch-mediated pathway	• Tip cell morphology • Stalk cell morphology • Filopodia extension		• Numerical results validated by qualitative similarities to experimental observations
Merks et al. (2004)	2D	• Chemoattractant concentration	• Endothelial cell morphology • Endothelial cell adhesion • Endothelial cell elongation		• Numerical results consistent with time-lapse videomicroscopy experiments of endothelial cell culture (Dougherty and Lotufo, 2003)
Scianna et al. (2015)	2D	• VEGF concentration • Oxygen concentration	• Endothelial cell (quiescent, stalk, tip) phenotype • Endothelial cell - ECM interaction	• Vessel network formation • Sprouting angiogenesis	• The vascular network obtained in the simulations has a good degree of realism
van Oers et al. (2014)	2D		• Endothelial cell motility • Substrate deformation • Endothelial cell - ECM interaction	• Vessels network formation • Sprouting angiogenesis	• Numerical results in accordance with <i>in vitro</i> experimental observations (Reinhart-King et al., 2008; Takakuda and Miyairi, 1996)

DII4, delta-like ligand 4; ECM, extracellular matrix; PDGF, platelet derived growth factor; TGF- β , transforming growth factor β ; VEGF, vascular endothelial growth factor.

Table 3: Hybrid models of wound healing angiogenesis on multiple biological scales.

Authors	Dimension	Scale of interest exploring in the model			Experimental validation
		Molecular	Cellular	Tissue	
Sun et al. (2005)	2D	• Chemotactic growth factors	• Endothelial cell sprout • Endothelial cell extension • ECM	• Capillary network formation • Anastomosis	• Vascular network obtained in the numerical simulation was morphological similar to those observed in vivo
Machado et al. (2011)	2D	• VEGF concentration • MMPs density	• ECM • Endothelial cell migration • Endothelial cell perfusion	• Capillary network formation • Vessel junction density • Vessel density • Wound area	• Wound areas, vessel densities and vessel junction densities obtained in the numerical simulation were in accordance with experimental studies
Bookholt et al. (2016)	3D	• VEGF concentration • DLL4 concentration • MMPs concentration • uPA concentration	• Tip cell - stalk cell interaction • Cell - matrix interaction • Substrate degradation • Cell motility	• Sprouting angiogenesis	• Effect of VEGF concentrations in the area of sprouted was similar both in numerical simulation and in experimental assay • Vascular morphology obtained in the numerical simulation was similar to experimental observations
Pillay et al. (2017)	2D/1D	• TAF concentration	• Tip cell density • Endothelial cell density	• Vessel formation • Anastomosis • Branching	

DLL4, delta-like ligand 4; ECM, extracellular matrix; MMPs, matrix metalloproteinases; TAF, tumour angiogenic factor; uPA, urokinase plasminogen activator; VEGF, vascular endothelial growth factor.

Table 4: Biological features analysed in the mathematical models of wound healing angiogenesis.

	Dimension			Biological features													
	1D	2D	3D	Chemoattractant effect	Cell densities	Capillary /vessel densities	Healing time	Wound contraction	Wound closure	Wound area	Capillary network	Sprouting	Branching	Anastomosis	EC-ECM interaction	Vascular remodelling	Substrate deformation
Pettet et al. (1996)	●			●	●	●											
Byrne et al. (2000)	●			●	●	●	●			●							●
Maggelakis (2003)	●			●		●											
Schugart et al. (2008)	●			●	●												
Flegg et al. (2010)	●			●	●		●			●							
Xue et al. (2009)	●			●	●	●			●								
Olsen et al. (1997)	●	●			●										●		
Gaffney et al. (2002)	●				●	●											
Javierre et al. (2008)		●		●		●			●								
Vermolen and Javierre (2011)		●		●	●	●		●	●								
Valero et al. (2013)		●		●		●	●	●		●							
Stokes and Lauffenburger (1991)		●		●							●		●	●			
Matsuya et al. (2016)		●									●	●	●				
Peirce et al. (2004)		●		●							●					●	
Bentley et al. (2008)			●	●							●						
Merks et al. (2004)		●		●							●						
Scianna et al. (2015)		●		●							●	●	●	●	●		
Van Oers et al. (2014)		●									●	●			●		●
Sun et al. (2005)		●		●							●		●	●			
Machado et al. (2011)		●		●		●				●	●						
Bookholt et al. (2016)			●	●						●		●			●		●
Pillay et al. (2017)	●	●		●	●								●	●			

Chapter III

Original Studies

3.1. Introductory Remark

Angiogenesis is a very complex process and computational simulation is a helpful tool to fully detail and understand this process. As discussed previous, some authors have already developed computational models to study the endothelial cell behaviour and the capillary network formation during angiogenesis. Regarding the advanced discretization technique used, the Finite Element Method (FEM) is the most applied. The application of meshless methods to model angiogenesis in the context of wound healing is less explored. However, since meshless methods allow to analyse any phenomenon that is modulated by differential equation [33] and allow to deal with higher mesh distortion characteristic of biological processes, their application in the field of angiogenesis was analysed in this thesis. Accordingly, the second contribution (3.2.2. Contribution II) presents the meshless-based model developed to simulate the endothelial cells migration modulated by chemical diffusion of VEGF, which is the main regulator of angiogenesis [7]. This work presents the full description of the numerical method used, the Radial Point Interpolation Method (RPIM), as well as the discrete formulation. In the third contribution (3.3.2. Contribution III) the branching process was implemented in the algorithm, allowing to simulate the capillary network obtained during an *in vivo* angiogenesis assay with a realistic morphology and structure. In the fourth contribution (3.4.2. Contribution IV) the predictive capacity of the meshless-based model to simulate the angiogenesis response for several VEGF concentrations is presented, as well as the quantitative and the qualitative evaluation of angiogenesis, compared with *in vivo* angiogenesis assay.

The modulation of angiogenesis sprouting due to growth factor gradients has already been extensively described [35]. However, mechanical signals can also regulate angiogenesis and endothelial cell behaviour but less is known about the mechanical stimuli in angiogenesis [36, 37]. Therefore, in the fifth contribution (3.5.2. Contribution V) the effect of compressive loading in angiogenesis was analysed and its response was quantified.

The present chapter includes the main highlights of each contribution presented as an extended abstract, followed by the corresponding complete publication.

3.2. Contribution II: A preliminary study of endothelial cell migration during angiogenesis using a meshless method approach

3.2.1. Highlights of Contribution II

This contribution focused on the meshless method approach used to analyse the effect of chemical diffusion of VEGF in endothelial cell migration.

Therefore, the main contents of this publication include:

- I. Brief description of the angiogenesis process and the regulatory mechanisms involved;
- II. Literature review on wound healing angiogenesis models;
- III. Concise summary with the advantages of using meshless methods to simulate biological processes;
- IV. Full description of the RPIM formulation used in this work;
- V. Full description of the discrete formulation used to analyse the chemical diffusion of VEGF and the endothelial cell migration;
- VI. Optimization of the model's parameters (integration scheme and influence domain) in order to decrease the computational cost without losing accuracy;
- VII. Analysis of the individual endothelial cell sprouting migration pattern. As demonstrated in Figure 3.1, endothelial cells migrate from the endothelial cell monolayer to the tissue damaged area according to the VEGF diffusion gradient;

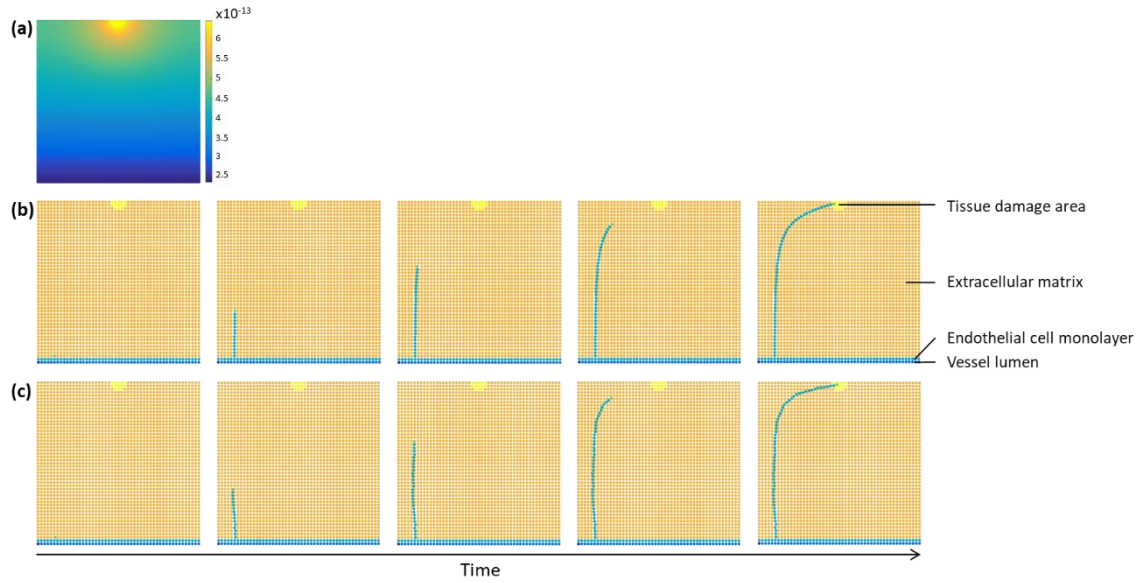


Figure 3.1: Tip cell migration profile. **(a)** VEGF diffusion gradient (VEGF concentration in g mm^{-3}); **(b)** and **(c)** Simulation results from individual tip cell migration in different time steps, respectively, with no random motion and with random motion, using a regular nodal discretization mesh.

VIII. Analysis of the triple endothelial cell sprouting migration in the presence of wounds placed at different locations, using a regular nodal discretization mesh (Figure 3.2);

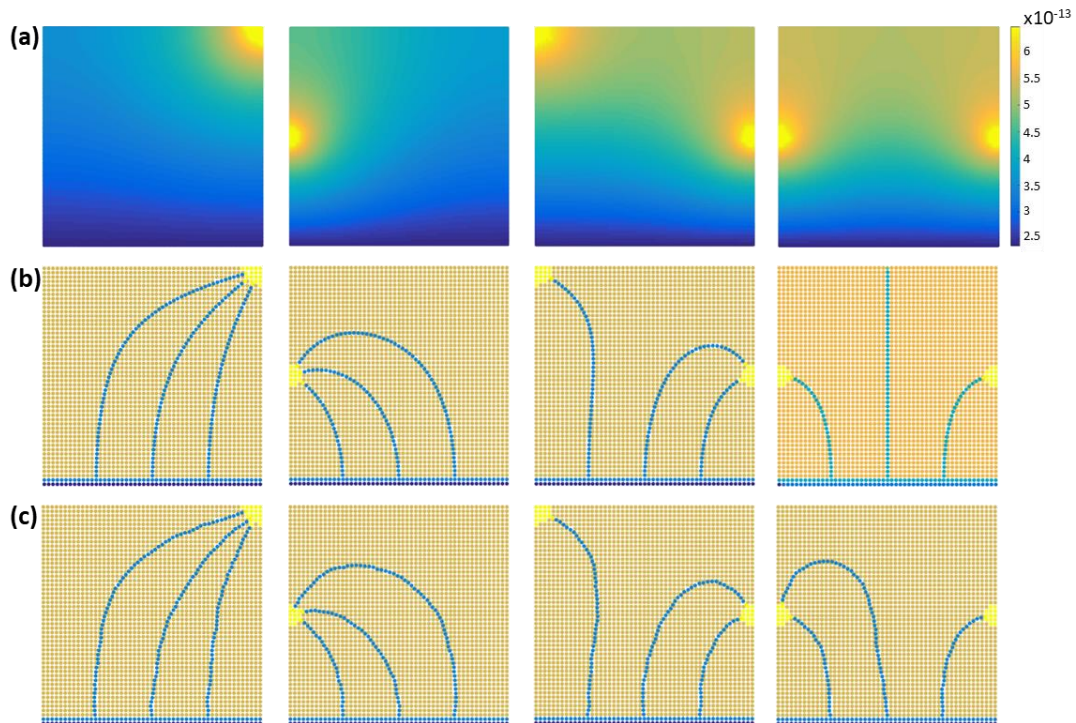


Figure 3.2: Tip cell migration profile in direction of two wounds placed at different location. **(a)** VEGF diffusion gradient (VEGF concentration in g mm^{-3}); **(b)** Final simulation results for the three tip cell migration with no random motion; **(c)** Final simulation results for the three tip cell migration with random motion.

- IX. Analysis of the capillary network pattern obtained using different domain geometries in order to simulate different hypothetical biological scenarios;
- X. Conclusion on the accuracy of the used meshless method to simulate endothelial cell migration. In all the performed simulations it was possible to verify that the tip cells migrated from the endothelial cell monolayer to the tissue damaged zone, indicating that the migration condition is well implemented in the code. Although the proposed model, in its present stage, is not capable to mimic the whole angiogenic process, the numerical formulation presented in this contribution is wide and robust enough to support further and more complex techniques required to fully simulate angiogenesis, such as branching and anastomosis processes.

The complete document can be found in the next sub-chapter.

3.2.2. Contribution II

A preliminary study of endothelial cell migration during angiogenesis using a meshless method approach

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A preliminary study of endothelial cell migration during angiogenesis using a meshless method approach

Ana Guerra^a, Jorge Belinha^b and Renato Natal Jorge^{c,d}

^aInstitute of Science and Innovation in Mechanical and Industrial Engineering (INEGI), Porto, Portugal.

^bSchool of Engineering, Polytechnic of Porto (ISEP), Mechanical Engineering Department, Porto, Portugal.

^cAssociated Laboratory For Energy, Transports and Aeronautics (LAETA – INEGI), Porto, Portugal.

^dFaculty of Engineering of the University of Porto (FEUP), Mechanical Engineering Department, Porto, Portugal.

ABSTRACT

Angiogenesis, the development of new blood capillaries, is crucial for the wound healing process. This biological process allows the proper blood supply to the tissue, essential for cell proliferation and viability. Several biological factors modulate angiogenesis, however the vascular endothelial growth factor (VEGF) is the main one. Given the complexity of angiogenesis, in the last years, computational modelling aroused the interest of scientists since it allows to model this process with different, more economic and faster methodologies, comparatively to experimental approaches. In this work, a mathematical model motivated by the analysis of the effect of VEGF diffusion gradient in endothelial cell migration is presented. This is the process that allows capillary formation and it is essential for angiogenesis. The proposed mathematical model is combined with the Radial Point Interpolation Method, being the area discretized considering an unorganized nodal cloud and a background mesh of integration points, without predefined relations. The nodal connectivity was achieved using the ‘influence-domain’ approach. The interpolation functions were constructed using the Radial Point Interpolators techniques. This method combines a radial basis functions with a polynomial functions to obtain the approximation. This preliminary work does not account for the whole complexity of cell and tissue biology, and numerical results are presented for an idealised two-dimensional setting. Nevertheless, the developed RPIM software is a valid numerical tool that can be adjusted to biological problems and may also be able to complement the biological and medical subjects.

KEYWORDS

Endothelial cell migration; Chemo-attractant effect; Meshless methods; Radial Point Interpolation Method.

1. INTRODUCTION

Angiogenesis is characterized by the formation of new blood capillaries from pre-existing vessels and it is an indispensable process during human life (1). This biological process occurs during embryogenesis and in adult life, in particular situations such as wound healing, when it is required to promote the blood supply to a particular tissue or organ (2). In fact, angiogenesis promotes the tissue homeostasis, it permits the re-establishment of a healthy blood vessel network and it supports the nutrients and oxygen supply and the metabolic's waste elimination (3).

In healthy tissues, the vasculature is preserved in a stable condition. Functional blood vessels are composed by a basement membrane covered with pericytes and smooth muscle cells. This basement membrane allows endothelial cell survival and vessel stability (4). However, during wound healing, blood vessels are vulnerable to compounds that promote angiogenesis and endothelial cells start to migrate and to proliferate. Then, the sprouting vessel's elongation in the direction of the tip cell occurs (3). The vascular density rises and anastomosis takes place, the fusion between the new and the previously blood vessels. Accordingly, anastomosis permits the re-establishing of the blood flow. The vessels that do not suffered anastomosis will degenerate by apoptosis. Finally, the vasculature undergoes stabilization and maturation and the basement membrane is restored in order to stabilize the vessel (3). If the tissue wound healing process was properly completed, the vessels' number standardizes (5).

As described above, the tip cell migration is an essential process during angiogenesis (6). This process includes three mechanisms: chemotaxis, haptotaxis and mechanotaxis. Chemotaxis is the cell migration in the direction of soluble chemical compounds' gradient. Haptotaxis is the directional migration toward an immobilized ligands' gradient. Mechanotaxis is the cell movement governed by mechanical forces (7). In angiogenesis, the endothelial cell migration occurs due to these three mechanisms.

Angiogenesis is regulated by genomic and proteomic modulation. The vascular endothelial growth factor (VEGF) is the best know proangiogenic factor and it stimulates new blood vessel formation by promoting endothelial cells' migration, proliferation, differentiation and viability. The binding of VEGF to its receptor (VEGFR2) is the most studied pathway responsible for stimulating endothelial cells to proliferate, to migrate and to increase vascular permeability (8).

Numerous biological parameters, such as growth factor concentration, oxygen or nutrients regulate the new blood vessel formation. Therefore, it is an arduous task to associate the variation between one biological parameter and its effect on wound healing. Accordingly, *in*

silico models in which computer models are developed to characterized biological experiments carried out entirely in a computer aroused the interest of the scientific community given its possibility to reproduce and to study biological systems, and also to modulate the behaviour of the biological system when a parameter is changed (9,10). In fact, some models that describe the effect of chemo-attractant species in the endothelial cell behaviour during angiogenesis had already been established. Some authors (11,12) developed the cellular Potts model, which are computational models able to reproduce the cooperative behaviour of cellular structures, and combined them with partial differential equations (PDEs). Merks *et al.* (11) study the cell morphology and the adhesion between cells and demonstrated that for the stable blood vessels' formation the endothelial cell adhesion is necessary. Scianna *et al.* (12) reproduce the development of a vascular network by sprouting angiogenesis. Other authors (13-16) established hybrid models in order to simulate the individual cell behaviour and also the effect of chemical gradients using separate space and time scales. Sun *et al.* (13,14) replicated the influence of growth factors gradients in the vascular pattern, creating capillary configuration with accurate organization and morphology, comparatively to experimental data. Machado *et al.* (15) virtually analysed the migration of endothelial cells by chemotaxis, due to soluble gradients of VEGF, and by haptotaxis, due to gradients of immobilized ligands in the extracellular matrix (ECM). Bookholt *et al.* (16) studied the influence of chemical compounds in the switch between tip cell and stalk cell.

The numerical method selected to perform the numerical simulation is determinant for its success. Usually, advanced discretization techniques, regardless of being mesh-dependent or meshless, are the most used to simulate biological processes (17). In the field of angiogenesis simulation, cell-based models are very useful to model the behaviour of a reduced number of cells. These models allow us to study the biological mechanisms that regulate individual endothelial cell behaviour during angiogenesis (18). Indeed, Drasdo and co-workers, used cell-based models to efficiently simulate cell behaviour (19). The authors developed a single-cell-based model in which the basic units were individual cells. In their model, each cell was modelled as an elastic, sticky particle of limited compressibility and deformability, capable to active migrate, growth and divide. With this approach, the authors provided a useful framework able to model the growth of cell populations *in silico*. Nevertheless, a limitation of these models is that they may not provide useful information at the tissue scale being difficult to ensure the description of angiogenesis as a whole. In order to overcome this aspect other authors developed hybrid models. Vilanova et al. (20), using a numerical method based on isogeometric analysis, developed a model that captures the vascular network formation around a tumour. In their hybrid model, the continuous equations were based on the phase-field method and

allowed to describe the complex interaction between the capillary network and the host tissue. The discrete formulation, at the cellular scale, presented the endothelial cells as mobile agents. With this approach, the authors showed that the isogeometric analysis is able to numerically simulate the angiogenesis process. In this work, we study the capillary growth following the tip cell migration due to VEGF diffusion gradients by using meshless methods. In the last years, the application of meshless methods to simulate biologic systems began to be addressed (21,22). In these numerical methods, the field functions are constructed through an approximation within a flexible influence-domain. Comparatively to other numerical methods, meshless methods have some benefits (17,23). Namely, meshless techniques allow studying structures with complex geometries, such as soft tissues; to construct the test functions using high-order continuous weight functions, permitting to obtain smoother variables fields; to simulate moving discontinuities and large deformations problems; and to perform mesh refinement in order to obtain accurate local solutions by insert locally more nodes, with the same computational cost (17). The model proposed in this study inserts new nodes, or changes the position of existent nodes, to simulate endothelial cell migration during angiogenesis, which induces mesh distortion. Accordingly, meshless methods are advantageous in order to deal efficiently with this mesh distortion (17). Furthermore, the literature shows that meshless methods converge faster than mesh dependent methods such as the finite element method (17). Previous works have shown that meshless methods have a good performance in models that analyse great mesh distortion (24). Nevertheless, the use of these numerical methods to model wound healing angiogenesis has been less explored. Since meshless methods allow to analyse any phenomenon that is governed by a differential equation, their application in the field of angiogenesis can be extremely promising.

In this paper, our numerical model able to reproduce the endothelial cell migration during wound healing was fully detailed. Our numerical results demonstrated that endothelial cells migrate following the VEGF gradient concentration allowing the formation of capillaries and the vascularization of the tissue. Briefly, in the next section, the numerical method used and the computational model developed will be entirely explained and detailed. Then, in the "Results" section, the simulation results concerning the endothelial cell migration governed by VEGF diffusion will be presented. In the end, a global discussion will be addressed.

2. METHODS

2.1. Radial point interpolation method

In this work, an iterative original code was developed in order to analyse the effect of chemical diffusion of VEGF in tip cell migration. Thus, the code combines the Radial Point Interpolation Method (RPIM) (17) with a chemical diffusion model and additional mathematical assumptions aiming to simulate the tip cell migration.

2.1.1. Numerical integration and nodal connectivity

RPIM is a discrete numerical method. In order to discretize the problem domain an unstructured nodal cloud and a background mesh of integration points are used. Thus, as a first step, the problem physical domain is discretized using a nodal set fitting the domain area. Then, a background integration lattice is defined. In this work, the integration lattice is built with quadrilateral regular cells perfectly fitting the domain area. Then, it is possible to insert inside each quadrilateral cell integration points. There are several numerical integration rules available, this work uses the Gauss-Legendre quadrature rule. Each integration point is defined by its Cartesian coordinates $\mathbf{x}_I \in \mathbb{R}^2$ and by its own integration weight, $\hat{w}_I$, which corresponds to the physical portion (area) that integration point occupies in the physical domain. The set of integration points establishes the background integration mesh, which is essential to discretely integrate the continuous equations ruling the physical phenomenon. After setting the integration points, the nodal connectivity can be defined. Comparatively to the finite element method, the nodal connectivity is not predefined in meshless methods. Such connectivity has to be obtained using the concept of “influence-domain”. To each integration point $\mathbf{x}_I$ it is univocal associated an “influence-domain”, representing the set of nodes closer to the integration point $\mathbf{x}_I$. The influence-domain of $\mathbf{x}_I$ is achieved by checking a sufficient number of nodes within a fixed area around an integration point $\mathbf{x}_I$. The nodes forming the influence-domain of $\mathbf{x}_I$ are used to build the shape function of integration point $\mathbf{x}_I$, which will then be applied to the weak form to build the system of equations. In the literature, both the topics “numerical integration” and “nodal connectivity” for meshless methods are described with detail (17).

2.1.2. Radial point interpolators

In RPIM, the interpolation functions use a Radial Point Interpolation (RPI) technique that combines two functions, the polynomial function, $\mathbf{p}(\mathbf{x}) = \{p_1(\mathbf{x}), p_2(\mathbf{x}), \dots, p_m(\mathbf{x})\}^T$, and a radial function, $\mathbf{r}(\mathbf{x}) = \{r_1(\mathbf{x}), r_2(\mathbf{x}), \dots, r_n(\mathbf{x})\}^T$. Being m the monomial terms of the polynomial function and n defines the number of nodes belonging to the influence-domain of integration point $\mathbf{x}_I$. Therefore, at the specific point $\mathbf{x}_I \in \mathbb{R}^2$, the interpolation function $u^h(\mathbf{x})$ can be determined by

$$u^h(\mathbf{x}_I) = \sum_{i=1}^n r_i(\mathbf{x}_I) a_i + \sum_{j=1}^m p_j(\mathbf{x}_I) b_j = \mathbf{r}(\mathbf{x}_I)^T \mathbf{a} + \mathbf{p}(\mathbf{x}_I)^T \mathbf{b} = u(\mathbf{x}_I) \quad (1)$$

where a_i and b_j represent, respectively, the non-constant parameters of $r_i(\mathbf{x}_I)$ and $p_j(\mathbf{x}_I)$. Each one of these vectors can be represented as follows:

$$\mathbf{a}^T = \{a_1, a_2, \dots, a_n\} \quad (2)$$

$$\mathbf{b}^T = \{b_1, b_2, \dots, b_m\} \quad (3)$$

$$\mathbf{r}(\mathbf{x})^T = \{r_1(\mathbf{x}), r_2(\mathbf{x}), \dots, r_n(\mathbf{x})\} \quad (4)$$

$$\mathbf{p}(\mathbf{x})^T = \{p_1(\mathbf{x}), p_2(\mathbf{x}), \dots, p_m(\mathbf{x})\} \quad (5)$$

being $\mathbf{x}_i = \{x_i, y_i\}^T$. It is possible to combine with the RPI formulation several distinct radial basis functions (RBF). Nevertheless, previous works have shown that the most efficient one is the multiquadrics radial basis function (MQ-RBF) (25,26), which can be generically expressed as,

$$R_{il} = (r_{il}^2 + c^2)^p \quad (6)$$

Thus, this work also uses the MQ-RBF combined with the RPI. Notice that c and p are shape parameters. These shape parameters were already optimized in previous works (17,23,27), which suggest $c = 0.0001$ and $p = 0.9999$. The term r_{il} is the distance between the node $\mathbf{x}_i = \{x_i, y_i\}^T$ and the integration point $\mathbf{x}_I = \{x_I, y_I\}^T$ and can be represented by,

$$r_{il} = \sqrt{(x_i - x_I)^2 + (y_i - y_I)^2} \quad (7)$$

A complete polynomial function is enforced by the RPI formulation, for instances, for a $\mathbb{R}^2$ domain,

$$\mathbf{p}(\mathbf{x}_i)^T = \{1, x_i, y_i, x_i^2, xy_i, y_i^2, x_i^3, x_i^2 y_i, x_i y_i^2, y_i^3, \dots\} \quad (8)$$

Nevertheless, previous works (17) found that it is not necessary to use a large polynomial basis function, stable and accurate results are obtained with a small constant polynomial basis function: $\mathbf{p}(\mathbf{x}_i) = \{1\}$, which allows to significantly reduce the computational

cost and maintain the same level of accuracy. Thus, in this case, the number of monomials terms is just $m = 1$. In order to assure a single solution, an additional equation is required (17),

$$\sum_{i=1}^n p_j(x_i, y_i) a_i = 0, \quad \text{with} \quad j = 1, 2, \dots, m \quad (9)$$

Hence, the following system of equations can be established,

$$\begin{bmatrix} \mathbf{R} & \mathbf{P} \\ \mathbf{P}^T & 0 \end{bmatrix} \begin{Bmatrix} \mathbf{a} \\ \mathbf{b} \end{Bmatrix} = \begin{Bmatrix} \mathbf{u} \\ 0 \end{Bmatrix} \Leftrightarrow \mathbf{G} \begin{Bmatrix} \mathbf{a} \\ \mathbf{b} \end{Bmatrix} = \begin{Bmatrix} \mathbf{u} \\ 0 \end{Bmatrix} \Leftrightarrow \begin{Bmatrix} \mathbf{a} \\ \mathbf{b} \end{Bmatrix} = \mathbf{G}^{-1} \begin{Bmatrix} \mathbf{u} \\ 0 \end{Bmatrix} \quad (10)$$

being $\mathbf{G}$ the complete moment matrix, $\mathbf{Z}$. The vector for function values is defined as $u_i = u(\mathbf{x}_i), \forall \{i \in N: i \leq n\}$. The radial matrix $\mathbf{R}$ is defined as $R_{ij} = r_i(\mathbf{x}_j, \mathbf{x}_j)$, being the matrix $\mathbf{R}$ symmetric, and the column matrix $\mathbf{P}$ as $P_j = 1$. Moreover, $r_i(\mathbf{x}_j, \mathbf{x}_j) = r_j(\mathbf{x}_i, \mathbf{x}_i)$, i.e., $R_{ij} = R_{ji}$, being the distance directionless. Thus, with equation (10) it is possible to obtain both $\mathbf{a}$ and $\mathbf{b}$.

Whereas $\text{rank}(p) = m \leq n$ (28), the solvability of this system is ensured. In order to address the mentioned conditions, the number of nodes inside the influence-area will always be adequate. In the end, the interpolation function is obtained with

$$u^h(\mathbf{x}_I) = \{r(\mathbf{x}_I)^T; p(\mathbf{x}_I)^T\} \mathbf{G}^{-1} \begin{Bmatrix} \mathbf{u} \\ 0 \end{Bmatrix} = \{ \Phi(\mathbf{x}_I)^T; \Psi(\mathbf{x}_I)^T \} \begin{Bmatrix} \mathbf{u} \\ 0 \end{Bmatrix} \quad (11)$$

where $\psi_1(\mathbf{x}_I)$ is a residual scalar (without relevant physical meaning) and the interpolation function vector $\Phi(\mathbf{x}_I)$ can be expressed as:

$$\Phi(\mathbf{x}_I) = \{\varphi_1(\mathbf{x}_I) \quad \varphi_2(\mathbf{x}_I) \quad \dots \quad \varphi_n(\mathbf{x}_I)\} \quad (12)$$

Thus, the field interpolation is obtained with

$$u^h(\mathbf{x}_I) = \Phi(\mathbf{x}_I)^T \mathbf{u} \quad (13)$$

The RPI shape functions' partial spatial derivatives are achieved straightforwardly, as disclosed in previous works (17,29). Moreover, the distribution of spread nodes is responsible for the RPI test functions $\Phi(\mathbf{x}_I)$ (17). It has already been demonstrated (17,25,27) that RPI functions have the Kronecker delta property, satisfying the following condition,

$$\varphi_i(\mathbf{x}_j) = \delta_{ij} \quad (14)$$

where δ_{ij} is the Kronecker delta, $\delta_{ij} = 1$ if $i = j$ and $\delta_{ij} = 0$ if $i \neq j$. Consequently, it is possible to directly enforce the essential boundary conditions in the equations' system, simplifying its numerical procedure and reducing the overall computational cost.

2.2. Discrete formulation

2.2.1. Equation system

In this work, the chemical diffusion in a homogeneous medium was assumed. Aiming to obtain the numerical solution with RPIM, the chemical diffusion should be represented as a discrete equation system. Thus, the chemical diffusion can be numerically simulated as a field problem (30). The general form of Helmholtz allows setting the equations system of 2D linear steady state field problems,

$$D_x \frac{\partial^2 \phi}{\partial x^2} + D_y \frac{\partial^2 \phi}{\partial y^2} - g\phi + Q = 0 \quad (15)$$

where ϕ is the field variable, which in this cases corresponds to the VEGF concentration. The parameters D_x and D_y represents, respectively, the VEGF diffusion coefficient along dimension x and y , g represents the matrix of chemical infusibility (in this work g is neglected, $g = 0$) and Q is the source VEGF concentration. Using the weighted residual approach, the meshless system equations can be formulated as,

$$[K_D + K_g]\Phi - f_q = 0 \quad (16)$$

where K_D , after the manipulation of equation (15), can be defined as,

$$K_D = \int_A B_G^T D B_G dA \quad (17)$$

being B_G and D respectively defined as follows:

$$B_G = \begin{bmatrix} \frac{\partial \phi}{\partial x} \\ \frac{\partial \phi}{\partial y} \end{bmatrix} = \begin{bmatrix} \frac{\partial \phi_1}{\partial x} & \frac{\partial \phi_2}{\partial x} & \dots & \frac{\partial \phi_n}{\partial x} \\ \frac{\partial \phi_1}{\partial y} & \frac{\partial \phi_2}{\partial y} & \dots & \frac{\partial \phi_n}{\partial y} \end{bmatrix} \quad (18)$$

$$D = \begin{bmatrix} D_x & 0 \\ 0 & D_y \end{bmatrix} \quad (19)$$

Additionally, K_g can be defined as,

$$K_g = \int_A g \{\phi_1 \quad \phi_2 \quad \dots \quad \phi_n\}^T \{\phi_1 \quad \phi_2 \quad \dots \quad \phi_n\} dA \quad (20)$$

and f_q , the VEGF concentration flux, is defined as,

$$f_q = \int_A Q \{\phi_1 \quad \phi_2 \quad \dots \quad \phi_n\}^T dA \quad (21)$$

Consequently, solving Equation (16) it is possible to obtain the final VEGF concentration in the medium, Φ .

The final equation system can be written as $\mathbf{K} \Phi - \mathbf{f}_q = 0$ being $\mathbf{K} = \mathbf{K}_D + \mathbf{K}_g$. Since the RPIM shape functions possess the delta Kronecker property, the periodic boundary conditions are imposed using the direct imposition method. Thus, imposing the same VEGF concentration in node n_i (belonging to the left essential boundary) and in node n_j (belonging to the right essential boundary), the stiffness matrix in i^{th} degrees of freedom is modified accordingly,

$$\begin{aligned} K_{im} &= 0 \rightarrow m = 1, \dots, N \\ K_{ii} &= 1 \wedge K_{ij} = -1 \end{aligned} \quad (22)$$

and in j^{th} degree of freedom as,

$$\begin{aligned} K_{jm} &= 0 \rightarrow m = 1, \dots, N \\ K_{jj} &= 1 \wedge K_{ji} = -1 \end{aligned} \quad (23)$$

Being N the total number of degrees of freedom (in this case, the only variable is the VEGF concentration, n is equal to the total number of nodes discretizing the problem domain).

2.2.2. Model parameters

In order to simulate the biological phenomenon under study, the $1 \times 1 \text{ mm}^2$ square domain was discretized with 51×51 nodes, forming a regular nodal distribution with 2601 nodes. We chose this dimensions considering the endothelial cell size of about $20 \mu\text{m}$. If we divided our domain size by our number of nodes in x and y , we obtain $19.6 \mu\text{m}$, in this way each node represents approximately an area with the size of one endothelial cell. Concerning the domain geometry, four distinct initial zones/sub-domains were identified: the lumen of the vessel, the endothelial cell monolayer, the extracellular matrix and the tissue damaged area that releases VEGF. The location of the wounds, the vessel lumen and the endothelial cell monolayer change in the performed simulations. An example of the model geometry is showed in Figure 1.

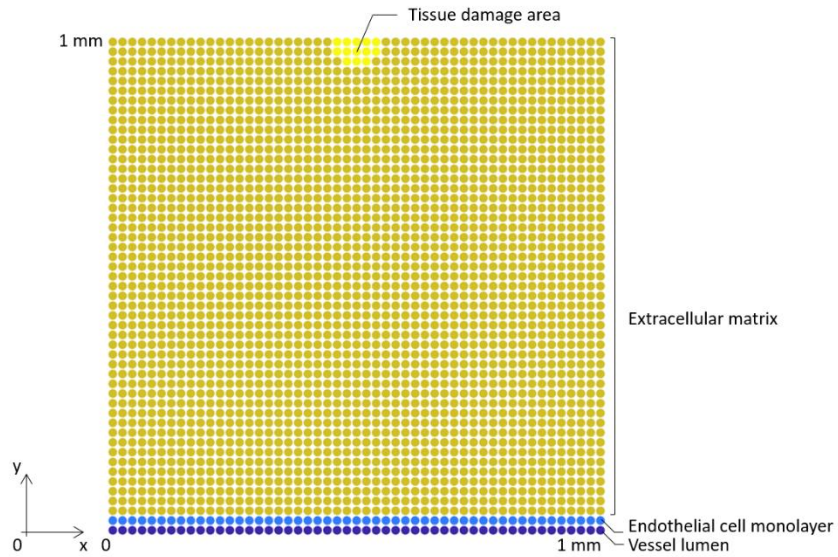


Figure 1: Example of a domain geometry with distinct initial zones/sub-domains identified.

2.2.3. Boundary conditions

Regarding the essential boundaries, a value for the VEGF basal concentration in the lumen vessel ($2.35 \times 10^{-13} \text{ g mm}^{-3}$) was applied and the VEGF concentration in the tissue damaged area ($6.43 \times 10^{-13} \text{ g mm}^{-3}$ and $5 \times 10^{-13} \text{ g mm}^{-3}$), following a periodic boundary condition linking the VEGF concentration of both sides, $\bar{\Phi}(x = 0) = \bar{\Phi}(x = 1)$, being $\bar{\Phi}$ the imposed VEGF concentration at the boundary. In the natural boundary conditions, the VEGF flux from the tissue damaged area was applied. With this methodology, it is possible to obtain the VEGF gradient concentration. The chemical diffusion coefficient ($1.16 \times 10^{-6} \text{ mm}^2\text{s}^{-1}$) and the VEGF concentration were obtained from available literature (31,32) (Table 1).

Table 1: Table of parameters.

Parameter	Dimensional value	Source
Basal VEGF concentration	$2.35 \times 10^{-13} \text{ g mm}^{-3}$	(32)
VEGF concentration in the wound	$6.43 \times 10^{-13} \text{ g mm}^{-3}$ and $5 \times 10^{-13} \text{ g mm}^{-3}$	(32)
VEGF diffusion concentration	$1.16 \times 10^{-6} \text{ mm}^2\text{s}^{-1}$	(31)
Model dimension	$1 \times 1 \text{ mm}^2$	-

As already mentioned, the background integration mesh is obtained using the square integration cell coming from a regular lattice and the influence domain is built using both the nodal and integration points distributions. The RPIM software proposed in this work has a

routine that generates the influence domain of each integration point and another routine that builds the shape functions.

2.2.4. Tip cell migration

To start the angiogenic sprouting, the tip cells in the sub-domain defined as endothelial cell monolayer were marked. In the first simulations, the tip cell positions using the Cartesian coordinates were chosen. Then, simulations with the tip cells marked in a random way were performed.

When the initial tip cells were marked, the RPIM software begins an iterative loop and in each step the tip cells are marked one after the other. The tip cell moves according to a diffusion-reaction equation. The direction of the tip cell movement is defined by the vector $\mathbf{u}$, that follows the gradient vector from the VEGF concentration field towards the VEGF source. Accordingly, the gradient of the VEGF concentration field is obtained ($\mathbf{n} = \nabla\Phi$), being $\mathbf{n}$ oriented towards the VEGF source (in opposition to its support cell). Since endothelial cell migration *in vivo* follows a random pattern (33), the vector $\mathbf{n}$ is slightly modified, taking into account a small random angle (this work assumed a random angle θ between -14° and 14° degrees: $\theta = \text{rand}(-14^\circ, 14^\circ)$ following a uniform random distribution). Thus, $\mathbf{n}_{mod} = \begin{bmatrix} \cos\theta & \sin\theta \\ -\sin\theta & \cos\theta \end{bmatrix} \mathbf{n}$. Then, the unit vector of $\mathbf{n}_{mod}$ is calculated: $\mathbf{u} = \mathbf{n}_{mod}/\|\mathbf{n}_{mod}\|$. Then, using the direction vector $\mathbf{u}$, the average distance between cells is considered and the new tip cell position is defined by summing the old tip cell position with the vector $\mathbf{u}$ multiplied by the average distance between cells. If a node exists in the new tip cell position, that node will become a tip cell. If in the new position there are no nodes, a new node is included in the model, and that new node becomes a tip cell. The vector $\mathbf{u}$ direction angle (θ) is calculated in each iteration, $\theta = \arctg(\mathbf{u}_x/\mathbf{u}_y)$. In each time step, the VEGF concentration is calculated and the new position of the tip cell is actualized, moreover, the VEGF concentration at the boundary domain is imposed. In our simulations, the time step represents the time that the capillary takes to migrate one single endothelial cell. Being the capillary growth rate of about 0.2mm per day and the endothelial cell size of about 20 μ mm, we considered that the time of one iteration step is 2.4h. The iterative process stops when the capillary arrives at the tissue damaged zone. The general algorithm implemented by the RPIM software is outlined in Figure 2.

Regular and irregular nodal meshes were performed. For both meshes, we obtained results were very similar (data are not shown). Since regular meshes normally produce accurate results (17), in the numerical results presented in this work only this nodal meshes are shown.

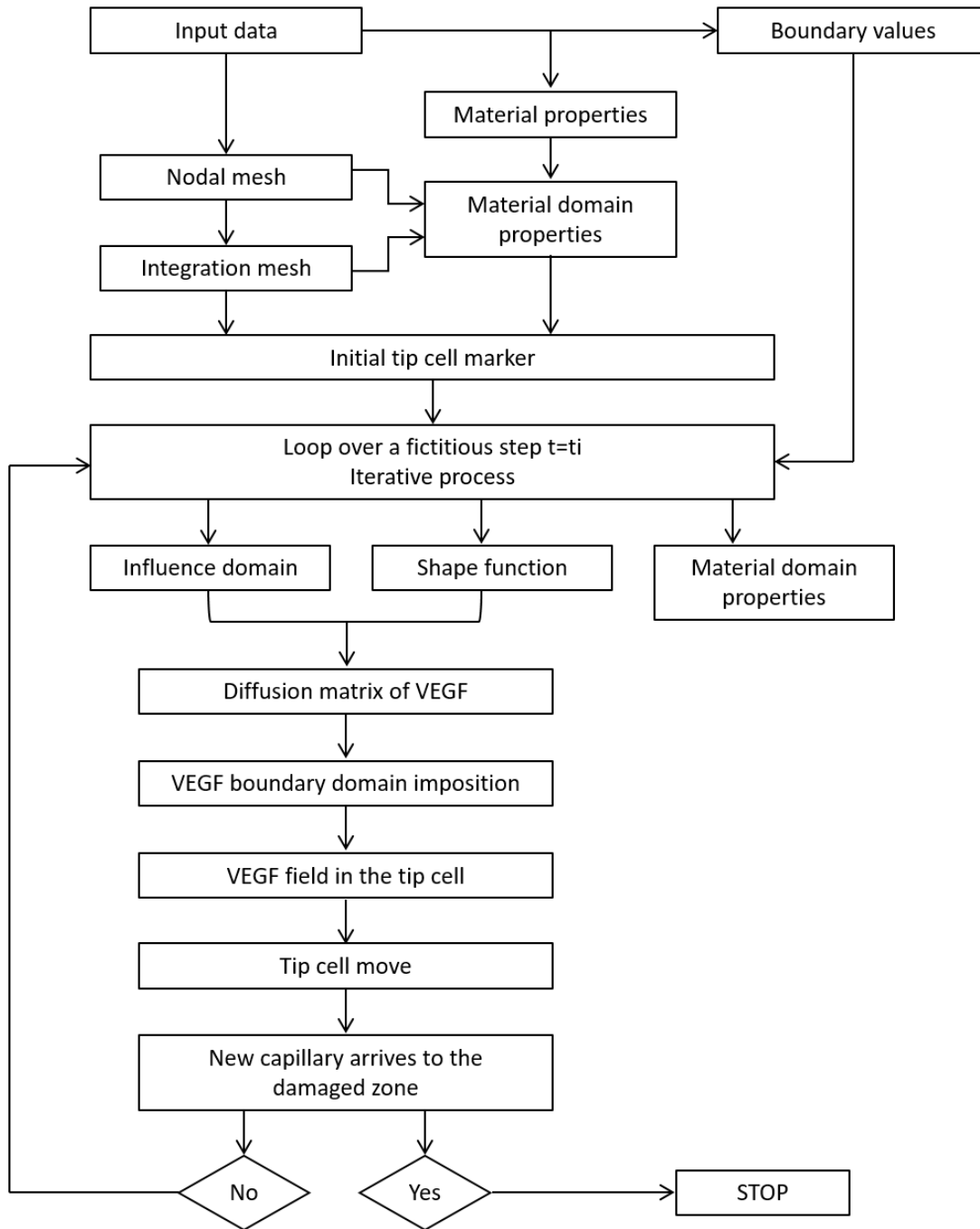


Figure 2: General representation of the algorithm used by the RPIM software tested in this work.

2.2.5. Model Parameters Optimization

With the purpose of reducing the computational cost without losing accuracy, some numerical parameters must be optimized first.

In order to study the integration scheme, the square integration cells were obtained from the background lattice. Each edge of the square integration cell is equal to the double of

the nodal distance. The integration points were distributed, inside of each integration cell, according to the Gauss-Legendre quadrature scheme. In this work, the following integration schemes were studied: 1×1 , 2×2 , 3×3 , 4×4 , 5×5 and 6×6 . Previous studies have shown that increasing the number of integration points inside of each integration cell also increases the solution accuracy. However, it also increases the computational cost. Accordingly, it is necessary to find an integration scheme that was capable of obtain accurate solutions but with an acceptable computational cost. Figure 3 shows the vector $\mathbf{u}$ direction angle (θ) and the VEGF concentration for each integration scheme under study, in five tip cells at different locations.

The tip cells were located at positions: $x = 0.02\text{mm}$, $x = 0.26\text{mm}$, $x = 0.50\text{mm}$, $x = 0.74\text{mm}$ and $x = 0.98\text{mm}$. Analysing Figure 3, it is possible to observe that the solution tends to stabilize after the 3×3 or the 4×4 integration scheme. Since the analysis becomes more expensive (in terms of computation cost) as the integration scheme becomes higher, we decided to use the 3×3 integration scheme in further simulations, because it produces acceptable results and it does not represent a very high computational cost.

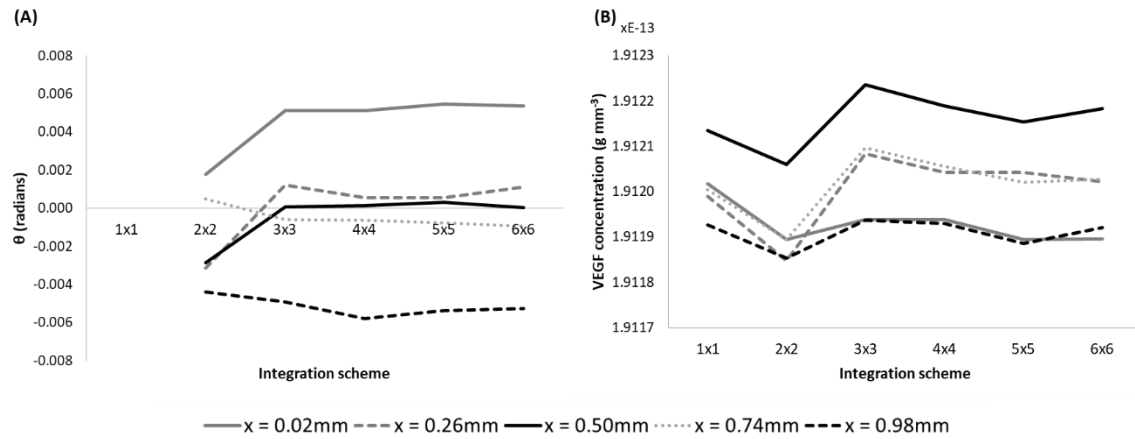


Figure 3: Integration scheme study. A, Vector $\mathbf{u}$ variation angle (θ), in radians, for each tip cell, for each integration scheme under study; B, VEGF concentration (g mm^{-3}) in each tip cell, for each integration scheme under study; The tip cells were located at positions: $x = 0.02\text{mm}$, $x = 0.26\text{mm}$, $x = 0.50\text{mm}$, $x = 0.74\text{mm}$ and $x = 0.98\text{mm}$.

The optimal number of nodes inside of each influence domain must also be obtained. In this work, nodal meshes with 4 to 32 nodes inside of each influence domain were tested. Figure 4 shows the vector $\mathbf{u}$ direction angle (θ) and the VEGF concentration for each number of nodes within each influence domain under study, in five tip cells at different locations. The tip cells were located at positions: $x = 0.02\text{mm}$, $x = 0.26\text{mm}$, $x = 0.50\text{mm}$, $x = 0.74\text{mm}$ and $x = 0.98\text{mm}$. Analysing Figure 4, it is possible to observe that 19 nodes inside of each influence domain

showed accurate results and presented less computational costs. Thus, in the following simulations this number of nodes inside of each influence area is used.

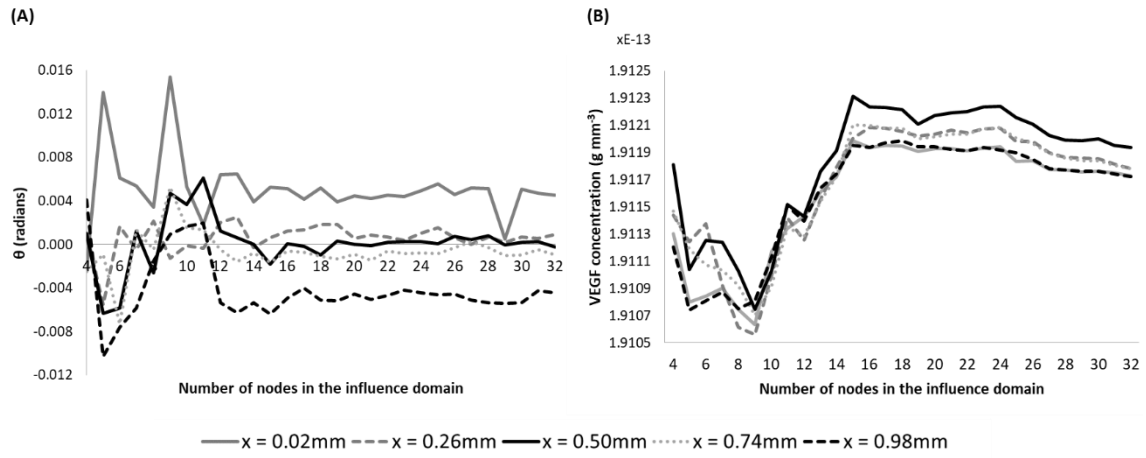


Figure 4: Influence domain study. A, Vector $\mathbf{u}$ variation angle (θ), in radians, for each tip cell, for each number of nodes in the influence domain under study; B, VEGF concentration (g mm^{-3}) in each tip cell, for each number of nodes in the influence domain under study; The tip cells were located at positions: $x = 0.02\text{mm}$, $x = 0.26\text{mm}$, $x = 0.50\text{mm}$, $x = 0.74\text{mm}$ and $x = 0.98\text{mm}$.

3. RESULTS

3.1. Individual endothelial cell sprouting

First, the individual tip cell migration pattern was analysed. *In vitro* studies indicate that endothelial cell follows a random walk during cell migration (33). Therefore, simulations using a random parameter for the vector's $\mathbf{u}$ movement direction were performed. Figure 5 A represents the VEGF diffusion gradient that goes from dark blue to yellow. The dark blue zone is located near to the endothelial cell monolayer and has the basal VEGF concentration. The yellow zone represents the wound, having the highest VEGF concentration. Figure 5 B and C presents the individual tip cell migration in consecutive time steps, using a regular nodal discretization mesh. Analysing the images, it is possible to verify that the tip cell migrates from the endothelial cell monolayer to the tissue damaged area. Moreover, the tip cell movement is done accordingly to the VEGF diffusion gradient. Taking into account the experimental results obtained with *in vivo* methodologies (13), the capillary network obtained in the simulations with the random motion was, as expected, more similar to the one obtained *in vivo*, mainly regarding the blood vessels network organization and structure (Figure 5 C).

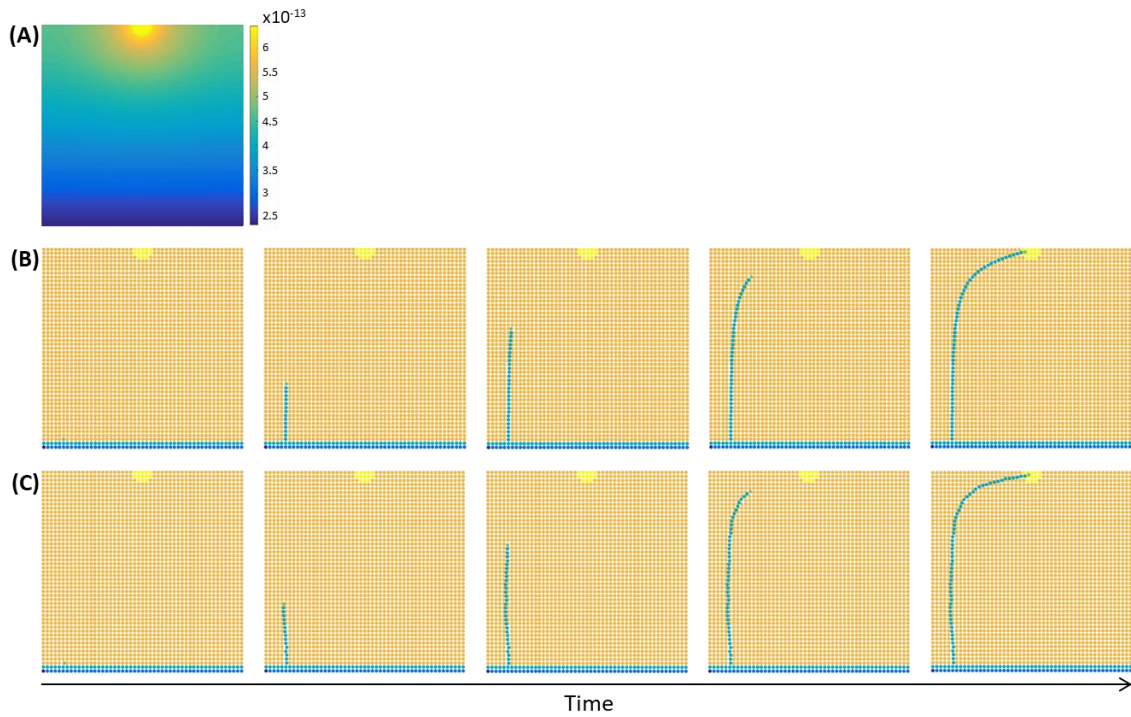


Figure 5: Tip cell migration profile. A, VEGF diffusion gradient (VEGF concentration in g mm^{-3}); B and C, Simulation results from individual tip cell migration in different time steps, with no random motion (B) and with random motion (C), using a regular nodal discretization mesh. The tip cell was located at position: $x = 0.1\text{mm}$.

3.2. Triple endothelial cell sprouting

In order to increase the model complexity the angiogenic sprouting from three tip cells in direction of wounds placed at different locations was also simulated. Figure 6 presents the final numerical simulation results for the three tip cell migration in the direction of wounds, regarding a non-random migration (B) or regarding a random migration (C). In these simulations the wounds are placed at: the centre, the top-middle, the top-right, the top-left, the middle-right and the middle-left. In all the tested possibilities, the tip cells migrate from the endothelial cell monolayer to the wound, following the VEGF diffusion gradient, as can be seen in Figure 6. Regarding the migration pattern obtained to the wounds located in the middle-right and the middle-left, it is possible to observe that the endothelial cell located further away from the wound, during its migration makes a bend. This occurs because the VEGF concentration increase in the zone upper the wound, because in the down part the VEGF concentration imposed is lower. It was also analysed how the VEGF concentration is distributed in each tip cell during the migration process (Figure 7). Analysing the graphs, it is possible to verify that the VEGF concentration is higher near the wound and decreases as the distance from the wound centre increases. As expected, in simulation with symmetric wounds (wounds located at top-right, top-

left, middle-right and middle-left) the obtained VEGF concentration as a function of the distance to the wound centre is also symmetric.

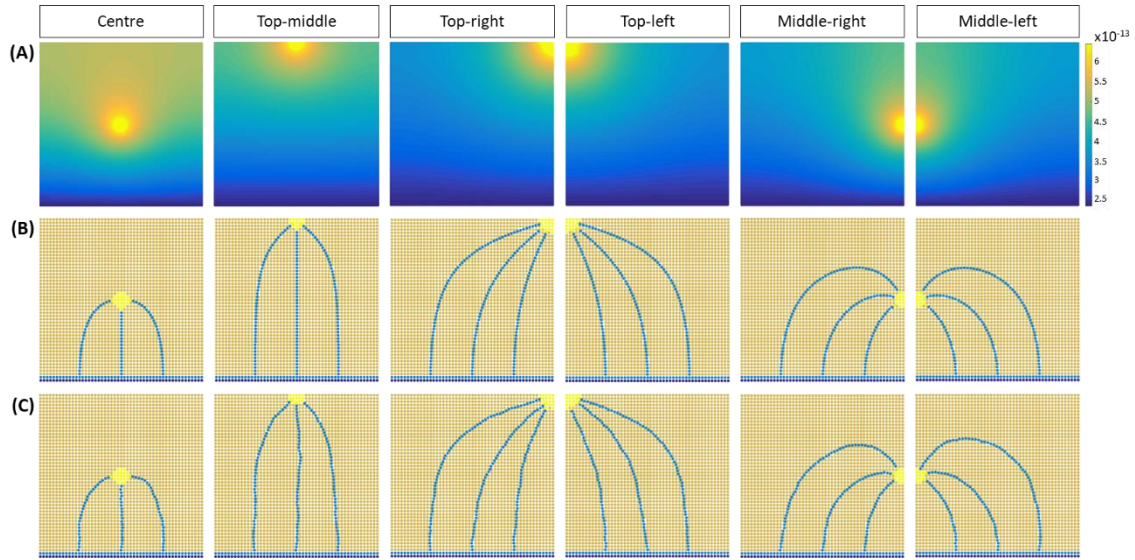


Figure 6: Tip cell migration pattern in direction of wounds placed at different location, using a regular nodal discretization mesh. A, VEGF diffusion gradient (VEGF concentration in g mm^{-3}); B, Final simulation results for the three tip cell migration with no random motion; C, Final simulation results for the three tip cell migration with random motion; The tip cells were located at positions: $x = 0.24\text{mm}$, $x = 0.50\text{mm}$ and $x = 0.76\text{mm}$.

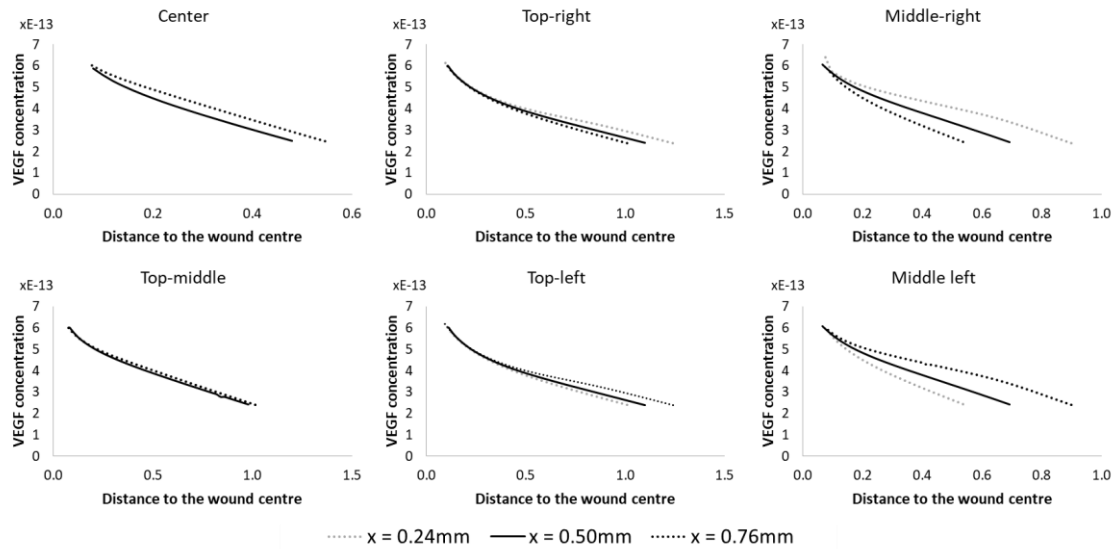


Figure 7: Numerical results showing how the VEGF concentration (in g mm^{-3}) is distributed in each tip cell, in the performed simulations. The tip cells were located at positions: $x = 0.24\text{mm}$, $x = 0.50\text{mm}$ and $x = 0.76\text{mm}$.

Figure 8 shows the simulations' results of the tip cell migration pattern in the presence of two wounds. In the first simulation, the three tip cells migrate up to the wounds, following the VEGF diffusion gradient, as in the previous results. However, in the other three simulations, with symmetric VEGF diffusion gradient, for the cell migration with no random motion (Figure 8 B), the tip cell in the centre does not achieve any of the wounds. Nevertheless, in the same simulations with symmetric VEGF diffusion gradient but with the random motion associated (Figure 8 C), all the tip cells achieve one of the wounds. This result is quite interesting and resembles more the observations occurring *in vivo*, the endothelial cells sense the VEGF gradients and migrate accordingly to this (34).

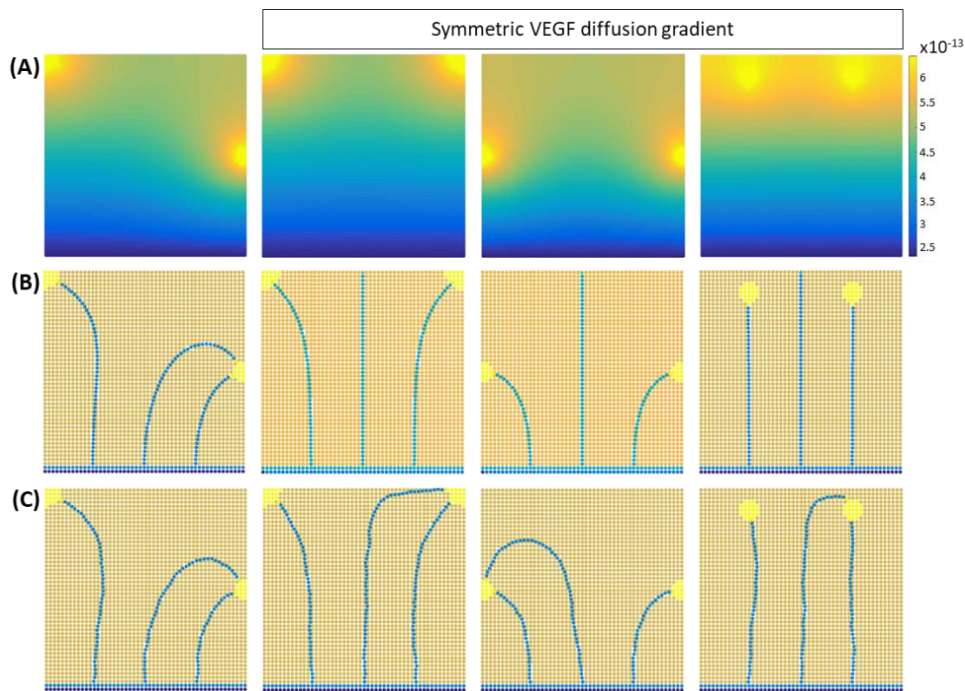


Figure 8: Tip cell migration profile in direction of two wounds placed at different location. A, VEGF diffusion gradient (VEGF concentration in g mm^{-3}); B, Final simulation results for the three tip cell migration with no random motion; C, Final simulation results for the three tip cell migration with random motion; The tip cells were located at positions: $x = 0.24\text{mm}$, $x = 0.50\text{mm}$ and $x = 0.76\text{mm}$.

3.3. Other numerical examples

The capillary network pattern obtained in different hypothetical biological scenarios was also analysed (Figure 9). Accordingly, the following simulations were performed: tip cell migration in the presence of three wounds, the tip cell migration from a circular capillary, tip cell migration in direction of two wounds with different VEGF concentration and located at the horizontal and at the vertical plane [two wounds (x) and two wounds (y), respectively] and tip cell migration in direction of a central wound. In all these simulations the tip cells were marked

in a random way and the cell migration with (Figure 9 C) and without random motion (Figure 9 B) was considered. Analysing the final numerical results it is possible to observe that all the tip cells migrate from the endothelial cell monolayer to the tissue damage area. Furthermore, this cell migration is done accordingly to the VEGF diffusion gradient. Additionally, the endothelial cell density in several iteration steps was quantified (Figure 10). The obtained results show that the simulation with the central wound presents the higher endothelial cell density, followed by the simulation with two wounds located at the horizontal plane [two wounds (x)]. These results indicate that the endothelial cell density is associated with the starting number of sprouting cells. Moreover, the endothelial cell density obtained for the same simulation but with and without cell random migration was very similar (Figure 10).

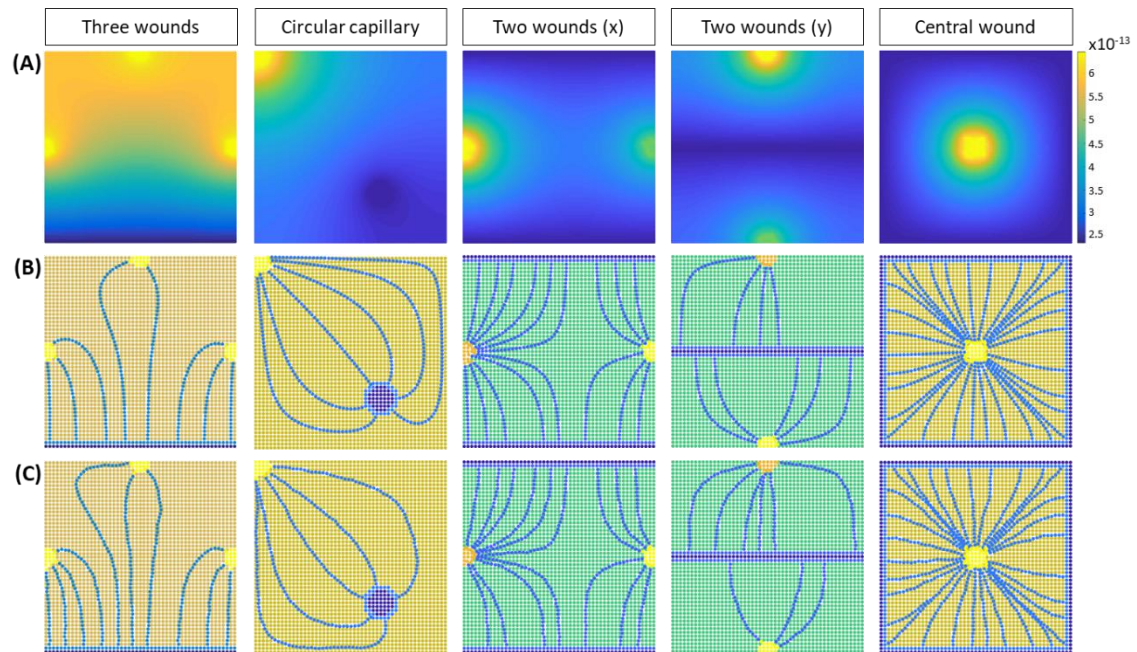


Figure 9: Tip cell migration profile. A, VEGF diffusion gradient (VEGF concentration in g mm^{-3}); B, Final simulation results for tip cell migration with no random motion; C, Final simulation results for tip cell migration with random motion. In all simulations the tip cells were marked in a random way and the wounds differ in number, location and VEGF concentration.

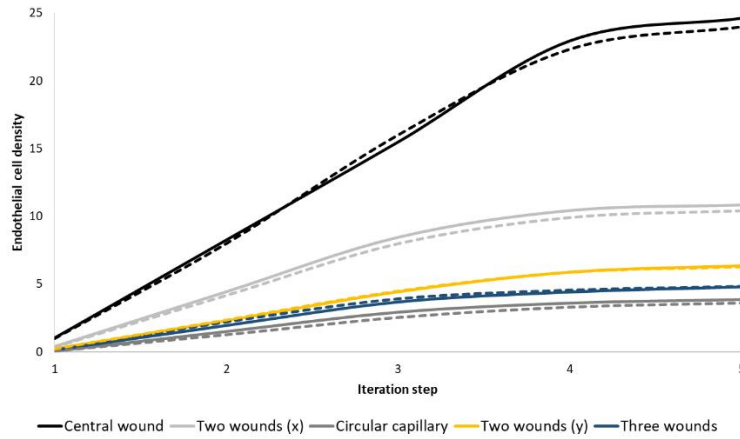


Figure 10: Numerical results showing the endothelial cell density in different iteration steps, in some performed simulations. In the simulations the tip cells were marked in a random way and the wounds differ in number, location and VEGF concentration. The solid lines represent the tip cell migration with no random motion and the dashed lines represent the tip cell migration with random motion.

4. DISCUSSION

The pertinence of using computational mathematical modelling of biological processes started to be addressed in the last years considering its potential to try new approaches and new therapies using non-invasive techniques. During wound healing, angiogenesis is an essential process and the effectiveness of this process is dependent on the migration of endothelial cells. Indeed, an important quality of endothelial cell is their motility response to growth factors, particularly to VEGF. In the human body, VEGF can be detected in plasma and serum (35), in diverse tissues especially during wound healing (36) and in tumours (37). Different cell types that participate in wound healing produce VEGF: fibroblasts, endothelial cells, smooth muscle cells, keratinocytes, macrophages, platelets and neutrophils (36). For this reason the release of VEGF from the tissue damaged area was simulated.

In this study, a computational model to analyse the tip cell migration due to chemotaxis was developed. In our computational model, the endothelial cell migration is done through chemotaxis, consequently the cells migrate following the highest VEGF concentration. Therefore, in all the performed simulations one verifies that the tip cell migrates from the endothelial cell monolayer to the tissue damage zone (Figures 5, 6, 8 and 9). This result indicate that the migration condition is well implemented in the code. These numerical results are in accordance with previous studies of endothelial cell culture *in vitro* that showed that VEGF induces cell migration (38,39) and chemotactic response (40,41). Additionally, the cell chemotactic response is frequently described as a range in the endothelial cell random walk in the direction of the higher growth factor gradient concentration (33). Indeed, in the simulations

performed with the random parameter associated to the vector's $\mathbf{u}$ movement direction, the endothelial cell also migrated according to the VEGF gradient concentration. Nevertheless, the capillary network obtained in these simulations was more similar to the one obtained *in vivo* experiments that identify the vascular pattern. Moreover, it had already been shown that endothelial cells, cultured in a proper matrix such as Matrigel, migrate in the direction of VEGF release. Accordingly, the gradient concentration of VEGF, formed in Matrigel, induces the migration of endothelial cell (42).

In this work, several numerical results (Figures 6, 8 and 9) are shown and in all of them the obtained capillary network presented some similarities with the vascular network obtained *in vivo* experiments. Schugart *et al.* (43) analysed the endothelial cell density due to different concentrations of oxygen and also chemoattractant concentration. For the normal oxygen concentration the endothelial cell density curve obtained in their study was similar to the one obtained in the simulations of the present work (Figure 10).

In order to develop this model some simplifications had to be made. First, the migration process was modelled using a 2D domain. Although 2D domains are simple domains at a first sight, they allow to test several numerical techniques and strategies. For instances, the 2D domain used in this work is actually an infinite 2D domain, since it uses periodic boundary conditions, which means that the left boundary condition is linked to the right boundary condition and the down boundary condition is linked to the up boundary condition. Therefore, with this approach, the growth of the capillary is more predictable, allowing to verify if the code is being well implemented. Second, the diffusion of VEGF was assumed as constant in all the iteration steps and the natural VEGF decay was not considered nor the uptake of VEGF by endothelial cells. Third, the proposed model neglects the anisotropic and heterogeneous behaviour of ECM. The incorporation of these characteristics would make the proposed model more close to the biological behaviour however would also increase the model parameters. Finally, the proposed computational model used a large number of parameters that were taken from the literature, when possible, or were estimated. The parameters' estimation is an important topic in computational modelling especially for human models for which *in vivo* studies are demanding to carry out.

In this work, a meshless-based model to analyse the endothelial cell migration due to chemotaxis was proposed. The model proposed in this work, in its present stage, is not yet capable to mimic the whole angiogenesis process that occurs *in vivo*. However, the authors believe that the numerical formulation here presented is wide and robust enough to support further and more complex techniques required to fully simulate the angiogenesis phenomenon, such as branching and anastomosis processes. The numerical implementation of these biological

processes will increase the mesh distortion and the use of meshless methods will reveal to be extremely useful. We are aware that not comparing our numerical results with experimental studies is a limitation of this study. Experimental methodologies that analyse angiogenesis *in vivo* contains different biological steps, such as the migration of endothelial cells and muscular cells that support the blood vessel formation, the branching of the growing capillaries that allows the wide spread of vascular network and anastomosis, the fusion of vessels from two distinct branches of the vascular system. Implement all these biological steps in a computation model is a hard and demanding task. Therefore, in this paper we developed an iterative code fitting merely the first step responsible for the formation of the vascular network that is the endothelial cell migration. The comparison with other numerical results is also difficult since, as far we are concern, there are not in the literature numerical studies that use the same kind of approach to study endothelial cell migration during angiogenesis. In the upcoming models, we intended to increase the model complexity and to include the anastomosis process and the vessel wall branching. Moreover, it is planned to integrate different cells populations and a complex capillary network in order to mimic the results described from the *in vitro* and *in vivo* studies. With these improvements in our model we believe that the RPIM formulation could be a valid numerical tool that can be adjusted to biological problems and may also be able to complement the biological and medical subjects.

In the future, it will be necessary to create a relationship between the mathematical models and the *in vitro* experiments, in order to decrease the time and costs of laboratory techniques, as well as justify and understand why some event occurs *in vitro*.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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3.3. Contribution III: Sprouting Angiogenesis: A Numerical Approach with Experimental Validation

3.3.1. Highlights of Contribution III

This contribution focused on simulate the sprouting angiogenesis regulated by the VEGF chemoattractant effect with realistic morphology and structure, comparatively to an *in vivo* angiogenesis assay.

Therefore, the main contents of this publication include:

- I. Brief description of the angiogenesis process;
- II. Advantages of using the Chick Chorioallantoic Membrane (CAM) assay as an *in vivo* methodology to study angiogenesis;
- III. Literature review on wound healing angiogenesis models;
- IV. Succinct description of the numerical method used and discrete formulation;
- V. Implementation of the branching process in the angiogenesis model, using data acquired during the CAM assay;
- VI. Simulation of sprouting angiogenesis modulated by VEGF diffusion gradient, as demonstrated in Figure 3.3;

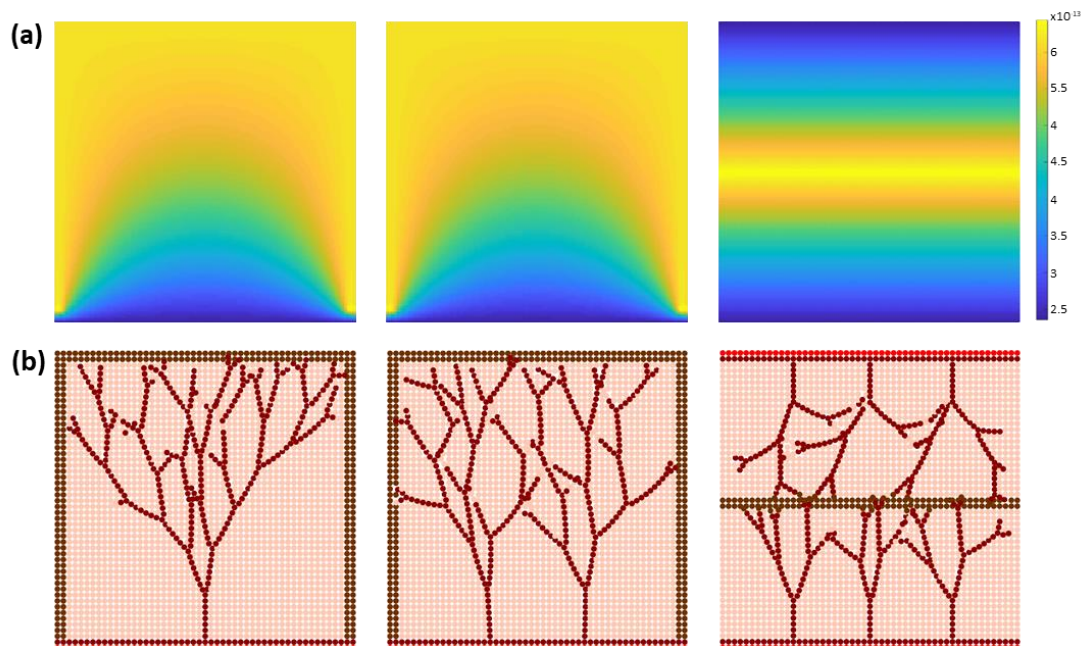


Figure 3.3: Capillary network. **(a)** VEGF diffusion gradient (VEGF concentration in g mm^{-3}); **(b)** Final simulation results for cell migration from individual sprouting tip (left), from two sprouting tips (middle) and from two parent vessels (right), using a regular nodal mesh.

- VII. Numerical validation of the computational model by comparison of several parameters between the simulation and the CAM images, presented in Table 3.1;

Table 3.1: Capillary network parameters analysed with both methodologies under study (*in silico* and *in vivo*).

	<i>In silico</i>	<i>In vivo</i>	p-value
Total branching number	25.50 ± 1.39	23.00 ± 2.01	0.37
Total vessel length (mm)	34.66 ± 0.98	30.27 ± 1.74	0.07
Branching angle (degree)	64.68 ± 3.59	70.27 ± 2.37	0.26

Values are expressed as mean ± SEM. Statistical analysis included t-test (n = 6).

- VIII. Estimation of the difference between the total capillary volume fractions assessed using the simulation and the CAM images (Figure 3.4);

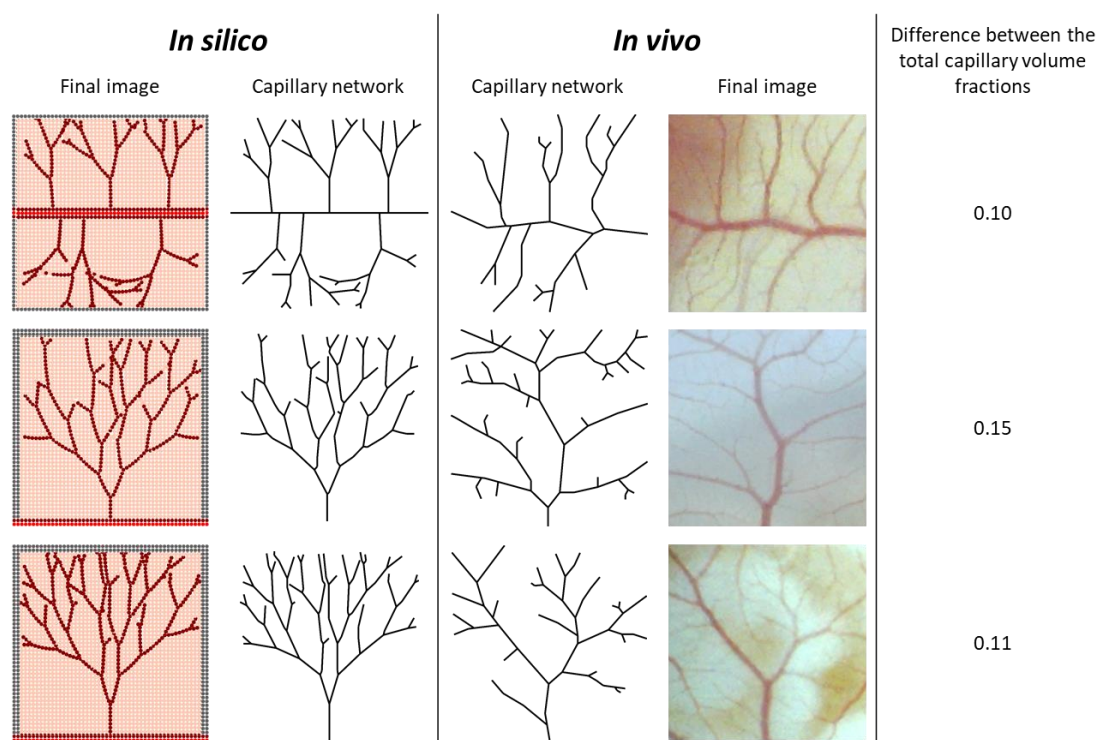


Figure 3.4: Capillary network profile obtained in simulations (1st column) and in CAM images (2nd column) for some of the performed examples and the corresponding difference between the total capillary volume fractions (3rd column). Images sized: 5 x 5 mm².

- IX. Conclusion regarding the realistic capillary network obtained in terms of structure and morphology. This was the first study that directly used data obtained during an *in vivo* angiogenesis assay to simulate the branching process.

The complete document can be found in the next sub-chapter.

3.3.2. Contribution III

Sprouting Angiogenesis: A Numerical Approach with Experimental Validation

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Sprouting Angiogenesis: A Numerical Approach with Experimental Validation

Ana Guerra^a, Jorge Belinha^b, Naside Mangir^{c,d}, Sheila MacNeil^c and
Renato Natal Jorge^{e,f}

^aInstitute of Science and Innovation in Mechanical and Industrial Engineering (INEGI), Porto, Portugal.

^bSchool of Engineering, Polytechnic of Porto (ISEP), Mechanical Engineering Department, Porto, Portugal.

^cKroto Research Institute, University of Sheffield, Sheffield, United Kingdom.

^dRoyal Hallamshire Hospital, Department of Urology, Sheffield, United Kingdom.

^eAssociated Laboratory For Energy, Transports and Aeronautics (LAETA – INEGI), Porto, Portugal.

^fFaculty of Engineering of the University of Porto (FEUP), Mechanical Engineering Department, Porto, Portugal.

ABSTRACT

A functional vascular network is essential to the correct wound healing. In sprouting angiogenesis, vascular endothelial growth factor (VEGF) regulates the formation of new capillaries from pre-existing vessels. This is a very complex process and mathematical formulation permits to study angiogenesis using less time-consuming, reproducible and cheaper methodologies. This study aimed to mimic the chemoattractant effect of VEGF in stimulating sprouting angiogenesis. We developed a numerical model in which endothelial cells migrate according to a diffusion-reaction equation for VEGF. A chick chorioallantoic membrane (CAM) bioassay was used to obtain some important parameters to implement in the model and also to validate the numerical results. We verified that endothelial cells migrate following the highest VEGF concentration. We compared the parameters – total branching number, total vessel length and branching angle – that were obtained in the *in silico* and the *in vivo* methodologies and similar results were achieved (*p-value* smaller than 0.5; *n* = 6). For the difference between the total capillary volume fractions assessed using both methodologies values smaller than 15% were obtained. In this study we simulated, for the first time, the capillary network obtained during the CAM assay with a realistic morphology and structure.

KEYWORDS

Vascular endothelial growth factor; Capillary network; Chick chorioallantoic membrane assay; Radial point interpolation method.

1. INTRODUCTION

The failure of some wounds to heal becomes a social and economic issue. The NHS spends every year between £4.5bn and £5.1bn on the treatment of acute and chronic wounds and the associated comorbidities.¹¹ Moreover, the United States estimates a total annual spending on wound treatment of between \$28.1 to \$96.8 billion.²² The causes of these non-healing wounds are predicted to increase as the population ages and the incidence of comorbidities that affect the wound healing process, such as diabetes, cardiac disease and obesity increase. A poor blood supply to the wound is probably the single most important factor adversely affecting the wound healing process. It is very important to address new strategies to understand wound healing angiogenesis.

Angiogenesis is the process by which new blood vessels grow from pre-existing vessels. This process mainly occurs during the proliferation phase of wound healing, when it is necessary to sustain the oxygen and nutrients supply for cells activity.⁸ Angiogenesis is stimulated by growth factors that are released during the previously inflammatory phase. Although there are several growth factors that play a role in angiogenesis, vascular endothelial growth factor (VEGF) is the most powerful angiogenesis stimulator.¹⁰ Indeed, VEGF is produced by several cell types that regulate wound healing, namely keratinocytes, smooth muscle cells, fibroblasts, endothelial cells and inflammatory cells.² Therefore, the VEGF gradient in the tissue is rapidly established and endothelial cells sense this gradient and start to proliferate and to migrate towards it.¹³ Then sprouting angiogenesis occurs and the new capillaries migrate in the direction of the cells at the tip of each sprout-tip cells. Finally, the newly formed blood vessels undergo stabilization and maturation. This process continues allowing new tip cells formation, capillary elongation and branching to occur. In this way a widespread blood vessel network is formed.⁸

Angiogenesis is a very complex process that often requires *in vivo* assays to study all the steps. Nowadays, one of the most used *in vivo* model for angiogenesis is the chick chorioallantoic membrane (CAM) assay. The CAM is an extraembryonic membrane that permits gas exchange between the embryo and the environment. This membrane is highly vascularized, with a dense capillary network, making the CAM assay a valuable bioassay to study angiogenesis *in vivo*.²⁶ Accordingly the CAM assay has been developed to be a quick, semi-quantitative, relatively economical and reliable methodology for the screening of pro- and antiangiogenic drugs.¹² Indeed, the CAM assay allows the direct visualization of sprouting angiogenesis and the measurement of parameters such as blood vessel number, density, branch point number, diameter and blood flow.²¹

Over the last 35 years, computational modelling of wound healing angiogenesis has aroused the interest of the scientific community. The mathematical formulation of angiogenesis could help researchers study this biological process using less time-consuming, reproducible and cheaper methodologies. Accordingly, some authors developed models to analyse the chemoattractant effect of growth factors in the capillary network morphology. Stokes and Lauffenburger²⁸ developed a simple probabilistic model of angiogenesis, showing that the movement of endothelial cells created by chemical gradients is essential in angiogenesis and affects the vascular network profile. The cellular automata model of Peirce et al.²⁴ reproduced an increase in vascular density similar to the one obtained in rodent model after exogenous VEGF treatment. Additionally, their model predicted the vascular pattern and the vascular remodelling with good agreement with direct experimental observations. Bentley et al.⁵ developed a hierarchical agent-based model and demonstrate that VEGF concentration determined the robustness of the tip cell morphology and they validated their numerical predictions with experimental observations. Merks et al.¹⁹ used a hybrid Cellular Potts (to model the endothelial cell) and partial differential equation (to model the chemoattractant) model to analyse the movement of endothelial cells along chemoattractant gradients. The numerical results showed that cell adhesion and cell morphology are important for formation of stable blood vessels. Moreover, the authors validated their model by comparing the model to time-lapse video microscopy experiments of endothelial cell culture.⁹ The Cellular Potts model of Scianna et al.²⁷ simulated the vessel network formation with a good degree of realism. The authors verified that, upon VEGF stimulation, the endothelial cells turn from a quiescent phenotype into a stalk or a tip one. Sun et al.^{29, 30} developed deterministic approaches that replicate the effect of growth factors distribution in endothelial cell sprouting and capillary network formation. The numerical results showed a capillary network that is representative of the structure and morphology of the blood vessels described *in vitro* assays. Machado et al.¹⁵ used a hybrid partial differential equation-discrete system to model endothelial cell migration due to VEGF gradients in the extracellular matrix. This was the first study that directly compared data obtained with a mathematical model and data obtained during an experimental wound healing assay. The obtained results for wound areas, vessel densities and vessel junction densities were in accordance in both methodologies. Although all these studies have contributed to the development of models capable of mimicking angiogenesis, none of them were obtained as a result of a comparison with *in vivo* methodologies of analysing the capillary network morphology and structure as in the current study. The aim of this study was to derive a predictable model of the effect of VEGF acting as a chemoattractant in stimulating sprouting angiogenesis studied in the CAM bioassay in order to get a better understanding of the biological

rules that govern angiogenesis. To the best of our knowledge this has not previously been studied.

The choosing of the numerical method is important in computational simulation in order to better adapt it to the biological aspects that it is being used to reproduce. Normally, advanced discretization techniques, regardless of being mesh-dependent or meshless, are the most commonly used.³ Regarding mesh dependent methods, the isogeometric analysis has been demonstrated to be capable of efficiently simulating the angiogenesis process.³³ Nevertheless, the application of meshless methods to biological simulation has been discussed as having some advantages^{4, 25} namely it allows one to work with complex geometries, such as soft tissues, to obtain smoother variable fields, to simulate moving discontinuities and large deformation problems and to insert locally more nodes allowing accurate local solutions, with the same computational cost.³ The model proposed in this study inserts new nodes, or changes the position of existing nodes, to simulate endothelial cell migration during angiogenesis, which induces mesh distortion. Therefore, meshless methods are advantageous in order to deal efficiently with this mesh distortion³ and they converge faster than mesh dependent methods such as the finite element method³. Moreover, meshless methods allow the modelling of any phenomenon that is governed by differential equations such as angiogenesis, thus its application in this study is extremely relevant.

In this study, we simulated the sprouting angiogenesis regulated by the VEGF chemoattractant effect and our numerical model simulates for the first time the capillary network obtained during the CAM assay with a realistic morphology and structure.

2. MATERIALS AND METHODS

In this study, we developed a computational model able to simulate sprouting angiogenesis. The model combines the Radial Point Interpolation Method (RPIM) with growth factor diffusion, endothelial cell migration and branching process in order to obtain a realistic capillary network. In this section, we will detail our algorithm implemented by the RPIM software. The general algorithm is outlined in Figure 1.

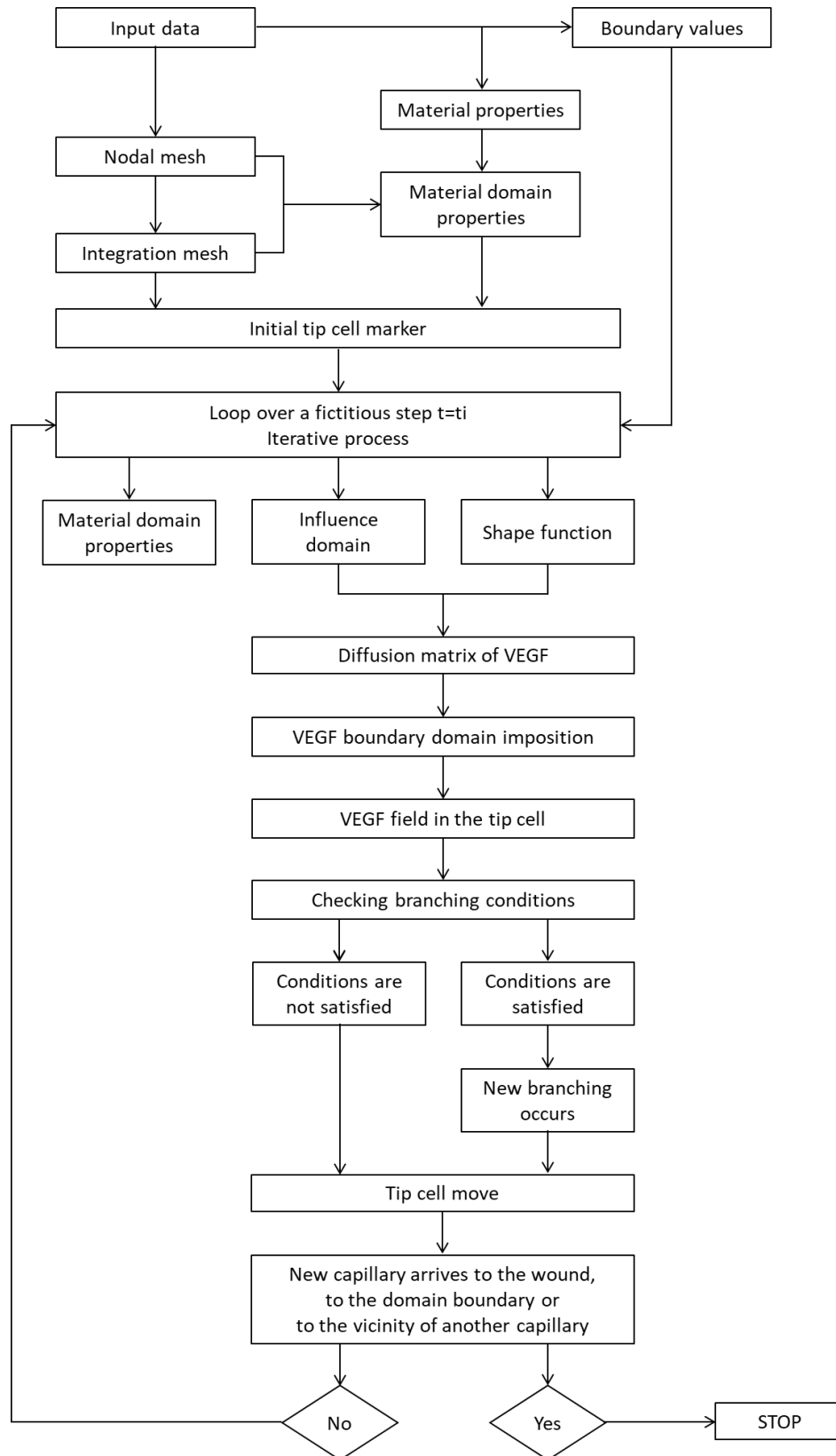


Figure 1: General representation of the algorithm used by the RPIM software tested in this work.

2.1. Numerical method

RPIM is a discrete numerical method, thus the first step is to discretize the problem physical domain using a nodal set fitting the domain volume/area. Then, a background integration lattice is built using quadrilateral regular cells fitting the domain volume/area. Now the numerical integration can be established and in this work we use the Gauss-Legendre quadrature rule. The integration point is defined by its Cartesian coordinates, $x_I \in \mathbb{R}^2$, and by its own integration weight, $\widehat{w}_I$, which corresponds to the volume that integration point occupies in the physical domain. This set of integration points establish the background integration mesh, which is necessary to discretely integrate the continuous equations ruling the physical phenomenon. Then, the nodal connectivity can be defined. In meshless methods the nodal connectivity is not predefined and needs to be obtained using the “influence-domain” concept. The set of nodes closer to each integration point x_I define its influence-domain. These nodes are then used to build the shape function of integration point x_I , and the weak form is applied to build the equations’ system. In RPIM, the Radial Point Interpolation (RPI) technique, combining a polynomial function with a radial function, is used to obtain the interpolation functions.

The RPIM numerical method used in this work was based in the one already described in the literature and extended information about the topics “numerical integration” and “nodal connectivity” can be found in Ref. 3.

2.2. Discrete equation system

In this work, we assumed that the endothelial cell migration is regulated by the chemical diffusion in a homogeneous medium. Accordingly, our formulation should be represented as a discrete equation system in order to obtain the numerical solution of the algorithm implemented in the RPIM software. The chemical diffusion can be numerically simulated as a field problem.¹⁴ The general form of the Helmholtz can be used to define the equation system of a 2D linear steady state field problem:

$$D_x \frac{\partial^2 \phi}{\partial x^2} + D_y \frac{\partial^2 \phi}{\partial y^2} - g\phi + Q = 0 \quad (1)$$

being ϕ the field variable, which in this case corresponds to the VEGF concentration, D_x and D_y represents the VEGF diffusion coefficient along dimension x and y , respectively, g represents the matrix of chemical infusibility (in this work g is neglected, $g = 0$) and Q is the VEGF release rate. The meshless system equations can be formulated using the weighted residual approach:

$$[K_D + K_g]\Phi - f_q = 0 \quad (2)$$

where K_D , after the manipulation of equation (1), can be represented as,

$$K_D = \int_A B_G^T D B_G dA \quad (3)$$

being B_G and D , respectively, defined as follows:

$$B_G = \begin{bmatrix} \frac{\partial \varphi}{\partial x} \\ \frac{\partial \varphi}{\partial y} \end{bmatrix} = \begin{bmatrix} \frac{\partial \varphi_1}{\partial x} & \frac{\partial \varphi_2}{\partial x} & \dots & \frac{\partial \varphi_n}{\partial x} \\ \frac{\partial \varphi_1}{\partial y} & \frac{\partial \varphi_2}{\partial y} & \dots & \frac{\partial \varphi_n}{\partial y} \end{bmatrix} \quad (4)$$

$$D = \begin{bmatrix} D_x & 0 \\ 0 & D_y \end{bmatrix} \quad (5)$$

Additionally, K_g can be defined as,

$$K_g = \int_A g\{\varphi_1 \quad \varphi_2 \quad \dots \quad \varphi_n\}^T \{\varphi_1 \quad \varphi_2 \quad \dots \quad \varphi_n\} dA \quad (6)$$

and f_q , the discrete VEGF release rate vector, is defined as,

$$f_q = \int_A Q\{\varphi_1 \quad \varphi_2 \quad \dots \quad \varphi_n\}^T dA \quad (7)$$

Therefore, solving equation (2) it is possible to obtain the final VEGF concentration in the medium, Φ .

2.3. Model parameters

The biological phenomenon under study was simulated using a $5 \times 5 \text{ mm}^2$ square domain, discretized with 51×51 nodes, forming a regular nodal distribution with 2601 nodes. Then, we defined the domain geometry trying to fit them with the biological situation and we initially identify four distinct zones/sub-domains: the capillary lumen, the endothelial cell monolayer, the extracellular matrix and the wound area that releases VEGF (Figure 2). The location of the wounds, the vessel lumen and the endothelial cell monolayer change in the performed simulations (Figure 2 C). Concerning the essential boundary conditions, we applied a VEGF basal concentration of $5.88 \times 10^{-24} \text{ M}$ in the capillary lumen and a concentration of $1.61 \times 10^{-23} \text{ M}$ in the wound (in the simulation the values applied were, respectively, $2.35 \times 10^{-13} \text{ g mm}^{-3}$ and $6.43 \times 10^{-13} \text{ g mm}^{-3}$) following a periodic boundary condition linking the VEGF concentration of both sides, $\bar{\Phi}(x = 0) = \bar{\Phi}(x = 5)$, being $\bar{\Phi}$ the imposed VEGF concentration at the boundary. In the natural boundary conditions, the VEGF flux from the wound was applied.

Accordingly, with this methodology, it is possible to obtain the VEGF gradient concentration. The chemical diffusion coefficient ($1.16 \times 10^{-6} \text{ mm}^2 \text{ s}^{-1}$) and the VEGF concentration were obtained from available literature.^{12, 32}

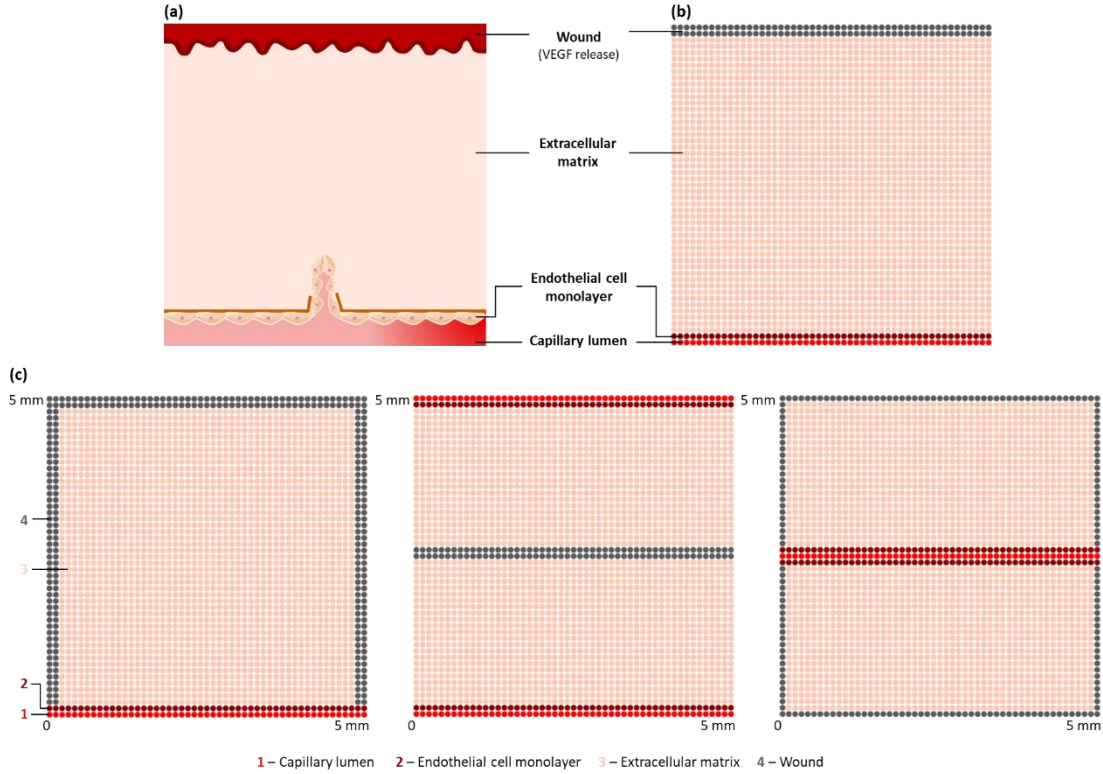


Figure 2: Initial domain geometry. **(a)** Schematic representation of the sprouting angiogenesis; **(b)** Discretization mesh obtained representing the domain geometry; **(c)** Domain geometries used in the simulations performed. The location of the wounds, the vessel lumen and the endothelial cell monolayer change in the simulations. Label 1 (red) represents the capillary lumen, label 2 (dark red) represents the endothelial cell monolayer, label 3 (light pink) represents the extracellular matrix and label 4 (grey) represents the wound.

The background integration mesh is obtained using the square integration cell coming from a regular lattice and the influence domain is built using both the nodal and integration points distributions. Concerning the integration scheme, the square integration cells were obtained from the background lattice. Each edge of the square integration cell is equal to the double of the nodal distance. The integration points were distributed, inside of each integration cell, according to the Gauss-Legendre quadrature scheme. In this work, the 3×3 integration scheme was used. Regarding the number of nodes inside the influence domain, we used 19 nodes inside of the integration cells. The integration scheme and the number of nodes inside the influence domain were optimized in our previous work. In this work the developed algorithm generates the influence domain of each integration point and another routine builds the shape functions.

2.4. Endothelial cell migration

In our *in silico* model the endothelial cell migration is governed by the growth factor gradient. To start the sprouting angiogenesis from the capillary lumen, the tip cells in the sub-domain defined as endothelial cell monolayer are marked using Cartesian coordinates. The location of that tip cell was chosen according to the CAM images in order to reduce the number of unknown variable in the initial conditions, such as the starting sprout location. After the initial tip cells marking, the algorithm begins an iterative loop and in each time step the tip cells are marked one after the other. In our model, each time step represents the time that the capillary takes to migrate one single endothelial cell. The direction of the tip cell movement is defined by the vector $\mathbf{u}$, that follows the gradient vector from the VEGF concentration field towards the VEGF source. Accordingly, the gradient of the VEGF concentration field is obtained ($\mathbf{n} = \nabla\Phi$), being $\mathbf{n}$ oriented towards the VEGF source (in opposition to its support cell). Therefore, $\mathbf{n}_{mod} = \begin{bmatrix} \cos\theta & \sin\theta \\ -\sin\theta & \cos\theta \end{bmatrix} \mathbf{n}$. Then, the unit vector of $\mathbf{n}_{mod}$ is calculated: $\mathbf{u} = \mathbf{n}_{mod}/\|\mathbf{n}_{mod}\|$. Then, using the direction vector $\mathbf{u}$, the average distance between cells is considered and the new tip cell position is defined by summing the old tip cell position with the vector $\mathbf{u}$ multiplied by the average distance between cells. If a node exists in the new tip cell position, that node will become a tip cell. If in the new position there are no nodes, a new node is included in the model, and that new node becomes a tip cell. The vector $\mathbf{u}$ variation angle (θ) is calculated in each iteration, $\theta = \arctg(\mathbf{u}_x/\mathbf{u}_y)$. In each step, the VEGF concentration is calculated and the new position of the tip cell is actualized, moreover, the VEGF concentration at the boundary domain is imposed. The iterative process stops when the capillary arrives at the wound, to the domain boundary or if the tip cell is near another endothelial cell.

2.5. Branching process

In order to implement the branching process in our model we used data obtained during the CAM assay. The CAM is high vascularized, with a dense capillary network, and because it is located on the top of the embryo it is easily accessible to be analysed. Accordingly, the CAM assay is a very convenient methodology to study angiogenesis *in vivo* and it is commonly used to analyse the angiogenic effect of different compounds and to study the morphology of the capillary network.¹² Accordingly, pathogen-free fertilized white leghorn chicken eggs (*Gallus gallus domesticus*) were obtained from Henry Stewart Co. Ltd (UK) and they were incubated. Care was consistent with the guidelines of the Home Office, UK. Chick embryos were cultured

as described previously.¹⁶ The egg shells were cracked and embryos were transferred into a square Petri dish on embryonic development day (EDD) 3. The ex ovo cultures were maintained in a humidified incubator at 38°C between EDDs 3 to 14. The survival of the embryos were checked daily and recorded. The detailed description of the *ex-vivo* CAM assay is presented in Ref. 17. Therefore, in order to extract valuable biological parameters to implement in our mathematical model we used images taken with a digital microscope during the CAM assay (Figure 3 A). In all the images used, the chick was between embryo development day (EDD) 10 to 13.

To implement in our model the branching process we analysed the capillary network in CAM accordingly to the capillary order concept (Figure 3 B). The capillary order was defined according to the capillary calibre, the 1st order represents the capillaries with higher calibre and the 3rd order the ones with lowest. To set the branching locations we measured the distance between consecutive branch points, for each capillary order, and we obtain:

$$d = 0.9286e^{-0.219 \times O_{cap}} \quad (8)$$

being d the distance between consecutive branch points and O_{cap} is the capillary order. Accordingly, a new branch occurs if the distance to the previous branch is greater than d , for the capillary order considered. To define the branching angle we measured several angles between capillaries with the same and different order. The obtained average branching angle for capillaries with same order was 68° (n = 36, maximum = 127°, minimum = 45°) and for capillaries with different order was 86° (n = 36, maximum = 117°, minimum = 44°). When we inputted these data into the model, some fluctuation in the branching angle was allowed. Accordingly, we used the CAM images to extract the function parameters and to create a phenomenological law (function). In this way, our model uses the developed phenomenological function to rule the branching location. Then, the tip cells migrate following the highest VEGF concentration.

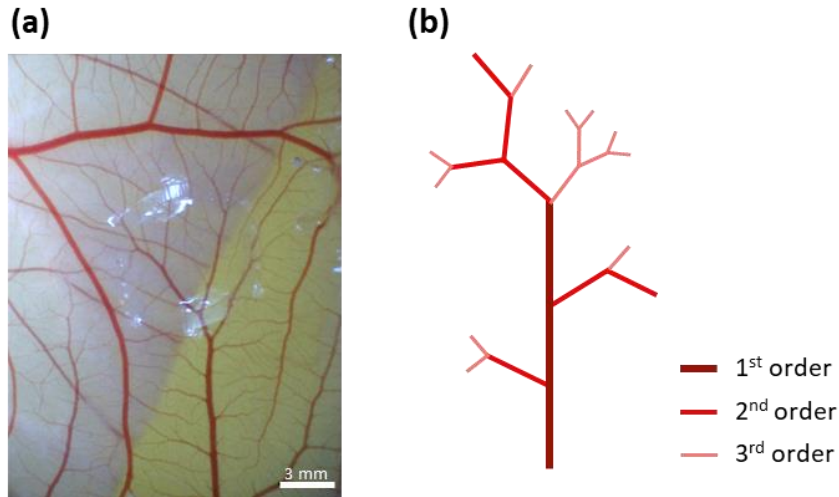


Figure 3: Capillary network profile observed in the chick chorioallantoic membrane (CAM). **(a)** Representative CAM image, at embryonic development day 10; **(b)** Capillary network scheme showing the capillary order concept used in the simulations. (Scale bar represents 3 mm).

2.6. Numerical model validation

In order to validate our numerical model, we compared some parameters between simulations and images obtained from the CAM experiments. Accordingly, we selected a region of interest in the CAM image of $5 \times 5 \text{ mm}^2$, equal to the model dimension. Then we performed simulations with identical domain geometries in which we looked at the initial number and location of sprouting tip cells and the location of the parent vessel. The parameters compared between the two groups, *in silico* and *in vivo*, were the total branching number, the total vessel length and the average branching angle. Differences between the groups were evaluated by T-Test for equal variances. The statistical analysis was performed using the data analysis of *Excel* for T-Test: Two-Sample Assuming Equal Variances and a statistical difference was considered significant, at a confidence interval of 95%, when $p < 0.05$. We also calculated the capillary volume fraction in order to compare the numerical and the *in vivo* results. For that, we divided the capillary network obtained in a grid of 10×10 (Figure 4) and we quantified the capillary volume fraction in each small square. Then, we compared the difference between the total capillary volume fraction obtained in the CAM image and in the simulation.

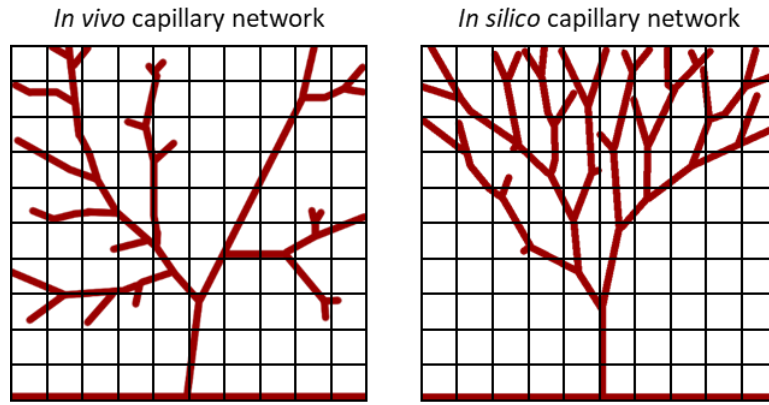


Figure 4: Schematic representation of the 10x10 grid used to calculate and to compare the capillary volume fraction obtained between the numerical and the *in vivo* results.

3. RESULTS

3.1. Capillary network formation

First, we simulated the sprouting angiogenesis from one parent vessel using a regular nodal mesh (Figure 5). Figure 5 A represents the VEGF diffusion gradient, that goes from dark blue to yellow. The dark blue zone is located near the parent vessel and has a VEGF concentration of around $2.5 \times 10^{-13} \text{ g mm}^{-3}$. The yellow zone is located near the wound and has a VEGF concentration around $6.0 \times 10^{-13} \text{ g mm}^{-3}$. Figure 5 B represents the endothelial cell migration pattern in sequential time steps. Analysing the VEGF gradient and the endothelial cell migration profile it is possible to verify that endothelial cells migrate from the parent vessel to the wound, following the highest VEGF concentration. Then we simulated the endothelial cell migration starting with two sprouting tips and also the cell migration from two parent vessels (Figure 6). Once again, the cells migrate from the parent vessel to the wound, following the VEGF gradient (Figure 6 A). Moreover, it is possible to verify that the endothelial cell number increases near the wound, where the VEGF concentration is the highest. In the bottom row of Figure 6 is possible to verify that the capillary network is not symmetric on both sides of the blood vessel. This occurs because, in our model, the direction of the branching to right or left is random and some fluctuation in the branching angle is allowed. In all the numerical results we observed that the capillary network and the branching profile follows the expected configuration.

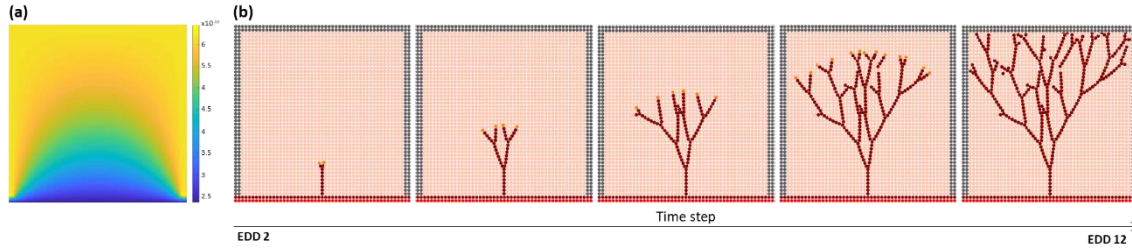


Figure 5: Capillary network. **(a)** VEGF diffusion gradient (VEGF concentration in g mm^{-3}); **(b)** Simulation results from tip cell migration in different time steps, using a regular nodal discretization mesh. The tip cell was located at position: $x = 2.5\text{mm}$.

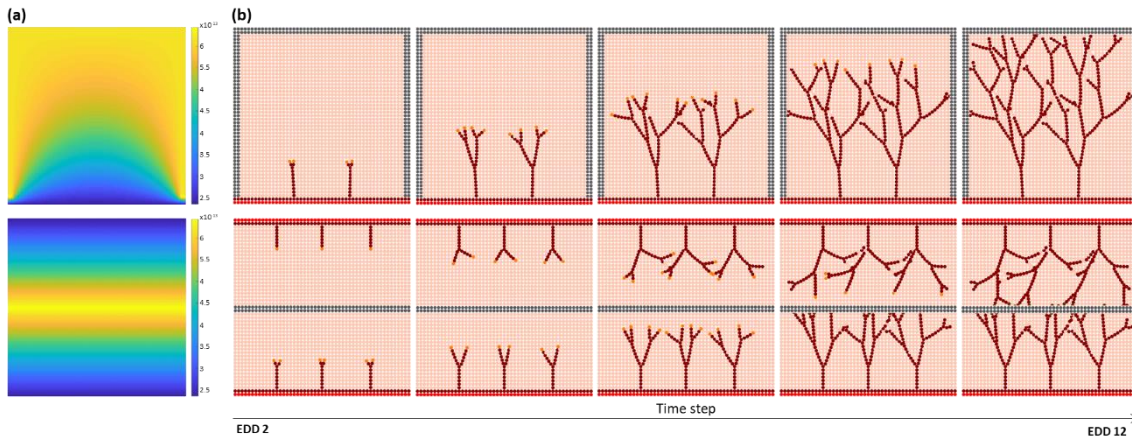
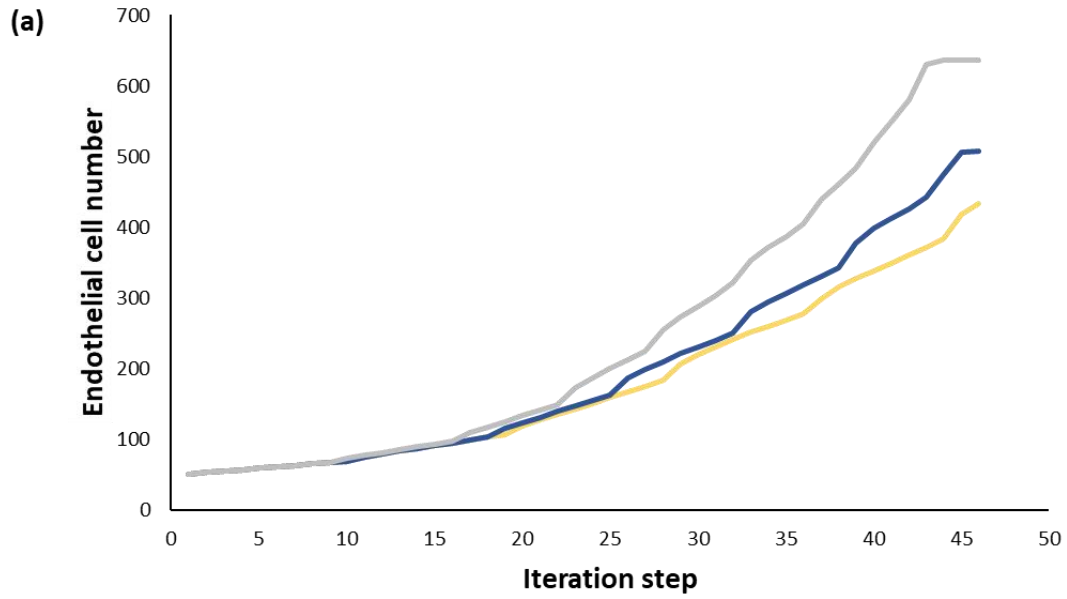


Figure 6: Capillary network. **(a)** VEGF diffusion gradient (VEGF concentration in g mm^{-3}); **(b)** Simulation results for cell migration from two sprouting tips (top) and from two parent vessels (bottom) in different time steps, using a regular nodal discretization mesh. The tip cells were located at positions: $x = 1.7\text{mm}$ and $x = 3.3\text{mm}$ (top) and $x = 1.2\text{mm}$, $x = 2.5\text{mm}$ and $x = 3.9\text{mm}$ (bottom).

3.2. Endothelial cell density

Experimental data have already shown that VEGF promotes endothelial cell sprouting which increases blood vessel density.³⁵ Accordingly, we performed simulations by increasing the VEGF concentration in the wound. In this study, we tested the following VEGF concentrations: $4.70 \times 10^{-13} \text{ g mm}^{-3}$, $9.40 \times 10^{-13} \text{ g mm}^{-3}$ and $1.41 \times 10^{-12} \text{ g mm}^{-3}$. Then, we quantified the endothelial cell number during each simulation (Figure 7). Analysing the graph (Figure 7 A) and the final simulation results (Figure 7 B), it is possible to observe that the endothelial cell density increases with the increase in the VEGF concentration in the wound.



VEGF concentration in the wound:

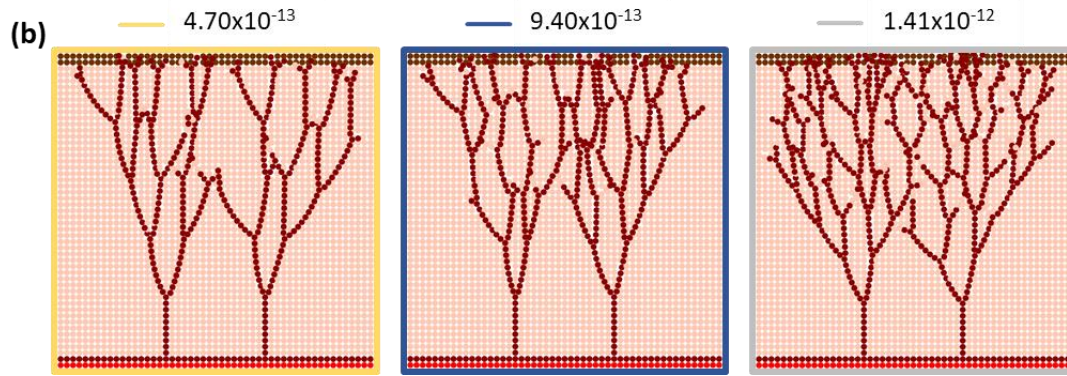


Figure 7: Endothelial cell density. **(a)** Endothelial cell quantification for each simulation with the different VEGF concentration tested (VEGF concentration: $4.70 \times 10^{-13} \text{ g mm}^{-3}$, $9.40 \times 10^{-13} \text{ g mm}^{-3}$ and $1.41 \times 10^{-12} \text{ g mm}^{-3}$); **(b)** Final simulation results for each VEGF concentration tested, using a regular nodal discretization mesh.

3.3. Comparison between numerical results and in vivo CAM assays

To validate our numerical model we used data obtained during the CAM assay. The MacNeil group have been using this assay extensively to study angiogenesis.¹⁷ Accordingly, we performed six different examples in order to compare the vascular network obtained in simulations and in CAM images. Then, we extracted the capillary network profile obtained in both methodologies (Figure 8). We used this capillary network profile to evaluate several parameters, namely the total branching number, the total vessel length and the branching angle. The results obtained for both methodologies (*in silico* and *in vivo*) are presented in Table 1. Analysing the obtained capillary networks and the parameters measurements, it was possible

to verify that the capillary network obtained in simulation was similar to the one obtained *in vivo*. With respect to the total branching number, the total vessel length and the branching angle the results obtained were similar for both methodologies ($p = 0.37$, $p = 0.07$ and $p = 0.26$, respectively). Estimating the difference between the total capillary volume fractions assessed using the simulation and the CAM assay we obtained values between 11% and 15% for the six examples as presented in the third column of Figure 8.

Table 1: Capillary network parameters analysed with both methodologies under study (*in silico* and *in vivo*).

	<i>In silico</i>	<i>In vivo</i>	<i>p-value</i>
Total branching number	25.50 $\pm$ 1.39	23.00 $\pm$ 2.01	0.37
Total vessel length (mm)	34.66 $\pm$ 0.98	30.27 $\pm$ 1.74	0.07
Branching angle (degree)	64.68 $\pm$ 3.59	70.27 $\pm$ 2.37	0.26

Values are expressed as mean $\pm$ SEM. Statistical analysis included t-test (n = 6).

4. DISCUSSION

Angiogenesis is an important cellular and biochemical process which is essential for wound healing. Sprouting angiogenesis is regulated by growth factors, with VEGF being the most important one.¹⁰ Given that VEGF is produced by different cell types that participate in wound healing², in this work, we simulated the release of VEGF by a wound. *In silico* models are useful tools that help scientists to understand cell behaviour. Some authors have already developed models to address angiogenesis during wound healing. However, some of them used continuum approaches and do not take into consideration the capillary network. In other models the implemented parameters are estimated with little approximation to reality or the numerical results are not validated with experimental data. In order to overcome some of these issues, in this study, we developed a new meshless model able to simulate the sprouting angiogenesis modulated by the chemotactic response to VEGF and we validated our numerical results with an *in vivo* angiogenesis assay.

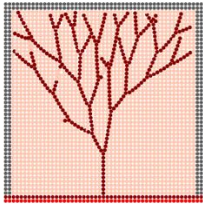
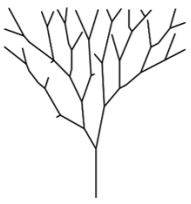
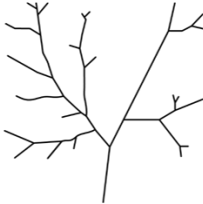
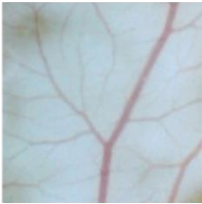
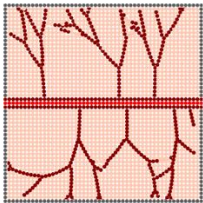
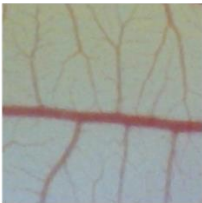
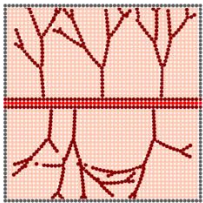
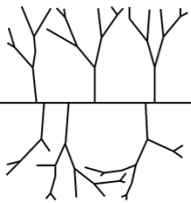
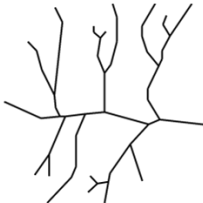
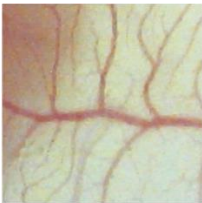
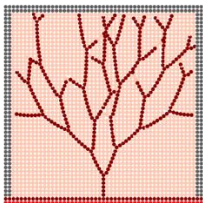
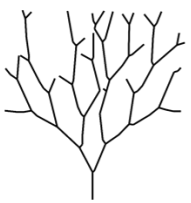
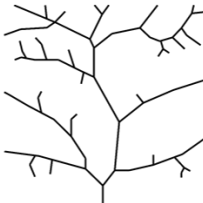
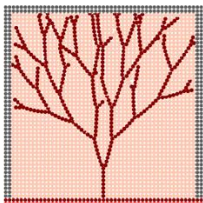
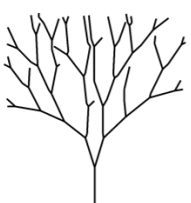
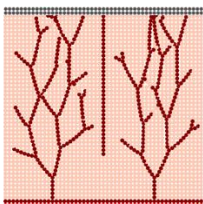
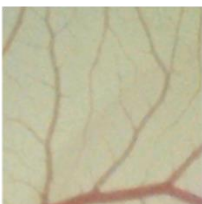
<i>In silico</i>		<i>In vivo</i>		Difference between the total capillary volume fractions
Final image	Capillary network	Capillary network	Final image	
Example 1 				0.14
Example 2 				0.15
Example 3 				0.10
Example 4 				0.15
Example 5 				0.11
Example 6 				0.11

Figure 8: Capillary network profile obtained in simulations (1st column) and in CAM images (2nd column) for the six performed examples and the corresponding difference between the total capillary volume fractions (3rd column). Images sized: $5 \times 5 \text{ mm}^2$.

In our model, endothelial cells migrate in response to the VEGF concentration gradient for all the different geometries tested (Figure 5 and Figure 6). These numerical results are in agreement with experimental studies. It has already been demonstrated that endothelial cells cultured in cell culture dishes migrate^{34, 36} and this migration is done in response to the VEGF concentration^{7, 23}. Furthermore, Del Amo et al., using microfluidic devices, showed that VEGF is

the main regulator of angiogenesis. The authors also concluded that the generation of a chemoattractant gradient of VEGF in the direction of sprout growth, promoted sprouting and cell reorganization.¹ In our study, we additionally tested the effect of VEGF concentrations in endothelial cell density. Accordingly, we performed simulations with the wound VEGF concentration of $4.70 \times 10^{-13} \text{ g mm}^{-3}$, $9.40 \times 10^{-13} \text{ g mm}^{-3}$ and $1.41 \times 10^{-12} \text{ g mm}^{-3}$ and we evaluated the endothelial cell number during the simulation (Figure 7). We verified that the endothelial cell density increased with the increasing VEGF concentration in the wound. This numerical result is in agreement with experimental data that shows that VEGF promotes the endothelial cell sprouts *in vitro*.⁶ We are aware that simulating angiogenesis using a 2D domain is a limitation of our study. Although 2D domains are simple domains at first sight, they allow one to test several numerical techniques and strategies and to analyse different biological behaviours. For this reason, several authors have analysed the effect of growth factors on the morphology of the capillary network using two-dimensional frameworks.^{18, 27} Also the 2D domain used in this work is actually an infinite 2D domain, since it uses periodic boundary conditions, which means that the left boundary condition is linked to the right boundary condition and the down boundary condition is linked to the up boundary condition. With this approach, the growth of the capillary is more predictable, allowing one to verify if the code is being well implemented. The branching is an essential process during angiogenesis since it ensures the capillary spread in the area that needs new vascularization. To implement the branching process in our model we used an *in vivo* angiogenesis assay. The CAM assay is a well-established method for the continuous *in vivo* visualization of the newly forming vessels, being a rapid, simple and cheaper methodology to study angiogenesis.³¹ Therefore, several CAM images were analysed in order to obtain important parameters to implement in the model namely, the branching angle and the distance between branches (Equation 8). With this data, we implemented the branching conditions in our computational code. With this methodology we could simulate the capillary network pattern presented in CAM images. However, if other biological situations are to be considered we should analyse the experimental data and establish a new equation similar to equation 8 and capable to define the branching location. At this moment it is not possible to make generalizations and this need can be understood as a limitation of the present algorithm.

Some authors have already developed models able to simulate the angiogenesis regulated by growth factor and to analyse the obtained global capillary network.^{29, 30} Nevertheless, they did not compare their numerical results with experimental data. To the best of our knowledge, this was the first study that compared the numerical results with data obtained during an *in vivo* angiogenesis assay. To validate the numerical model, we conducted six independent examples with different model geometries and we compared the capillary

network obtained with CAM images. The capillary network obtained in our numerical simulation had a very realistic structure and morphology (Figure 8). Moreover, for the parameters compared between the *in silico* and *in vivo* methodologies similar results were achieved – total branching number ($p = 0.37$), total vessel length ($p = 0.07$) and branching angle ($p = 0.26$) (Table 1). With respect to the difference between the total capillary volume fractions, we obtained values from 11% to 15% (Figure 8, 3rd column). We consider this is an acceptable result that supports the robustness of our numerical model. When looking to the capillary network obtained in the CAM and to that predicted from the *in silico*, in the simulations these are similar to the ones presented *in vivo* experiments but the network patterns do not totally overlap. However, it is necessary to consider that biological processes are very complex with several biological variables, different cell types and intricate regulatory mechanisms.

Combined numerical and experimental approaches are essential since *in silico* models require feedback from experimental assays in order to validate the computational model and to determine how close the model is to reality. To develop the current model several simplifications had to be done corresponding to a set of limitations of the computational model. The diffusion of VEGF was assumed as constant in all the iteration steps and the natural VEGF decay was not considered nor the uptake of VEGF by endothelial cells. However, the values used for the VEGF concentrations and for the diffusion coefficient were obtained from studies in man. The proposed model does not encompass the full complexity of the forces which influence angiogenesis *in vivo* as we have not begun to address the influence of the architecture and the anisotropic and heterogeneous behaviour of ECM on angiogenesis or the influence of mechanical stimuli on this process. Both will be challenges for the future now that we have this simple model established. The incorporation of these characteristics would make the proposed model closer to the biological behaviour however these would also increase the model parameters. In order to develop a model able to simulate the *in vivo* angiogenesis, we compared the numerical results we obtained with the ones obtained from the CAM assay. It has already been shown that the chicken genome shares a 75% similarity to that of the human genome, with similar sequences regulating basic cell structure and function.²⁰ Moreover, the clinical relevance of the CAM model for studying angiogenesis has been proven repeatedly. It tends to be used initially to study pro and anti-angiogenic agents prior to confirming the results with these in live rat or rabbit experiments.

In conclusion the meshless-based model presented in this study accurately simulates the sprouting angiogenesis due to chemical diffusion of VEGF during wound healing. The capillary network obtained was realistic in structure and morphology. As far as we are aware this is the first study that directly used data obtained during an *in vivo* angiogenesis assay in

order to simulate the branching process. Furthermore, the same experimental *in vivo* assay was used to validate the obtained numerical results and the accuracy of the developed computational model. The combination of *in vivo* protocols and mathematical modelling presented in this study showed the benefits of this collaboration. The data obtained with *in vivo* methodologies could be used to inform modelling and to test the veracity of the *in silico* predictions. This kind of approach will be extremely important in the context of wound healing, in order to optimize protocols and cell culture conditions and to decrease the time and cost of experiments.

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CONFLICT OF INTEREST

Author Ana Guerra, from Institute of Science and Innovation in Mechanical and Industrial Engineering, declare that she has no conflict of interest. Author Jorge Belinha, from School of Engineering, Polytechnic of Porto, declare that he has no conflict of interest. Author Naside Mangir, from Kroto Research Institute and Royal Hallamshire Hospital, declare that she has no conflict of interest. Author Sheila MacNeil, from Kroto Research Institute, declare that she has no conflict of interest. Author Renato Natal Jorge, from Faculty of Engineering of the University of Porto, declare that he has no conflict of interest.

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3.4. Contribution IV: Simulation of the Process of Angiogenesis: Quantification and Assessment of Vascular Patterning in the Chicken Chorioallantoic Membrane

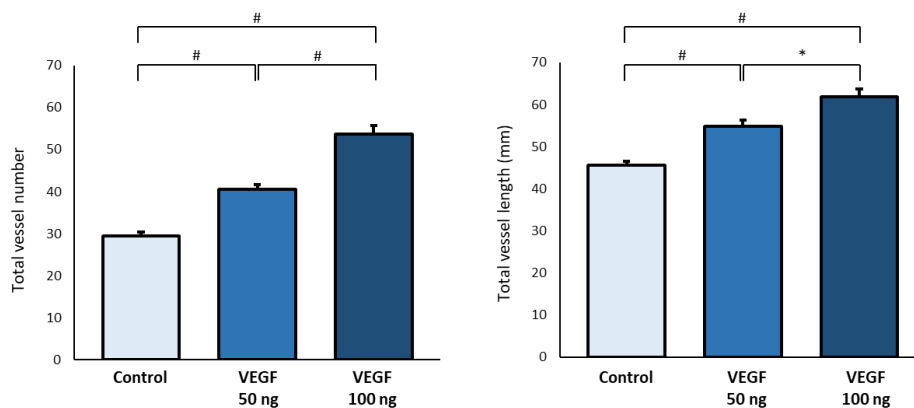
3.4.1. Highlights of Contribution IV

This contribution focused on mimic the angiogenesis response for several VEGF concentrations and on evaluating this response.

Therefore, the main contents of this publication include:

- I. Brief description of the angiogenesis process;
- II. Succinct explanation about the advantages of using the CAM assay to access angiogenesis *in vivo*;
- III. Brief literature review on wound healing angiogenesis models;
- IV. Succinct description of the numerical method used and discrete formulation;
- V. Prediction of angiogenesis response by the computational model for different VEGF concentrations – control, VEGF 50 ng, VEGF 75 ng, VEGF 100 ng and VEGF 200 ng:
 - a. The sprout tips migrate from the parent vessel to the biomaterial, following the VEGF diffusion gradient;
 - b. The total vessel number and the total vessel length were higher in the VEGF treatments, and this increase was proportional to the increasing VEGF concentration;
- VI. Quantitative comparison between the angiogenesis response predicted by the model and the one observed *in vivo* (Figure 3.5). The obtained quantitative results were very similar in the two methodologies used;

In vivo



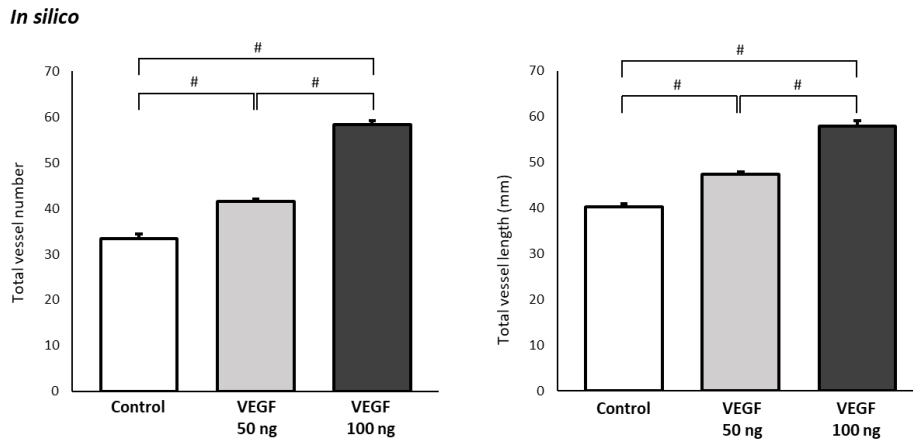


Figure 3.5: *In vivo* and *in silico* quantitative analysis of angiogenesis. Quantification of total vessel number and total vessel length for each treatment from CAM processed images (*in vivo*) and from simulation results (*in silico*) ($n_{\text{Control}} = n_{\text{VEGF 50 ng}} = n_{\text{VEGF 100 ng}} = 6$, One-way ANOVA, pairwise comparison Tukey's post-hoc test, *: $p < 0.05$, #: $p < 0.01$).

VII. Qualitative comparison between the angiogenesis response predicted by the model and *in vivo* (Figure 3.6), using the difference between the total capillary volume fractions. The agreement between the two methodologies was better for control and for the lowest VEGF concentration tested;

	<i>In vivo</i>		<i>In silico</i>		Difference between the total capillary volume fractions
	Final image	Capillary network	Capillary network	Final image	
Control					0.24
VEGF 50ng					0.22
VEGF 100ng					0.49

Figure 3.6: Capillary network profile obtained in CAM images (1st column) and in simulations (2nd column) for control, VEGF 50ng and VEGF 100ng and the corresponding difference between the total capillary volume fractions (3rd column). In all the images the biomaterial is located in the lower left corner. Images sized: $5 \times 5 \text{ mm}^2$.

- VIII. Conclusion regarding the capacity of the model to simulate the sprouting angiogenesis modulated by several VEGF concentrations. For all the tested concentrations, the model simulates the angiogenesis response regarding the blood vessel length, number and morphology with acceptable accuracy.

The complete document can be found in the next sub-chapter.

3.4.2. Contribution IV

Simulation of the process of angiogenesis: Quantification and assessment of vascular patterning in the chicken chorioallantoic membrane

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Simulation of the process of angiogenesis: Quantification and assessment of vascular patterning in the chicken chorioallantoic membrane

Ana Guerra^a, Jorge Belinha^b, Naside Mangir^{c,d}, Sheila MacNeil^c and Renato Natal Jorge^{e,f}

^aInstitute of Science and Innovation in Mechanical and Industrial Engineering (INEGI), Porto, Portugal.

^bSchool of Engineering, Polytechnic of Porto (ISEP), Mechanical Engineering, Porto, Portugal.

^cKroto Research Institute, University of Sheffield, Sheffield, United Kingdom.

^dHacettepe University School of Medicine, Department of Urology, Ankara, Turkey.

^eAssociated Laboratory For Energy, Transports and Aeronautics (LAETA – INEGI), Porto, Portugal.

^fFaculty of Engineering of the University of Porto (FEUP), Mechanical Engineering Department, Porto, Portugal.

ABSTRACT

Angiogenesis, the formation of new blood vessels from pre-existing ones, begins during embryonic development and continues throughout life. Sprouting angiogenesis is a well-defined process, being mainly influenced by vascular endothelial growth factor (VEGF). In this study, we propose a meshless-based model capable of mimicking the angiogenesis response to several VEGF concentrations. In this model, endothelial cells migrate according to a diffusion-reaction equation, following the VEGF gradient concentration. The chick chorioallantoic membrane (CAM) assay was used to model the branching process and to validate the obtained numerical results. To analyse the angiogenic response, the total vessel number and the total vessel length presented in the CAM images and in the simulations for all the VEGF concentrations tested were quantified. In both the CAM assay and simulation, the treatments with VEGF increased the total vessel number and the total vessel length. The obtained quantitative results were very similar between the two methodologies used. The proposed model accurately simulates the capillary network pattern concerning its structure and morphology, for the lowest VEGF concentration tested. For the highest VEGF concentration, the capillary network predicted by the model was less accurate when compared to the one presented in the CAM assay but this may be explained by changes in blood vessel width at higher VEGF concentrations. This remains to be tested.

KEYWORDS: Vascular endothelial growth factor; Angiogenesis evaluation; Capillary network analysis; Chick chorioallantoic membrane assay; Meshless methods.

1. INTRODUCTION

Blood vessels are required in all tissues in order to provide the diffusion of oxygen, nutrients and metabolites. Indeed each cell in the body cannot be more than a few hundred micrometers from a blood capillary in order to survive. *In vivo*, the formation of new blood vessels from pre-existent ones occurs due to angiogenesis. This process begins with embryonic development and continues throughout life [1]. Angiogenesis is also particularly important during wound healing since a poor blood supply will compromise this process. It is therefore very important to achieve a good understanding of angiogenesis to improve wound healing.

Angiogenesis involves multiple highly coordinated phases. Briefly, this process starts with the secretion of local growth factors from cells which form sprouts. Then, a subset of endothelial cells become migratory and breakdown the adjacent extracellular matrix. This allows endothelial cell migration and proliferation and the recruitment of other cells such as smooth muscle cell and pericytes that stabilize the newly formed vessel [2, 3]. This sprouting angiogenesis is due to chemotaxis and permits a widespread vascular network growth. Finally, these immature and leaky blood vessels are remodelled, allowing the formation of a mature vascular network. Because of its complexity, angiogenesis is regulated by several growth factors; of these, vascular endothelial growth factor (VEGF) is the most effective angiogenesis stimulator [4]. It regulates several aspects of angiogenesis – it promotes the permeability of blood vessels and vasodilation and stabilizes new blood vessel growth during wound healing. Accordingly many pro-angiogenic therapies designed to promote wound healing are based on the delivery of this growth factor from biomaterials [5].

Usually, to fully study angiogenesis it is necessary to use animal based assays as *in vitro* assays only reflect part of the complex tissue remodelling which occurs in this process. The chick chorioallantoic membrane (CAM) assay which shows the developing vascular net which supports the chick in the egg is one of the most commonly used *in vivo* assays for angiogenesis. The CAM assay allows the assessment of vascular patterning and the quantification of angiogenesis (by analysing the number of vessels and their hierarchy), of branching points/area, of capillary density and of degree of branching [6, 7]. This assay is widely used to screen pro- and antiangiogenic drugs [8]. As the CAM assay allows the direct visualization and quantification of sprouting angiogenesis *in vivo* without needing to sacrifice animals it has becoming a favourite assay for vascular biology studies and is also used in bioengineering, cosmetics testing, transplant biology, drug and vaccines development and cancer research [9, 10]. Another advantage of the CAM assay in studying angiogenesis is that the immune system of the developing chick embryo starts maturing only after 11th day of embryonic life, which allows one

to study angiogenesis that is not affected by factors secreted during any immune reaction [11]. It uses fertilized chicken eggs which are only considered living animals beyond 2 weeks of fertilization. In recent years, concerns about using animals in science has been increasing. The major aim of the "Three Rs" initiative is to find alternatives to animal testing in order to reduce the number of animals used in research [12].

Another approach to reduce the use of animals is to use *in silico* or computer simulation and mathematical modelling as alternative methodologies to study angiogenesis [13]. Mathematical formulation of angiogenesis permits one to reduce the number of animals used in experiments and to use less time-consuming, more reproducible and cheaper methodologies to study this process.

Since the first model of angiogenesis in 1985 [14], several mathematical models of wound healing angiogenesis have been developed. These models address different features of this process, use different model approaches and analyse angiogenesis on different scales. Some authors used continuum models [15-20] in which the behaviours of the cell population and chemical concentration are described, at the tissue scale, using a system of partial differential equations. In continuum models, the details of the model constituents, such as cells, are ignored and the new capillary growth is often modelled as changes in vascular density [15, 17]. On the other hand, cell-based models [21-24] allow one to determine the effect of growth factors and oxygen on the morphology of the capillary network. These models allow the study of individual endothelial cell behaviour, such as proliferation and migration [21], endothelial cell morphology [22, 23] and blood vessel length [21, 24]. However, in cell-based models the information obtained at tissue scale is limited and they are computationally more expensive. Accordingly, some authors have developed hybrid models to address these drawbacks. Hybrid models [25-28] permit one to combine the interaction between distinct spatiotemporal scales being able to describe the behaviour of vessel networks modulated by growth factors. With these models, it is possible to predict individual endothelial cell behaviour, such as proliferation, differentiation and migration [27], to capture specific phases of angiogenesis, such as branching and anastomosis [25, 26, 28] and to analyse the morphology and structure of the capillary networks [25, 26]. Due to their complexity, hybrid models are less explored than other models.

Despite the contributions of the previous models to describing angiogenesis, none of them were obtained by comparison with *in vivo* studies of angiogenesis. Accordingly, the present research is focused on developing a predictable model of the effect of VEGF as a chemoattractant in simulating sprouting angiogenesis studied using the CAM bioassay.

In recent work [29], we developed a meshless-based model capable of accurately simulating sprouting angiogenesis due to the chemoattractant effect of VEGF on the capillary

network structure and morphology. In the current study, this model is extended and the angiogenic response to several VEGF concentrations – 50, 75, 100 and 200 ng – is simulated. As in the previous study, the model we now propose uses data acquired during running the CAM assay to predict the branching process and to validate the numerical results. The predictive capacity of our model to simulate angiogenesis is presented as well as a quantitative and a qualitative evaluation of angiogenesis, compared with the CAM assay. In summary, we present a computational algorithm model which shows for the first time how *in vivo* angiogenesis modulated by different VEGF concentrations can be simulated.

2. MATERIALS AND METHODS

In this study, a computational algorithm able to simulate the angiogenic response to several VEGF concentrations is developed, and the numerical results are compared with experimental data. The proposed model combines the Radial Point Interpolation Method (RPIM) with a VEGF diffusion formulation, endothelial cell migration and capillaries branching in order to mimic sprouting angiogenesis modulated by chemotaxis. The proposed algorithm developed and implemented to analyse angiogenesis is detailed in Figure 1.

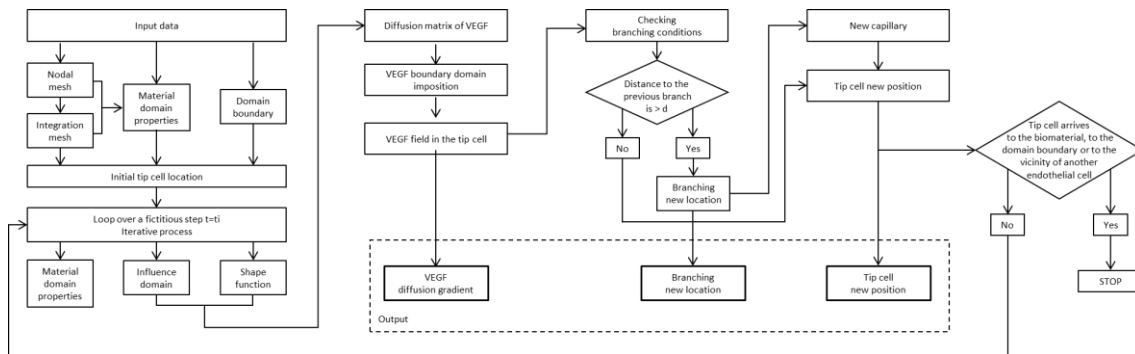


Figure 1: General representation of the algorithm describing the angiogenesis process. The algorithm describes the interrelationships between VEGF gradient, branching occurrence and endothelial cell migration. The arrows indicate the specific interaction between processes, the boxes indicate the specific sub-process calculations and the diamonds indicate the decisions steps, which determine the direction of processes.

2.1. Numerical method

The numerical method used in this work was the RPIM. This method was successfully used in previous studies to model endothelial cell migration due to growth factor diffusion [29,

30]. Briefly, the first step is to discretize the problem physical domain using a nodal set fitting the domain area. Then, the background integration lattice was defined using quadrilateral regular cells fitting the domain area. In this work, the Gauss-Legendre quadrature rule was used to establish the numerical integration. Each integration point is defined by its Cartesian coordinates, $\mathbf{x}_I \in \mathbb{R}^2$, and by its own integration weight, $\widehat{w}_I$ (the volume that the integration point occupies in the domain). With this set of integration points it is possible to obtain the background integration mesh, allowing to discretely integrate the continuous equations governing the physical phenomenon. At this point, the nodal connectivity can be determined. In RPIM, the nodal connectivity is obtained using the “influence-domain” concept, which is the set of nodes closer to each integration point $\mathbf{x}_I$. This set of n nodes are then used to build the shape function of integration point $\mathbf{x}_I$, and the weak form is applied to build the equations’ system. The shape functions allow to interpolate a field variable at a point within the physical domain under study. Using the radial point interpolating (RPI) technique, the concentration of VEGF at an integration point $\mathbf{x}_I$ can be defined as,

$$\phi^h(\mathbf{x}_I) = \boldsymbol{\varphi}(\mathbf{x}_I)^T \boldsymbol{\phi} \quad (1)$$

being $\boldsymbol{\varphi}(\mathbf{x}_I)$ the shape function vector at the integration point $\mathbf{x}_I$ defined as,

$$\boldsymbol{\varphi}(\mathbf{x}_I) = \{\varphi_1(\mathbf{x}_I) \quad \varphi_2(\mathbf{x}_I) \quad \dots \quad \varphi_n(\mathbf{x}_I)\}^T \quad (2)$$

Its terms are obtained with the following equation system:

$$\begin{Bmatrix} \varphi_1(\mathbf{x}_I) \\ \varphi_2(\mathbf{x}_I) \\ \vdots \\ \varphi_n(\mathbf{x}_I) \\ \lambda(\mathbf{x}_I) \end{Bmatrix} = \begin{bmatrix} r_1(\mathbf{x}_1) & r_2(\mathbf{x}_1) & \dots & r_n(\mathbf{x}_1) & 1 \\ r_1(\mathbf{x}_2) & r_2(\mathbf{x}_2) & \dots & r_n(\mathbf{x}_2) & 1 \\ \vdots & \vdots & \ddots & \vdots & \vdots \\ r_1(\mathbf{x}_n) & r_2(\mathbf{x}_n) & \dots & r_n(\mathbf{x}_n) & 1 \\ 1 & 1 & \dots & 1 & 0 \end{bmatrix}^{-1} \begin{Bmatrix} r_1(\mathbf{x}_I) \\ r_2(\mathbf{x}_I) \\ \vdots \\ r_n(\mathbf{x}_I) \\ 1 \end{Bmatrix} \quad (3)$$

being the scalar $\lambda(\mathbf{x}_I)$ a by-product of the equation system, without any relevant mathematical application and $r_i(\mathbf{x}_j)$ the multi-quadric radial basis function defined as,

$$r_i(\mathbf{x}_j) = ((x_i - x_j)^2 + (y_i - y_j)^2 + c^2)^p \quad (4)$$

being c and p shape parameters controlling the interpolation property of the obtained shape function. Previous work [31] demonstrated that the RPI shape functions achieve the delta Kronecker property with $c = 0.0001$ and $p = 0.9999$. The radial point interpolation meshless methods formulations are well described in the literature [31].

2.2. Discrete equation system

This model allows one to follow endothelial cell migration due to the chemical diffusion of VEGF in a homogeneous medium. To obtain the numerical solution of the algorithm implemented in the RPIM software, a discrete equation system must be established. The VEGF diffusion can be numerically simulated as a field problem [32], as already successfully assumed in previous works [29, 30]. The general form of the Helmholtz can be used to define the equation system of a 2D linear steady state field problem:

$$D_x \frac{\partial^2 \phi}{\partial x^2} + D_y \frac{\partial^2 \phi}{\partial y^2} - g\phi + Q = 0 \quad (5)$$

being ϕ the field variable, which in this case corresponds to the VEGF concentration, D_x and D_y represents the VEGF diffusion coefficient along dimension x and y , respectively, g represents the matrix of chemical infusibility (in this work g is neglected, $g = 0$) and Q is the VEGF release rate. The meshless system equations can be formulated using the weighted residual approach:

$$[K_D + K_g]\Phi - f_q = 0 \quad (6)$$

where K_D , after the manipulation of equation (5), can be represented as,

$$K_D = \int_A \mathbf{B}_G^T \mathbf{D} \mathbf{B}_G dA \quad (7)$$

being $\mathbf{B}_G$ and $\mathbf{D}$, respectively, defined as follows:

$$\mathbf{B}_G = \begin{bmatrix} \frac{\partial \phi}{\partial x} \\ \frac{\partial \phi}{\partial y} \end{bmatrix} = \begin{bmatrix} \frac{\partial \phi_1}{\partial x} & \frac{\partial \phi_2}{\partial x} & \dots & \frac{\partial \phi_n}{\partial x} \\ \frac{\partial \phi_1}{\partial y} & \frac{\partial \phi_2}{\partial y} & \dots & \frac{\partial \phi_n}{\partial y} \end{bmatrix} \quad (8)$$

$$\mathbf{D} = \begin{bmatrix} D_x & 0 \\ 0 & D_y \end{bmatrix} \quad (9)$$

Additionally, K_g can be defined as,

$$K_g = \int_A g \{\phi_1 \quad \phi_2 \quad \dots \quad \phi_n\}^T \{\phi_1 \quad \phi_2 \quad \dots \quad \phi_n\} dA \quad (10)$$

and f_q , the discrete VEGF release rate vector, is defined as,

$$f_q = \int_A Q \{\phi_1 \quad \phi_2 \quad \dots \quad \phi_n\}^T dA \quad (11)$$

Therefore, solving equation (6) it is possible to obtain the final VEGF concentration in the medium, Φ .

2.3. Computational modelling of angiogenesis

Model parameters: Angiogenesis was simulated using a 5x5 mm² square domain, discretized with 51x51 nodes, forming a regular nodal distribution with 2601 nodes. The domain geometry was defined in order to fit the CAM assay, as shown in Figure 2. The parent vessel is composed of the capillary lumen and endothelial cell monolayer. The sprouting angiogenesis was initiated in the endothelial cell monolayer domain.

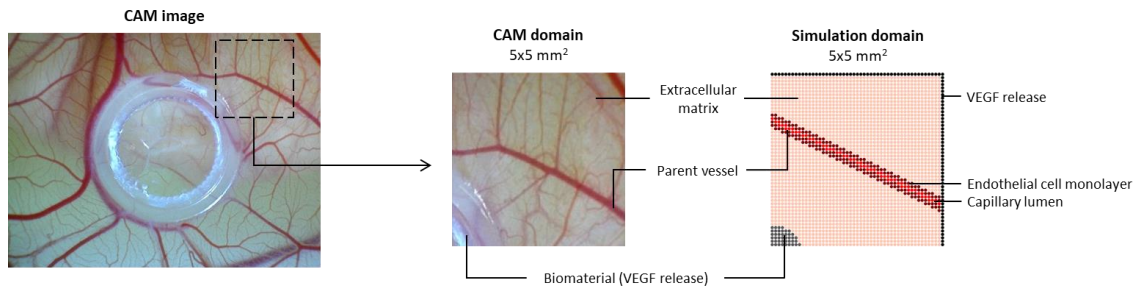


Figure 2: Initial domain geometry, showing the discretization mesh obtained. In the simulation domain the red label represents the capillary lumen, the dark red label represents the endothelial cell monolayer, the light pink label represents the extracellular matrix, the grey label represents the biomaterial that releases the highest VEGF concentration and the dark label represents an additional VEGF release zone.

The essential and natural boundary conditions, the VEGF concentration in the biomaterial and in the capillary lumen were obtained from experimental data. Accordingly, in essential boundary conditions, a VEGF basal concentration of $0.05\text{ng } \mu\text{L}^{-1}$ was used (in the simulation the value applied was $50 \times 10^{-6} \text{ g mm}^{-3}$) in the capillary lumen [33]. In the biomaterial (100 μL), VEGF concentrations of 50ng, 75ng, 100ng and 200ng were used (in the simulation the values applied were, respectively, $500 \times 10^{-6} \text{ g mm}^{-3}$, $750 \times 10^{-6} \text{ g mm}^{-3}$, $1000 \times 10^{-6} \text{ g mm}^{-3}$, $2000 \times 10^{-6} \text{ g mm}^{-3}$). At the domain boundary, on the top and on the right hand side, VEGF release was modelled in order to obtain the VEGF gradient for both sides of the parent vessel. The simulations follow a periodic boundary condition linking the VEGF concentrations of both sides, $\overline{\Phi}(x = 0) = \overline{\Phi}(x = 5)$, being $\overline{\Phi}$ the imposed VEGF concentration at the boundary. The chemical diffusion coefficient of VEGF used, in all the domains, was $1.16 \times 10^{-6} \text{ mm}^2\text{s}^{-1}$ [19]. With this methodology, it is possible to obtain the VEGF gradient concentration (Figure 3), which regulates the endothelial cell migration during angiogenesis. This gradient goes from dark blue to yellow. The dark blue zone is located near the parent vessel and has a VEGF concentration of

around $50 \times 10^{-6} \text{ g mm}^{-3}$. The yellow zone is located near the biomaterial and has the highest VEGF concentration that varies accordingly to the situation that is being tested ($500 \times 10^{-6} \text{ g mm}^{-3}$, $750 \times 10^{-6} \text{ g mm}^{-3}$, $1000 \times 10^{-6} \text{ g mm}^{-3}$, $2000 \times 10^{-6} \text{ g mm}^{-3}$).

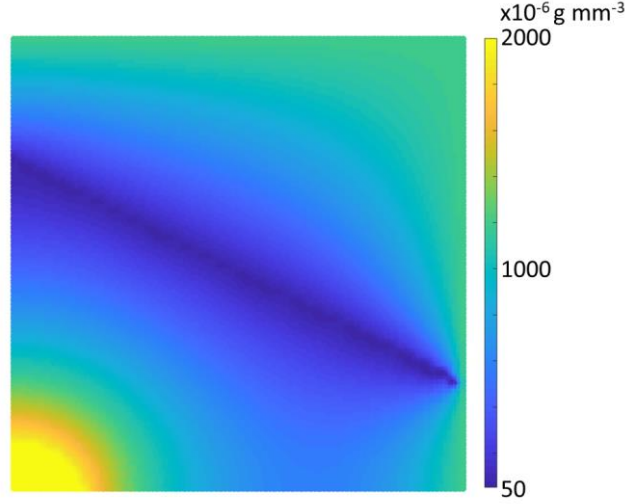


Figure 3: VEGF diffusion gradient. The dark blue zone is located near the parent vessel and has the basal VEGF concentration. The yellow zone is located near the biomaterial and has the highest VEGF concentration. The green zone is located at the domain boundary allowing the existence of the VEGF gradient for both sides of the parent vessel (VEGF concentration in g mm^{-3} . The scale bar used is linear).

Endothelial cell migration: To initiate sprouting angiogenesis from the parent vessel, the tip cells in the sub-domain defined as endothelial cell monolayer were marked using Cartesian coordinates. The location and the initial number of tip cells (one to six) changed in the simulations performed. After the initial tip cell marking, the algorithm begins an iterative loop and in each time step the tip cells are marked one after the other allowing the sprouting vessel growth. In this work, each time step represents the time that the capillary takes to migrate one single endothelial cell length. The final simulation result corresponds to the vascular pattern obtained in CAM at EDD14. Considering the total number of iterations, each iteration time corresponds to about 6.7 hours. The direction of the tip cell movement is defined by the vector $\mathbf{u}$, that follows the gradient vector from the VEGF concentration field towards the VEGF source. Accordingly, the gradient of the VEGF concentration field is obtained ($\mathbf{n} = \nabla \Phi$), being $\mathbf{n}$ oriented towards the VEGF source (in opposition to its support cell). Therefore, $\mathbf{n}_{mod} = \begin{bmatrix} \cos\theta & \sin\theta \\ -\sin\theta & \cos\theta \end{bmatrix} \mathbf{n}$, being θ a variation angle ranging between -0.24 and 0.24 radian and attributed randomly. Then, the unit vector of $\mathbf{n}_{mod}$ is calculated: $\mathbf{u} = \mathbf{n}_{mod} / \|\mathbf{n}_{mod}\|$. Then, using the direction vector $\mathbf{u}$, the average distance between cells (the actual tip cell and the

previous one) is considered and the new tip cell position is defined by summing the old tip cell position with the vector $\mathbf{u}$ multiplied by the average distance between the two cell (the actual tip cell and the previous one). If a node exists in the new tip cell position, that node will become a tip cell. If in the new position there are no nodes, a new node is included in the model, and that new node becomes a tip cell. In each step, the VEGF concentration is calculated and the new position of the tip cell is actualized. Moreover, the VEGF concentration at the boundary domain is imposed. The iterative process stops when the capillary arrives to the domain boundary, to the biomaterial or if the tip cell is near another endothelial cell.

Branching process: In this study, data obtained during the CAM assay was used to implement the branching process, applying the same methodology as previously described [29]. Thus, the capillary networks presented in control and VEGF treated eggs were analysed. The capillary order was defined according to the capillary calibre, the 1st order represents the capillaries with higher calibre and the 3rd order the ones with lowest. To set the branching locations, the distance between consecutive branch points in control and VEGF treatments was measured, for each capillary order, and the rules presented above were obtained. Equation (12), presented for control, was defined in a previous work [29]. In the present work, using the same methodology, equation (13) was defined to set the branching for the VEGF treatments.

$$d_{control} = 0.9286e^{-0.219 \times O_{cap}} \quad (12)$$

$$d_{VEGF} = 1.0927e^{-0.357 \times O_{cap}} \quad (13)$$

being d the distance between consecutive branch points and O_{cap} the capillary order. Consequently, a new branch occurs if the distance to the previous branch is greater than d , for the capillary order considered. When a branch occurs, the mother capillary maintains its order and the daughter increases its own. The mother and daughter orders were adjusted in the simulations considering the tested VEGF concentration. The direction of branching whether to right or left is random. However, in the same capillary, after the first branch, the direction of the following branch is in the opposite direction and so forth. To define the branching angle in control and VEGF treatments, several angles between capillaries (with the same and different order) were measured. A previous work [29] defines the data for control, being the average branching angle of 68° (n = 36, maximum = 127°, minimum = 45°) for capillaries with same order and 86° (n = 36, maximum = 117°, minimum = 44°) for capillaries with different order. In the VEGF treatment, established in the current work, the average branching angle was 72° (n = 126, maximum = 130°, minimum = 13°) for capillaries with same order and 76° (n = 146, maximum = 150°, minimum = 32°) for capillaries with different order. The n changed between the

measurements since it represents the total number of branching angles measured for each situation. When this data was inserted into the model, a random fluctuation in the branching angle was allowed in order to modify the angle in each new branch. Accordingly, the CAM images of control and VEGF treatments were used to extract the function parameters and to create a phenomenological law. In this way, the proposed model uses the developed phenomenological function to rule the branching location. To perform the simulation for different VEGF concentrations (50ng, 75ng, 100ng and 200ng) the branching location for each VEGF concentration was adjusted and then the tip cells were modelled as migrating following the highest VEGF concentration.

2.4. *In vivo* angiogenesis

To analyse the predictive capacity of our computational model, the CAM assay was used to validate the model's predictions. The CAM assay is a widely used methodology to study angiogenesis *in vivo* [34]. Pathogen-free fertilized white leghorn chicken eggs (*Gallus gallus domesticus*) were obtained from Henry Stewart Co. Ltd (UK) and they were incubated. Care was consistent with the guidelines of the Home Office, UK. Chick embryos were cultured as described previously [35]. The egg shells were cracked and embryos were transferred into a square Petri dish on embryonic development day (EDD) 3. The ex-ovo cultures were maintained in a humidified incubator at 38°C between EDDs 3 to 14. The survival of the embryos were checked daily and recorded. The detailed description of the *ex-ovo* CAM assay can be accessed in a previous work [36]. Unloaded (control), VEGF 50ng and VEGF 100ng loaded hydrogels (100 μ L) with an internal diameter of 8 mm were prepared, as described previously [37]. At EDD7 the hydrogels were placed on the CAM surface. At EDD14, pictures of hydrogels and surrounding CAM area were taken with a digital microscope.

2.5. Angiogenesis evaluation

Quantification of angiogenesis was performed on 6 digital images from each tested condition using online Image J plug-in, NeuronJ. In CAM images, the quantification was performed in 5x5 mm² regions of interest areas, always containing the biomaterial in one of the corners. In the simulations, the quantification was also performed in 5x5 mm² areas, being the biomaterial located in the lower left corner. Then, the total vessel number and the total vessel length of all the traced vessels were calculated. In the CAM assay, the tested conditions groups

were control, VEGF 50ng and VEGF 100ng. In the simulation, the groups were control, VEGF 50ng, VEGF 75ng, VEGF 100ng and VEGF 200ng.

The capillary network profile obtained in control, VEGF 50 ng and VEGF 100ng was also compared between the CAM images and the simulation. Accordingly, simulations with identical CAM domain geometries were performed. For each one, the initial number and location of sprouting tip cells and the location of the parent vessel were identified. Then, the capillary volume fraction was calculated to compare the numerical and the *in vivo* results. For that, the capillary network was divided in a grid with 10x10 equal square patches. Then, the capillary volume fraction in each square patch of the grid was quantified. Finally, the difference between the total capillary volume fraction obtained in the CAM image and in the simulation were compared.

2.6. Statistical analysis

Statistical analysis was performed using the data analysis sheets. Comparisons of more than two groups were performed with One-way ANOVA with Tukey's post-hoc test, to determine significant differences. All values are presented as the mean $\pm$ standard error of the mean (SEM). Differences were considered to be significant, at a confidence interval of 95%, for $p < 0.05$.

3. RESULTS

3.1. Angiogenesis response predicted by the model

In vivo, endothelial cells migrated and organized themselves allowing the directional growth of capillaries towards the hydrogel (Figure 4). To analyse the angiogenic response due to chemotaxis, simulations were performed by increasing the VEGF concentration in the biomaterial domain (Figure 5). Figure 6 indicates that the four sprout tips migrated from the parent vessel, localized in the top and in the right domain boundary, to the biomaterial. The endothelial cell migration follows the VEGF gradient. Moreover, the density of capillaries increased with the increase in the VEGF concentration. To confirm this, the total vessel number and the total vessel length, for each simulated condition (control, VEGF 50ng, VEGF 75ng, VEGF 100ng and VEGF 200ng), was quantified. Figure 7 clearly demonstrates that the number and length of blood vessels were higher in the VEGF treatments, and this increase was proportional to the increasing VEGF concentration.

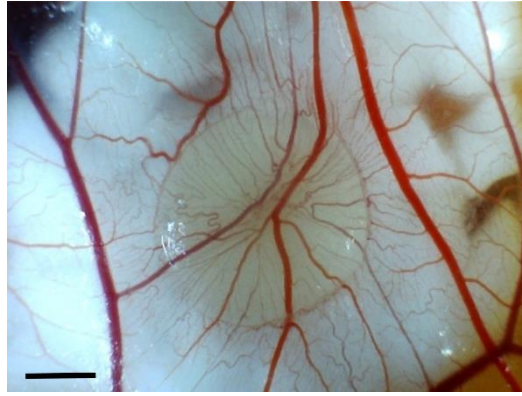


Figure 4: CAM image on EDD 14 showing the directional growth of capillaries towards the hydrogel, located in the image centre. Scale bar: 3mm.

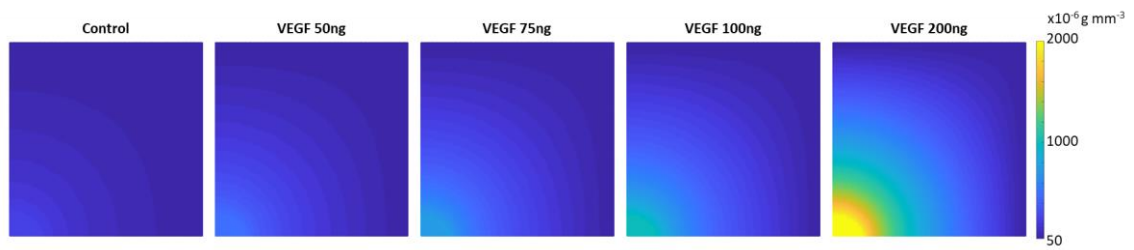


Figure 5: VEGF diffusion gradient obtained in the performed simulations. The biomaterial is located in the lower left corner (VEGF concentration in g mm^{-3} . The scale bar used is linear).

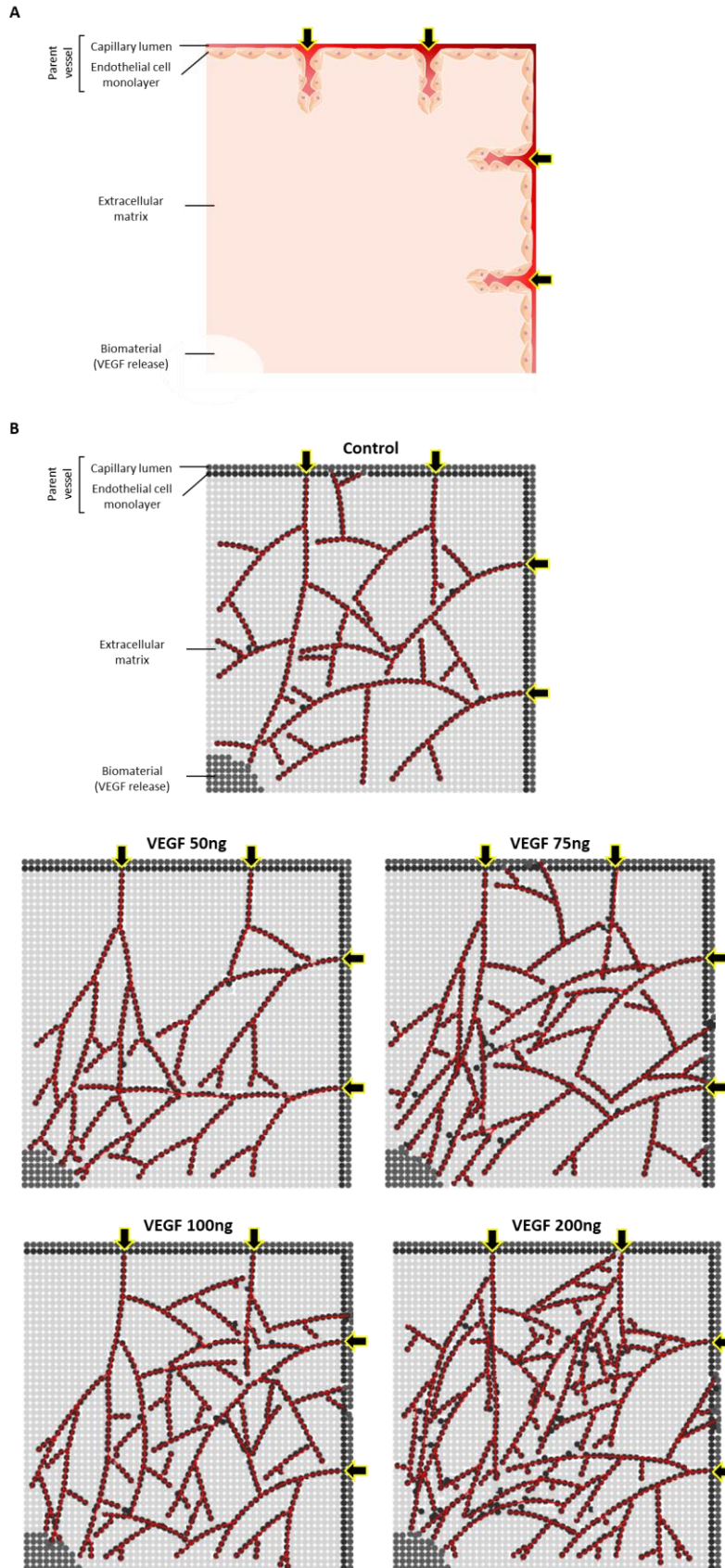


Figure 6: Capillary network obtained for control and for VEGF concentrations tested. **(A)** Schematic representation of the simulated sprouting angiogenesis; **(B)** Final simulation results obtained for control and for VEGF concentrations tested (50ng, 75ng, 100ng and 200ng), using a regular nodal discretization mesh. The arrows mark the initial sprout tips location. Images sized: $5 \times 5 \text{ mm}^2$.

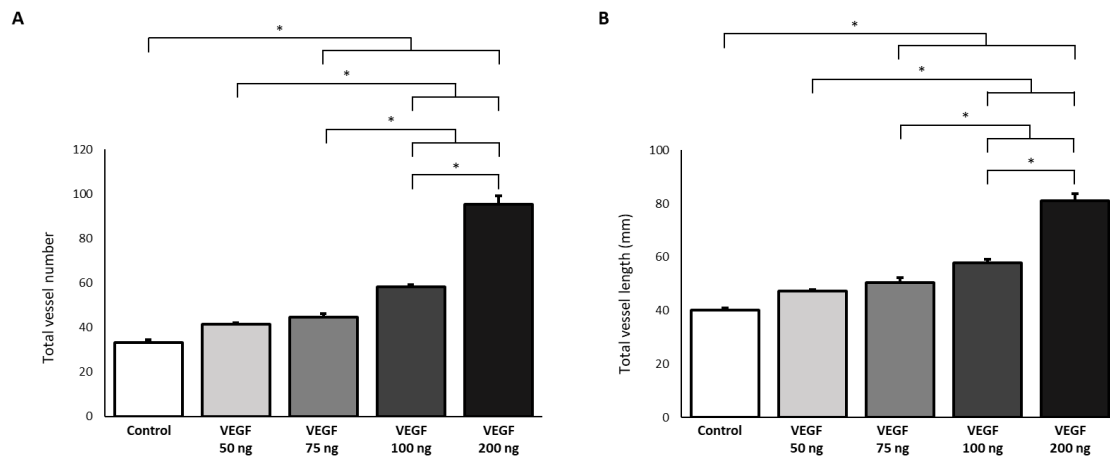


Figure 7: Evaluation of angiogenesis response for each VEGF concentration tested. Quantification of total vessel number **(A)** and total vessel length **(B)** for each treatment from final simulation results ($n_{\text{Control}} = n_{\text{VEGF 50ng}} = n_{\text{VEGF 75ng}} = n_{\text{VEGF 100ng}} = n_{\text{VEGF 200ng}} = 6$, One-way ANOVA, pairwise comparison Tukey's post-hoc test, *: $p < 0.05$).

3.2. Angiogenesis compared between *in vivo* and *in silico* methodologies

To compare the angiogenesis response predicted in the proposed model and the one observed *in vivo*, we used the CAM assay (Figure 8). This assay has been extensively used by the MacNeil group to analyse the effect of several compounds on angiogenesis and it is well documented in the literature [36, 38, 39]. Accordingly, the total vessel number and the total vessel length presented in the CAM area (Figure 8) and in the simulation for control, VEGF 50ng and VEGF 100ng were quantified. Figure 9 shows that the obtained quantitative results were very similar between the two methodologies used. In the CAM assay, the treatment with VEGF 50ng and VEGF 100ng increased the total vessel number, respectively, by 37% (vs. control, $p < 0.01$) and by 82% (vs. control, $p < 0.01$). In the simulation, the treatment with VEGF 50ng and VEGF 100ng increased the total vessel number, respectively, by 25% (vs. control, $p < 0.01$) and by 75% (vs. control, $p < 0.01$). Concerning the total vessel length, in CAM, the treatment with VEGF 50ng and VEGF 100ng increased this by 20% (vs. control, $p < 0.01$) and by 36% (vs. control, $p < 0.01$). In the simulation, the treatment with VEGF 50ng and VEGF 100ng increased the total vessel length, respectively, by 18% (vs. control, $p < 0.01$) and by 44% (vs. control, $p < 0.01$).

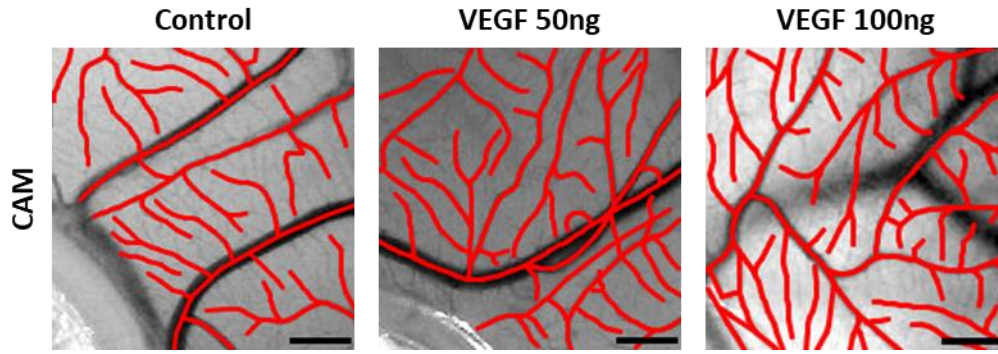


Figure 8: CAM images on EDD14 obtained after processing for control, VEGF 50ng and VEGF 100ng. In all the images the biomaterial is located in the lower left corner. Scale bar: 1mm.

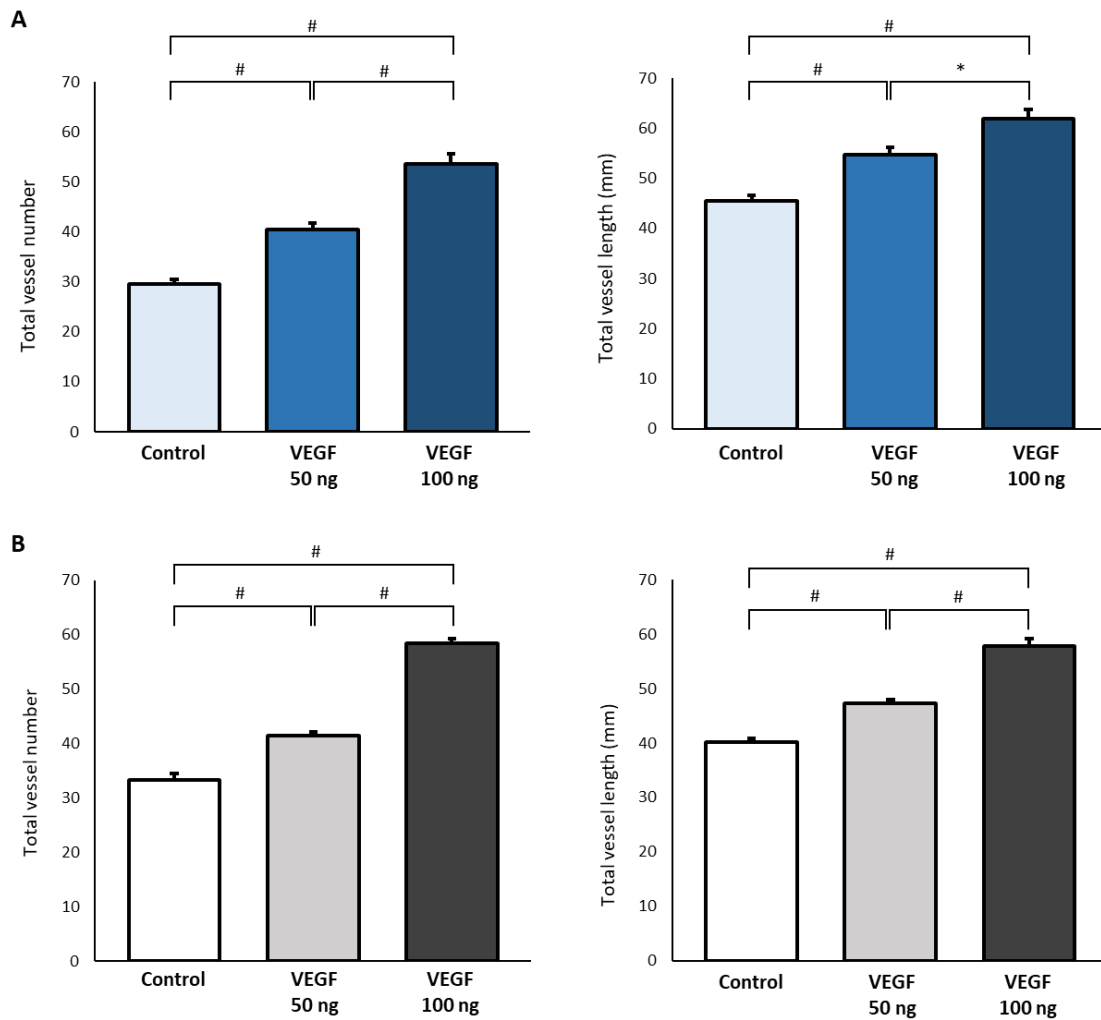


Figure 9: *In vivo* and *in silico* quantitative analysis of angiogenesis. **(A)** Quantification of total vessel number and total vessel length for each treatment from CAM processed images; **(B)** Quantification of total vessel number and total vessel length for each treatment from simulation results ($n_{\text{Control}} = n_{\text{VEGF 50ng}} = n_{\text{VEGF 100ng}} = 6$, One-way ANOVA, pairwise comparison Tukey's post-hoc test, *: $p < 0.05$, #: $p < 0.01$).

3.3. Capillary pattern analysis

The capillary network profile presented in the CAM was also analysed, as well as the one obtained in the simulations. Accordingly, six different tests were performed in order to compare the vascular network found in simulations and in CAM images (two for control, two for VEGF 50ng and two for VEGF 100ng). Then, the capillary network profile presented in the images of both methodologies (*in vivo* and *in silico*) was extracted. Analysing Figure 10, we verified that the capillary network presented in simulations reveals similarities to the one obtained *in vivo*.

To qualitatively analyse the capillary pattern predicted by our model, the difference between the total capillary volume fractions accessed using the CAM assay and the simulation was estimated. For control and for the lowest VEGF concentration tested, the differences between the total capillary volume fractions were 22% and 32% (Figure 10, 3rd column). For the highest VEGF concentration tested, we obtained values of 24% and 49% for the difference between the total capillary volume fractions (Figure 10, 3rd column). Considering that the mathematical model is only driven by the VEGF concentration (and there are many other factors influencing angiogenesis), these results can be considered satisfactory, showing that the proposed model is capable of predicting the angiogenic response. It was noticeable that agreement was best for lower VEGF concentrations.

4. DISCUSSION

Angiogenesis is an indispensable biological process that occurs in all living tissues. This process is even more important during wound healing when it is necessary to re-establish the normal blood flow and support cell viability. Several *in vivo* [40, 41] and *in vitro* [42, 43] studies have been conducted in order to describe wound healing angiogenesis. However, these methodologies are time consuming and very expensive. Moreover, working with animals involves ethical considerations. *In silico* methodologies stimulated the interest of the scientific community since they allow us to simulate biological processes with different levels of complexity and to better understand the underlying phenomenon. Accordingly, several wound healing angiogenesis models have been developed. However, in many of them the capillary network morphology is not considered, the parameters are estimated with little approximation to the biological situation and the numerical results are not compared with experimental data.

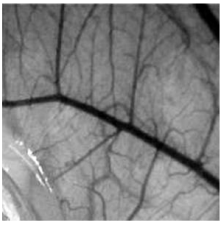
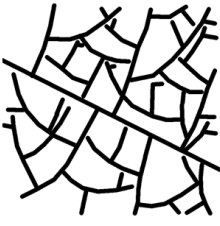
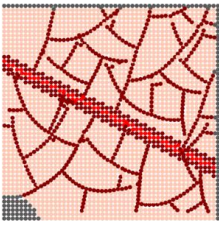
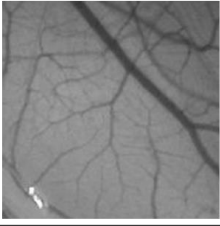
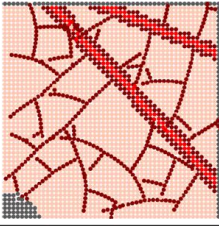
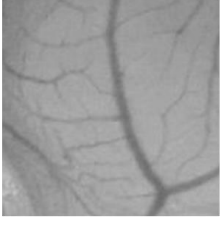
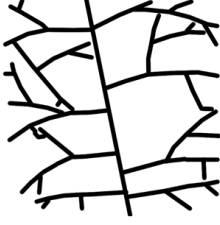
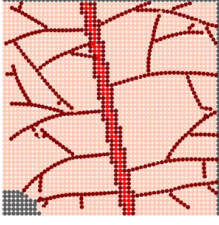
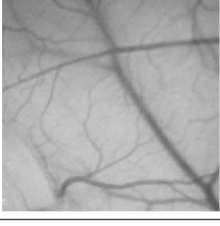
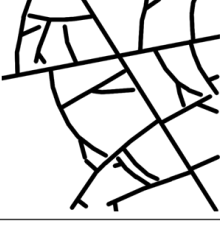
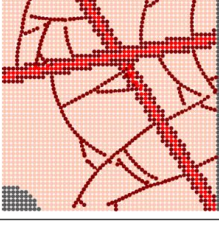
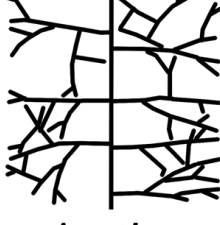
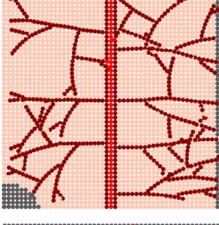
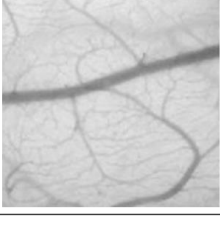
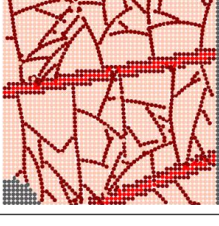
	<i>In vivo</i>		<i>In silico</i>		Difference between the total capillary volume fractions
	Final image	Capillary network	Capillary network	Final image	
Control					0.24
					0.29
VEGF 50 ng					0.22
					0.32
VEGF 100ng					0.24
					0.49

Figure 10: Capillary network profile obtained in CAM images (1st column) and in simulations (2nd column) for control, VEGF 50ng and VEGF 100ng and the corresponding difference between the total capillary volume fractions (3rd column). In all the images the biomaterial is located in the lower left corner. Images sized: $5 \times 5 \text{ mm}^2$.

Aiming to overcome some of these limitations, this work intends to further develop a meshless model combined with phenomenological law (from *in vivo* angiogenesis assays) to simulate sprouting angiogenesis regulated by several VEGF concentrations. In this work, as in our previous study [29], the data obtained during the CAM assay was used to simulate the branching process. The model proposed in this work was capable of predicting angiogenesis modulated by several VEGF concentrations and to reproduce a realistic capillary network. Moreover, the evaluation of angiogenesis (quantitative and qualitative) compared between the *in silico* and the *in vivo* methodologies showed good agreement between both.

One of the strategies used in tissue engineering to improve wound healing consists in using biomaterials to increase the VEGF concentration in the wound area [5]. Thus, in this work, the release of VEGF from a biomaterial was simulated. The biomaterial location was chosen considering the *in vivo* situation. In this study, the angiogenic response to different VEGF concentrations (0, 50ng, 75ng, 100ng and 200ng VEGF) (Figure 6 and Figure 7) was analysed. Endothelial cells migrated from the parent vessel to the biomaterial following a VEGF diffusion gradient. For angiogenesis evaluation, we verified that the total vessel number and the total vessel length increased with the increase in the VEGF concentration. These results are in agreement with experimental ones that show that VEGF release induces the migration of endothelial cells based on the concentration gradient [44]. For each treatment condition, six simulations were performed with increasing numbers of sprout tips (1 to 6) and with different initial tip cell locations. The results obtained (total vessel number and total vessel length) were identical for the same tested condition. This suggests that the process is being well implemented in the algorithm, since the angiogenic response does not vary with the initial number or location of sprouting tips, corroborating the good performance of the current model.

The CAM assay is a useful, relatively economical, semi-quantitative and reliable methodology to study angiogenesis. This assay provides the continuous *in vivo* visualization of the newly forming vessels allowing the measurement of several parameters: capillary density, blood vessel number, length and diameter, branch point number and blood flow [7, 8]. To ensure the vascularization of a substantial area it is necessary that branching occurs. Several CAM images of control and VEGF were analysed in order to obtain significant parameters to implement in the model, namely the branching angle and the distance between branches [Equations (12) and (13)]. With this methodology, the capillary network pattern presented in CAM images was simulated. The angiogenic response predicted by our model was evaluated. For this, CAM images of control, VEGF 50ng and VEGF 100ng were used (Figure 8) and the total vessel number and the total vessel length, for each tested condition, were quantified (Figure 9 A). Then, simulations for the same conditions (control, VEGF 50 ng and VEGF 100ng) were

performed and the total vessel number and the total vessel length were, once again, quantified (Figure 9 B) in order to compare the numerical and experimental results. Figure 9 demonstrates that the numerical results were in agreement with experimental ones, allowing us to confirm that the proposed model could efficiently simulate the angiogenesis response for different VEGF concentrations. Although the comparison presented only for two different VEGF concentration seems limited, this is the first study that directly compared the numerical results obtained for the quantitative evaluation of angiogenesis with the ones obtained using an *in vivo* angiogenesis assay. In the *in vivo* angiogenesis assay, VEGF 80ng increased the number of branches and the vessel length by 73% and 36%, respectively [38]. In this study the model gave values of VEGF 100ng increasing the number of branches and the vessel length by 75% and 44%, respectively.

An analysis of the capillary network profile modulated by growth factors has already been conducted in other studies [25, 26]. However, the comparison with experimental data was not determined in these prior studies. The capillary network profile presented in CAM images, for the same treatment conditions, was analysed. The qualitative evaluation between the two methodologies used (*in vivo* and *in silico*) was done using the difference between the total capillary volume fractions. These results, presented in Figure 10, showed that the capillary network obtained *in silico* had a very realistic structure and morphology. For the lower VEGF concentrations tested, the model correctly mimicked the vascular network profile. However, for the highest VEGF concentration tested, the difference between the total capillary volume fractions increased to 49%. For this concentration, the ability of our model to predict the *in vivo* results was not as good. This is not surprising because it is well established that VEGF mediated angiogenesis (e.g. tumour angiogenesis) is characterized by the formation of disorganized, poorly remodelled (and therefore fragile and leaky) and dysfunctional blood vessels which are more prominent at higher concentrations [1, 45, 46]. Indeed, in the CAM assay with VEGF 100ng, it was possible to observe the increase in the blood vessels number, however, given their very thin thickness it is difficult to individualize them, worsening the comparison with the simulation. Nevertheless, we considered that we obtained a satisfactory result, supporting the robustness of our numerical model. Although the capillary network presented in simulations is similar to the one presented in CAM images, they do not completely overlap. Despite that, it is necessary to consider that angiogenesis presents *in vivo* variability, being a complex process with different cell types, many biological variables and complex regulatory processes.

To develop a computational model several assumptions and simplifications had to be considered. In the current study, angiogenesis was simulated using an infinite 2D domain, with periodic boundary conditions, meaning that the left boundary condition is linked to the right boundary condition and the down boundary condition is linked to the up boundary condition.

With this methodology, the growth of the capillary is more predictable, allowing us to verify if the code is being well implemented. Moreover, some authors have established models to analyse the effect of growth factors on capillary network morphology using 2D domains [24, 47]. Other assumptions were that the VEGF diffusion was constant in time and the VEGF natural decay and the uptake of VEGF by endothelial cells were not considered and the biomaterial was assumed to give a continuous source of VEGF. Additionally, the physiochemical characteristics of applied hydrogels were not taken into consideration. In our model, endothelial cell migration was modelled only with respect to the VEGF chemoattractant effect and the role of anisotropic and heterogeneous behaviour of the extracellular matrix on angiogenesis was not considered, and the rate of blood flow and the diameter variation of blood vessels were also not considered. Moreover, we assumed that the VEGF concentration did not affect the branching occurrence, as the branching locations were defined by Equations (12) and (13), that consider the distance to the previous branch. The sheer number of factors that can influence angiogenesis indicate the complexity of this process. In the future, we intend to overcome some of these limitations in order to develop a model as close to reality as possible. The CAM assay is also widely used to screen antiangiogenic drugs [8] which is extremely interesting in the field of cancer treatment. We did not attempt to design a model that could perform this kind of study but this is also something that could be developed in the future.

In conclusion, the computational model we present simulates the sprouting angiogenesis which is seen following the addition of several concentrations of VEGF. The model simulates with acceptable accuracy the angiogenic response regarding blood vessel length, number and morphology. In the present model the width of blood vessels was not considered. In the CAM images, identifying and isolating the very thin blood vessels is a demanding task. For this reason, for the highest VEGF concentration, the capillary network extracted from the *in vivo* images is less approximated to the one that actually exists. This may have hindered the comparison of the capillary network pattern obtained between the *in silico* and *in vivo*, mainly for the highest VEGF concentration tested. The development of *in silico* methodologies alongside *in vivo* methodologies is essential since computational models require input from experimental assays in order to extract model parameters, to validate the model performance and to define how close the model is to reality. In the future, the development of robust *in silico* wound healing angiogenesis models will allow one to make predictions concerning cell behaviour, the best culture conditions and experimental outputs and to test different experimental protocols. This will allow researcher to reduce the time and the cost of experimental assays, as well as the number of animals used, making *in silico* simulation a valuable tool in angiogenesis research.

DECLARATION OF COMPETING INTEREST

Author Ana Guerra, from Institute of Science and Innovation in Mechanical and Industrial Engineering, declare that she has no conflict of interest. Author Jorge Belinha, from School of Engineering, Polytechnic of Porto, declare that he has no conflict of interest. Author Naside Mangir, from Kroto Research Institute and Hacettepe University School of Medicine, declare that she has no conflict of interest. Author Sheila MacNeil, from Kroto Research Institute, declare that she has no conflict of interest. Author Renato Natal Jorge, from Faculty of Engineering of the University of Porto, declare that he has no conflict of interest.

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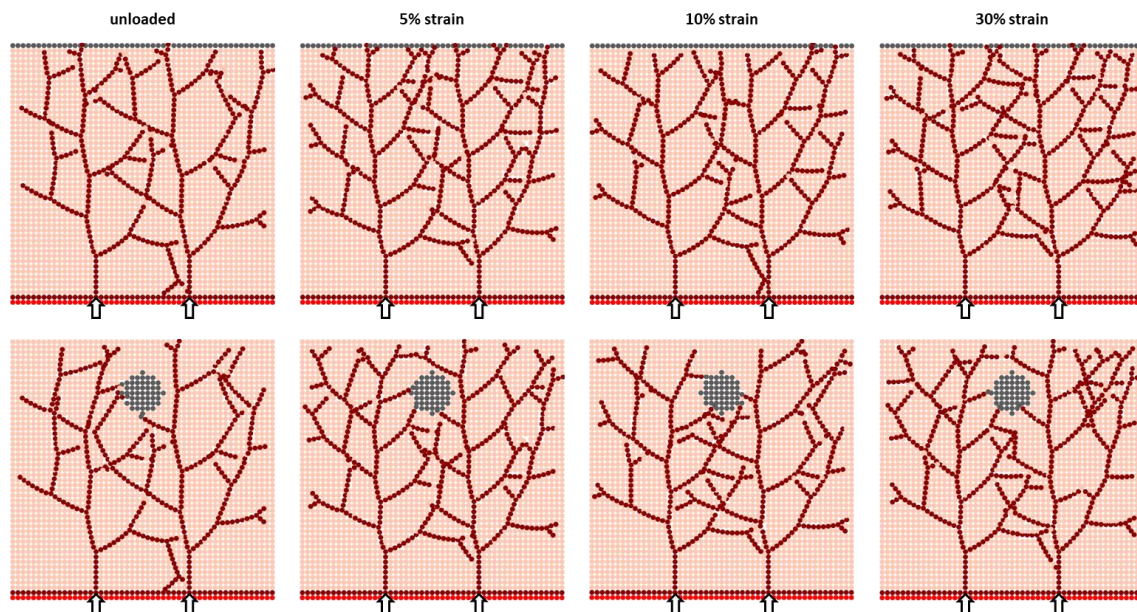
3.5. Contribution V: Using a meshless method to assess the effect of mechanical loading in angiogenesis

3.5.1. Highlights of Contribution V

This contribution focused on studying the effect of compressive loading in angiogenesis. In this model, the vascular endothelial growth factor gradient regulates the endothelial cell migration and the compressive loading affects the branching process.

Therefore, the main contents of this publication include:

- I. Brief description of the angiogenesis process and its regulation;
- II. Succinct literature review on loading parameters that regulate angiogenesis;
- III. Analysis of the effect of compressive loading 5%, 10% and 30% strain, in capillary network growth for three different VEGF release regions locations – in the superior boundary, circular and in the middle domain – and with different initial number of sprouting cells, one to four (Figure 3.7):
 - a. The sprouting cells migrate from the parent vessel to the VEGF release region, following the VEGF gradient;
 - b. The density of capillaries increased in the simulations under 5%, 10% and 30% strain;



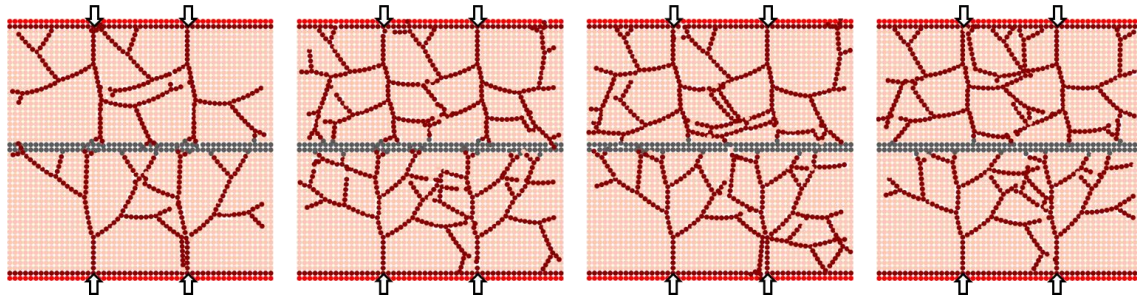
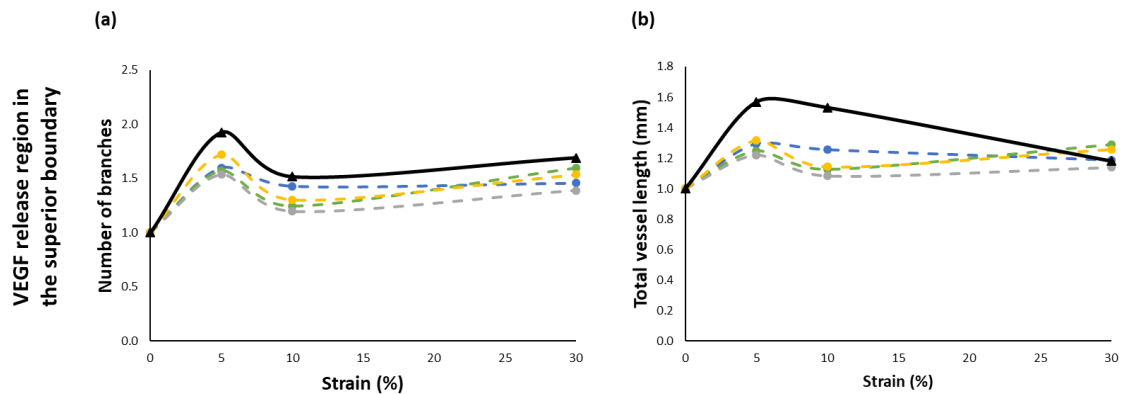


Figure 3.7: Final simulation results obtained for unloaded and under 5%, 10% and 30% strain loading, for a VEGF release region located in the superior boundary (1st row), a circular VEGF release region (2nd row) and a VEGF release region located in the middle (3rd row), using a regular nodal discretization mesh. The arrows mark the initial sprouting cells location. Images sized: $5 \times 5 \text{ mm}^2$.

- IV. Quantitative analyses of the angiogenesis stimulation for each simulated condition – unloaded, 5%, 10% and 30% strain:
 - a. All the strain magnitudes tested increased the number of branches, for all the domains tested;
 - b. All the strain magnitudes tested increased the total vessel length, for all the domains tested;
 - c. The 5% strain loading was the most effective one;
- V. Comparison between the angiogenesis response predicted by the model and the one described in an experimental study [38]:
 - a. The number of branches between the numerical results and the experimental data are similar, mainly when the VEGF release region is located in the superior boundary and in the circular VEGF release region domain [Figure 3.8 (a)];
 - b. Regarding the total vessel length, the numerical and the experimental results differ a little more. Nevertheless, it is possible to observe some similarity [Figure 3.8 (b)].



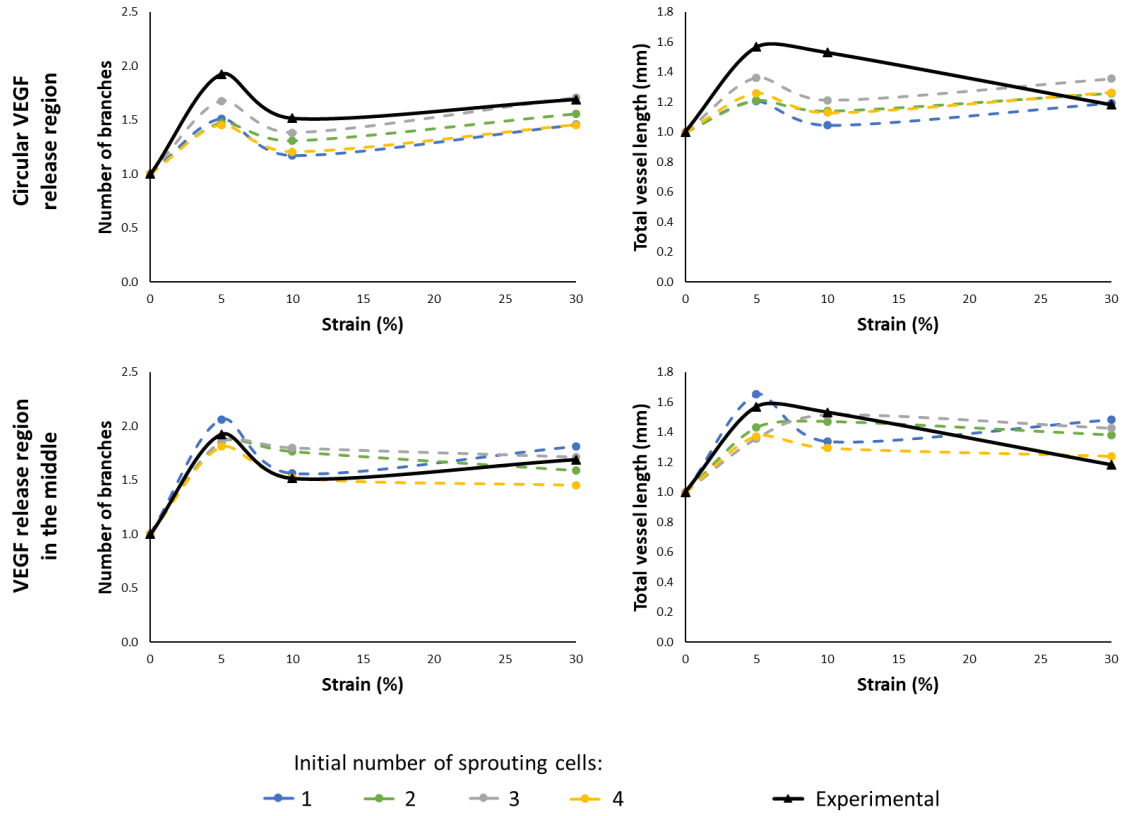


Figure 3.8: Quantification of the number of branches (a) and the total vessel length (b) for unloaded and under 5%, 10% and 30% strain loading, for the three different initial geometry domains tested: VEGF release region in the superior boundary (1st row), circular VEGF release region (2nd row) and VEGF release region in the middle (3rd row). All the results were normalized to the unloaded situation. The experimental data was obtained in [38].

VI. Analysis of the model's accuracy to mimic the effect of compressive loading in the branching process. The numerical results show that all the compressive loadings tested increased the vascular network length and the number of branches. However, the 5% strain loading was the most effective one. The capillary network obtained resembles the one presented in experimental assays and the obtained numerical results agree with the experimental ones.

The complete document can be found in the next sub-chapter.

3.5.2. Contribution V

Using a Meshless Method to Assess the Effect of Mechanical Loading in Angiogenesis

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Using a Meshless Method to Assess the Effect of Mechanical Loading in Angiogenesis

Ana Guerra^a, Jorge Belinha^b and Renato Natal Jorge^{c,d}

^aInstitute of Science and Innovation in Mechanical and Industrial Engineering (INEGI), Porto, Portugal.

^bSchool of Engineering, Polytechnic of Porto (ISEP), Mechanical Engineering Department, Porto, Portugal.

^cAssociated Laboratory in Energy, Transport and Aeronautics (LAETA – INEGI), Porto, Portugal.

^dFaculty of Engineering of the University of Porto (FEUP), Mechanical Engineering Department, Porto, Portugal.

ABSTRACT

Although it is known that blood vessels can be found in mechanically active environments, less is known about the effect of mechanical stimulus in angiogenesis. Therefore, understanding how endothelial cells respond to a mechanical stimulus is essential to improve tissue vascularization and promote wound healing and tissue engineering development. In this work, a meshless method is used to combine an elasticity formulation with a capillary growth algorithm. The final numerical model is capable to simulate the effect of compressive loading in angiogenesis, using three strain magnitudes (5, 10 and 30% strain). In this proposed model, the vascular endothelial growth factor gradient regulates the endothelial cell migration and the compressive loading affects the branching process. The numerical results show that all the compressive loadings tests increased the vascular network length and the number of branches, being 5% strain magnitude the most effective one. The capillary network obtained resembles the one presented in experimental assays and the obtained numerical results agree to the experimental ones. Nevertheless, this study possesses some limitations since the viscoelastic properties of the tissue, the dynamic loading effect and the effect of the time variable were not considered. In the future, the combination of computational and experimental studies will be very useful to understand and to define which are the mechanical cues that promote angiogenesis, allowing to improve tissue vascularization and, consequently, the wound healing process.

KEYWORDS

Angiogenesis quantification; Compressive loading; Radial Point Interpolation Method.

1. INTRODUCTION

A functional vasculature is essential to support cell viability. Angiogenesis is the primary process responsible for the formation of new blood vessels from the pre-existent vasculature, being characterized by the infiltration and extension of endothelial cells from a parent blood vessel [6]. This process is essential during wound healing as well as in tissue engineering [8]. In both situations, it is necessary the existence of a functional vascular network to supply the cells with nutrients and oxygen.

Angiogenesis is regulated by oxygen levels, growth factor gradients and mechanical cues [25]. The modulation of sprouting angiogenesis due to growth factor gradients, namely by the vascular endothelial growth factor (VEGF), has already been extensively described [23]. However, less is known about the mechanical stimuli in angiogenesis. Nevertheless, blood vessels are constantly exposed to mechanical forces due to blood flow or external loading applied in the tissue. In fact, it has already been demonstrated that mechanical signals can regulate angiogenesis and endothelial cell behaviour [19, 28].

Although some studies have already been conducted, the specific parameters of loading that regulate angiogenesis are poorly defined. Yung et al. demonstrated that 7% cyclic tensile strain promotes the autocrine and paracrine secretion of growth factors and enhances endothelial cell migration and sprout formation in 2D cell culture [30]. Sharifpanah et al. showed that 10% mechanical strain upregulates the expression of angiogenesis guidance molecules and of several angiogenic growth factors *in vitro* [24]. Studies performed *in vivo*, in bone, revealed that delaying loading enhances vascular remodelling by increasing the number of large vessels and by decreasing the number of small vessels [3]. Accordingly, defining which mechanical conditions promote neovascular network formation and remodelling is mandatory in order to improve the healing of chronic wounds and the development of tissue engineered scaffolds with relevant size.

In silico models are very useful to understand the dynamics of biological systems. These models are capable of reproduce distinct cell behaviour, different growth factors and extracellular matrix compositions with different levels of complexity. Numerical models permit to test several laboratorial protocols and deduce which ones present best outcomes, using non-invasive, cheaper and less time-consuming approaches. Moreover, *in silico* models are often useful methods to dissociate how mechanical cues can affect endothelial cell behaviour, which would be complex to perform using experimental techniques alone. However, *in silico* models require the proper feedback from experimental assays in order to validate the computational model and to determine how close the model is to reality. Some *in silico* models analysed the

effect of mechanics in endothelial cell behaviour, namely in its migration and proliferation [5] and the effect of mechanical loading in angiogenesis during bone formation [9]. Nevertheless, there are still scarce computational models described in the literature capable of analysing the effect of mechanics in angiogenesis in soft tissues as well as its effect in the capillary network profile. In this work, a meshless discretization technique combined with a reaction-diffusion formulation is enhanced to simulate angiogenesis under the effect of compressive loading. Thus, three compressive strain magnitudes were considered: 5, 10 and 30% strain. In this proposed model, the VEGF gradient regulates the endothelial cell migration and the compressive loading affects the branching process. The angiogenic response was analysed concerning the number of branches and the total vessel length quantifications.

2. MATERIAL AND METHODS

2.1. Numerical method

The Radial Point Interpolation Method (RPIM) was the meshless discretization method used in this work. This method was adequately employed in previous studies to model angiogenesis [11, 13], and the full description of RPIM formulation can be found in the literature [1]. Briefly, being RPIM a meshless method the first step consists in discretization of the biological physical domain under study using a nodal set fitting the domain area. Then, using the quadrilateral regular cells fitting the domain area, the background integration mesh was defined. In this work, the Gauss-Legendre quadrature rule was used to establish the numerical integration. Each integration point is defined by its Cartesian coordinates, $\mathbf{x}_I \in \mathbb{R}^2$, and by its own integration weight, $\widehat{w}_I$ (the volume that the integration point occupies in the domain). Then, it is possible to obtain the background integration mesh, using this set of integration points, and to discretely integrate the continuous equations governing the angiogenic process. The nodal connectivity can now be determined. In RPIM, the nodal connectivity is obtained using the “influence-domain” concept, which is the set of nodes closer to each integration point $\mathbf{x}_I$. This set of n nodes are then used to build the shape function of integration point $\mathbf{x}_I$, and the weak form is applied to build the equations’ system. The shape functions allow to interpolate the VEGF at a point within the physical domain under study. Using the radial point interpolating (RPI) technique, the concentration of VEGF at an integration point $\mathbf{x}_I$ can be defined.

2.2. Discrete equation system

As established in previous works [11, 13], the endothelial cell migration during angiogenesis was modelled due to chemical diffusion of VEGF using a homogeneous medium. Moreover, the VEGF diffusion can be numerically simulated as a field problem [17]. The general form of the Helmholtz [Equation (1)] can be used to define, with the meshless formulation, the discrete equation system for the variable field under analysis,

$$D_x \frac{\partial^2 \phi}{\partial x^2} + D_y \frac{\partial^2 \phi}{\partial y^2} - g\phi + Q = 0 \quad (1)$$

being ϕ the field variable, which in this case corresponds to the VEGF concentration, D_x and D_y represents the VEGF diffusion coefficient along dimension x and y , respectively, g represents the matrix of chemical infusibility (in this work g is neglected, $g = 0$) and Q is the VEGF release rate. The meshless system equations can be formulated using the weighted residual approach:

$$[K_D + K_g]\Phi - f_q = 0 \quad (2)$$

where K_D , after the manipulation of equation (1), can be represented as,

$$K_D = \int_A B_G^T D B_G dA \quad (3)$$

being B_G and D , respectively, defined as follows:

$$B_G = \begin{bmatrix} \frac{\partial \phi}{\partial x} \\ \frac{\partial \phi}{\partial y} \end{bmatrix} = \begin{bmatrix} \frac{\partial \phi_1}{\partial x} & \frac{\partial \phi_2}{\partial x} & \dots & \frac{\partial \phi_n}{\partial x} \\ \frac{\partial \phi_1}{\partial y} & \frac{\partial \phi_2}{\partial y} & \dots & \frac{\partial \phi_n}{\partial y} \end{bmatrix} \quad (4)$$

$$D = \begin{bmatrix} D_x & 0 \\ 0 & D_y \end{bmatrix} \quad (5)$$

Additionally, K_g can be defined as,

$$K_g = \int_A g \{\phi_1 \quad \phi_2 \quad \dots \quad \phi_n\}^T \{\phi_1 \quad \phi_2 \quad \dots \quad \phi_n\} dA \quad (6)$$

and f_q , the discrete VEGF release rate vector, is defined as,

$$f_q = \int_A Q \{\phi_1 \quad \phi_2 \quad \dots \quad \phi_n\}^T dA \quad (7)$$

With this methodology, and after solving Equation (2) it is possible to determine the final VEGF in the medium, Φ , which rule the endothelial cell migration process and, consequently, the capillary network formation.

2.3. Model parameters

The capillary network growth was simulated using a square domain of $5 \times 5 \text{ mm}^2$, discretized with 2601 nodes, forming a regular nodal mesh. Three different initial domain geometries were defined, as shown in Figure 1, in accordance with previous works [11, 13]. Notice that the location of the capillary lumen, the endothelial cell monolayer and the VEGF release region change in each analysed model. Concerning the essential boundary conditions, a VEGF basal concentration of $2.35 \times 10^{-13} \text{ g mm}^{-3}$ in the capillary lumen and a concentration of $6.43 \times 10^{-13} \text{ g mm}^{-3}$ in the VEGF release region [16] was applied. The simulations follow a periodic boundary condition linking the VEGF concentration of both sides, $\bar{\Phi}(x = 0) = \bar{\Phi}(x = 5)$, being Φ the imposed VEGF concentration at the boundary. Regarding the natural boundary conditions, a VEGF flux from the VEGF release region was applied, being the VEGF chemical diffusion coefficient used $1.16 \times 10^{-6} \text{ mm}^2 \text{ s}^{-1}$ [27]. With this methodology, the VEGF gradient concentration that rules the endothelial cell migration during angiogenesis was obtained (Figure 2). This gradient goes from dark blue to yellow. The dark blue zone is located near the endothelial cell monolayer and has a VEGF concentration of around $2.35 \times 10^{-13} \text{ g mm}^{-3}$. The yellow zone is located near the VEGF release region and has the highest VEGF of around $6.43 \times 10^{-13} \text{ g mm}^{-3}$.

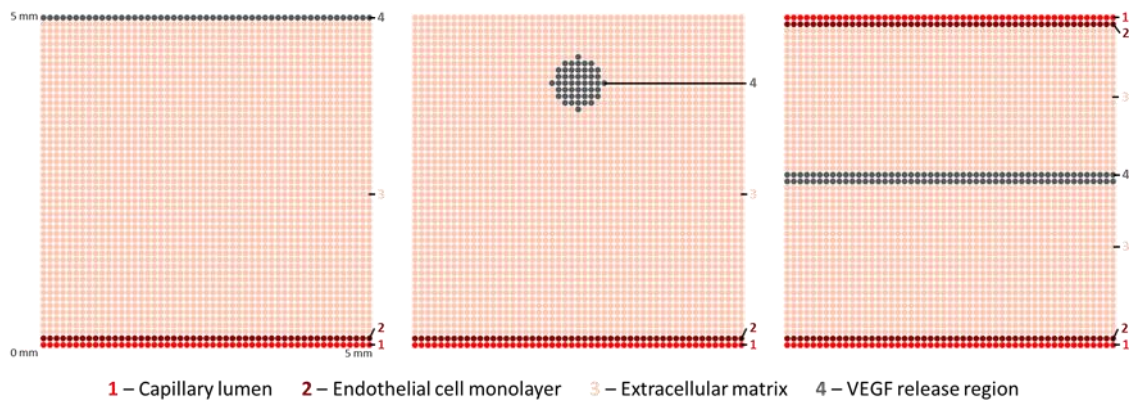


Figure 1: Initial domain geometries used in the performed simulations. The location of the capillary lumen, the endothelial cell monolayer and the VEGF release region change in the performed simulations. Label 1 (red) represents the capillary lumen, label 2 (dark red) represents the endothelial cell monolayer, label 3 (light pink) represents the extracellular matrix and label 4 (grey) represents the VEGF release region.

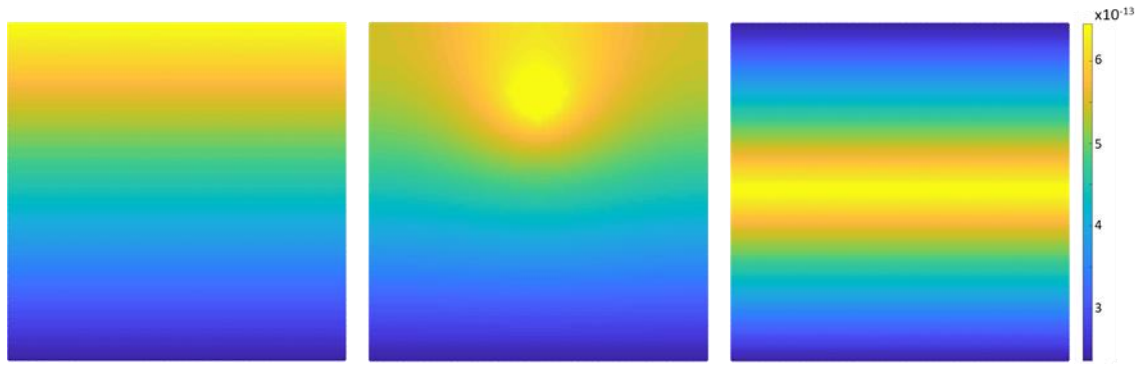


Figure 2: VEGF diffusion gradient. The dark blue zone is located near the endothelial cell monolayer and has the basal VEGF concentration. The yellow zone is located near the VEGF release region and has the highest VEGF concentration (VEGF concentration in g mm^{-3} . The scale bar used is linear).

2.4. Capillary network growth modulation

In the current model, angiogenesis was initiated by marking the starting sprouting cells, in the sub-domain defined as endothelial cell monolayer, using the Cartesian coordinates. The location and the initial number of sprouting cells (one to four) changed in the performed simulations. After the initial sprouting cells marking, the algorithm begins an iterative loop and in each time step the tip cells are marked one after the other allowing the capillary network growth. In the current model, each time step represents the time that the capillary takes to migrate one single endothelial cell. The direction of the endothelial cell movement is defined by vector $\mathbf{u}$, that follows the gradient vector from the VEGF concentration field towards the VEGF source. Accordingly, the gradient of the VEGF concentration field is obtained ($\mathbf{n} = \nabla\Phi$), being $\mathbf{n}$ oriented towards the VEGF source (in opposition to its support cell). Consequently, $\mathbf{n}_{mod} = \begin{bmatrix} \cos\theta & \sin\theta \\ -\sin\theta & \cos\theta \end{bmatrix} \mathbf{n}$. Then, the unit vector of $\mathbf{n}_{mod}$ is calculated: $\mathbf{u} = \mathbf{n}_{mod}/\|\mathbf{n}_{mod}\|$. Therefore, using the direction vector $\mathbf{u}$, the average distance between the two cells is considered and the new tip cell position is defined by summing the old tip cell position with the vector $\mathbf{u}$ multiplied by the average distance between cells. If a node exists in the new tip cell position, that node will become a tip cell. If in the new position there are no nodes, a new node is included in the domain, and that new node becomes a tip cell. In each step, the VEGF concentration is calculated and the new position of the tip cell is actualized. Moreover, the VEGF concentration at the boundary domain is imposed. The iterative process stops when the tip cell arrives to the domain boundary, to the VEGF release region or if the tip cell is near another endothelial cell.

The branching process was implemented using the same methodology detailed in a previous work [11]. Therefore, it was analysed the capillary network presented in an *in vivo*

angiogenesis assay, using the capillary order concept. The capillary order was defined according to the capillary calibre, the 1st order represents the capillaries with higher calibre and the 3rd order the ones with lowest. In this study, the effect of compressive loading in capillary branching was analysed for three strain magnitudes (5, 10 and 30% strain). We chose these strain magnitudes based in an experimental work conducted by Ruehle et al. [22]. In its work, gels containing microvascular fragments were subjected to loading applied in a triangle wave with amplitudes corresponding to 5%, 10%, or 30% strain at a frequency of 1 Hz. Then, the previous equation that regulates the branching location [11] was adjusted using this experimental data. Consequently, it was possible to obtain the following equations that regulate the number of branches points for unloaded, 5%, 10% and 30% strain:

$$\text{If } \varepsilon < 10\% \quad Nr_{branch} = -0.027\varepsilon^2 + 0.32\varepsilon + 1 \quad (8)$$

$$\text{If } \varepsilon \geq 10\% \quad Nr_{branch} = 0.009\varepsilon + 1.43 \quad (9)$$

being Nr_{branch} the number of branches points and ε the strain magnitude considered. Equations (8) and (9) were multiplied to the previous branching equation that rules the distance between consecutive branch points. With this methodology and based on the implemented computational model, the effect of compressive loading for different strain magnitudes in angiogenesis was simulated.

To analyse the effect of unloaded, 5%, 10% and 30% strain in capillary network, the total number of vessels and the total vessel length of all the traced vessels were calculated. The obtained numerical results were compared with experimental data available in the literature.

3. RESULTS

3.1. Compressive loading influences capillary network growth

To analyse the effect of compressive loading in capillary network growth, simulations under 5%, 10% and 30% strain loading were performed in all models. In the current work, we simulate the sprouting angiogenesis process for three different VEGF release regions locations. Accordingly, Figure 3 presents the capillary network pattern obtained when the VEGF release region is located in the superior boundary, Figure 4 presents the capillary network pattern obtained when a circular VEGF release region was considered and Figure 5 presents the capillary network pattern for a VEGF release region located in the domain middle. Different initial number of sprouting cells, one to four, were also considered in all the geometry domains analysed.

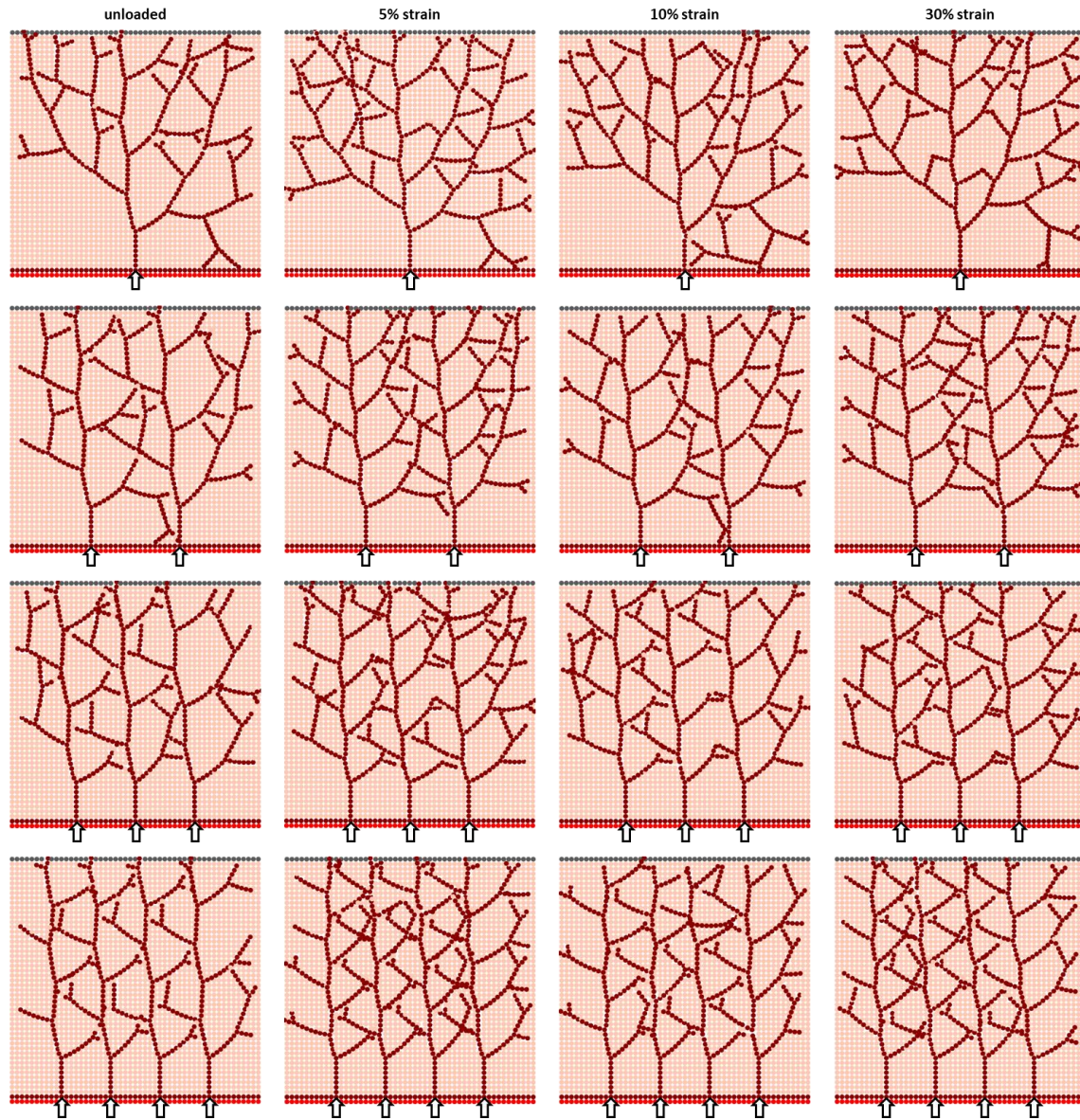


Figure 3: Final simulation results obtained for unloaded and under 5%, 10% and 30% strain loading, for a VEGF release region located in the superior boundary, using a regular nodal discretization mesh. In the first row is represented the migration for one initial sprouting cell, in the second row is represented the migration for two initial sprouting cells, in the third row is represented the migration for three initial sprouting cells and in the fourth row is represented the migration for four initial sprouting cells. The arrows mark the initial sprouting cells location. Images sized: $5 \times 5 \text{ mm}^2$.

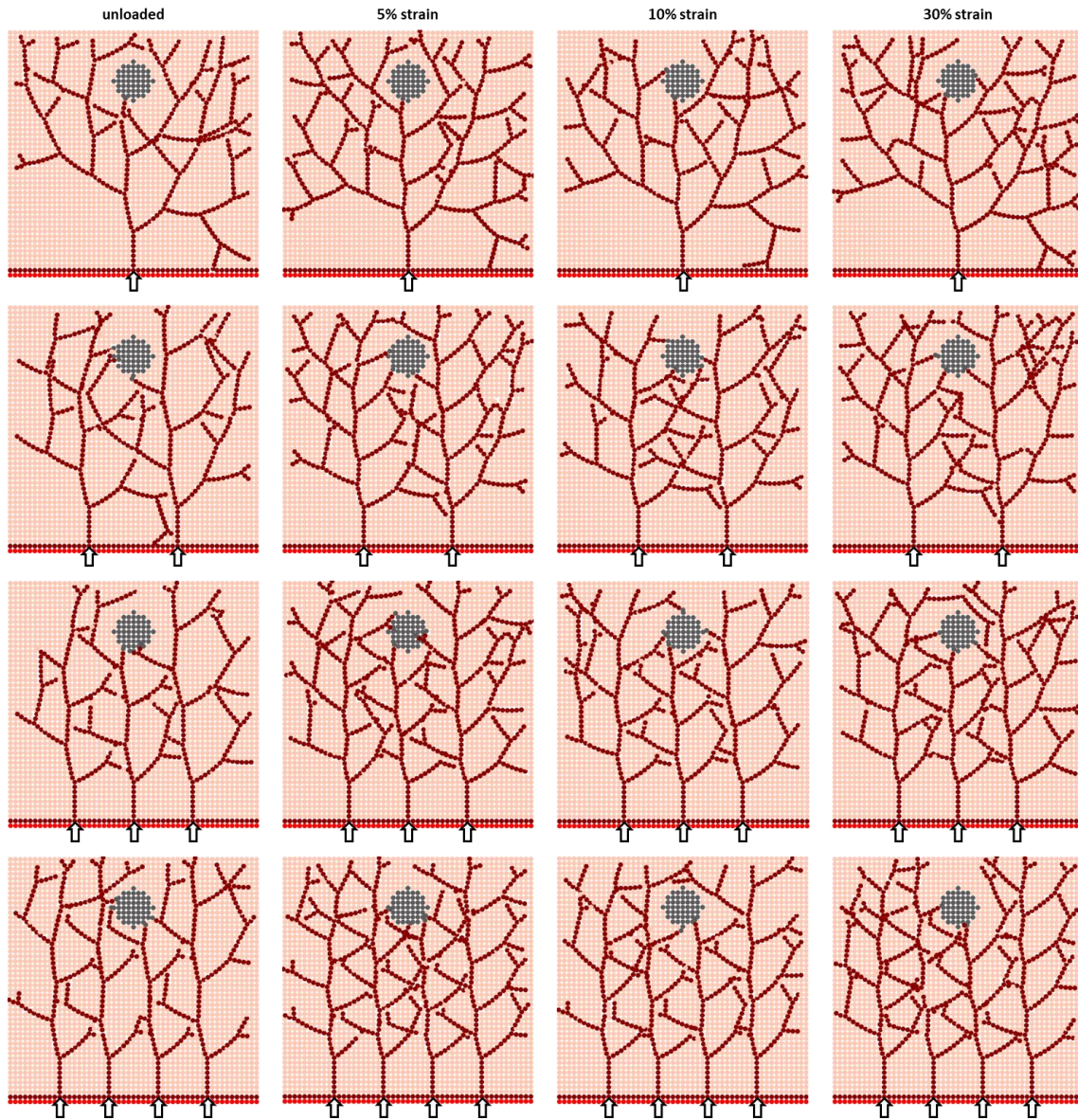


Figure 4: Final simulation results obtained for unloaded and under 5%, 10% and 30% strain loading, for a circular VEGF release region, using a regular nodal discretization mesh. In the first row is represented the migration for one initial sprouting cell, in the second row is represented the migration for two initial sprouting cells, in the third row is represented the migration for three initial sprouting cells and in the fourth row is represented the migration for four initial sprouting cells. The arrows mark the initial sprouting cells location. Images sized: $5 \times 5 \text{ mm}^2$.

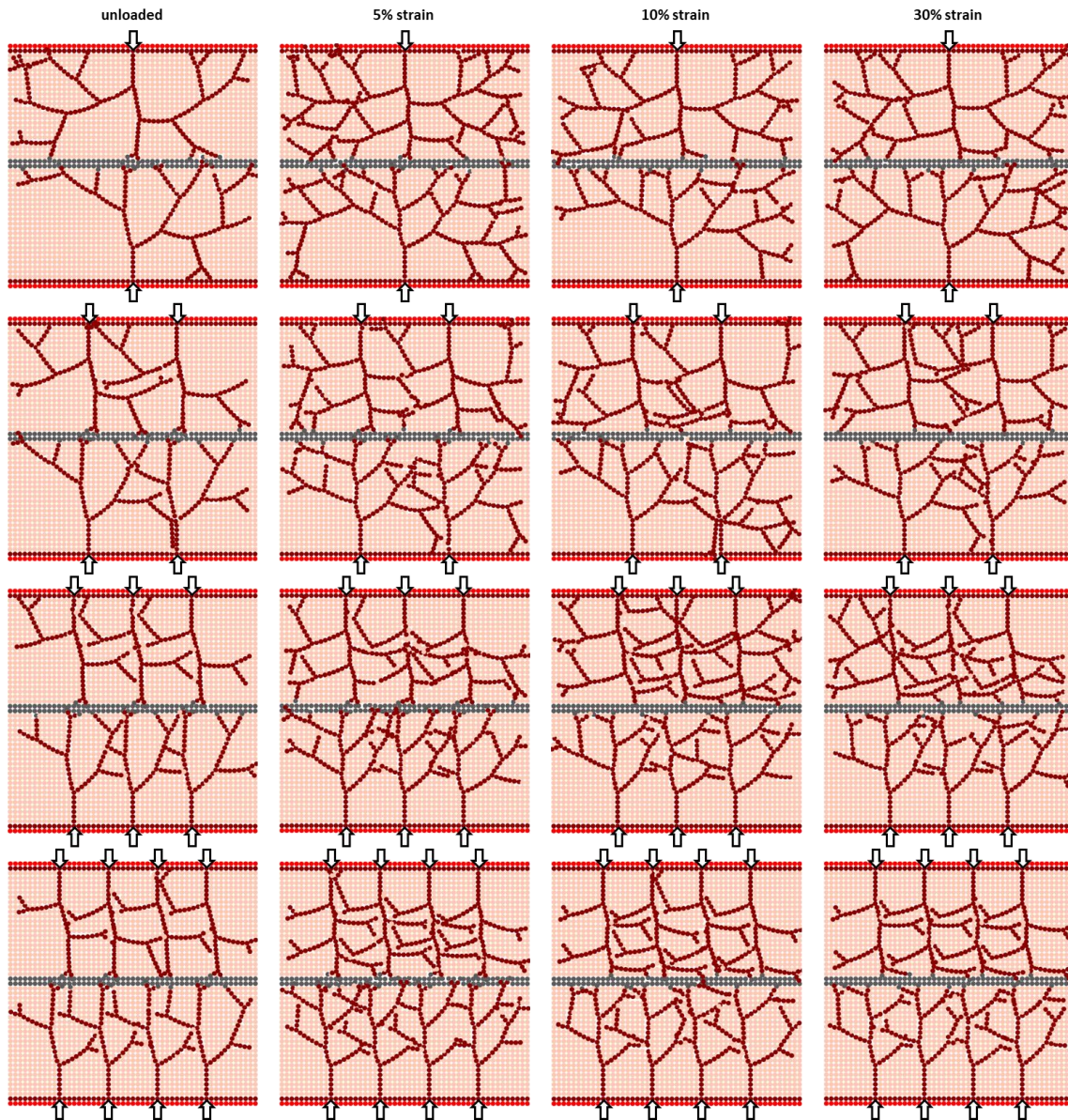


Figure 5: Final simulation results obtained for unloaded and under 5%, 10% and 30% strain loading, for a VEGF release region located in the middle, using a regular nodal discretization mesh. In the first row is represented the migration for one initial sprouting cell (each side), in the second row is represented the migration for two initial sprouting cells (each side), in the third row is represented the migration for three initial sprouting cells (each side) and in the fourth row is represented the migration for four initial sprouting cells (each side). The arrows mark the initial sprouting cells location. Images sized: $5 \times 5 \text{ mm}^2$.

Analysing Figure 3, Figure 4 and Figure 5 it is possible to verify that all the sprouting cells migrate from the parent vessel to the VEGF release region, following the VEGF gradient. Moreover, the density of capillaries increased in the simulations under 5%, 10% and 30% strain. To confirm this, it was quantified the number of branches (Table 1) and the total vessel length (Table 2) for each simulated condition (unloaded, 5%, 10% and 30% strain). Analysing Table 1, we verified that, for the VEGF release region located in the superior boundary, 5% strain loading increased the number of branches by 60% (vs. unloaded, 1 initial sprouting cell), by 57% (vs.

unloaded, 2 initial sprouting cells), by 54% (vs. unloaded, 3 initial sprouting cells) and by 72% (vs. unloaded, 4 initial sprouting cells). The 10% strain loading increased the number of branches by 43% (vs. unloaded, 1 initial sprouting cell), by 24% (vs. unloaded, 2 initial sprouting cells), by 20% (vs. unloaded, 3 initial sprouting cells) and by 30% (vs. unloaded, 4 initial sprouting cells). The 30% strain loading increased the number of branches by 46% (vs. unloaded, 1 initial sprouting cell), by 59% (vs. unloaded, 2 initial sprouting cells), by 39% (vs. unloaded, 3 initial sprouting cells) and by 53% (vs. unloaded, 4 initial sprouting cells). Concerning the circular VEGF release region, the 5% strain loading increased the number of branches by 51% (vs. unloaded, 1 initial sprouting cell), by 47% (vs. unloaded, 2 initial sprouting cells), by 68% (vs. unloaded, 3 initial sprouting cells) and by 45% (vs. unloaded, 4 initial sprouting cells). The 10% strain loading increased the number of branches by 17% (vs. unloaded, 1 initial sprouting cell), by 31% (vs. unloaded, 2 initial sprouting cells), by 38% (vs. unloaded, 3 initial sprouting cells) and in 20% (vs. unloaded, 4 initial sprouting cells). The 30% strain loading increased the number of branches by 46% (vs. unloaded, 1 initial sprouting cell), by 56% (vs. unloaded, 2 initial sprouting cells), by 71% (vs. unloaded, 3 initial sprouting cells) and by 45% (vs. unloaded, 4 initial sprouting cells). In the VEGF release region located in the middle, the 5% strain loading increased the number of branches by 106% (vs. unloaded, 1 initial sprouting cell), by 85% (vs. unloaded, 2 initial sprouting cells), by 86% (vs. unloaded, 3 initial sprouting cells) and by 81% (vs. unloaded, 4 initial sprouting cells). The 10% strain loading increased the number of branches by 56% (vs. unloaded, 1 initial sprouting cell), by 76% (vs. unloaded, 2 initial sprouting cells), by 80% (vs. unloaded, 3 initial sprouting cells) and by 52% (vs. unloaded, 4 initial sprouting cells). The 30% strain loading increased the number of branches by 81% (vs. unloaded, 1 initial sprouting cell), by 59% (vs. unloaded, 2 initial sprouting cells), by 71% (vs. unloaded, 3 initial sprouting cells) and by 45% (vs. unloaded, 4 initial sprouting cells).

Table 1: Quantification of the number of branches for unloaded and under 5%, 10% and 30% strain loading, for the three different initial geometry domains tested (VEGF release region in the superior boundary, circular VEGF release region and VEGF release region in the middle).

		Number of branches			
VEGF release region in the superior boundary					
		unloaded	5% strain	10% strain	30% strain
Initial number	1	35	56	50	51
of sprouting	2	37	58	46	59
cells	3	41	63	49	57
	4	43	74	56	66
Circular VEGF release region					
		unloaded	5% strain	10% strain	30% strain
Initial number	1	35	53	41	51
of sprouting	2	36	53	47	56
cells	3	34	57	47	58
	4	44	64	53	64
VEGF release region in the middle					
		unloaded	5% strain	10% strain	30% strain
Initial number	1	32	66	50	58
of sprouting	2	34	63	60	54
cells	3	35	65	63	60
	4	42	76	64	61

The results obtained for the effect of strain loading in total vessel length are presented in Table 2. Accordingly, it was verified that, in the VEGF release region located in the superior boundary, 5% strain loading increased the total vessel length by 30% (vs. unloaded, 1 initial sprouting cell), by 25% (vs. unloaded, 2 initial sprouting cells), by 22% (vs. unloaded, 3 initial sprouting cells) and by 32% (vs. unloaded, 4 initial sprouting cells). The 10% strain loading increased the total vessel length by 26% (vs. unloaded, 1 initial sprouting cell), by 13% (vs. unloaded, 2 initial sprouting cells), in 8% (vs. unloaded, 3 initial sprouting cells) and by 14% (vs. unloaded, 4 initial sprouting cells). The 30% strain loading increased the total vessel length by 19% (vs. unloaded, 1 initial sprouting cell), by 29% (vs. unloaded, 2 initial sprouting cells), by 14% (vs. unloaded, 3 initial sprouting cells) and by 26% (vs. unloaded, 4 initial sprouting cells). Concerning the circular VEGF release region, the 5% strain loading increased the total vessel length by 21% (vs. unloaded, 1 initial sprouting cell), by 21% (vs. unloaded, 2 initial sprouting cells), by 36% (vs. unloaded, 3 initial sprouting cells) and by 26% (vs. unloaded, 4 initial sprouting cells). The 10% strain loading increased the total vessel length by 5% (vs. unloaded, 1 initial sprouting cell), by 14% (vs. unloaded, 2 initial sprouting cells), by 21% (vs. unloaded, 3 initial sprouting cells) and by 13% (vs. unloaded, 4 initial sprouting cells). The 30% strain loading increased the total vessel length by 19% (vs. unloaded, 1 initial sprouting cell), by 26% (vs. unloaded, 2 initial sprouting cells), by 35% (vs. unloaded, 3 initial sprouting cells) and by 26% (vs.

unloaded, 4 initial sprouting cells). For the VEGF release region located in the middle, the 5% strain loading increased the total vessel length by 65% (vs. unloaded, 1 initial sprouting cell), by 43% (vs. unloaded, 2 initial sprouting cells), by 36% (vs. unloaded, 3 initial sprouting cells) and by 37% (vs. unloaded, 4 initial sprouting cells). The 10% strain loading increased the total vessel length by 34% (vs. unloaded, 1 initial sprouting cell), by 47% (vs. unloaded, 2 initial sprouting cells), by 51% (vs. unloaded, 3 initial sprouting cells) and by 29% (vs. unloaded, 4 initial sprouting cells). The 30% strain loading increased the total vessel length by 48% (vs. unloaded, 1 initial sprouting cell), by 38% (vs. unloaded, 2 initial sprouting cells), by 43% (vs. unloaded, 3 initial sprouting cells) and by 24% (vs. unloaded, 4 initial sprouting cells).

Table 2: Quantification of the total vessel length (mm) for unloaded and under 5%, 10% and 30% strain loading, for the three different initial geometry domains tested (VEGF release region in the superior boundary, circular VEGF release region and VEGF release region in the middle).

		Total vessel length (mm)			
VEGF release region in the superior boundary					
		unloaded	5% strain	10% strain	30% strain
Initial number	1	39.3	50.9	49.3	46.6
of sprouting	2	41.4	51.6	46.6	53.2
cells	3	45.7	55.7	49.4	52.0
	4	46.1	60.7	52.6	58.0
Circular VEGF release region					
		unloaded	5% strain	10% strain	30% strain
Initial number	1	39.8	48.0	41.6	47.4
of sprouting	2	40.1	48.5	45.7	50.4
cells	3	38.9	53.0	47.2	52.7
	4	45.4	57.1	51.2	57.3
VEGF release region in the middle					
		unloaded	5% strain	10% strain	30% strain
Initial number	1	31.9	52.7	42.7	47.4
of sprouting	2	34.3	49.0	50.3	47.3
cells	3	37.2	50.5	56.3	53.1
	4	40.8	56.0	52.8	50.6

Accordingly, it was possible to conclude that all strain magnitudes tested promoted angiogenesis, being the 5% strain loading the most effective one.

3.2. Comparison between numerical and experimental results

To validate the robustness of the proposed model at predicting the angiogenic response for different strain loadings, it was considered data from an experimental study. In the work of Ruehle et al. [22], gels containing microvascular fragments were loaded in homogeneous compression to generate an initially homogeneous deformation. Moreover, loading was applied in a triangle wave with amplitudes corresponding to 5%, 10%, or 30% strain at a frequency of 1 Hz. Then, the microvascular network length and number of branches under 5%, 10%, and 30% strain loading were quantified. Figure 6 and Figure 7 show the comparison between the numerical results obtained in the previous described simulations (coloured dashed lines) and the experimental data presented in the work of Ruehle et al. (black solid line). In these images, the results were normalized to the unloaded situation. Figure 6 clearly demonstrates that the number of branches agreed between the numerical results and the experimental data, mainly when the VEGF release region is located in the superior boundary and in the circular VEGF release region domain. Regarding the total vessel length (Figure 7), the numerical results and the experimental ones are more distant; however, it is possible to observe some similarity.

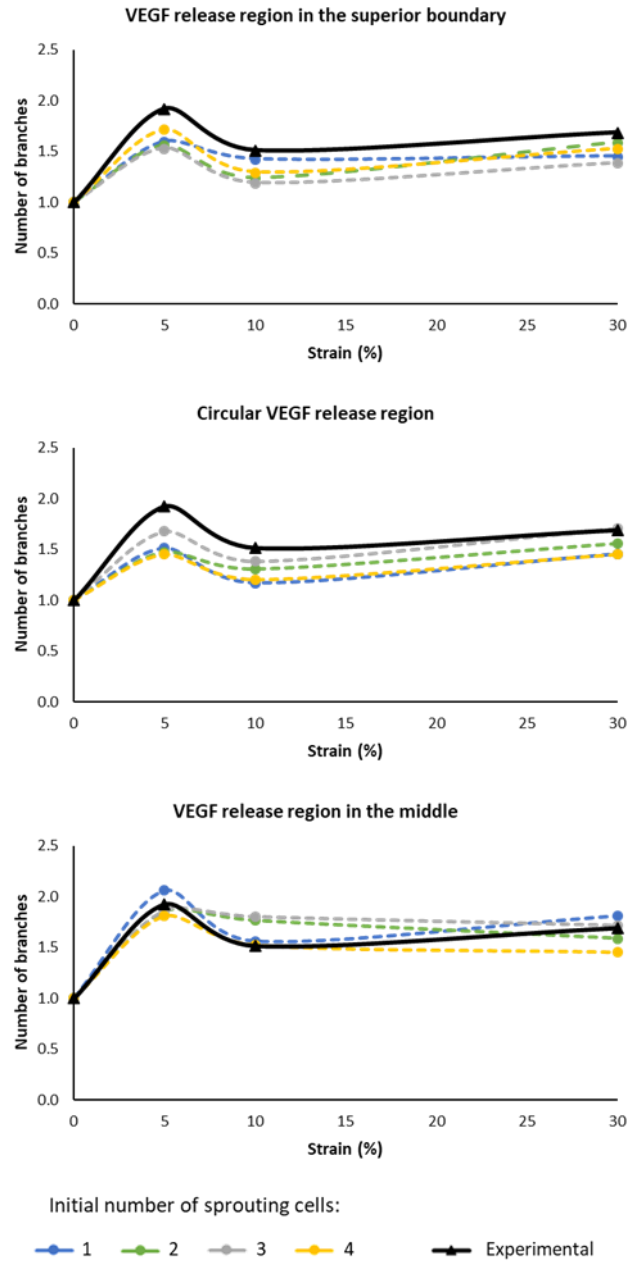


Figure 6: Quantification of the number of branches for unloaded and under 5%, 10% and 30% strain loading, for the three different initial geometry domains tested (VEGF release region in the superior boundary, circular VEGF release region and VEGF release region in the middle). All the results were normalized to the unloaded situation. The experimental data was obtained in [22].

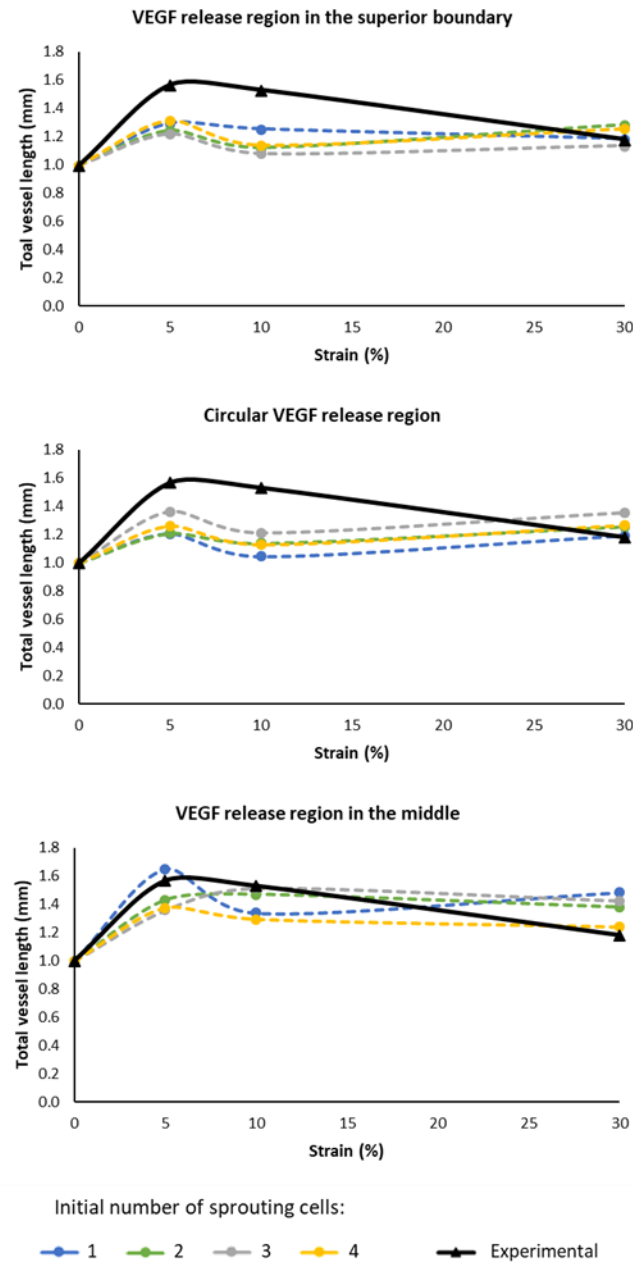


Figure 7: Quantification of the total vessel length (mm) for unloaded and under 5%, 10% and 30% strain loading, for the three different initial geometry domains tested (VEGF release region in the superior boundary, circular VEGF release region and VEGF release region in the middle). All the results were normalized to the unloaded situation. The experimental data was obtained in [22].

Finally, it was analysed the effect of compressive loading in two different experimental domains (Example 1 and 2) that were already validated in previous works [11, 12]. In these works, it was compared the capillary network profile obtained in the simulation with the ones obtained in an *in vivo* angiogenesis assay and it was found a good agreement between the two methodologies used (numerical and experimental) [11, 12]. Figure 8 shows the effect of 5%, 10% and 30% strain in the number of branches and total vessel length for the two different

experimental domains already performed. As in the previous results, it was verified that all the strain magnitudes tested promoted angiogenesis, by increasing the number of branches and the total vessel length. As expected, in these two experimental domains the 5% strain was the most effective strain magnitude. Once again, the obtained numerical results were similar to the ones reported by Ruehle et al., mainly the ones related to the number of branches.

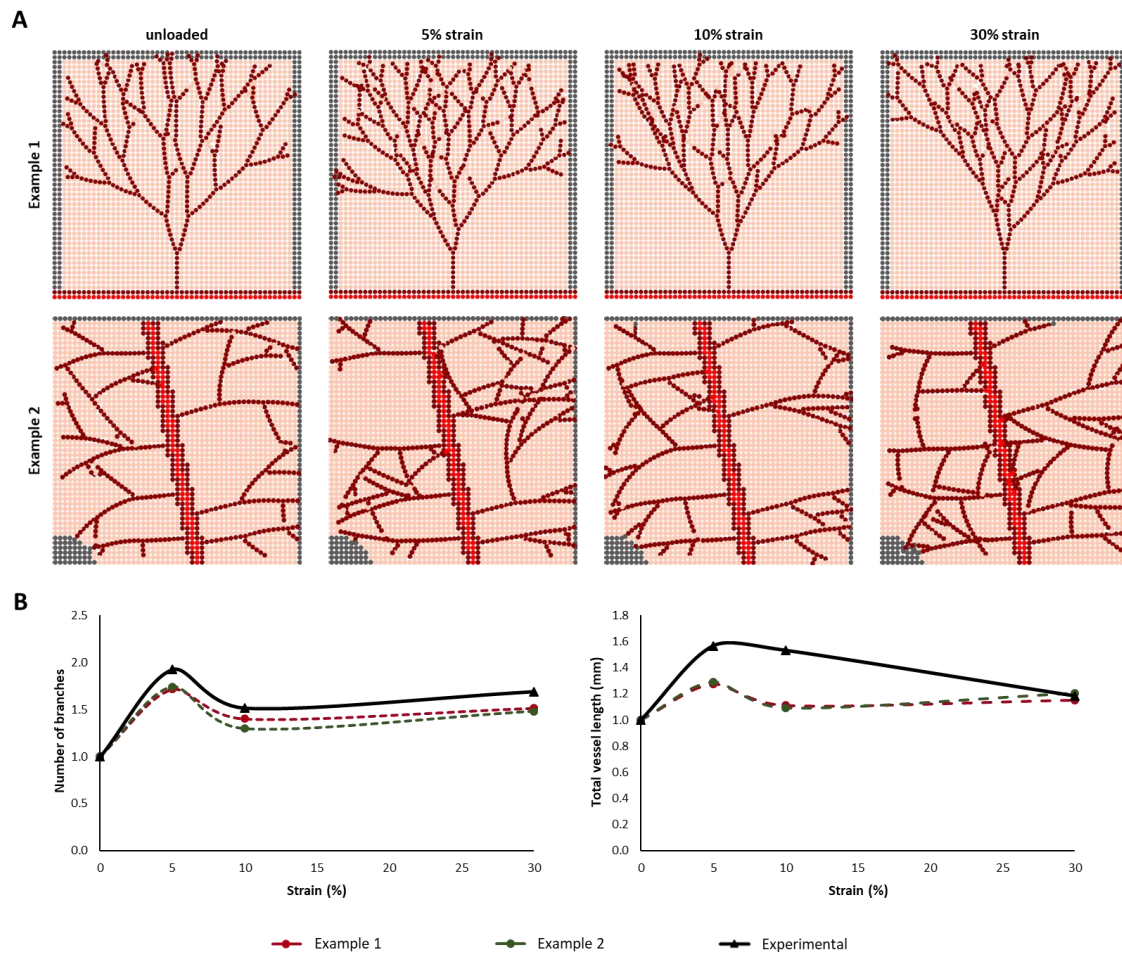


Figure 8: Effect of compressive loading in angiogenesis. **A)** Final simulation results obtained for unloaded and under 5%, 10% and 30% strain loading, using a regular nodal discretization mesh. Images sized: $5 \times 5 \text{ mm}^2$; **B)** Quantification of the number of branches and the total vessel length (mm) for unloaded and under 5%, 10% and 30% strain loading, for the two experimental domains tested. All the results were normalized to the unloaded situation. The experimental data was obtained in [22].

4. DISCUSSION

Blood vessels, and consequently endothelial cells, reside in mechanically active environments and are constantly exposed to stress and strain. It has already been demonstrated that fluid shear stress, stretch and pressure regulate cytoskeletal organization, signal transduction, gene expression and endothelial cell function, including proliferation, migration

and extracellular matrix remodelling [10, 15, 21, 29]. Understand the cellular and molecular behaviour of endothelial cells responding to mechanical stimulus is essential to improve tissue vascularization and promote wound healing and tissue engineering development.

In this work, we simulated the effect of compressive loading in angiogenesis, for three different strain magnitudes (5, 10 and 30% strain), using a meshless computational model. The pertinence of using computational models to address biological process, such as angiogenesis, started to be studied in the last decades given its potential to decouple complex processes using reproductive and relatively economic approaches. Several models analysed the effect of growth factors and oxygen in endothelial cell behaviour, such as proliferation, differentiation and migration [18, 20], others simulated specific phases of angiogenesis, such as branching and anastomosis [2, 4] and also studied the morphology of the vascular network [26], with different levels of complexity. Nevertheless, the effect of mechanical cues in angiogenesis was less explored.

In this work, it was analysed the effect of compressive loading in endothelial cell migration, considering different domains geometries – VEGF release region located in the superior boundary, circular VEGF release region and VEGF release region located in the middle. In all the performed simulations, endothelial cells migrate from the parent vessel to the regions with higher VEGF concentration. This migration profile was similar to the one reported in previous works [11, 13]. Figure 3, Figure 4 and Figure 5 indicate that compressive loading promote angiogenesis. To confirm this, the number of branches (Table 1) and the total vessel length (Table 2) for each simulated condition (unloaded, 5%, 10% and 30% strain) were quantified. Despite the different tested domains and the initial number of sprouting cells, it was verified that, approximately, 5%, 10% and 30% strain increased the number of branches, respectively, by 68%, by 41% and by 56%. It was also verified that, approximately, 5%, 10% and 30% strain increased the total vessel length, respectively, by 33%, by 23% and by 29%. Accordingly, all the tested strain magnitudes promoted angiogenesis, being the 5% strain the most effective one. Then, the obtained numerical results were compared with experimental data presented in the literature [22]. Ruehle et al. showed that compressive loading increase the vascular network length and branching at all the three tested strain magnitudes, including the high 30% strain. Accordingly, the simulations performed in this work allowed to achieve the same findings. Analysing Figure 6, it was verified that the numerical results for the branching number are quite similar to the ones presented in the experimental study. Regarding the total network length (Figure 7) the results are less comparable. Nevertheless, it is important to be aware that angiogenesis is a very complex process with many biological variables, different cell types and sophisticated regulatory mechanisms, being difficult to capture the overall process in

just one computational model. Therefore, the proposed model is only capable to simulate the effect of mechanical loading in angiogenesis to some extent. It was also analysed the effect of compressive loading in two simulation domains extracted for an experimental assay and already validated in our previous works (Figure 8). Also in these two situations, all the strain magnitudes tested promoted angiogenesis, being the 5% strain the most effective one. As in the previous findings, the obtained numerical results were similar to the experimental ones reported by Ruehle et al. [22], mainly the ones related to the number of branches.

In our model, the endothelial cell migration was governed by a diffusion-reaction equation for VEGF. Moreover, the VEGF release rate was assumed as constant in time and its natural decay was not considered. The branching process was implemented in the presented model using the same methodology carefully detailed in a previous work [11]. Then, to implement the effect of compressive loading in branching occurrence, it was used data obtained in an experimental study [22], and Equations (8) and (9) were obtained. These equations allowed us to actualize the branching process in order to study the effect of strain in angiogenesis. Nevertheless, the use of experimental curves to obtain these equations brings limitations from a mechanical perspective since the viscoelastic properties of the tissue, the dynamic loading effect and the effect of the time variable were not considered. In this work, the loading was applied as homogeneous compression in all domain to generate an initially homogeneous deformation that continued from the beginning to the end of the simulation. However, it was already demonstrated, in bone studies, that early and delayed loading have different effects in angiogenesis. Early mechanical loading inhibits vascular invasion but delayed loading stimulates vascular remodelling by increase the number of large vessels and by decrease the number of small vessels [3]. This suggests that not only the strain magnitude but also the initial time of force application influence angiogenesis (a future research topic). Mechanical forces generated by blood flow, specifically shear stress and cyclic strain activate endothelial cell signalling cascades and consequently gene expression that promote angiogenesis [7, 14]. Accordingly, in the upcoming models it is intended to include the blood flow in order to analyse the effect of blood flow, pressure and vessel diameter in the shear stress and its resultant effect in angiogenesis.

In conclusion, the model proposed in this study simulates the effect of compressive loading in the branching process. The numerical results show that all the compressive loadings tested increased the vascular network length and number of branches, including the high 30% strain. The capillary network obtained resembles the one presented in experimental assays and the obtained numerical results correspond to the experimental ones. The combination of computational and experimental studies will be very useful, in the future, to understand and to

define which are the mechanical cues that promote angiogenesis, in order to optimize laboratory protocols and cell culture conditions and to decrease the time and cost of experiments. This will allow to improve tissue vascularization and, consequently, the wound healing process.

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DECLARATION OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter IV

Thesis Considerations

4.1. General Discussion

Angiogenesis is a complex and well-coordinated process. In order to promote cell viability and tissue homeostasis, it is necessary to fully understand this process. To do so, it is often required to join the knowledge of different fields such as biology, chemistry, engineering, mathematics and medicine. Conventionally, researchers use experimental assays to study endothelial cell behaviour. However, in recent years, computational models arouse the interest of scientific community, since they allow to analyse biological processes in different scales by using distinct approaches. Accordingly, it has already been demonstrated that meshless methods could efficiently be used to model biological process such as bone remodelling [39], cellular growth [40] and vestibular system dynamics [41]. Moreover, meshless methods allow the analysis of any phenomenon that is governed by a differential equation, such as the cell migration modulated by growth factors gradient. Indeed, in Contributions II, III, IV and V it was demonstrated that RPIM formulation can be used to proper simulate sprouting angiogenesis.

Angiogenesis can be induced in response to tissue injury. During wound healing, endothelial cells sense the angiogenic growth factors present in the medium and start to proliferate and to migrate, allowing the formation of the new sprouting vessel due to chemotaxis. Considering all the growth factors involved in this process, VEGF is the main pro-angiogenic one [42, 43]. Moreover, VEGF is produced by different cell types that participate in wound repair such as endothelial cells, fibroblasts, keratinocytes, smooth muscle cells and immune cells [44]. Accordingly, the endothelial cell migration regulated by VEGF diffusion

gradient created from the release of wounds was modelled in Contributions II and III. One of the strategies used in the field of pro-angiogenic therapies to promote wound healing is the delivery of VEGF from biomaterials [45]. Thus, this condition was addressed in Contribution IV. In the three previous mentioned works, the endothelial cell migration follows the VEGF diffusion gradient and accordingly to the chemotaxis effect described in experimental studies [46-48]. Indeed, several computational models described in the literature analysed the chemoattractant effect in angiogenesis. However, many of them are continuum models in which the individual endothelial cell behaviour and the vascular pattern are not considered. Moreover, the mathematical assumptions that regulate processes such as branching are not conducted using experimental data or the numerical results are not compared with experimental results. Accordingly, Contributions II, III and IV were conducted in order to overcome some of these drawbacks.

In Contribution II, it was demonstrated that the RPIM formulation could be combined with the VEGF diffusion gradient and with the endothelial cell migration in order to simulate cell migration due to chemotaxis. The model presented in this work addressed only the endothelial cell migration, which is the first step of angiogenesis. Nevertheless, it was demonstrated that the numerical formulation presented in Contribution II is wide and robust, capable enough to support the inclusion of other processes such as branching and anastomosis. The inclusion of these processes in further models will increase the mesh distortion and it was already demonstrated that meshless methods are extremely useful in these situations [33, 49]. Then, in Contribution III in order to increase the computational model complexity, the branching process was implemented. To simulate biological processes, it is necessary to make some assumptions and to insert some parameters in the computational model. Ideally, these parameters should be obtained using direct *in vivo* measurements; however, this is not always practicable due to the lack of technical support or due to ethical issues. Nevertheless, in this study the branching process was implemented in the computational model using data acquired with an *in vivo* angiogenesis assay. Since this reduces the parameters estimation and makes the model closer to reality it represents an important advance comparatively to the existent models. Afterwards, in order to validate the computational model and to confirm that the branching process was correct implemented in the algorithm, the numerical results were compared with the ones obtained with the CAM assay. For total branching number (*in silico* = 25.50 ± 1.39 ; *in vivo* = 23.00 ± 2.01 ; $p = 0.37$), total vessel length (*in silico* = 34.66 ± 0.98 mm; *in vivo* = 30.27 ± 1.74 mm; $p = 0.07$) and branching angle (*in silico* = 64.68 ± 3.59 degree; *in vivo* = 70.27 ± 2.37 degree; $p = 0.26$) no statistical differences were obtained between the *in vivo* and the *in silico* methodologies used. Moreover, a qualitatively analysis regarding the capillary network pattern using the

difference between the total capillary volume fractions was performed. Differences between 11% and 15% were obtained between the two methodologies used. Therefore, in Contribution III, a CAM assay was used for the first time to simulate the sprouting angiogenesis during wound healing modulated by the VEGF diffusion. This methodology allows to achieve capillary networks with realistic structure and morphology. The combination between mathematical formulation and *in vivo* protocols presented in this study will enable the optimization of cell culture conditions and to decrease the time and cost of experimental assays, allowing to improve knowledge on the wound healing process.

In Contribution IV, the previous model was extended and the angiogenic response to several VEGF concentrations was simulated. As in the previous study, the CAM assay was used to obtain the phenomenological laws that rule the branching process for control and for VEGF treatments and to validate the numerical results. Thus, this study presents the novelty of addressing the predictive capacity of the computational model to simulate angiogenesis modulated by different VEGF concentrations as well as provides a quantitative and a qualitative evaluation of angiogenesis compared with an *in vivo* angiogenesis assay. Regarding the quantitative analysis, the total vessel number and the total vessel length were quantified between the *in silico* and the *in vivo* methodologies and a reliable agreement between them were obtained. The qualitative analysis of the capillary network pattern was done, once again, using the difference between the total capillary volume fractions. For the lower VEGF concentrations tested (control and VEGF 50ng), the capillary network achieved with the model presented a very realistic structure and morphology, with differences between 22% and 32%. For the highest VEGF concentration tested (100ng), the difference between the total capillary volume fractions increased to 49% in one of the examples performed. *In vivo*, it was already demonstrated that higher VEGF concentrations promotes the rapid formation of disorganized, poorly remodeled and dysfunctional blood vessels [50, 51]. Additionally, to identify and to isolate the very thin blood vessels presented in the CAM area is a demanding task. Accordingly, in the highest VEGF concentration, the capillary network extracted from the CAM images is more distant from reality, which impairs the comparison of the capillary network pattern obtained between the two methodologies. Despite all the advantages addressed by *in vivo* models to understand angiogenesis, concerns about using animals in science has been increasing over the last years [52]. Accordingly, *in silico* models emerge as alternative methodologies to study angiogenesis [53] and to promote wound healing, allowing to reduce the number of animals used in science. Since biomaterials are commonly used in tissue engineering to delivery VEGF to the wound site in order to promote wound repair, Contribution IV is one example of how computer models can be successfully used in this field.

The effect of oxygen and growth factors, mainly the VEGF, in endothelial cell behaviour and in angiogenesis has been extensively studied [35]. However, less is known about the effect of mechanical stimulus in angiogenesis. Since endothelial cells are constantly exposed to stress and strain fields the insertion of its effect in angiogenesis modelling is essential in order to address new strategies to promote angiogenesis and wound healing. Contribution V was conducted as an effort to overcome these blanks. In this study, it was demonstrated that compressive loading, 5%, 10% and 30% strain, promotes angiogenesis by increasing the number of branches and the total vessel length for different domain geometries. When the numerical results were compared with the experimental ones [38], a good agreement between them was found especially concerning the number of branches. Moreover, the effect of compressive loading in angiogenesis was modelled using experimental data. For this, the previous established phenomenological laws that regulate the branching occurrence were adjusted, allowing to study the effect of strain in angiogenesis. The effect of strain was implemented using experimental curves which brings limitations considering the tissue viscoelastic properties, the dynamic loading effect and the rule of the time variable. Even though, Contribution V demonstrates the pertinence of using computational approaches to study the effect of mechanical laws in angiogenesis.

Although the previous discussed contributions were conducted in order to overcome some drawbacks presented by the numerical models in the field of wound healing angiogenesis described in the literature, the models addressed during the conduction of this thesis present limitations. First, angiogenesis was modelled using a 2D domain, which despite allowing to test different numerical approaches and to explore distinct biological processes is a simple domain. Indeed, the 2D domain used in all the contributions here presented is actually an infinite 2D domain in which periodic boundary conditions were used. With this methodology it is easier to verify if the code is being well implemented since the growth of the capillary is more predictable. Second, the VEGF diffusion from the wound or from the biomaterial was assumed as constant during all the iterative process and the VEGF uptake by endothelial cells and its natural decay was not included in the code. Third, the branching process was implemented using data extracted from the analysis of several CAM images, such as the distance between consecutive branches and the branching angle, for control and for VEGF treatments. Then, the phenomenological laws that rule the branching occurrence were obtained and implemented in the computational model, allowing to reproduce the capillary network displayed in the CAM images. However, if another biological condition was studied, the new experimental data should be analysed and the phenomenological laws need to be updated. The current model is not yet generalizable which constitutes a limitation of this thesis.

The results of this thesis highlight the pertinence of using computational models to address biological processes, such as angiogenesis. Moreover, it was demonstrated that the collaboration between biochemistries, biologists, engineers and physicians is very useful in order to develop truthful models, as closer to reality as possible.

4.2. Conclusions and Future Work

With this thesis, the proposed aims and challenges were successfully achieved contributing to increase the knowledge in the field of wound healing angiogenesis, and the resultant research outputs were published in international journals. Moreover, a review paper addressing the existing wound healing angiogenesis models was developed and published being this an achievement of the present work.

The established models demonstrated that the RPIM formulation could efficiently simulate angiogenesis modulated by the VEGF diffusion, allowing to obtain capillary networks with realistic structure and morphology. The obtained numerical results were in accordance with the ones obtained with the CAM assay, providing the accuracy of the developed models. Moreover, the effect of compressive loading in the branching process was well simulated.

The combination between computational models and experimental assays demonstrated in this thesis will contribute, in the future, to build more complex models able to improve wound repair. The development of robust *in silico* approaches will allow to study individual cell behaviour as well as the interaction between different cells and its substrates, to test several experimental protocols and culture conditions in order to achieve the best results and to reduce the time and cost of experimental methodologies as well as the number of animals used in science.

This thesis should not be viewed as a finalized work, but rather as the beginning of several research possibilities. Accordingly, in the upcoming models it is intended to include the effect of heterogeneous and anisotropic behaviour of the ECM on angiogenesis. Moreover, the VEGF diffusion from the wound or from the biomaterial should also be modelled as a variable in time which better mimics the natural VEGF decay and its consumption by the endothelial cells. It has already been demonstrated that shear stress is a crucial factor to stimulate cells and activate downstream signaling. Once stimulated by shear stress, cells transform it in biochemical signals and adjust their physiological response. Previous studies have shown that laminar flow stimulates mechanosensors and also the carries of VEGF. Moreover, regions of positive shear

stress are also regions of high VEGF concentration and sprouts formation. Nevertheless, the effect of shear stress on network morphogenesis is still unclear, and this should be addressed. Accordingly, computational fluid dynamics will be applied in future models to simulate the blood flow. The blood flow will then be used to estimate the fluid shear stress effect on the endothelial cells and to calculate solute fields of growth factors and oxygen. Moreover, the effect of pressure and vessel diameter in angiogenesis will also be studied. The inclusion of all these parameters and processes in the model will make it more complex, bringing extra computational cost but will bring it closer to reality, allowing to extract important numerical results that after experimental validation will possible contribute to improve angiogenesis in wound healing.

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Appendix I

Meshless Methods

1.1. Radial Point Interpolation Method

In the last years, the use of meshless methods for numerically solving partial differential equations start to be under focus. Accordingly, meshless methods can be successfully used to solve computational mechanical problems. Briefly, the generic meshless method procedure starts with the discretization of the problem domain $\Omega \in \mathbb{R}^2$, bounded by a physical boundary $\Gamma \subset \Omega$, using a nodal set $\mathbf{N} = \{n_0, n_1, \dots, n_N\}$ distributed in the space domain, $\mathbf{X} = \{x_1, x_2, \dots, x_N\} \in \Omega$. Then, a nodal dependent or independent background integration mesh is constructed, regarding the meshless method used. After that, the nodal connectivity is obtained for each integration point using the influence-domain concept. Finally, the shape functions are constructed using approximation or interpolation functions, allowing to obtain the global system of equations. In this appendix, a detailed description of these steps is presented for the Radial Point Interpolation Method (RPIM) [1], which was the meshless method addressed during the development of this thesis.

1.1.1. Nodal Connectivity

In RPIM, the nodal connectivity needs to be determined after the nodal discretization since there is no predefined nodal interdependency. Accordingly, the nodal interdependency is established with the “influence-domain” geometric construction, which is obtained after the

nodal discretization and the construction of the background integration mesh [2]. The overlap of all the influence domains allows to determine the nodal connective in RPIM.

The influence-domain of each integration point is obtained by searching radially enough nodes inside a fixed area (two-dimensional problems) or volume (three-dimensional problems) centred in the integration point under study, as can be seen in Figure 1. It has already been observed that variations in the influence-domains size and shape affect the final solution of the meshless method. To overlap this problem, all the influence-domains should have similar number of nodes. For this reason, RPIM uses variable size influence-domains, with constant number of nodes inside of each domain. In the literature, it is recommended to use between 9 and 16 nodes inside of each two-dimensional influence-domain [1, 3].

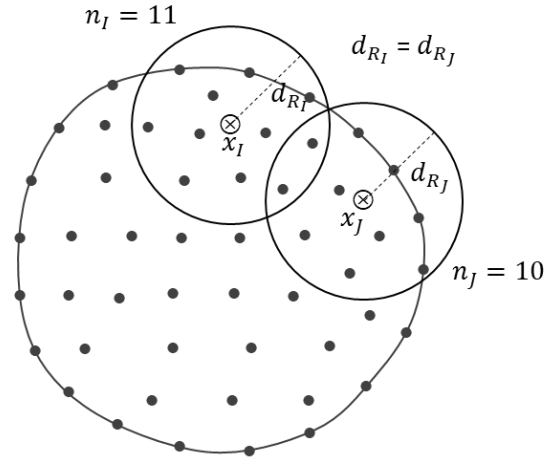


Figure 1: Influence-domain in RPIM for a two-dimensional problem.

1.1.2. Numerical Integration

In RPIM, that is a discrete numerical method that uses the Galerkin weak formulation, to perform the numerical integration of the integro-differential equations that governs the physical phenomenon under study it is necessary to construct the background integration mesh. A substantial fraction of the total computational cost of the analysis is due to the numerical integration process.

In RPIM, the background integration mesh is obtained by dividing the solid domain in a regular grid forming quadrilateral integration cells [1, 4], and subsequently each cell is filled with integration points, following the Gauss–Legendre quadrature rule [2]. This technique is briefly described in Figure 2. Accordingly, the solid domain is divided in a regular grid allowing to divide the problem domain with integration cells [Figure 2 a)]. Then, each integration cell is

transformed into an isoparametric square [Figure 2 b)] and filled with Gauss points, respecting the Gauss-Legendre quadrature scheme [Figure 2 c)]. As can be seen in Figure 2 c), the grid cover more than the physical domain. Consequently, it is necessary to remove the integration points that are outsider the solid domain in order to obtain an integration cell grid adjusted to the physical domain [Figure 2 d)] [2]. RPIM uses regular quadrature integration meshes because it is simpler to apply.

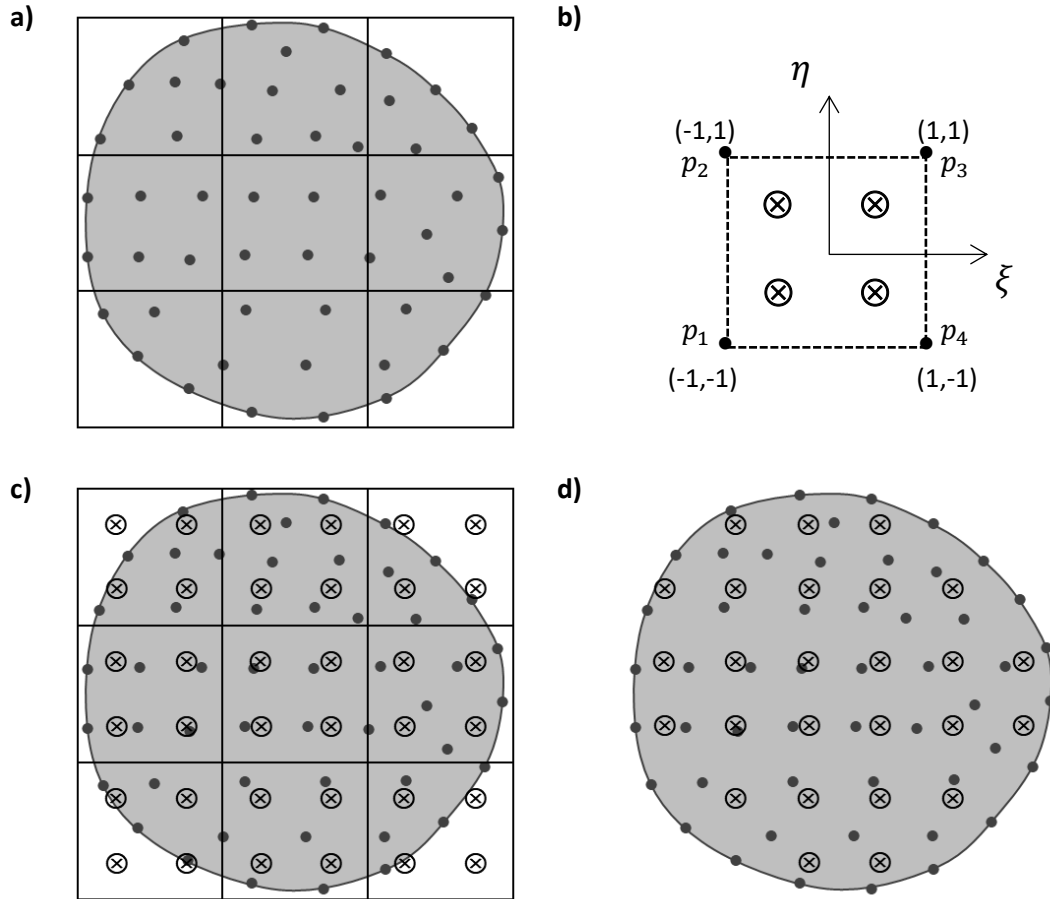


Figure 2: Schematic representation of the construction of the background numerical integration mesh following the Gauss-Legendre quadrature scheme. **a)** The solid domain is divided in a regular grid; **b)** Gauss-Legendre quadrature following a 2×2 scheme per integration cell; **c)** Gauss-Legendre quadrature points are distributed inside the isoparametric square using a 2×2 scheme per integration cell; **d)** Final integration cell grid adjusted to the physical domain.

The Cartesian coordinates of the quadrature points are obtained using isoparametric interpolation functions, N_i . The isoparametric interpolation functions for quadrilaterals are presented in Equation (1) and for triangles in Equation (2).

$$\begin{aligned}
N_1(\xi, \eta) &= \frac{1}{4} (1 - \xi)(1 - \eta) \\
N_2(\xi, \eta) &= \frac{1}{4} (1 - \xi)(1 + \eta) \\
N_3(\xi, \eta) &= \frac{1}{4} (1 + \xi)(1 + \eta) \\
N_4(\xi, \eta) &= \frac{1}{4} (1 + \xi)(1 - \eta)
\end{aligned} \tag{1}$$

$$\begin{aligned}
N_1(\xi, \eta) &= 1 - \xi - \eta \\
N_2(\xi, \eta) &= \eta \\
N_3(\xi, \eta) &= \xi
\end{aligned} \tag{2}$$

Moreover, the Cartesian coordinates of the integration points are presented in Equation (3).

$$\begin{aligned}
x &= \sum_{i=1}^m N_i(\xi, \eta) \cdot x_i \\
y &= \sum_{i=1}^m N_i(\xi, \eta) \cdot y_i
\end{aligned} \tag{3}$$

where m represents the number of nodes inside the grid-cell and x_i and y_i represent the Cartesian coordinates of the cells nodes.

The integration weight of the quadrature point is obtained multiplying the isoparametric weight of the quadrature point with the inverse of the Jacobian matrix determinant of the respective grid-cell, as presented in Equation (4).

$$[J] = \begin{pmatrix} \frac{dx}{d\xi} & \frac{dx}{d\eta} \\ \frac{\partial y}{\partial \xi} & \frac{\partial y}{\partial \eta} \end{pmatrix} \tag{4}$$

Accordingly, the global integration, considered a function $\mathbf{F}(\mathbf{x})$ defined in the domain Ω , can be represented as a sum, as shown in Equation (5).

$$\int_{\Omega} \mathbf{F}(\mathbf{x}) d\Omega = \sum_{i=1}^{n_g} \omega_i \mathbf{F}(\mathbf{x}_i) \tag{5}$$

where ω_i is the weight of the integration point $\mathbf{x}_i$.

1.1.3. Shape Functions

The RPIM uses the Radial Point Interpolators (RPI) technique to construct the respective shape functions. In RPIM, a polynomial basis function is combined with a radial basis function (RBF) in order to build an approximation function, which exactly interpolates the nodal data. This formulation permits to obtain numerical solutions with high accuracy. Here, it will be considered the interpolation function $u^h(\mathbf{x})$ defined for an influence-domain $\Omega_I \subset \Omega$ of an interest point $\mathbf{x}_I \in \mathbb{R}^2$. The influence-domain of $\mathbf{x}_I$ is defined by n nodes: $\mathbf{N}_I = \{n_1, n_2, \dots, n_n\}$, whose the Cartesian coordinates are $\mathbf{X} = \{\mathbf{x}_1, \mathbf{x}_2, \dots, \mathbf{x}_n\} \wedge \mathbf{x}_i \in \mathbb{R}^2$.

Using the RPI it is possible to construct an interpolation function, $u^h(\mathbf{x}) \in T$, capable to pass through all nodes inside the influence-domain. Thus, since the nodal function value is assumed to be u_i at the node $\mathbf{x}_i$, $u_i = u(\mathbf{x}_i)$, and consequently, $u^h(\mathbf{x}_i) = u(\mathbf{x}_i)$. The interpolation function $u^h(\mathbf{x}) \in T$ can be defined at the interest point $\mathbf{x}_I \in \mathbb{R}^d$ (not necessarily coincident with any $\mathbf{x}_i \in \mathbf{X}$) using a RBF $r(\mathbf{x})$ and a polynomial basis function $p(\mathbf{x})$, as demonstrated in Equation (6).

$$u^h(\mathbf{x}_I) = \sum_{i=1}^n r_i(\mathbf{x}_I) a_i + \sum_{j=1}^m p_j(\mathbf{x}_I) b_j = \mathbf{r}(\mathbf{x}_I)^T \mathbf{a} + \mathbf{p}(\mathbf{x}_I)^T \mathbf{b} = u(\mathbf{x}_I) \quad (6)$$

where $\mathbf{a}^T = \{a_1, a_2, \dots, a_n\}$ are the non-constant coefficients of $\mathbf{r}(\mathbf{x})^T = \{r_1(\mathbf{x}), r_2(\mathbf{x}), \dots, r_n(\mathbf{x})\}$ and $\mathbf{b}^T = \{b_1, b_2, \dots, b_m\}$ the non constant coefficients of $\mathbf{p}(\mathbf{x})^T = \{p_1(\mathbf{x}), p_2(\mathbf{x}), \dots, p_m(\mathbf{x})\}$. The integer n represents the number of nodes inside the influence-domain of the interest point $\mathbf{x}_I$ and the integer m is the number of monomial terms of the polynomial basis $\mathbf{p}(\mathbf{x})^T$.

In RPIM, the RBF uses to construct the shape function the Multiquadrics Radial Basis Function (MQ-RBF) [1, 5, 6], which is defined in Equation (7).

$$\mathbf{r}_i(\mathbf{x}_I) = \mathbf{s}(d_{iI}) = (d_{iI}^2 + c^2)^p \quad (7)$$

where d_{iI} is the distance between the interest point $\mathbf{x}_I = \{x_I, y_I\}^T$ and a node $\mathbf{x}_i = \{x_i, y_i\}^T$ inside the influence-domain. Accordingly, it can be defined as

$d_{il}^2 = (x_i - x_l)^2 + (y_i - y_l)^2$. The variables c and p are the shape parameters of the MQ-RBF and the efficiency of the meshless method is affected by the variation of these shape parameters. These variables were already optimized in previous works and it was demonstrated that c and p should have, respectively, the values of 0.0001 and 0.9999 in order to maximize the methods' performance [2, 5].

The original RPI formulation requires a complete polynomial basis function, which for the two-dimensional space can be defined as $\mathbf{p}(\mathbf{x}_i)^T = \{1, x_i, y_i, x_i^2, x_i y_i, y_i^2, \dots\}$. However, it has already been demonstrated in the literature that when a simple constant basis is used ($\mathbf{p}(\mathbf{x}_i) = \{1\}$), the efficiency of the RPI formulation is increased by reducing its computational cost while preserving the same accuracy level [2, 5, 6]. Using the constant basis, the number of monomial terms is defined by $m = 1$.

To obtain the non-constant coefficients a_i and b_j [Equation (6)], it is necessary to force the interpolation function to pass through all the n nodes inside the same influence-domain. Thus, at the k^{th} node, the interpolation function is defined by Equation (8).

$$u^h(x_k, y_k) = \sum_{i=1}^n r_i(x_k, y_k) a_i + \sum_{j=1}^m p_j(x_k, y_k) b_j = u_k, \quad k = 1, 2, \dots, n \quad (8)$$

In order to assure a unique solution, the following polynomial equation has to be included in the system of equations [5], as shown in Equation (9).

$$\sum_{i=1}^n p_j(x_i, y_i) a_i = 0, \quad j = 1, 2, \dots, m \quad (9)$$

In Equation (10), it is presented the computation of the shape functions in a matrix form.

$$\begin{bmatrix} \mathbf{R} & \mathbf{P} \\ \mathbf{P}^T & \mathbf{Z} \end{bmatrix} \begin{Bmatrix} \mathbf{a} \\ \mathbf{b} \end{Bmatrix} = \begin{Bmatrix} \mathbf{u} \\ \mathbf{z} \end{Bmatrix} \Leftrightarrow \mathbf{G} \begin{Bmatrix} \mathbf{a} \\ \mathbf{b} \end{Bmatrix} = \begin{Bmatrix} \mathbf{u} \\ \mathbf{z} \end{Bmatrix} \quad (10)$$

where $\mathbf{G}$ is the complete moment matrix, $\mathbf{Z}$ is a null matrix defined by $Z_{ij} = 0, \forall \{i, j\} \in \mathbb{N}: \{i, j\} \leq m\}$ and the null vector $\mathbf{z}$ can be represented by $z_i = 0, \forall i \in \mathbb{N}: i \leq m$. The radial moment matrix $\mathbf{R}$ and the polynomial moment matrix $\mathbf{P}$ are defined, respectively, in Equations (11) and (12).

$$\mathbf{R}_{[n \times n]} = \begin{bmatrix} r_1(x_1, y_1) & r_1(x_2, y_2) & \cdots & r_1(x_n, y_n) \\ r_2(x_1, y_1) & r_2(x_2, y_2) & \cdots & r_2(x_n, y_n) \\ \vdots & \vdots & \ddots & \vdots \\ r_n(x_1, y_1) & r_n(x_2, y_2) & \cdots & r_n(x_n, y_n) \end{bmatrix} \quad (11)$$

$$\mathbf{P}_{[n \times m]} = \begin{bmatrix} p_1(x_1, y_1) & p_2(x_1, y_1) & \cdots & p_m(x_1, y_1) \\ p_1(x_2, y_2) & p_2(x_2, y_2) & \cdots & p_m(x_2, y_2) \\ \vdots & \vdots & \ddots & \vdots \\ p_1(x_n, y_n) & p_2(x_n, y_n) & \cdots & p_m(x_n, y_n) \end{bmatrix} \quad (12)$$

The distance is directionless, $r_i(x_j, y_j) = r_j(x_i, y_i)$, and $\mathbf{R}_{ij} = \mathbf{R}_{ji}$ thus the matrix $\mathbf{R}$ is symmetric. Accordingly, a unique solution is obtained if the inverse of the radial moment matrix $\mathbf{R}$ exists, as demonstrated in Equation (13).

$$\begin{Bmatrix} \mathbf{a} \\ \mathbf{b} \end{Bmatrix} = \mathbf{G}^{-1} \begin{Bmatrix} \mathbf{u} \\ \mathbf{z} \end{Bmatrix} \quad (13)$$

The solvability of this system is usually assured by the requirement that $rank(p) = m \leq n$ [2]. This condition is largely satisfied since the constant polynomial basis is being used. Accordingly, the RPI function can be obtained, as in Equation (14).

$$u^h(\mathbf{x}_I) = \{\mathbf{r}(\mathbf{x}_I)^T; \mathbf{p}(\mathbf{x}_I)^T\} \mathbf{G}^{-1} \begin{Bmatrix} \mathbf{u} \\ \mathbf{z} \end{Bmatrix} = \{\boldsymbol{\varphi}(\mathbf{x}_I)^T; \boldsymbol{\Psi}(\mathbf{x}_I)^T\} \begin{Bmatrix} \mathbf{u} \\ \mathbf{z} \end{Bmatrix} \quad (14)$$

where $\boldsymbol{\varphi}(\mathbf{x}_I)$ represents the interpolation function vector, and it is defined in Equation (15).

$$\boldsymbol{\varphi}(\mathbf{x}_I) = \{\varphi_1(\mathbf{x}_I) \quad \varphi_2(\mathbf{x}_I) \quad \dots \quad \varphi_n(\mathbf{x}_I)\} \quad (15)$$

The residual vector, $\boldsymbol{\psi}(\mathbf{x}_I)$, with no relevant physical meaning, is expressed as presented in Equation (16).

$$\boldsymbol{\psi}(\mathbf{x}_I) = \{\psi_1(\mathbf{x}_I) \quad \psi_2(\mathbf{x}_I) \quad \dots \quad \psi_m(\mathbf{x}_I)\} \quad (161)$$

Since $u^h(\mathbf{x}_I)$ can be expressed as presented in Equation (17), it is possible to obtain the partial derivatives of the interpolated field variable with respect to x and y as demonstrated in Equation (18).

$$u^h(\mathbf{x}_I) = \mathbf{\Phi}(\mathbf{x}_I)^T \mathbf{u} = \{\boldsymbol{\varphi}(\mathbf{x}_I)^T; \boldsymbol{\Psi}(\mathbf{x}_I)^T\} \begin{Bmatrix} \mathbf{u} \\ \mathbf{z} \end{Bmatrix} \quad (17)$$

$$\begin{cases} \frac{\partial u^h(\mathbf{x}_I)}{\partial x} = \frac{\partial \mathbf{\Phi}(\mathbf{x}_I)^T}{\partial x} \mathbf{u} = \left\{ \frac{\partial \boldsymbol{\varphi}(\mathbf{x}_I)^T}{\partial x}; \frac{\partial \boldsymbol{\Psi}(\mathbf{x}_I)^T}{\partial x} \right\} \begin{Bmatrix} \mathbf{u} \\ \mathbf{z} \end{Bmatrix} \\ \frac{\partial u^h(\mathbf{x}_I)}{\partial y} = \frac{\partial \mathbf{\Phi}(\mathbf{x}_I)^T}{\partial y} \mathbf{u} = \left\{ \frac{\partial \boldsymbol{\varphi}(\mathbf{x}_I)^T}{\partial y}; \frac{\partial \boldsymbol{\Psi}(\mathbf{x}_I)^T}{\partial y} \right\} \begin{Bmatrix} \mathbf{u} \\ \mathbf{z} \end{Bmatrix} \end{cases} \quad (18)$$

Considering that the moment matrix $\mathbf{G}$ does not depend on the variable $\mathbf{x}_I$, the interpolation function term of Equation (18) can be rewritten as presented in Equation (19).

$$\begin{cases} \left\{ \frac{\partial \boldsymbol{\varphi}(\mathbf{x}_I)^T}{\partial x}; \frac{\partial \boldsymbol{\Psi}(\mathbf{x}_I)^T}{\partial x} \right\} = \frac{\partial (\{\mathbf{r}(\mathbf{x}_I)^T; \mathbf{p}(\mathbf{x}_I)^T \mathbf{G}^{-1}\})}{\partial x} = \left\{ \frac{\partial \mathbf{r}(\mathbf{x}_I)^T}{\partial x}; \frac{\partial \mathbf{p}(\mathbf{x}_I)^T}{\partial x} \right\} \mathbf{G}^{-1} \\ \left\{ \frac{\partial \boldsymbol{\varphi}(\mathbf{x}_I)^T}{\partial y}; \frac{\partial \boldsymbol{\Psi}(\mathbf{x}_I)^T}{\partial y} \right\} = \frac{\partial (\{\mathbf{r}(\mathbf{x}_I)^T; \mathbf{p}(\mathbf{x}_I)^T \mathbf{G}^{-1}\})}{\partial y} = \left\{ \frac{\partial \mathbf{r}(\mathbf{x}_I)^T}{\partial y}; \frac{\partial \mathbf{p}(\mathbf{x}_I)^T}{\partial y} \right\} \mathbf{G}^{-1} \end{cases} \quad (19)$$

The partial derivatives of the MQ-RBF vector, $\mathbf{r}(\mathbf{x}_I)$, with respect to x and y , can be obtained for each component $\partial r_i(\mathbf{x}_I)/\partial x$ and $\partial r_i(\mathbf{x}_I)/\partial y$ using the Equation (20).

$$\begin{cases} \frac{\partial r_i(\mathbf{x}_I)}{\partial x} = 2p(x_i - x_I)(d_{II}^2 + c^2)^{p-1} \\ \frac{\partial r_i(\mathbf{x}_I)}{\partial y} = 2p(y_i - y_I)(d_{II}^2 + c^2)^{p-1} \end{cases} \quad (20)$$

The RPI test functions $\boldsymbol{\varphi}(\mathbf{x}_I)$ depend exclusively on the distribution of dispersed nodes and are linearly independent in the influence-domain [1, 2]. Moreover, the RPI test functions for RPIM possess the Kronecker delta property, satisfying the condition presented in Equation (21).

$$\varphi_i(\mathbf{x}_j) = \delta_{ij} \quad (21)$$

where δ_{ij} is the Kronecker delta, $\delta_{ij} = 1$ if $i = j$ and $\delta_{ij} = 0$ if $i \neq j$. This property simplifies the imposition of the essential boundary conditions because allows to directly apply them in the stiffness matrix. Additionally, if a polynomial basis is included, the RPI test functions have reproducing an unity partition properties [2].

1.2. Discrete Formulation System

The presented thesis assumes that the endothelial cell migration is modulated by the chemical diffusion of the vascular endothelial growth factor (VEGF) in a homogenous medium. Aiming to obtain the numerical solution with the RPIM, the chemical diffusion should be represented as a discrete equation system. The chemical diffusion can be numerically simulated as a field problem [7]. The general form of Helmholtz allows to set the equation system of a two-dimensional linear steady state field problem, as presented in Equation (22).

$$D_x \frac{\partial^2 \phi}{\partial x^2} + D_y \frac{\partial^2 \phi}{\partial y^2} - g\phi + Q = 0 \quad (22)$$

where ϕ is the field variable, which in this cases corresponds to the VEGF concentration. The parameters D_x and D_y represents, respectively, the VEGF diffusion coefficient along dimension x and y , g represents the matrix of chemical infusibility (in this work g is neglected, $g = 0$) and Q is the VEGF release rate. Using the weighted residual approach, the meshless system equations can be formulated as in Equation (23).

$$[K_D + K_g]\Phi - f_q = 0 \quad (23)$$

where K_D , after the manipulation of equation (22), can be defined as presented in Equation (24).

$$K_D = \int_A \mathbf{B}_G^T \mathbf{D} \mathbf{B}_G dA \quad (24)$$

$\mathbf{B}_G$ and $\mathbf{D}$ can respectively be obtained as presented in Equation (25) and Equation (26).

$$\mathbf{B}_G = \begin{bmatrix} \frac{\partial \phi}{\partial x} \\ \frac{\partial \phi}{\partial y} \end{bmatrix} = \begin{bmatrix} \frac{\partial \phi_1}{\partial x} & \frac{\partial \phi_2}{\partial x} & \dots & \frac{\partial \phi_n}{\partial x} \\ \frac{\partial \phi_1}{\partial y} & \frac{\partial \phi_2}{\partial y} & \dots & \frac{\partial \phi_n}{\partial y} \end{bmatrix} \quad (25)$$

$$\mathbf{D} = \begin{bmatrix} D_x & 0 \\ 0 & D_y \end{bmatrix} \quad (26)$$

Additionally, K_g can be defined as in Equation (27).

$$\mathbf{K}_g = \int_A g\{\varphi_1 \ \varphi_2 \ \dots \ \varphi_n\}^T \{\varphi_1 \ \varphi_2 \ \dots \ \varphi_n\} dA \quad (27)$$

The discrete VEGF release rate vector, $\mathbf{f}_q$, is defined as presented in Equation (28).

$$\mathbf{f}_q = \int_A Q\{\varphi_1 \ \varphi_2 \ \dots \ \varphi_n\}^T dA \quad (28)$$

Consequently, solving Equation (23) it is possible to obtain the final VEGF concentration in the medium, Φ .

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Appendix II

Compilation Authorization

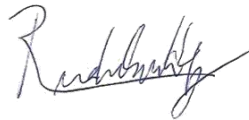
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Jorge Américo Oliveira Pinto Belinha and Renato Manuel Natal Jorge, Co-Authors of the research paper "Modelling skin wound healing angiogenesis: A review", published in the *Journal of Theoretical Biology*, declare that they authorize its inclusion in the PhD Thesis of the candidate Ana Catarina Couto Soares Guerra, entitled "Computational Methods To Simulate Angiogenesis".

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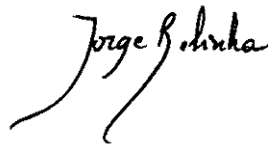


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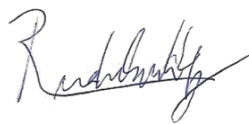
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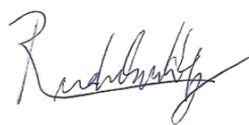
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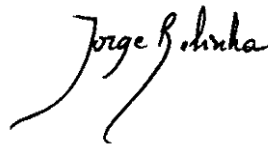


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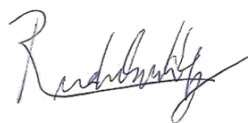
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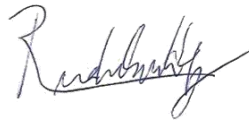
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