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Bone metastatic growth as a phase transition

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MECHANICS OF BIOLOGICAL TISSUES

Relatore

Prof. Luca Cristofolini

Correlatore

Dr. Giuseppe Zurlo

Presentata da

Nanni Davide

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CONTENTS

ABSTRACT

Bone is one of the most common sites of cancer spread. A secondary bone tumour is defined as a cancer that originated in another part of the body and has spread (metastasised) to the bone through the bloodstream or lymph nodes.
Metastasis is a complex, multistep process responsible for more than 90% of cancer-related deaths.
We will see that skeletal metastases are frequent complications of many cancers and cause bone complications (fractures, bone pain, disability) that negatively affect the quality and life expectancy of the patient.
With the exception of some relatively rare malignancies, such as high-grade lymphomas or germ cell tumours affecting bone, metastatic bone disease is currently incurable.
These are the reasons why it is of paramount importance to investigate novel solutions to try to counteract the progression of metastases.
The aim of this thesis will be to demonstrate, by means of a 1D model, that the tumour can be described as a phase transition. We will show that if the free energy of the system is not convex with respect to an appropriately chosen order parameter, then homogeneous configurations (representing mixtures of healthy and cancerous cells) become unstable, favouring instead the emergence of inhomogeneous configurations, in which the tumour is localised. To arrive at this, we will go through a detailed discussion of bone tissue and the metastatic process, which are essential for a critical and informed view of the results that we will take as the basis for our idea. At last, a study of the theory of variation calculation necessary to develop our model.

This thesis is organized as follows. In the first chapter we describe the main characteristics of bone tissue, from skeletogenesis to bone remodelling. In the second chapter we describe the metastatic process, a multistep process involving the colonisation of bone by tumour cells. Tumour cells in order to detach themselves from the primary tumour and invade foreign tissue will have to migrate through very different microenvironments and undergo changes in their mechanical and physical properties. These first two chapters are the basis for understanding the continuation of the thesis, in fact in the third chapter, through the analysis of the results obtained from recent experiments conducted at the Rizzoli Orthopaedic Institute, we have developed the idea that the tumour propagation and spread can be described as a non-linear dynamic system with two attractors, where the transition from the stable attractor of the 'normal state' to the stable attractor of the 'cancerous state' can be described by a first-order phase transition, as shown in the work of Davies et al., taken as a reference for this chapter.

Then a fourth chapter deals with the basic theory of the calculus of variations, giving us the necessary tools for the model that we will propose in the fifth chapter.
In the last chapter, we propose a simple 1D model describing the energy of the system in which healthy and tumour cells are present. The aim of the chapter is to show that the tumour can be described as a phase transition. In this setting, we show that if the free energy of the system is non-convex with respect to a suitably chosen order parameter, then homogeneous configurations (representing mixtures of healthy and cancerous cells) become unstable, thus favoring instead the onset of inhomogeneous configurations, where the tumor localizes.
In this first stage, the analysis of the theory of 1D phase transitions is abstract and does not account for important elements such as the tissue elasticity, which is well known to play a major role. At the same time, however, the results of this thesis provide an interesting direction for future studies, where considerations of energetic type could bring a deeper physical and biological understanding of the complex process of tumor invasion in bones.

INTRODUCTION

This thesis aims at providing a description of tumor propagation according to an energetic perspective, that allows to describe the spread of cancer cells as a form of instability. This perspective is novel in the literature on cancer propagation, and it ultimately results into the understanding that if the free energy of the aggregate is defined by a free energy is non-convex with respect to a suitably chosen order parameter, that describes the regions occupied by healthy and cancerous cells, then instabilities can take place, leading to localizations of cancer invasion. This is in line with experimental evidence of tumour invasion of bone tissue. This line of thinking is well known in the study of phase transitions in elastic solids, as per pioneering works of Jerry L. Ericksen ("Equilibrium of bars", Journal of Elasticity, volume 5, 191-201, 1975).

The thesis starts from an in-depth study on bone tissue, where the main mechanical, metabolic and hematopoietic functions will be highlighted. The structural organization will be described, starting from the macrostructure where we will talk about the differences between cancellous and cortical bones, passing through the microstructure to describe how the lamellae are arranged in the various osteons that form the bone matrix and finally a look at the nanostructure where we will see that collagen fibers are the main element present in the matrix. In the second half of the chapter we will describe the whole process of formation of the bone structure from skeletogenesis to bone maintenance and remodeling.

After the part concerning bone tissue, we focus attention on bone tumors, which are divided into primary and secondary tumors. A primary tumor is a tumor confined to the site of origin, while a secondary tumor is a tumor that has spread to other regions of the body. In the course of the thesis we will focus on secondary tumors and the process of metastasis, since bone is one of the most common sites of cancer spread. A secondary bone tumor is defined as cancer that originated elsewhere in the body and spread (metastasized) to bone through the bloodstream or lymph nodes. Cancer cells enter the circulation and begin to spread to distant organs. The cancer can spread to the bones of the spine, ribs and pelvis, upper arms and legs, for example. Pioneers in the field of cancer and bone, they have shown that skeletal complications associated with bone metastases are the consequence of altered bone remodelling caused by interactions between cancer cells and cells of the bone microenvironment.

There are two main types of metastases: osteolytic, in which the bone is damaged, or osteoblastic, in which new bone has formed but has grown abnormally, weakening the bone.

We will see that skeletal metastases are frequent complications of many tumors and cause bone complications (fractures, bone pain, disability) that will negatively affect the quality and life expectancy of the patient. With the exception of a few relatively rare malignancies, such as high-grade lymphomas or germ cell tumors that affect the bone, metastatic bone disease is currently incurable. However, for many patients the average prognosis after the development of bone metastases can be measured in years, especially in patients with metastatic breast or prostate cancer or multiple myeloma who, with modern therapeutic approaches, can often survive more than 5 years after the diagnosis of bone involvement. In addition, new drugs, such as tyrosine kinase inhibitors and immune checkpoint inhibitors, have significantly prolonged the control of primary disease in patients, resulting in longer survival and, consequently, a sufficiently long lifespan for bone metastases to become clinically relevant. The epidemiology of bone metastases is therefore evolving. In the coming years, we could therefore expect the appearance of bone metastases in patients who until a few years ago would never have developed clinically detectable bone metastases, because they would have died from their tumor at a time when they presented only bone marrow micrometastases (subclinical). As a result, the possibility of studying the behavior of bone metastases is increasing. A fundamental step forward, because metastases are a complex and multistep process, responsible for more than 90% of cancer-related deaths.

It is a gradual sequence of events that will involve the colonization of bone by cancer cells: The formation of a premetastatic niche in the bone marrow to attract circulating tumor cells (CTCs); Tumor cells of the primary tumor must undergo an epithelium-mesenchymal transition (EMT) to invade the surrounding tissue and enter the microvasculature (intravasation) of the blood and / or lymphatic system; Once in the bloodstream, cancer cells can disseminate into distant organs, exit blood vessels (extravasation) and settle in the foreign microenvironment, where they enter a quiescent state or proliferate to subsequently form macroscopic secondary tumors (metastases). We will analyze each of these events in detail. We will see that in all these phases that make up the metastatic process, cancer cells will migrate through very different microenvironments, including the stroma, the endothelium of blood vessels, the vascular system and the tissue of a secondary site. The ability to successfully go through each of these stages and advance toward secondary tumor formation and growth will depend, in part, on the physical interactions and mechanical forces between the cancer cells and the microenvironment. For example, physical interactions between a cell and the extracellular matrix, the collagen-rich scaffold on which it grows, play a critical role in allowing cells to migrate from a tumor to nearby blood vessels. During intravasation and extravasation, the cells will have to undergo large elastic deformations to penetrate the endothelial cell-to-cell junctions. In the vascular system, the interaction between cell speed and adhesion affects the binding of cancer cells to the walls

of blood vessels, and thus the localization of sites where a secondary tumor can form and grow. Naturally, the presence of metastases will entail a number of changes in the cell. From the genetic point of view, changes in cellular metabolism, morphology, alterations of signaling and protein expression will occur, while the physical interactions of tumor cells with their microenvironment and their modulation by mechanical forces will be modified. These alterations will be described in detail during the thesis.

This first part of the thesis aims at understanding in detail the main mechanisms involved in the process of bone metastases. This study will be necessary to understand the behavior of cancer cells, the various genetic changes and the physical forces to which they are subjected.

The idea that will be developed in the following is that there is the possibility that actually the bone tumor may present those characteristics that are somehow already described by the theory of phase transitions, and hence the intention to establish a link between this very theoretical aspect and an experimental aspect that still lacks a theoretical description. The idea will be to apply the theory of phase transitions to describe tumor progression in bone tissue stems from the study of two experiments conducted at the Rizzoli Orthopedic Institute, in which 3D models were used in an attempt to restore the native tumor and its microenvironment to mimic as much as possible the biological processes that occur in patients and thus obtain satisfactory results. A model made in vitro using human bone tissue taken from patients undergoing total hip replacement surgery, cultured with human breast or prostate cancer cells (MCF-7 and PC-3) and then established a "proof of concept" to recapitulate bone metastases and their microenvironment. Promising results have been obtained from this 3D model, from which we will extrapolate data on the localization of cancer cells in bone tissue. We will see breast cancer and prostate cancer that have metastasized to the vertebrae. From these images we will see that there are two phases, one composed of healthy cells and the other of cancerous cells. We will notice that healthy and cancerous areas have well-defined boundaries and this invasion of cancer cells into healthy tissue can be explained as a loss of stability of the phase boundary between the two zones.

Cancerous tissue will invade healthy tissue, just as it happens in phase transitions, where one phase (e.g., solid) invades another phase (e.g., liquid).

We will see that, while physical phase transitions typically occur at or near thermodynamic equilibrium, the transition from normal to tumor (NTC) is a dynamic phenomenon of non-equilibrium, which will depend on the supply of metabolic energy and local physiological conditions. This theory will be based on the hypothesis that the energy of biological tissues can be described as non-convex energy. The hypothesis of non-convexity is consistent with many of the phenomena already found in tumor propagation, in particular the instability of the phase front, invasion guided by a control parameter, the presence of fine mixtures, etc.

In the continuation of the thesis, we will try to demonstrate that describing tumor propagation from an energetic perspective will allow us to describe the spread of cancer cells as a form of instability. The latter can occur if the free energy of the aggregate is expressed by a non-convex energy with respect to an order parameter, describing the regions occupied by healthy and cancerous cells. The idea will be to use phase transition theory to explain key stages of cancer progression, providing an alternative route to more common approaches, based on biochemistry, genomics and cell biology.

We will see that in the case of a transition from normal cells to cancer cells, the nature of the change taking place is that of a molecular and cellular reorganization that will lead to a drastic elimination of various checkpoints of the cell cycle and to a simplification of the functional program of the cell that appears aimed exclusively at survival and proliferation. Therefore, depending on the type of cancer, there may be different control parameters that bring living cells from a normal state to a cancerous state. These control parameters may lead to external influences that will destabilize the normal metabolism of the cell (oxygen reduction, increased concentration of carcinogens, pH reduction, increase in harmful ionizing radiation, etc.). On the other hand, the order parameter of the system will define the response to external perturbations. The other fundamental aspect will concern the energy of the system. It will be observed that cancer progression must also involve a change at the population level, due to a better adaptation of cancer cells to the prevailing conditions and competition between the two coexisting phenotypes: normal and cancerous. This change will undeniably involve natural selection, which may favor the new phenotype, but natural selection will not cause the transition, it will be a consequence. Eventually, given the resources and time available, cancer cells will usually outperform healthy ones in the organ or tissue where they coexist, competing for space and resources. In the work of Davies et al., which will be used as the basis for this chapter, it will be argued that this shift of the population in the direction of greater malignancy can be seen as a trend towards greater stability (and therefore maximum entropy or minimum free energy). Static physical systems will achieve thermodynamic equilibrium by achieving the lowest free energy within certain physical constraints (e.g., constant temperature, volume, or pressure), while living systems achieve dynamic stability (or robustness) as a competitive advantage over other species within the externally imposed environment, such as a variable rate of nutrient intake, lower oxygen concentration, or increased acidity. The work of Davies et al. will allow us to demonstrate that cancer propagation can be described through a first-order phase transition and system energy that takes on a non-convexity profile. The idea of the model that we are going to describe will be based on the description of cancer in terms of a nonlinear system, in which a living cell will be represented as a stable attractor of a complex dynamic system. The transition from the stable attractor of the "normal state" to the stable attractor of the "cancerous state" can be described by a phase transition of the first order (irreversible and

discontinuous). Under normal conditions, healthy cells represent a more stable state, while cancer is a metastable state (less stable but not necessarily unstable). With increased cell damage caused by external events or internal dysregulation due to alterations in metabolism or accumulated genetic changes, the relative stability between these two types of states (normal and cancerous) shifts. This will be similar to a first-order phase transition in physics, where two equilibrium states (ordered and disordered) reverse roles (stable versus metastable) as the value of the control parameter changes. We will also see that they are characterized by extreme sensitivity to small perturbations and zero correlations throughout the system, which determine the global character of the phase transition. This property of phase transitions in the context of cellular transformations will indicate an increased sensitivity and global nature of the NTC transition. For example, below 100°C liquid water is stable and water vapor is metastable, while above 100°C the situation is reversed. In the case of cells, the turning point between the two sets of conditions marks precisely the phase transition in which the fate of the biological system hangs in the balance.

Before moving on to the final chapter, where we will demonstrate that the results deriving from the experiments conducted by the Rizzoli team on bone cancer, which show the presence of localizations, can be described as phase transitions, and we will describe in detail the theory of the calculus of variations accordingly. Calculus of variations is a branch of mathematics that deals with the optimization of functionals, that is, functions of functions. The goal of calculus of variations is to find the function that minimizes or maximizes a given functional, subject to certain constraints. One of the most well-known problems of calculus of variations with is finding the curve, or path, that minimizes the distance between two points. In the chapter on the calculus of variations we will describe the concept of perturbation, we will state the fundamental lemma of the calculus of variations and the concept of functional derivative, which is a generalization of the concept of derivative in calculus. This concept will be closely related to the Euler-Lagrange equation, which is a differential equation used to find the function that minimizes or maximizes a given functional. This is a fundamental chapter, because the theory of the calculus of variations will then be used to show that the mixture of healthy and cancerous cells has an energetic preference to create "phases" rather than creating a homogeneous (fine) mixture. We will show that the tumor is localized because there is an energy convenience, which can be guided by the control parameters.

In the concluding chapter, starting from the hypotheses of Davies et al. on the actual possibility that cancer tissue is described by a non-convex free energy density, we will illustrate how this general theory produces effects are compatible with observations,

although at this stage the analytical model is still general. So for now, we will set aside bone tissue and talk about cancer in general.

As shown in this final chapter, the special form of the free energy density is the main player towards cancer localizations. In this, albeit rather general, perspective, the aim of the chapter is to demonstrate that the forms of localization observed in the experiments performed by the Rizzoli Institute are strongly connected with the study of problems of minimization of non-convex energy functionals.

In this perspective, cancer eventually localizes because there is an energetic convenience, where non-convexity is externally modulated by suitable parameters, such as temperature and pH. Here we are not interested in describing energetics in detail, a topic that could be investigated in future studies. A subsequent phase of this thesis work will be necessary for the derivation of a free energy in the metastatic bone, including three-dimensional and mechanical aspects.

1. THE BONE TISSUE

1.1 THE MAIN FUNCTION OF BONE TISSUE

The human skeleton consists of 206 bones. These bones are classified into four categories: long bones, flat bones, short bones and irregular bones. Bone tissue has three main functions[1] :

- Mechanical: it supports and protects vital organs and the nervous system. Bones enable the transfer of forces from one part of the body to another. The mechanical properties of a bone are a compromise between rigidity, low elasticity to reduce stress and ductility, to absorb shocks and reduce the risk of fractures [2].
- Metabolic: bone tissue is a dynamic tissue that is constantly renewing itself under the effect of mechanical stress. This remodelling leads to the storage or release of mineral salts. Bone therefore participates in the body's phosphocalcic homeostasis [2].
- Haematopoietic: The haematopoietic medulla, enclosed in the medullary space of the bone, is responsible for the production of all three types of blood cell lines [2].

1.2 STRUCTURAL ORGANIZATION

The vertebrate skeleton is composed of specialized connective tissues, which are structures composed of cells embedded in an abundant extracellular matrix. All forms of connective tissue are composed of stationary and migrating cells and an amorphous matrix called ground substance [3]. The proportions of these components vary from one part of the body to another depending on local structural requirements.
The extracellular matrix that keeps the cells apart is composed of three types of fibers: collagen fibers(the most abundant), reticular fibers, and elastic fibers [3].
The ground substance is a clear, colorless, viscous fluid that fills the space between the cells and the fibers. It is composed of proteoglycans and cell adhesion proteins that allow it to act as glue for cells to attach to the matrix. In bone the ground substance includes minerals [3].
Finally, in connective tissue there are two types of cells: stationary cells (fibrocytes and fat cells) and migrating cells (macrophages, monocytes, lymphocytes, plasma cells and eosinophils) [3].

Knowing the structure and functions of connective tissue is of paramount importance because, as mentioned earlier, the skeleton is an organ composed of two distinct connective tissues, cartilage and bone (Fig.1.1A-1.1B) [4].

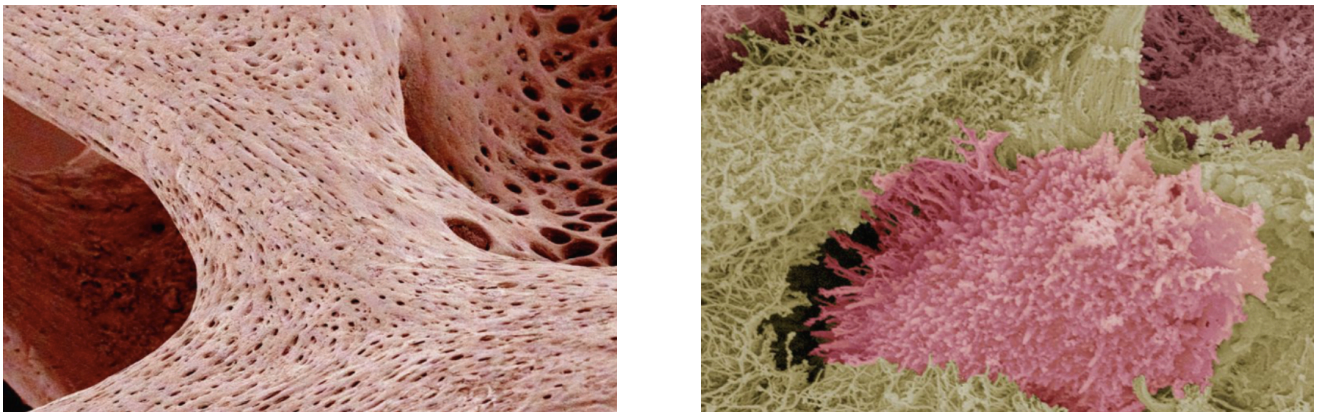

Figure 1.1: A) This micrograph shows hyaline cartilage, a semi-rigid connective tissue from a human trachea.
B) This micrograph shows cancellous (spongy) bone from a vertebra. Cancellous bone is characterized by a honeycomb arrangement, comprising a network of trabeculae. These structures provide support and strength to the bone [4].

Bone, or bone tissue, is a composite material consisting of two different types of matrix: inorganic and organic. The inorganic matrix, which constitutes approximately 66% of the total, consists of mineral salts, mainly calcium and hydroxyapatite [$Ca_{10}(PO_4)6(OH)_2$] (HA), which impart hardness to the tissue [5]. The inorganic matrix is also broken down into a mosaic of tiny microcrystals, which create a large surface area for ion exchange and limit crack diffusion. These crystals are deposited and embedded in the collagen fibres [6]. They are arranged parallel to each other and to the collagen fibres. This organisation enables good mechanical strength of the bone tissue [5,6].
In addition to calcium and phosphate, the mineral phase also consists of sodium and magnesium ions. They are released when the body needs them, to maintain the plasma concentration necessary for functions such as nerve conduction or muscle contraction [7].
The organic matrix, on the other hand, is materially similar to other connective tissues, and is composed mainly of collagen (90%), The remaining 10% represents noncollagenous proteins, such as glycoproteins, proteoglycans, osteopontin, osteocalcin, etc. [8], proteins that control the deposition of HA . This gives strength and flexibility to the tissue. Collagen is a fibrous, insoluble protein present in the extracellular matrix of many connective tissues. In bone tissue, the organic proteins in the extracellular matrix are mainly collagen type I (95%) [5,8]. Collagen fibres regulate the nucleation and spatial orientation of HA crystals. In fact, the collagen triple helix is aligned in the collagen fibrils with a shift between the ends of the two consecutive subunits. These shifts are the nucleation site of the HA crystals, whose size and orientation are controlled by the structure and organisation of the collagen fibrils (Fig. 1.2) [6,9].
The extracellular organic matrix also consists of collagen types III and V in small amounts, which play a role in fibrillogenic regulation and modulation of fibril diameter [10].

Without adequate organic material in the matrix, the tissue breaks down; without adequate inorganic material in the matrix, the tissue folds. The structural organisation of bone is adapted to provide maximum strength for its load-bearing function with minimum weight.

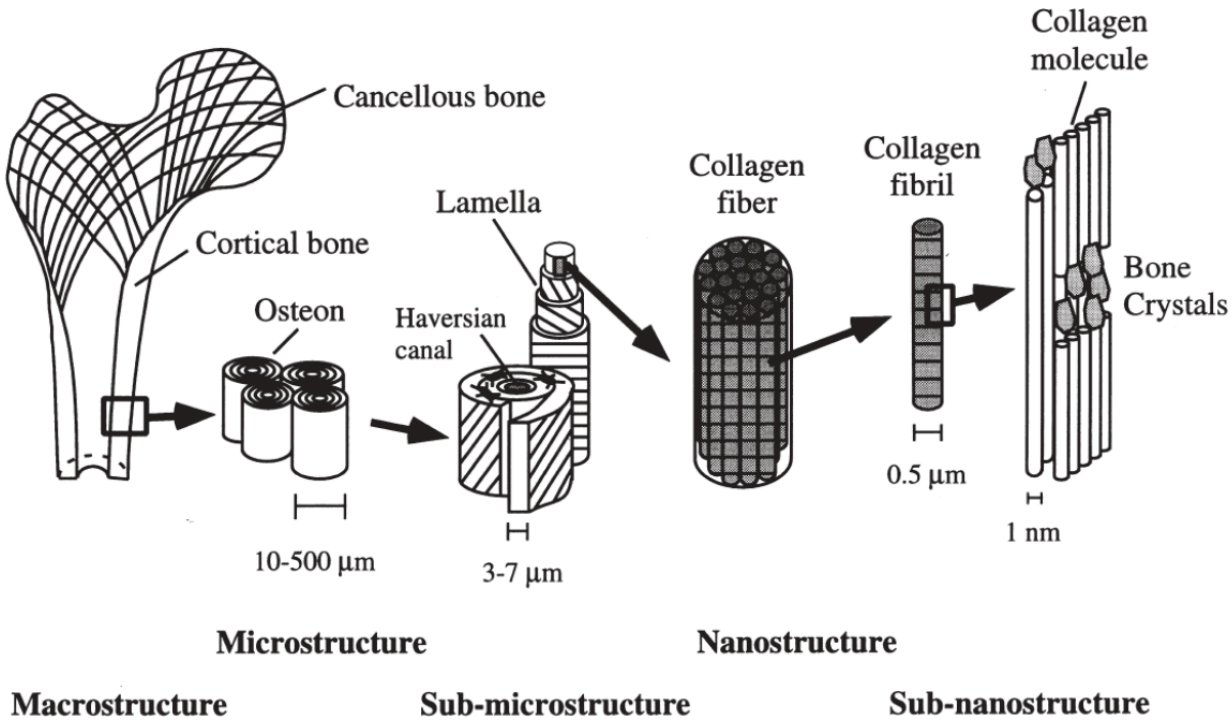

Figure 1.2: Hierarchical structural organization of bone: (A) cortical and cancellous bone; (B) osteons with Haversian systems; (C) lamellae; (D) collagen fiber assemblies of collagen fibrils; (E) bone mineral crystals, collagen molecules, and non-collagenous proteins [11].

Once it has been pointed out that bone serves as a structure for the body and as a metabolic reserve of calcium and phosphate in the event of mineral deficiencies, the next step is to describe what types of cells it consists of. It is made up of three types of cells: osteoblasts, osteocytes, and osteoclasts [12]. Osteoblasts are active in the production of bone for growth and remodeling [12]. They deposit bone material into the matrix and, after the matrix has surrounded them, continue to live, but in a reduced metabolic state as osteocytes. Osteocytes are found in the gaps in the bone and help maintain the bone [13]. Osteoclasts are active in breaking down bone for bone remodeling, providing access to calcium stored in the tissues to release it into the bloodstream [14]. Osteoclasts are usually found on the surface of the tissue [14].

Bone is commonly classified according to its gross appearance as spongy bone (bone with numerous, macroscopic interconnected cavities, or trabeculae, also known as trabecular bone) or compact bone (dense lamellar bone without trabeculae); despite this, both types have the same basic histological structure [15]. The diaphysis of long bones (shaft; a typical long mammalian bone) is composed mostly of compact bone, with spongy bone confined to the inner surface around a central medullary cavity, while the epiphyses (joint ends) are composed mostly of spongy bone overlaid by a thin, smooth layer of compact bone [15].
Compact bone, the stronger but heavier of the two forms, comprises the outer wall of all bones and performs a primarily mechanical function. It consists of parallel cylinders of matrix (osteons) arranged along the load-bearing axis of the bone(diaphysis) [1,15].
The central (or Haversian) canal of each osteon contains bone cells, blood vessels, and nerve fibers. Within each osteon the matrix is deposited in concentric layers, each 2-3 μm thick, with a predominant fiber direction (like a multilayered plywood), known as lamellae [15]. The small spaces between these circles are called lacunae. Between the lacunae are microchannels called canaliculi; they connect the lacunae to help diffusion between cells [15]. The various Haversian channels communicate with each other through other channels placed, however, transversely or obliquely, called Wolkmann channels, which also contain blood capillaries. Nutrients reach the osteocytes through the capillary network and are distributed through the dense network of cytoplasmic extensions that connect them. The spaces created between the different osteons are occupied by fragments of lamellar bone of variable shape and size called interstitial systems [15]. The boundaries between the osteons and the interstitial systems are easily detected by a layer of refractive connective tissue called the cementing line with much higher HA content than the lamellae. The bone surface in contact with the periosteum and endosteum is formed by lamellae arranged parallel to the free surface of the bone; these lamellae are called circumferential lamellae [1,16].
The trabecular bone, however, it is found at the ends of the long bones (epiphysis) and at the center of the vertebrae. It consists of a network made of small plates called trabeculae, which act as struts, giving strength to the bone [16]. The trabeculae of bone tissue are made up of lamellae, distinct layers of fibers and bone cells held together by collagen fibers. In spongy bone tissue they form two families of trabeculae, roughly perpendicular to each other, corresponding to the principal direction of strain for the most frequent loading [17]. The trabecula is therefore the macroscopic structural unit typical of spongy bone tissue. There are no Haversian canals, but internally the trabeculae are variously anastomosed to delimit intercommunicating spaces, called medullary cavities as they are occupied by bone marrow, vessels and nerves [1,17]. Although weaker than cortical bone, it is more cellular and therefore more metabolically active (Fig.1.3).

Vertebrates have joint structures to interconnect these elements [17]. They have synovial joints between limb elements to show large ranges of motion; intervertebral discs to give ductility to the spine; and fibrous joints to minimize movement between skull bones. These different types of joints are made of different tissues [18]. Synovial joints, for example, consist of articular cartilage that lines the bony surfaces, a fibrous capsule that surrounds the joint and seals its cavity, a synovial membrane that internally lines the capsule and produces a lubricating fluid, tendons that transmit muscle strength to the bones, and ligaments that stabilize the joints by connecting the bones [19]. The vertebrate skeleton is thus a complex and highly advantageous edifice [18,19].

We will now go into detail about those mechanisms that lead to the formation of cartilage and bone tissue.

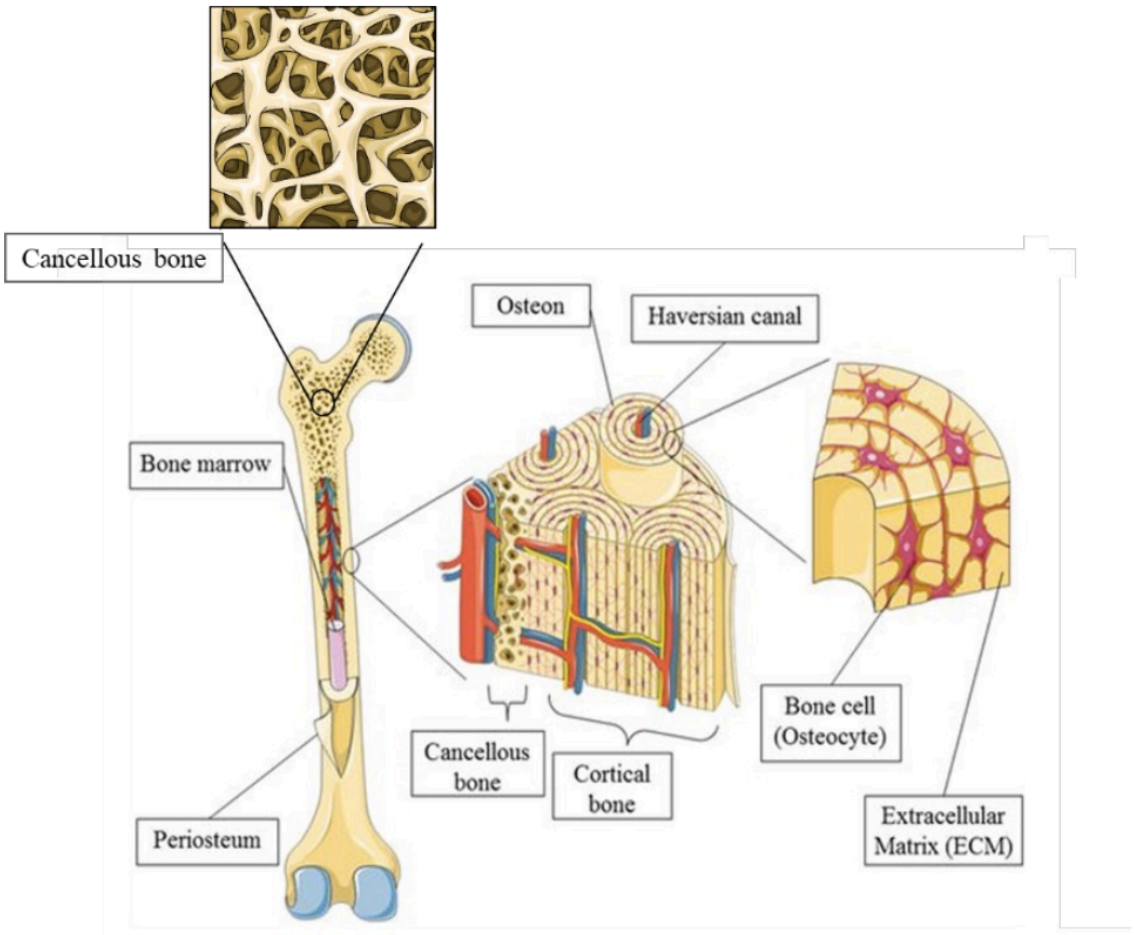

Figure 1.3: Structure and composition of bone (from Servier medical art) [2].

1.3 SKELETOGENESIS

Bone and cartilage differ in composition and regulation, but their associated developments are closely coordinated [20]. Knowledge of the cellular and molecular events that govern skeletogenesis has increased dramatically over the past two decades through the identification of disease-causing mutations, gene manipulations in animals, and new molecular and cellular approaches [20,21]. It is now clear that an incredibly large number of factors are involved in skeletogenesis. In fact, no other process, except perhaps brain development, can recruit so many factors. These factors are hormones, growth factors, receptors, signaling mediators, transcription factors, extracellular matrix components, and enzymes [21]. Factors that determine the identity of skeletal cells are called differentiation factors, and factors that specify the number, size, and shape of skeletal elements are called patterning factors [21]. The latter far outweigh the former, and contribute to the incredible skeletal variations that exist between individuals both within and across species [20,21].

Vertebrate skeletogenesis is therefore a fascinating process to study for developmental and evolutionary biologists who want to understand how the skeleton and its variations are generated in developing vertebrates. In addition, it is also a mandatory process to study for geneticists and clinical scientists who want to decipher the molecular basis of skeletal diseases in humans and develop much needed therapies for these diseases [20,21]. Key aspects of the composition and development of the vertebrate skeleton are reviewed below. We review the fundamental discoveries made toward understanding the mechanisms underlying skeletal cell fate determination, chondrogenesis, osteogenesis, and joint formation.

1.4 DEVELOPMENT OF SKELETOGENIC CELLS

The first step in skeletogenesis consists in generating skeletogenic cells [21]. The origin of these cells can be tracked back to the onset of organogenesis, when the vertebrate embryo is comprised of three germ layers: ectoderm, mesoderm, and endoderm (Fig. 1.4A) [20]. These layers transform themselves into multiple early derivatives. These include the ectoderm-derived neural tube, mesoderm-derived notochord, paraxial mesoderm, and lateral plate mesoderm, which give rise to the skeleton as well as other organs. The neural crest is a population of cells that delaminates from the neural tube, undergoes epithelial-to-mesenchymal transformation, and migrates to numerous locations in the embryo (Fig. 1.4B) [20]. Once there, the cells develop into various types, such as neuronal cells, melanocytes, and skeletogenic cells. The latter give rise to several throat and craniofacial skeletal elements (Fig. 1.4C) [20]. The lateral plate mesoderm gives rise to the other craniofacial

skeletal structures, the limb skeletal elements (appendicular skeleton), the sternum (part of the axial skeleton), and non-skeletal structures [20,22]. The paraxial mesoderm gives rise to somites, which develop into dermomyotomes and sclerotomes [20,23]. The latter form most of the axial skeleton, i.e., the ribs and vertebrae. As they develop around the notochord, the vertebrae force the notochord cells to change phenotype, migrate to the intervertebral spaces and develop the nuclei pulposi of intervertebral discs. Many factors control neural crest and mesoderm cells before they reach skeletal sites. will not be reviewed, but excellent recent reviews on this topic are suggested [22,23].

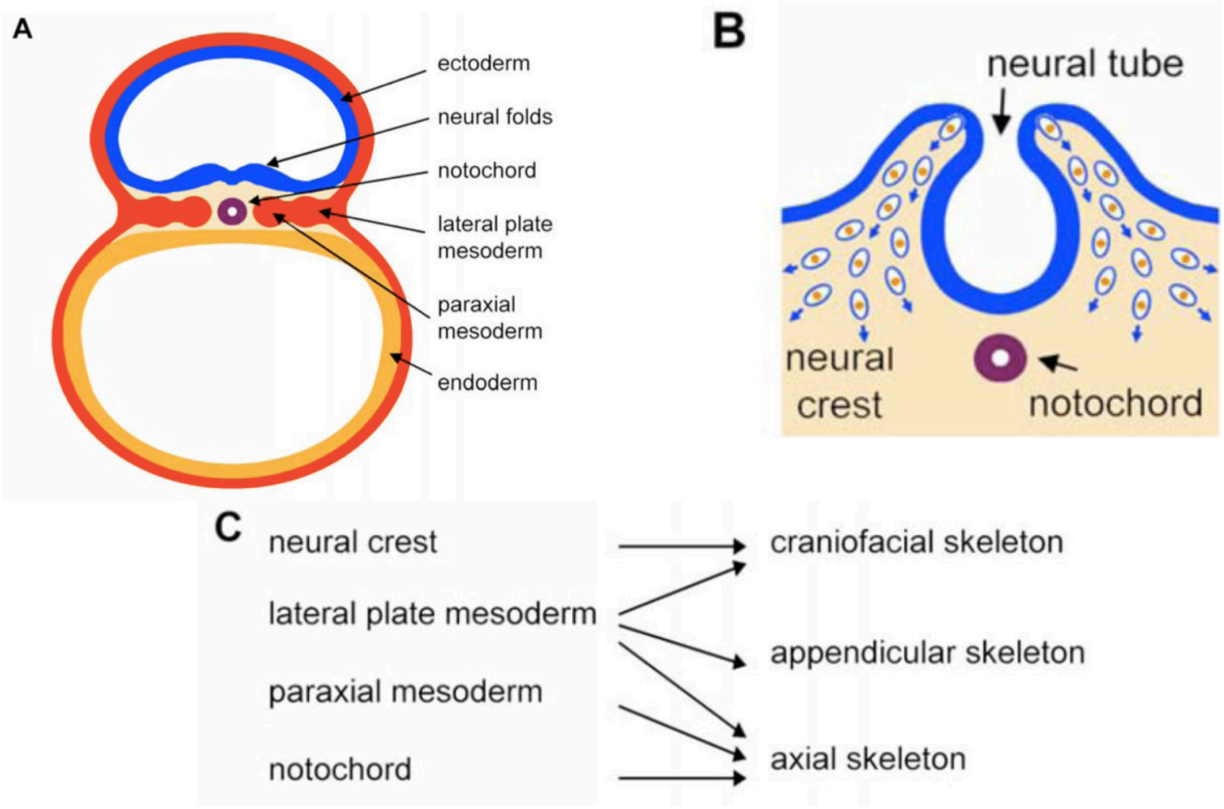

Figure 1.4: Origin of skeletal cells in the vertebrate embryo [20] :
A. Schematic of a cross section through a mouse embryo soon after gastrulation at day 8 of development (equivalent to gestation day 17 in humans). The three germ layers are seen: ectoderm, endoderm, and mesoderm. Ectoderm-derived neural folds are rising. The mesoderm has formed the notochord and is starting to form lateral plate and paraxial derivatives on either sides of the midline.
B. Schematic showing the delamination of neural crest cells from the neural folds at the time of neural tube closure. These cells are starting to migrate inside the embryo (blue arrows), where they will participate in the formation of various structures.
C. Schematic showing the contribution of the neural crest, lateral plate mesoderm, paraxial mesoderm, and notochord to the three major parts of the skeleton.

Upon settling in skeletal sites, neural crest- and mesoderm-derived cells produce a matrix rich in collagen-1, fibronectin, and hyaluronan, and they proliferate or die in a tightly controlled spatial and temporal manner [24]. They thereby establish mesenchymal structures that prefigure the future skeletal elements. They are often called osteochondroprogenitors because most of them give rise to osteoblasts and chondrocytes, and are able to switch fate under specific conditions. Some, however, give rise to synovial cells, tenocytes, bone marrow stromal cells, endothelial cells, and presumably mesenchymal stem cells. therefore they are called skeletogenic cells [20,24].

1.5 DEVELOPMENT OF CARTILAGE STRUCTURE

Chondrogenesis is the process by which cartilage forms from mesenchymal tissue, differentiates into chondrocytes, and begins to secrete the molecules that form the extracellular matrix. This is the fate of most skeletal cells, leading to the construction of a multitude of primordial cartilage cells, which collectively constitute the primary skeleton of the vertebrate embryo (Fig.1.5) [20]. This process occurs in five stages [25,26]:

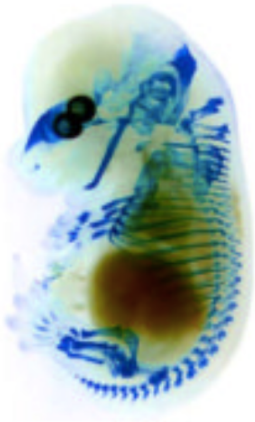

Figure 1.5: Chondrocyte early differentiation and development of cartilage primordia
Alcian blue staining of a mouse embryo at demonstrates that chondrocyte differentiation of skeletogenic cells leads to the formation of a primary skeleton that is entirely cartilaginous [20].

The cells become encased in a matrix: when the chondroblast becomes completely encased in its own matrix, the cartilage cells turn into chondrocytes. The new chondroblasts stand out from the membrane surface (perichondrium), resulting in an increase in cartilage size (cartilage can increase in size through growth by apposition) [25].

The chondrocytes enlarge, divide and produce a matrix. The cell growth continues and produces a matrix that causes an increase in the size of the cartilage mass from within. Growth that causes an increase in size from the inside is called interstitial growth [25].

The matrix remains non-calcified: the cartilage matrix is rich in chondroitin sulphate, associated with non-collagenous proteins. Nutrition and metabolic wastes are discharged directly through the soft matrix to and from the cell. Therefore, blood vessels are not necessary in cartilage [25].

The membrane covers the surface but is not essential: cartilage has a closed membrane vasculature (perichondrium), but cartilage can exist without any of these elements. This property makes cartilage able to grow and adapt where it needs pressure (in joints), so that cartilage can receive pressure [25,26].

1.6 BONE DEVELOPMENT

In humans by the end of the eighth week after conception, the skeletal model is formed into cartilage and connective tissue membranes and ossification begins [1,3].
Bones are formed through two processes: intramembranous (or osteogenesis) and endochondral ossification [3]. During intramembranous (Fig.1.6) bone formation, the connective tissue membrane of undifferentiated mesenchymal cells is transformed into bone cells and bone matrix [27].
An ossification centre appears in the fibrous connective tissue membrane. The mesenchymal cells of the embryonic skeleton come together and begin to differentiate into specialised cells. Some of these cells differentiate into capillaries, while others become osteogenic cells and osteoblasts, eventually forming an ossification centre [1,3].

The bone matrix (osteoid) is secreted within the fibrous membrane. Osteoblasts produce osteoid tissue, differentiating from the ectomesenchyma condensation centre and producing bone fibrous matrix (osteoid). The osteoid then mineralises within a few days and the trapped osteoblasts become osteocytes. As a result, interlocking bone and periosteum are formed. Encapsulation of cells and blood vessels occurs [1,3].
The accumulating osteoid is deposited between the embryonic blood vessels, which form a random network (instead of lamellae) of trabeculae [1]. The vascularised mesenchyme condenses on the outer face of the intertwined bone and becomes the periosteum. The production of osteoid tissue by membrane cells occurs with the loss of the ability of osteocytes to contribute directly to the increase in bone size, but osteoblasts on the surface

of the periosteum produce more osteoid tissue that thickens the layer of tissue on the existing bone surface (appositional bone growth) [1,3].
Osteoid calcification occurs with mineralisation of the bone matrix that makes bones relatively impermeable to nutrients and metabolic wastes. Trapped blood vessels have the function of supplying nutrients to the osteocytes and bone tissue and eliminating waste products [3].
An essential bone membrane is formed, which includes an outer membrane called the bone endosteum. The endosteum is very important for the survival of bone. Disruption of the membrane or its vascular tissue can cause bone cell death and bone loss [27].
The matrix or intercellular substance of bone calcifies and eventually becomes bone. Bone tissue found in the periosteum, endosteum, suture and periodontal membrane (ligaments) is an example of intramembranous bone formation [28,29].

Intramembranous bone formation occurs in two types of bone: bundle bone and lamellar bone. Bundle bone develops directly into non-calcified connective tissue [29]. Osteoblasts, which differentiate from the mesenchyme, secrete an intercellular substance containing collagen fibrils. This osteoid matrix calcifies by precipitating apatite crystals. Primary ossification centres show only a minimal density of bone calcification. The deposits of apatite crystals are mostly irregular and structured like networks, contained in the medullary and cortical regions [1,3]. Mineralisation occurs very rapidly (several tens of thousands of millimetres per day) and can occur simultaneously in large areas. These apatite deposits increase with time. Bone tissue is only considered mature when the crystallised area is arranged in the same direction as the collagen fibrils [3,29].

In adults, the bone bundle usually only forms during rapid bone remodelling. This is reinforced by the presence of lamellar bone. In contrast to bundle bone formation, lamellar bone development only occurs in mineralised matrix (e.g. calcified cartilage or bony spicules of the bundle). The networks of the bundle bone are filled to strengthen the lamellar bone, until a compact bone is formed. Osteoblasts appear in the mineralised matrix, which then form a circle with intercellular matter surrounding the central vessels in several layers (Haversian system). Lamellae bone forms from 0.7 to 1.5 microns per day. The network is formed by complex fibre arrangements, which are responsible for its mechanical properties. The arrangement of apatites in the concentric layer of fibrils ultimately fulfils the functional requirements. Lamellar bone depends on continuous deposition and resorption, which can be influenced by environmental factors [30,31].

On the other hand, concerning the process of endochondral bone formation, mesenchymal tissue first differentiates into cartilaginous tissue. Endochondral bone formation is a morphogenetic adaptation that produces continuous bone in certain areas that are significantly stressed. Thus, this endochondral bone formation is found in bones associated with joint movement and in certain parts of the skull base. In hypertrophic cartilage cells, the matrix calcifies and the cells degenerate [30,31].

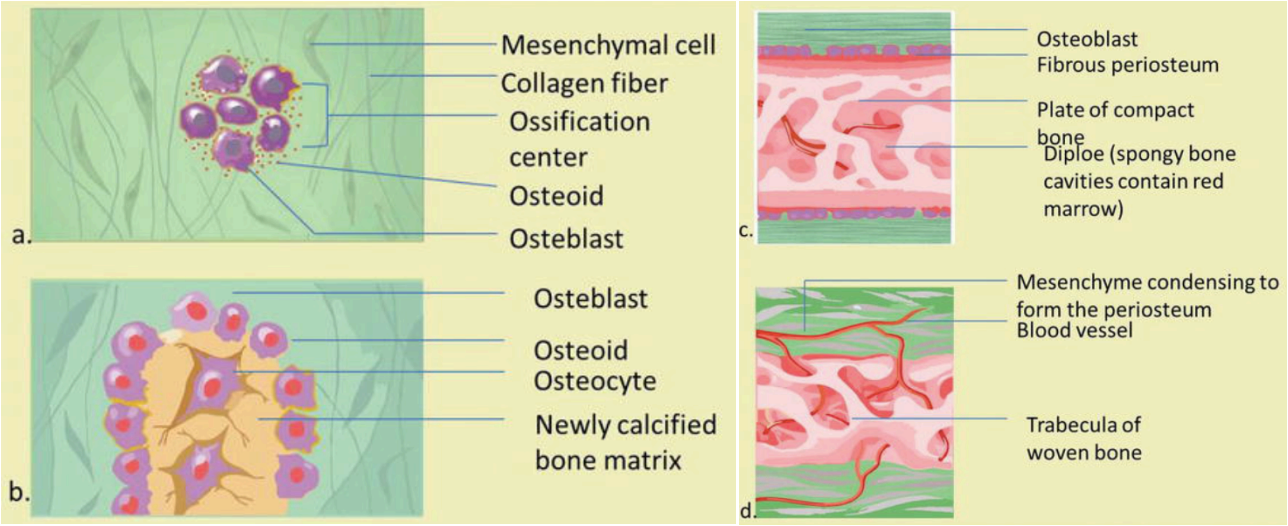

Figure 1.6: The stage of intramembranous ossification. The following stages are (a) Mesenchymal cells group into clusters, and ossification centers form. (b) Secreted osteoid traps osteoblasts, which then become osteocytes. (c) Trabecular matrix and periosteum form. (d) Compact bone develops superficial to the trabecular bone, and crowded blood vessels condense into red marrow [32].

There are important aspects of cartilage in endochondral bone formation [30]:
Cartilage has a rigid and solid, but usually non-calcified structure, which confers three fundamental functions for growth: its flexibility can support an appropriate network structure, pressure tolerance at a particular location where compression occurs, and localisation of growth in conjunction with bone enlargement.
Cartilage grows in two adjacent locations (due to chondrogenic membrane activity) and grows in tissue (division of chondrocyte cells and addition of intercellular matrix).
Bone tissue is not the same as cartilage in terms of tension adaptation and cannot grow directly in areas of high compression because its growth depends on the vascularisation of the bone formation covering the membrane [31,33].

Cartilage growth occurs where linear growth towards the direction of pressure is required, which allows the bone to stretch towards the area of resistance and has not yet grown elsewhere, by ossification of the membrane in combination with all periosteal and endosteal surfaces [33].

1.7 BONE REMODELING

Although bones stop growing in length early in adulthood, they may continue to change in thickness or diameter throughout life in response to stress from loads, muscle activity, or weight change. This process is called bone remodeling [3,34].

Bone remodeling is a continuous process of synthesis and destruction that gives bone its mature structure and maintains normal calcium levels in the body. The destruction, or resorption, of bone by osteoclasts releases calcium into the bloodstream to meet the body's metabolic needs and simultaneously allows bone, which is inhibited by its inorganic component from growing by cell division like other tissues, to change size and shape as it grows to adult proportions [34]. While osteoclasts resorb bone at various sites, osteoblasts produce new bone to maintain skeletal structure. During childhood, bone formation overcomes destruction as growth proceeds. After reaching skeletal maturity, the two processes maintain an approximate balance [34,35].

2. BONE TUMOURS : THE METASTASIS PROCESS

Having concluded the part of the thesis that goes from skeletogenesis to bone remodelling, which is necessary to understand the continuation of the thesis, we will now discuss bone tumours, which are differentiated into primary and secondary tumours. A primary tumour is a tumour confined to the site of origin, while a secondary tumour is a tumour that has diffused to other regions of the body. In the course of the thesis, we will focus on secondary tumours and the process of metastasis, because bone is one of the most common sites of cancer diffusion.

2.1 SECONDARY BONE TUMOURS

A secondary bone tumour is defined as a cancer that originated in another part of the body and has spread (metastasis) to the bone through the bloodstream or lymph nodes. Tumour cells enter the circulation and begin to diffuse to distant organs [36]. The tumour can spread to the bones of the spine, ribs and pelvis, upper arms and legs. One case could be a lung tumour that has metastasised to the ribs due to proximity [36].

In adults, bone mass is maintained by continuous shaping and remodelling of the overall bone structure through the process of bone remodelling, which is a balance between the resorption of mineralised bone by the cells that resorb it (osteoclasts) and the formation of new bone by the cells that form it (osteoblasts) [34]. Bone remodelling is tightly regulated by systemic and local factors to maintain this balance at a physiological steady state [37]. Greg Mundy [38] was a pioneer in the field of cancer and bone, demonstrating that skeletal complications associated with bone metastases are the consequence of altered bone remodelling caused by interactions between tumour cells and cells in the bone microenvironment [39].
There are two main types of metastases: osteolytic, in which the bone is damaged, or osteoblastic, in which new bone has formed but has grown abnormally, weakening the bone. Apart from osteoblastic bone metastases in prostate cancer, bone metastases from other tumours are mainly osteolytic or a mix of lytic and blastic changes in bone structure (Fig. 2.1) [39].

Metastases are more common than primary bone cancer because bone is one of the most common sites of metastases in cancer. Much of the work done to describe the history of bone metastases is based on autopsy studies and large case series from individual institutions conducted several decades ago [39]. Although bone is a frequent site for metastases of many malignancies, there are specific types of cancer that have a predilection

for metastases to the skeleton [40,41]. In particular, bone metastases are frequent complications of breast (especially oestrogen receptor (ER) positive) and prostate cancer. Previous studies have found a 70% incidence of bone metastases in breast cancer [42]. These results are consistent with Galasko's post-mortem examination, which reported an incidence of bone metastases of 73% and 68% in breast and prostate cancer, respectively [43]. Other studies, again post-mortem, have identified that any type of cancer can spread, metastasising, to bone, but the most common besides breast and prostate are lung cancer with an incidence of 30-40%, thyroid cancer with 60% and renal cancer with 30% [44].

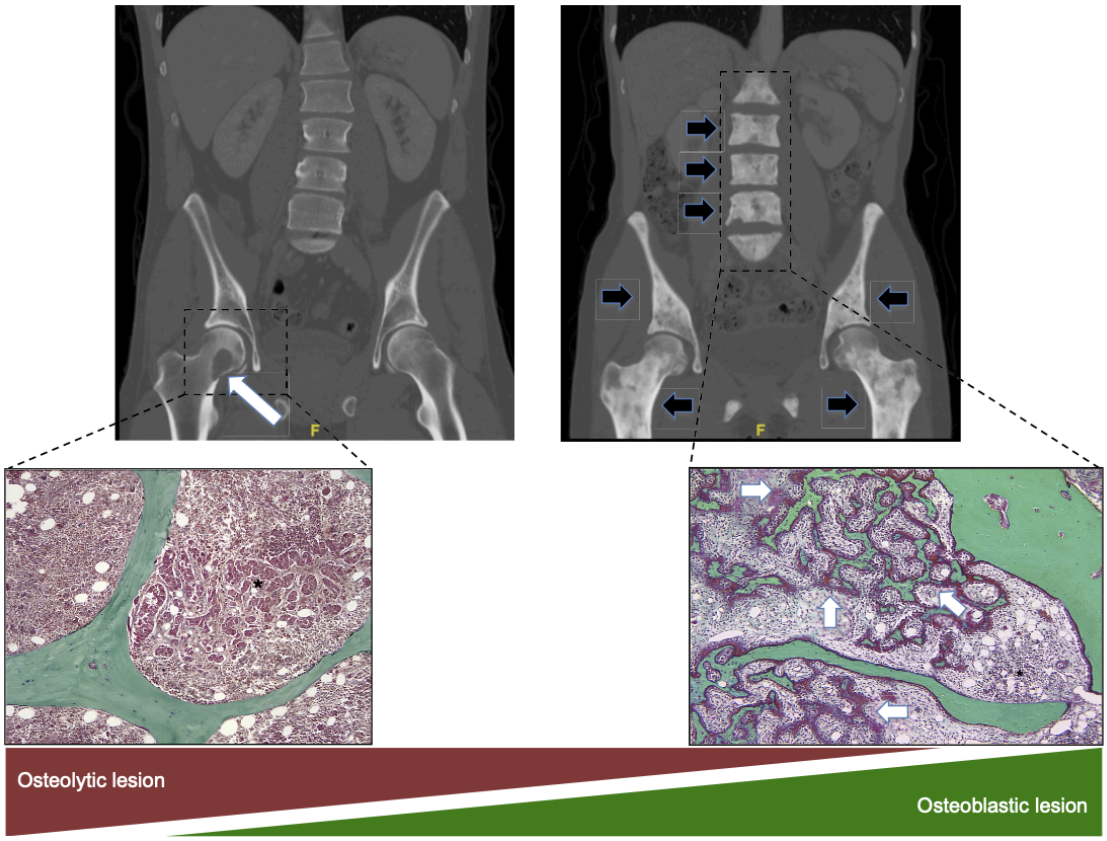

Figure 2.1: Patterns of bone metastases from solid tumors ranging from mostly destructive (osteolytic) to mostly bone forming (osteoblastic). Representative radiographs and histology of bone metastases with osteolytic (white arrow) or osteoblastic (black arrow) lesions are shown. For bone tumor sections, mineralized bone is stained green, whereas bone marrow and tumor cells are stained red. Of note, in osteoblastic lesions, extensive new woven bone (stained dark red) can be observed, leading to the formation of new trabecular bone that fills the bone marrow cavity (white arrow) [39].

Skeletal metastases are frequent complications of many cancers and cause bone complications (fractures, bone pain, disability) that negatively affect the patient's quality and expectancy of life [44].

With the exception of some relatively rare malignancies, such as high-grade lymphoma or germ cell tumours affecting bone, metastatic bone disease is currently incurable.

However, for many patients the average prognosis after the development of bone metastases can be measured in years, especially in patients with metastatic breast or prostate cancer or multiple myeloma who, with modern therapeutic approaches, can often survive more than 5 years after the diagnosis of bone involvement [42,44]. Moreover, new drugs, such as tyrosine kinase inhibitors and immune checkpoint inhibitors, have significantly prolonged the control of primary disease in patients, resulting in longer survival and, consequently, a sufficiently long life span for bone metastases to become clinically relevant [45]. The epidemiology of bone metastases is therefore evolving. In the coming years, we can therefore expect the occurrence of bone metastases in patients who a few years ago would never have developed clinically detectable bone metastases, because they would have died from their tumour at a time when they only had bone marrow micrometastases (subclinical) [45]. Consequently, the possibility of studying the behaviour of bone metastases is increasing[46]. A crucial step forward because metastasis is a complex, multistep process responsible for >90% of cancer-related deaths [45,46].

Naturally, the presence of metastases entails a number of variations in the cell. Genetically, there are changes in cell metabolism, morphology, alterations in signalling and protein expression; while there is a modification in the physical interactions of tumour cells with their microenvironment, as well as their modulation by mechanical forces. These alterations will be described in detail below.

2.2 MAIN CHANGES IN A CANCER CELL

Cells, the fundamental units of organisation of living matter [47], can exist in two main physiological states: normal, well-differentiated cells, which reproduce faithfully, undergo apoptosis when damaged (or stimulated to do so by their internal clock) and adhere to each other to form regular tissues or organs, and tumour cells, poorly differentiated, which reproduce in an unfaithful and sometimes unlimited manner, escape apoptosis, colonise organs where they do not belong and associate in relatively disordered assemblages (tumours) rather than forming well-defined tissues and organs [48]. If a normal cell undergoes a transition such that it evades apoptosis due to the accumulation of genetic mutations [49] or sometimes due to somatic damage (e.g. from ionising radiation or toxins), two classes of organisational changes are triggered: at the cellular level (cancer initiation) and at the population level (cancer progression) [50].

The first category includes changes in cellular metabolism, such as the transition from oxidative phosphorylation to glycolysis (the so-called Warburg effect [51,52]), the epithelial-mesenchymal transition (EMT) characterised by changes in cell morphology and

motility [47], as well as the activation of a series of alterations in signalling and protein expression [51]. These three classes of changes (physiological, morphological and molecular) are intimately related and probably result from epigenetic transformations [55]. Changes at the population level involve the replacement of one group of cells, which adhere to each other to form a differentiated tissue, with another group of cells, which form a highly heterogeneous and more mobile aggregate: a tumour or neoplasm [55].
It should be noted that although most investigations in the past have focused on biochemical and chemical carcinogens, it is becoming increasingly clear that living cells are also profoundly affected by physical forces, such as shear stress, surface adhesive forces and pressure [53]. Moreover, different cell types respond differently to these stimuli [54]. Understanding how normal and cancer cells respond to macroscopic chemical and physical variables is one of the most interesting developments, which promises not only to lead to a better understanding of the origin of cancer, but also to offer new diagnostic techniques and therapeutic recommendations [55]. A classification of cancer cells in terms of the variables described above should provide a deeper level of understanding of the processes that lead from normality to malignancy, as well as the factors that can stop and even return a cell from a malignant to a normal state [55].

Once the main features of the tumour cell have been highlighted, the next step will be to reconstruct all the various steps that a tumour cell has to take to invade the surrounding tissues, the metastatic process. Going on to describe the importance of genetic, physical and mechanical processes in each step of the cascade.

2.3 THE METASTASIS PROCESS : BONE COLONIZATION BY TUMOR CELLS

Bone colonisation by tumour cells is a gradual sequence of events involving: The formation of a premetastatic niche in the bone marrow to attract circulating tumour cells (CTC) [56]; Tumour cells from the primary tumour must undergo an epithelial-mesenchymal transition (EMT) to invade the surrounding tissue and enter the microvasculature (intravasation) of the blood and/or lymphatic system [57,58]; Once in the bloodstream, tumour cells can

disseminate to distant organs, exit the blood vessels (extravasation) and establish themselves in the foreign microenvironment, where they enter a dormant state or proliferate to later form macroscopic secondary tumours (metastases) [57].

In all these stages that make up the metastatic process, tumour cells migrate or flow through very different microenvironments, including the stroma, the endothelium of blood vessels,

the vascular system and the tissue at a secondary site [57,58] (Figure 2.2). The ability to successfully navigate each of these steps and advance to secondary tumour formation and growth depends, in part, on the physical interactions and mechanical forces between tumour cells and the microenvironment [56]. For example, the physical interactions between a cell and the extracellular matrix, the collagen-rich scaffold on which it grows, play a key role in allowing cells to migrate from a tumour to nearby blood vessels. During intravasation and extravasation, cells must undergo large elastic deformations to penetrate endothelial cell-cell junctions [56]. In the vascular system, the interaction between cell speed and adhesion influences the binding of tumour cells to blood vessel walls and thus the location of sites where a secondary tumour can form and grow [56].

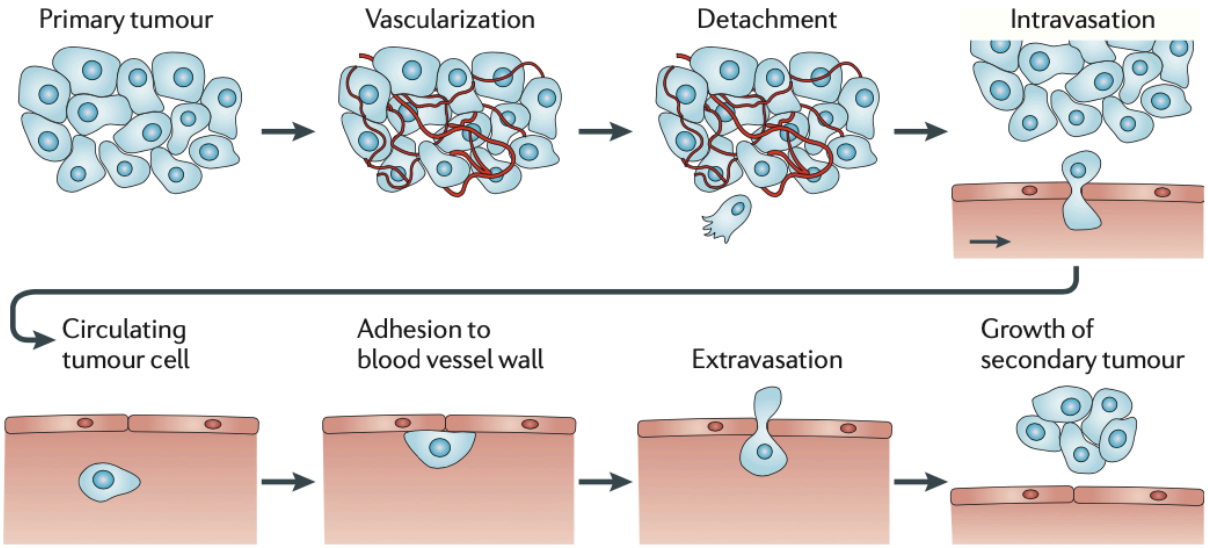

Figure 2.2: The metastatic process. In this complex process, cells detach from a primary, vascularized tumour, penetrate the surrounding tissue, enter nearby blood vessels (intravasation) and circulate in the vascular system. Some of these cells eventually adhere to blood vessel walls and are able to extravasate and migrate into the local tissue, where they can form a secondary tumour [56].

2.3.1 The Pre-metastatic Niche

A key phase of metastasis is the entry of circulating tumour cells (CTCs) into secondary or distant organ sites to become disseminated tumour cells for subsequent metastasis; however, this phase is critically influenced by the local microenvironment that CTCs encounter, which determines whether or not they can colonise tumour cells [59]. Primary tumours prepare the local microenvironment (pre-metastatic niche) by releasing soluble molecules such as TDSFs (Tumour Derived Secreted Factors), extracellular vesicles and other

molecular components that act at a distance, in distant organs for colonisation of tumour cells even before their arrival [59]. The role of TDSFs is mainly to recruit bone marrow myeloid cells into the pre-metastatic niche, which in turn secrete pro-inflammatory, pro-angiogenic molecules and growth factors [60]. Other factors are derived from the local stroma, i.e. the tissue that forms the supporting scaffold of the target organ. The fibroblasts or endothelial cells that make it up can be reprogrammed by the tumour to produce pro-inflammatory cytokines or metalloproteinases, which favour the establishment of metastatic cells [59,60].

For the understanding of tumour metastasis and for the explanation of organotropism in metastasis the 'seed and soil' hypothesis has been determinant: pro-metastatic tumour cells (the 'seed') colonise at specific sites in the organ (the 'soil') where the microenvironment is favourable for metastasis [41,59]. Increasing evidence shows that the primary tumour can promote metastasis by inducing the formation of a supportive microenvironment at a secondary organ site, termed the pre-metastatic niche [41].
The pre-metastatic niche can be defined as a supportive and receptive tissue microenvironment that undergoes a series of molecular and cellular changes to form the designated sites for metastasis, or the fertile 'soil' in preparation for the colonisation of the 'seed' of metastatic tumour cells, thus supporting tumour establishment in the distant organ and promoting tumour metastasis. This concept is similar to the physiological 'niche' in that a paradigm is established to confer specific functions to the cells within this niche [41].
The characteristics that the niche must possess include, for example, immunosuppression, inflammation, hypoxia (oxygen deprivation) and the formation and increased permeability of new blood or lymphatic vessels [41].

2.3.2 Physical interactions in the invasion

After growth of a primary tumour and definition of the pre-metastatic niche, the combination of continued tumour proliferation, angiogenesis, accumulated genetic transformations and activation of complex signalling pathways trigger the metastatic cascade (Figure 2.3) [56]. In particular, the detachment of carcinoma cells from the epithelium and the subsequent invasion of the underlying stroma resemble, both on a cellular and molecular level, the epithelial-mesenchymal transition (EMT) well characterised in embryogenesis [61]. The role of EMT in tumour metastasis is currently being explored [62]. The loss of E-cadherin (an intercellular adhesion molecule) and cytokeratins (structural fibrous protein) is a critical factor in EMT, leading to drastic changes in the physical and mechanical properties of cells: in particular, reduced intercellular adhesion and a morphological change from cuboidal epithelial to mesenchymal

[63]. A consequence of these changes is detachment from the primary tumour and the acquisition of a motile phenotype [64]. These cells also begin to express matrix metalloproteinases (MMPs) on their surface, which promote digestion of the laminin (Fibrous protein that performs mainly adhesive functions) and collagen IV rich basement membrane [65]. After leaving the tumour microenvironment, mobile tumour cells encounter the architecturally complex extracellular matrix (ECM), which is rich in collagen I and fibronectin[56]. In the vicinity of the tumour, the matrix is often stiffer than in normal tissue, due to increased collagen deposition and lysyl-oxidase (LOX)-mediated crosslinking of collagen fibres by tumour-associated fibroblasts [66]. Collagen crosslinking increases integrin (Glycoprotein capable of promoting the adhesion of similar cells to each other) signalling and aggregation of individual fibres [67]. These changes in the physico-chemical properties of the matrix may increase cell proliferation and invasion in a positive feedback loop [56]. However, despite recent technological advances, very little is still known about the molecular and physical mechanisms that drive mobile tumour cells away from the primary tumour and into the stromal space, especially at the subcellular level [56].

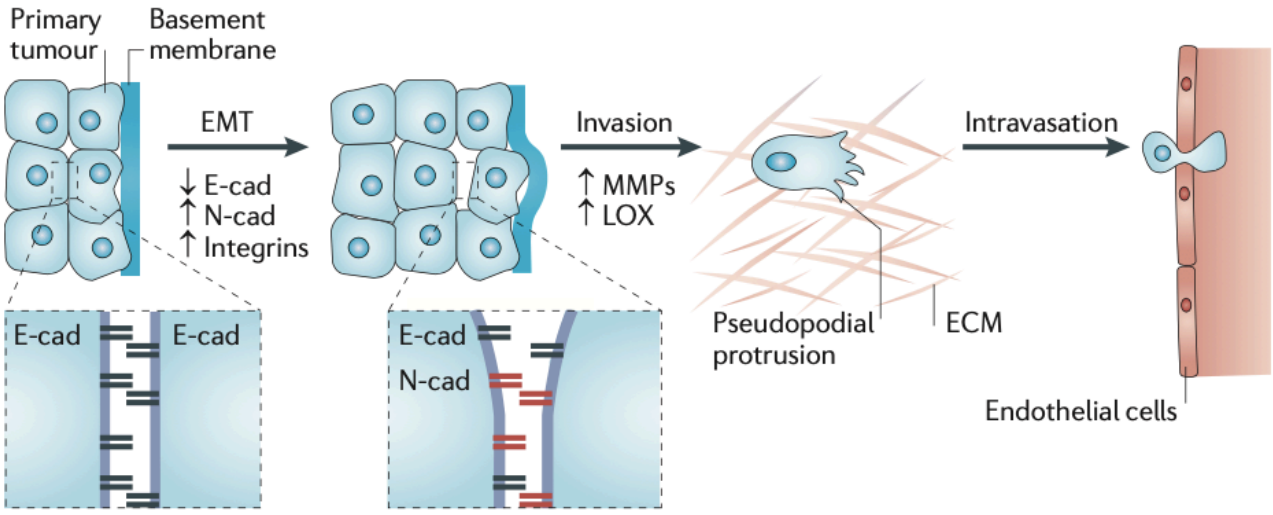

Figure 2.3: The physics of invasion and intravasation. The epithelial-to-mesenchymal transition (EMT) is associated with a loss of adhesion through downregulation of E-cadherin (E-cad) and a change in morphology. Invasion by tumour cells of the surrounding tissue and subsequent motion is dictated by the physicochemical properties of the extracellular matrix (ECM). By squeezing between blood vessel endothelial cells, tumour cells can enter the vascular system. All of these steps involve physicochemical processes, such as adhesion and deformation, that are dependent on the local environment [56].

2.3.3 The role of cell mechanics in intravasation

During entry and exit from the vascular system, tumour cells undergo drastic shape changes, driven by cytoskeletal remodelling, which allow them to penetrate the endothelial cell-cell junctions [56]. The cytoplasm is a complex composite system that behaves like an elastic material at high strain rates, but more like a viscous material that exhibits yield stress at low strain rates [67]. Elasticity reflects the ability of cytoplasm to rebound upon the application of a force, while viscosity measures the ability of cytoplasm to flow under the action of an external shear [56]. However, since MMP-mediated matrix digestion appears to be only partial, the step that limits the speed of cancer cell migration within a matrix or through an endothelium could be the deformation of the interphase nucleus (encloses the genomic DNA, as well as the machinery for regulation of gene expression, RNA synthesis, and DNA replication), which is the largest organelle in the cell and is about ten times stiffer than the cytoplasm [68,69] . The elasticity of the nucleus appears to be determined by the nuclear lamina underlying the nuclear envelope and the organisation of chromatin (a complex structure composed of DNA and proteins, located in the cell nucleus) and LINC complexes (linkers of the nucleus and cytoskeleton) [69,71] . LINC complexes are protein assemblies that cover the nuclear envelope and mediate physical connections between the nuclear lamina and cytoskeleton [72]. These connections are mediated by interactions between SUN domain-containing proteins (including SUN1 and SUN2) and KASH (Klarsicht homology) domain-containing proteins on the outer nuclear membrane (including the giant isoform nesprin 2 and nesprin 3, which can bind actin directly or indirectly) [56] . Indeed, depletion of LINC complex components, including nesprins(a family of proteins that are found primarily in the outer nuclear membrane) and SUN proteins, leads to nuclear shape defects and associated softening of the nucleus and cytoplasm [73].

Biophysical measurements comparing the mechanical properties of normal and tumour cells have consistently shown that tumour cells are softer than normal cells and that this cell softness correlates with a higher metastatic potential [74]. In cancer cells, a softer cytoplasm correlates with a less organised cytoskeleton. However, the softening of the cytoskeleton still needs to be verified in vivo or in a 3D matrix in the presence and absence of interstitial flow [56]. This is important because the physical properties of the environment, such as the stiffness and dimensionality of the ECM and the presence of interstitial flow, regulate cell mechanics [56]. The development of new methods, such as particle-tracking micro-networkology, will allow these measurements to be made in animal models, making it possible to directly test the hypothesis that tumour cells exhibit lower stiffness than non-transformed cells. It is likely that migration through a 3D matrix and penetration through an endothelium require optimal mechanical properties: if they are too stiff or too soft, the cells

cannot deform the highly cross-linked collagen fibres of the matrix to migrate efficiently [56]. However, measurements of single cells have consistently revealed that single cells of a particular cell type are usually heterogeneous and exhibit a wide range of mechanical properties. This suggests that cells with the optimal mechanical properties for invasion and intravasation into blood vessels are likely to maintain this phenotype for several generations. If mechanical properties are randomly determined by cell division, the wide distribution of mechanical properties implies that migration and intravasation would be unlikely events [56]. Therefore, an important question is whether the physical attributes of cancer cells, such as stiffness, are transmitted from generation to generation. If these physical properties are inherited, then it may be possible to alter them, through pharmacological inhibition or activation of proteins that influence cell mechanics, so that they are not optimal for stromal invasion and intravasation [56].

Different optimal mechanical properties are probably required for each stage of the metastatic cascade. For example, the optimal mechanical properties for invasion into the stromal matrix near the primary tumour site might be different from the mechanical properties of cells with optimal (efficient) intravasation [56]. Therefore, the mechanical properties of tumour cells could dynamically change during the metastatic process to successfully survive the difficult and changing environment of blood vessels, lymphatic vessels and stromal space. These differences in mechanical properties could also be modulated by biochemical gradients, interstitial fluxes and endogenous electric fields [56].

2.3.4 Shear stress and the circulatory system

During transit through the circulatory system, tumour cells are subject to haemodynamic forces, immunological stress and collisions with host cells, such as blood cells and endothelial cells lining the vessel wall [56]. All these stresses can affect cell survival and the ability to create metastatic foci. Only circulating tumour cells (CTCs) that overcome or even exploit the effects of fluid shear and immunosurveillance adhere to the vascular endothelium of distant organs, leave the circulation and successfully penetrate these tissues. A small fraction of CTCs survive and generate metastases; most CTCs die or remain inactive [56].

When they enter the circulatory system, the trajectory or path of a cancer cell is influenced by a number of physical and mechanical parameters: the pattern of blood flow, the diameter of the blood vessels and the complex interaction between shear stress and intercellular adhesion that leads to the arrest of cell movement in larger vessels [56]. Shear stress (τ) occurs between adjacent layers of fluid (in this case blood) of different viscosity (μ) moving at different speeds. The velocity of a fluid in a cylindrical tube is greatest at the centre and null at the cylinder walls, and the relative velocities of adjacent parallel layers of fluid in laminar flow define the shear velocity ($d\gamma/dt \equiv \dot{\gamma}$) where γ is the strain amplitude and t is the

elapsed time. Shear stress is defined by the product of fluid viscosity and shear rate and has as its unit the force per unit area (Newton per square metre (Nm^{-2}) or dynes per square centimetre (dyn cm^{-2})) [56].

At shear rates above 100 s^{-1}, blood is considered a Newtonian fluid, which implies that shear stress increases linearly with shear rate. The normal time-averaged levels of shear stress vary between 1-4 dyn cm^{-2} in the venous circulation and 4-30 dyn cm^{-2} in the arterial circulation [75]. The maximum shear stress is experienced at the vessel wall.
Shear flow influences the translational and rotational movement of CTCs (see next section) and thus determines the orientation and time constant associated with receptor-ligand interactions leading to adhesion [56]. Shear flow can also induce CTC deformation and margination towards the vessel wall. However, the extent of these effects and their influence on occlusion and adhesion remain to be determined [56].

2.3.5 Extravasation of circulating tumour cells

For a circulating tumour cell to leave the circulatory system, it must first bind to the wall of a blood vessel. There are two mechanisms of arrest, physical occlusion and cell adhesion; the relative prevalence of these mechanisms depends on the diameter of the local blood vessel (Fig. 2.4) [56].
Physical occlusion. If a circulating tumour cell enters a vessel whose diameter is smaller than that of the circulating tumour cell ($d_{vessel} < d_{cell}$), arrest may occur by mechanical entrapment. Since circulating tumour cells of epithelial origin are usually >10 μm in size, physical occlusion occurs in small vessels or capillaries of <10 μm. Branching arrest in brain blood vessels, resulting in extravasation and metastasis formation, was observed by intravital microscopy in a mouse model [56].
Adhesion. Extravasation of a tumour cell from a large blood vessel ($d_{cell} < d_{vessel}$) requires adhesion of the cell to the vessel wall through the formation of specific bonds. The probability (P) of arrest in a large vessel can be written as $P \propto ft$, where f is the collision frequency between membrane-bound receptors and endothelial ligands and t is the residence time [76]. The residence time depends on the shear force exerted on the cell and the adhesive forces associated with the ligand-receptor pairs between the circulating tumour cell and the endothelial cells of the blood vessel wall [56]. An increase in fluid shear is expected to increase the frequency of collision with the endothelium, but decrease the residence time of the receptor-ligand pairs [56].

A cell moving along the wall of a blood vessel has both translational and tangential (angular) velocity. The translational speed of a cell is always greater than the tangential speed of the surface, resulting in a sliding movement relative to the stationary blood vessel wall [56]. This gliding motion increases the encounter speed between a single receptor on a CTC and ligands on the vessel wall [77]. For a rotating cell, the rotation brings successive receptors on the surface of the CTC into contact with ligands on the vessel wall. The total adhesion strength depends non-trivially on the tensile strength of the single receptor-ligand bond and the number of receptor-ligand pairs involved [56].

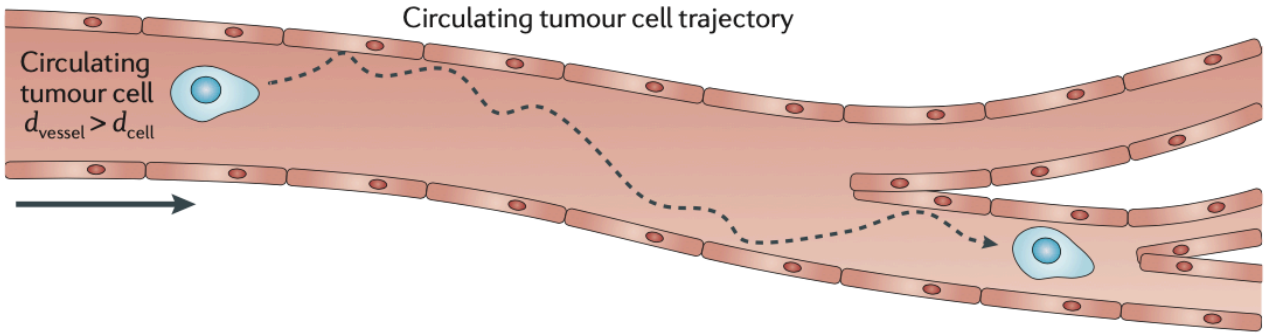

Figure 2.4: Arrest of circulating tumour cells. Tumour cells with a diameter (d_{cell}) less than the diameter of the blood vessel wall (d_{vessel}) will follow a trajectory that is determined by the local flow pattern and by collisions with host cells and blood vessel walls. Collisions with a blood vessel wall may lead to arrest. Tumour cells with diameter greater than the diameter of a blood vessel will be arrested owing to mechanical trapping (physical occlusion) [56].

The probability of arrest, leading to extravasation, should be highest at intermediate values of shear stress. The kinetic (rate of activation and deactivation) and micro-mechanical (tensile strength) properties of a single receptor-ligand bond determine the formation of a bond at a given level of shear stress and the macroscopic pattern of cell adhesion (Figure 2.5a) [56]. For example, the initiation of receptor-mediated cell adhesion under shear stress requires: a relatively fast activation rate, which allows receptor-ligand binding at relatively short interaction (encounter) times; sufficient tensile strength to withstand the dispersive hydrodynamic force; and a relatively slow deactivation rate, which will provide adequate bonding duration, thereby facilitating further bond formation [56]. Receptor-ligand pairs, such as selectins and their ligands discussed below, that exhibit high tensile strength, a fast ON rate and a relatively fast OFF rate can initiate binding under shear stress and mediate transient rolling interactions. Molecules with slower ON speed, such as integrins, can only bind after selectin-mediated cell binding or, in the absence of selectin-dependent interactions, at very low shear stress[56]. The clustering of integrins is responsible for the firm, multi-bond adhesion of cells to surfaces[78]. Thus, integrins are involved in tumour cell dissemination and may also control angiogenesis and metastatic growth [79].

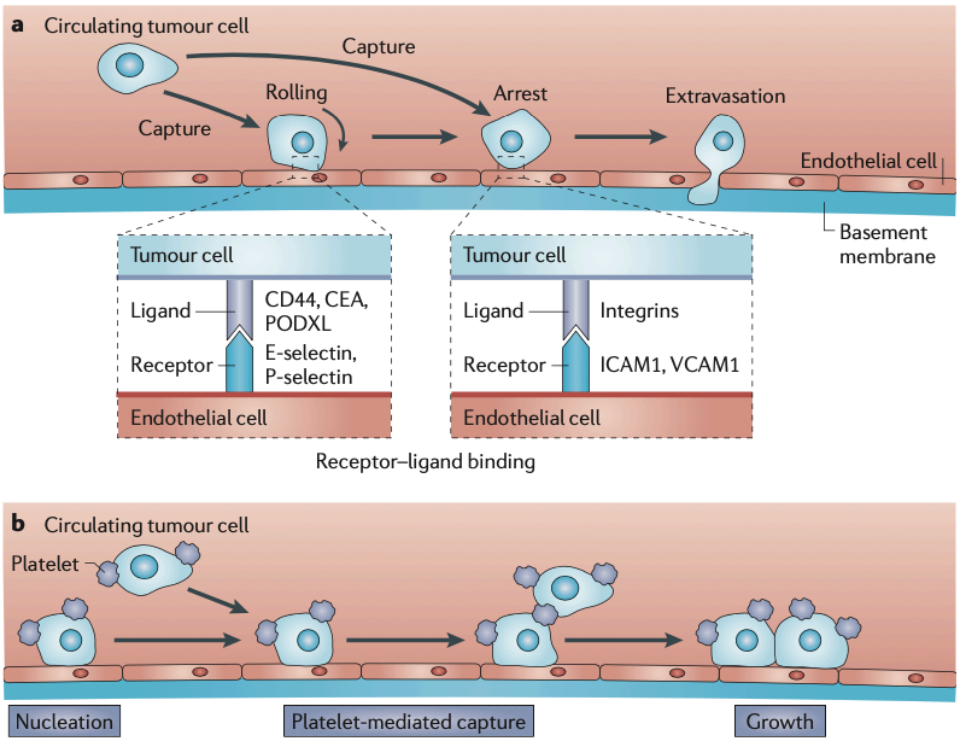

Figure 2.5: Capture and arrest of circulating tumour cells. a) A collision between a cell and a vessel wall may lead to transient and/or persistent (firm) adhesion as a result of ligand–receptor interactions. Persistent adhesion either follows transient binding or is initiated at very low shear stress and involves interactions between integrins and their receptors. b) The association of tumour cells with platelets may enhance arrest through platelet-mediated capture, a process analogous to nucleation and growth. The growth process is achieved by a platelet-bridging mechanism, whereby platelets adherent to an endothelium-bound carcinoma cell serve as a 'nucleus' to capture free-flowing cells that subsequently attach to the blood vessel wall downstream or next to the already adherent cell [56].

Evidence suggests that CTCs may escape immune surveillance and promote their exit from the circulatory system by associating with platelets [56]. Direct evidence of the role of platelets in metastasis comes from studies in a mouse model demonstrating inhibition of metastasis by pharmacological or genetic depletion of platelets and restoration of metastatic potential by platelet infusion [56]. By forming heterotypic adhesive clusters with CTCs, platelets are thought to mask and protect CTCs from immune-mediated elimination mechanisms [56]. Platelets may also facilitate the adhesion of tumour cells to the vessel wall (Figure 2.5b) and release a number of bioactive compounds such as vascular endothelial growth factor (VEGF) at the sites of attachment to the endothelium, thus promoting vascular hyperpermeability and extravasation [80]. After tumour cells have left the circulation, factors secreted by activated platelets can induce angiogenesis and stimulate growth at the metastatic site [79,80]. CTCs can also hijack polymorphic nuclear leukocytes (PMNs) to arrest in the endothelium of distant organs. Microscopy studies have shown PMNs in close association with metastatic tumour cells during tumour cell arrest and extravasation in vivo [81].

It is believed that tumour cells can form multicellular complexes with platelets and leukocytes (via P- and L-selectin-dependent mechanisms). These multicellular complexes may then arrest in the microvasculature of distant organs and may eventually extravasate and establish metastatic colonies [56].

2.3.6 The location of metastatic sites

Models of metastasis have been explained in terms of two hypotheses. The 'seed and soil' hypothesis states that a cancer cell will metastasise at a site where the local microenvironment is favourable [41,56], just as a seed released from a plant will only grow if it lands in a site where the soil is fertile. The 'mechanical' hypothesis states that metastasis is likely to occur at sites based on the pattern of blood flow [82]. It is believed that both blood flow (mechanical hypothesis) and the local microenvironment (seed and soil hypothesis) have complementary roles in influencing the location of a metastatic site [82].
Based on the previous discussion of circulating tumour cell arrest, we can elaborate on the physics of metastatic site localisation [56]. Blood circulates from most organs to the heart and then to the lungs through the venous system, and then back to the heart and circulates to the organs through the arterial system. The capillary beds of organs are characterised by a network of small blood vessels. If a cancer cell encounters a capillary with a diameter smaller than the size of the cell (dcell > dvessel), the probability of entrapment of the cell by physical occlusion at that site is very high [76]. For metastasis to occur, the cancer cell must still extravasate and colonise the local tissue [77]. In one study, more than 50 per cent of metastases could be explained by the pattern of blood flow between the primary and secondary sites [83]. Since cell entrapment, extravasation and colonisation occur in series, we can hypothesise that the probability of a metastasis occurring at a specific site, in accordance with the mechanistic hypothesis, can be expressed as $P \propto P_t * P_{e,i} * P_{c,i}$, where P_t is the probability of encountering a vessel with a diameter smaller than the cell diameter, $P_{e,i}$ is the probability of extravasation at that site and $P_{c,i}$ is the probability of colonisation [56]. The probability of extravasation and colonisation should depend on the local microenvironment [56].
Every collision between a circulating tumour cell and the wall of a blood vessel, where $d_{cell} < d_{vessel}$, has the possibility of causing adhesion [76]. If the dwell time is long enough, the tumour cell can adhere to the blood vessel wall and thus extravasate. The probability of the dwell time being long enough for extravasation to occur is related to the local shear stress [56]. A further complexity is that the expression levels of adhesion proteins are different in different organs and thus the strength of receptor-ligand adhesion may also be organ-specific [84]. For the seed-soil hypothesis, we write the probability of metastasis

occurring at a specific site i as $P_i \propto P_{a,i} * P_{e,i} * P_{c,i}$, where $P_{a,i}$ is the probability that a collision at site i leads to adhesion and $P_{e,i}$ and $P_{c,i}$ are the same as defined above [56]. It is evident from these considerations that the probability of metastasis occurring in a specific organ, in both the mechanical and the seed and soil hypotheses, has common elements related to extravasation and colonisation, both of which are dependent on the local microenvironment [56].

As described above, it is likely that there is an optimal range of shear stress, to obtain a sufficiently long residence time [56]. For example, in vitro adhesion assays reveal that metastatic tumour cells bind to the vascular endothelium in the presence of venous but not arterial shear stress levels [85] . Furthermore, it has been reported that high shear stress (12 dyn cm^{-2}), similar to that found in the arterial circulation, causes cell cycle arrest of metastatic tumour cells, facilitating their eradication by the immune system [86]. Conversely, it has been shown that low levels of shear stress, typical of the venous system, can have opposite effects on intracellular signalling and tumour cell function [56].

2.4 MAIN CHARACTERISTICS

Bone is one of the most common sites of metastases, especially for breast, prostate and lung cancer. These skeletal metastases contribute substantially to morbidity and mortality in patients with advanced cancer. It is therefore essential to better understand the pathophysiology of bone metastases in order to improve therapies for the treatment and prevention of bone metastases and to predict the risk of disease relapse.

We list below what may be the hallmarks of bone metastases and most cancers : self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limited replicative potential, sustained angiogenesis and avoidance of apoptosis [55]. From a physical point of view, other common and important features of cancer initiation and progression can be listed, as seen above [87]. These can be divided into two groups:

I. Mechanical and Structural:
1. Change in the viscoelasticity of cells: a higher level of stiffness of the ECM and a lower level of stiffness of cancer cells compared to normal cells. These changes are accompanied by major changes in cell and nuclear morphology and chromatin architecture, which facilitate cell motility and invasive potential.
2. Changes in membrane composition (e.g. overexpression of signalling proteins and/or p-glycoproteins).

3. Epithelial to mesenchymal (ETM) transition in cell morphology, resulting in reduced synchronisation of cellular functions and a higher level of motility.
4. De-differentiation and elimination of various signalling pathways, especially apoptotic ones, which allow tumour cells to survive, spread and thrive in 'foreign' organs,
5. Production and secretion of specialised proteins that dissolve base and other membranes to facilitate cell motility [55].

II. Metabolic:
1. A glycolytic switch, also called the Warburg effect, which results in increased metabolic energy production using the glycolytic pathway rather than the oxidative phosphorylation pathway.
2. Hypoxia, which is related to the glycolytic switch.
3. A decrease in the trans-membrane potential.
4. A decrease in cellular pH values, probably also related to the Warburg effect [55].

3. THE TUMOUR SEEN AS A PHASE TRANSITION

This first part of the work aimed to understand in detail the main mechanisms involved in the process of bone metastasis. This study was necessary to understand the behaviour of tumour cells, the various genetic changes and the physical forces to which they are subjected.
Hence the idea of the thesis, through an in-depth study of the literature, of the possibility that indeed the bone tumour could present those characteristics that are in some way already described by the theory of phase transitions, thus to issues related to the coexistence of different phases in equilibrium and the loss of stability in the phase boundary. Hence my intention to establish a link between this very theoretical aspect and an experimental aspect that still lacks a theoretical description.

3.1 RELEVANT EXPERIMENTS

The idea of applying the theory of phase transitions to describe tumour progression in bone tissue stems from the study of two experiments [88,89] that took place at the Rizzoli Orthopaedic Institute, in which they used 3D models in an attempt to re-establish the native tumour and its microenvironment in order to mimic the biological processes occurring in patients as closely as possible and then derive satisfactory results.

They developed a three-dimensional (3D) in vitro model of metastases using human bone tissue taken from patients undergoing total hip replacement surgery, cultured with human breast or prostate cancer cells (MCF-7 and PC-3) and then established a 'proof of concept' to recapitulate bone metastases and their microenvironment. The main reason for the development of this dynamic 3D in vitro model was the need for a suitable model that takes into account the critical importance of species-specific osteotropism, which is essential in the study of bone metastases [88,89]. The development of this model has direct relevance in the study of bone metastasis because it more closely mimics metastatic microenvironments in humans and provides a compromise between the reductionist approach that isolates tumour cells as a 2D monolayer and the complexity produced by the growth of human tumours in xenogeneic hosts [88]. Furthermore, a model that so closely mimics human metastases could be important for evaluating new therapeutic interventions to prevent and treat bone metastases [88,89].
The three-dimensional model of tumour and bone metastases devised in this study was developed using a rolling apparatus system, in which suspended human breast or prostate cancer cells were cultured with free-floating human female and male bone fragments

isolated from discarded total hip replacement surgeries. The use of the rolling apparatus equally exposes all surfaces of human bone fragments to tumour cells [88]. In addition, to mirror in vivo conditions, the hypoxic condition was also considered to mimic the nutrient and oxygen deficiency in the tumour-host interaction. Indeed, bone is a hypoxic microenvironment (pO_2 between 1-7%) [90], which increases the growth of metastatic tumour cells that have adapted to survive in low O_2 conditions [88]. A brief description of the model is given in Figure 3.1 [88] .

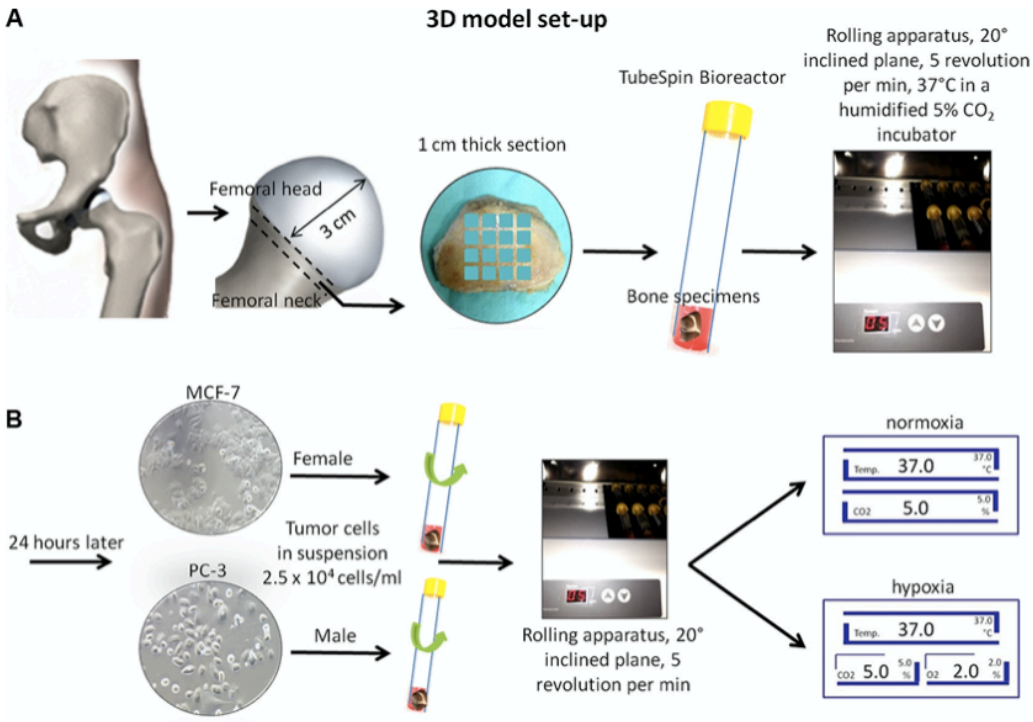

Figure 3.1: Schematic representation of the 3D model set-up. A) Femoral head and neck resection and cut of 1-cm thick section. Bone femoral samples (1 × 1 cm) were placed in a TubeSpin Bioreactor and with a 20° inclined plane in a rolling apparatus at 5 revolutions per minute for 24 hours at 37°C in a humidified 5% CO_2 incubatorn[88]. B) After 24 hours 2.5 × 104 cells/ml in suspension (MCF-7 in female bone specimens and PC-3 in male bone specimens) were added. Rolling TubeSpin were cultured for 7 days in a rolling tube apparatus (20° inclined plane and 5 revolutions/min) at 37°C in hypoxic conditions: in a humidified 2% O_2, 5% CO_2 and 88% N2 incubator or in normoxic conditions: in a humidified 5% CO_2 incubator [88].

The model used aimed to study the tumour/bone cell interaction over a relatively long period, up to 7 days. No decline in bone marrow viability was observed after 7 days of culture, as revealed by the Alamar Blue test and the appearance of histological sections. In fact, the bone viability results obtained immediately after surgery were maintained after 7 days of culture with and without tumour cells (MCF-7 and PC-3), both under normoxic and hypoxic conditions.

Gene expression profiling, protein levels, histological, immunohistochemical and four-dimensional (4D) micro-CT analyses were also conducted, which showed a remarkable specificity of breast and prostate cancer cells for bone colonization and growth, thus highlighting species-specific and sex-specific osteotropism and the need to expand current knowledge on the spread of cancer metastases in human bone tissue [88].

Promising results were obtained from this 3D model; however, some limitations of the study and a possible future update using a larger sample should be considered. Firstly, they followed the cell/bone interaction up to 7 days, so an essential improvement would be to optimize the culture conditions for longer experimental times also in order to study microstructural and bone density parameters. Secondly, any molecular changes should be further explored to identify other pathways and key factors that could contribute to bone metastasis. Finally, the lack of a functioning circulatory system in this model prevents the study of tumour cell extravasation [88].
In conclusion, this dynamic 3D system supports the 'proof of concept' for the application of this model to recapitulate the spread of bone metastases in vivo, particularly by monitoring and controlling hypoxia, which seems to better mimic the physiological conditions of tumours. Compared to other models, the proposed system appears to be cost-effective and, as a result, more experiments can be performed to obtain extended data sets for reliability, reproducibility and statistical analysis.
The versatility of this 3D model offers the possibility to further explore the application of the model for other clinical applications, e.g. increasing the biological complexity of the system by adding more cell types or increasing the culture time [88,89].
We leave aside the merits and defects of the 3D model to focus on the actual distribution of cancerous cells in relation to healthy ones, the starting point for developing our idea.

3.2 TUMOUR ONSET AS A PHASE TRANSITION

Let us begin by looking at two significant images, where we have respectively a breast tumour (Fig. 3.2A) and a prostate tumour (Fig. 3.2B) that have metastasized in the vertebrae, in which we can see what we discussed earlier. When we described the metastatic process, we saw that the first step in the invasion of tumour cells into distant organs is the preparation by the primary tumour of a 'soil', i.e. a pre-metastatic niche is formed that will be favourable to receiving tumour cells once they have entered the circulation. In the proposed images, we can see that the tumour is localized in a precise location, tumour cells entering the blood vessels tend to be attracted to these pre-metastatic niches and then tumour growth will take place from there.

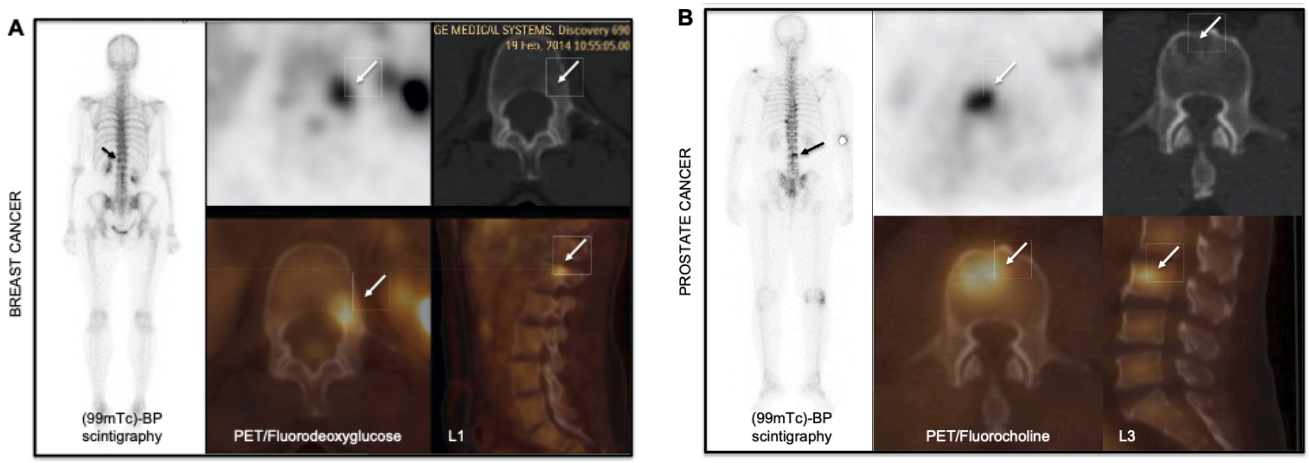

Figure 3.2 A: clinical presentation of a solitary asymptomatic bone metastasis in a 54-yr-old patient with breast cancer. B: clinical presentation of a solitary asymptomatic bone metastasis in a 67-yr-old patient with prostate cancer [56].

Now having a more detailed view of what is happening, we can use the results of the 3D model made by the Rizzoli team to get a microscopic view of what is happening at the cellular level (Fig. 3.3A and Fig. 3.3B).

From these images we can see that there are two phases, one consisting of healthy cells and the other of cancerous cells.

We can see that the healthy and cancerous zones have well-defined borders and this invasion of the cancerous cells into the healthy tissue can be explained as a loss of stability of the phase border between the two zones.

Cancerous tissue invades healthy tissue, just as happens in phase transitions, where one phase (e.g. solid) invades another phase (e.g. liquid).

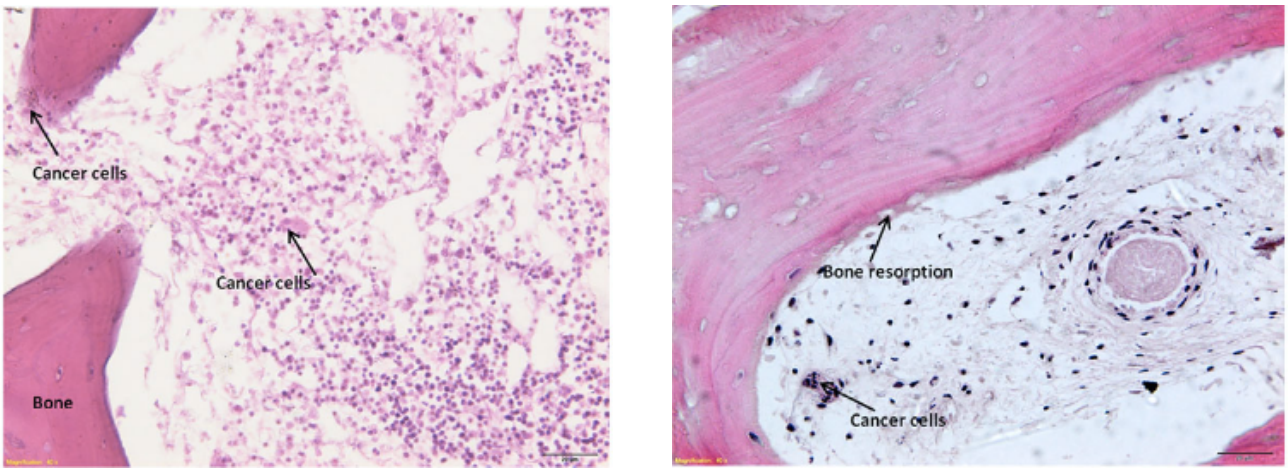

Figure 3.3: stained sections of human bone femoral head specimens grown in the presence of human breast cancer cells. 40 magnification (A), 80 magnification (B) [89].

We will see that while physical phase transitions typically occur at or near thermodynamic equilibrium, the transition from normal to cancer (NTC) is a dynamic non-equilibrium phenomenon, which depends on the metabolic energy supply and local physiological conditions [55]. This theory is based on the hypothesis that biological tissue energy can be described as non-convex energy. The non-convexity hypothesis is consistent with many of the phenomena already encountered in tumour propagation, notably phase front instability, invasion driven by a control parameter, the presence of fine mixtures, etc [55,91].
In the continuation of the thesis, we will attempt to show that describing cancer propagation from an energy perspective allows us to describe the spread of cancer cells as a form of instability. The latter can take place if the free energy of the aggregate is tracked by a non-convex energy with respect to an order parameter describing the regions occupied by healthy and cancerous cells.
The idea is to use phase transition theory to explain the key stages of cancer progression, providing an alternative to the more common approaches based on biochemistry, genomics and cell biology.

Although up to now we have talked about bone, bone-related metastatic process, we realized that what we have said can be extended to all tumour types and we can extend the concept of tumour propagation as a phase transition to all tumour types. So from now on when we talk about tumour, it will be understood as a general tumour and not as a bone tumour.

3.3 PHASE TRANSITIONS

The physics of phase transitions is part of statistical mechanics. A phase transition of a physical system is defined as the transition from one phase to another phase of the system. This involves a change in physical properties such as density, electrical conductivity, magnetization, crystal structure and so on. In the presence of a phase transition, there are singularities of free energy and/or its derivatives [92].

We give a first, albeit restrictive, classification of phase transitions. It is based on the thermodynamics of the system undergoing the phase transition at the time of the transition. A fundamental quantity that gives a precise indication of the thermodynamics of a system is entropy S. A change in entropy ΔS always corresponds to a change in heat ΔQ that occurs at a given temperature T, i.e. we have in general $\Delta Q = T\Delta S$. In the case of phase transitions, we speak of latent heat variation ΔQ_l .

First-order phase transitions are defined as those transitions in which there is a release or absorption of latent heat at the transition point. Since latent heat is related to entropy we have in general $\Delta Q = T\Delta S$ where T is the temperature of the transition,

$$\Delta Q_l = Q_{l2} - Q_{l1} \qquad \text{and} \qquad \Delta S = S_2 - S_1$$

where 1 and 2 denote the two phases. Phase 1 is the initial phase and phase 2 is the final phase. The relationship $\Delta S = S_2 - S_1$ implies that $S_1 \neq S_2$ at the transition point. Thus, first-order phase transitions are characterized by an entropy jump or discontinuity. The entropy jump is finite.

It should be noted that the release or absorption of latent heat by the system under consideration at the transition point is caused by an external pressure change at a certain temperature.

On the other hand, second-order phase transitions are those transitions in which there is no release or absorption of latent heat, i.e. $\Delta Q_l = 0$. In this case at the transition point there is no entropy change between phase 1 and phase 2, i.e. $S_1 = S_2$. Thus it is said that in this class of transitions the entropy varies continuously during the transition. We will deal with first-order phase transitions in the course of the thesis.

However, in order to frame phase transitions in a more general classification within which the classification just discussed remains valid, we start with the fundamental quantity that characterises a system from the point of view of statistical thermodynamics, i.e. free energy, which is the amount of macroscopic work that the system can perform on the environment. Its behaviour and that of its derivatives are studied. Free energy depends on the intensive variables of the system such as temperature T and pressure P. The intensive variables enjoy the property that, for an increase in the number N of the particles constituting the system, they tend to a finite limit. These variables can be controlled from the outside. Extensive conjugate variables such as volume V (conjugate of pressure P) and entropy S (conjugate of temperature T) are obtained by deriving the free energy with respect to the corresponding intensive variables. Extensive variables enjoy the property whereby, for N that grows, they grow as N. In other words, a quantity is extensive when, under conditions of thermodynamic equilibrium, the quantity of matter, i.e. the number N of particles in the system, increases linearly with N. Free energy and internal energy U are also extensive quantities.

To classify phase transitions, reference is made to the thermodynamic potential at constant pressure (referring to a fluid where pressure is an important intensive variable), also called Gibbs free energy or Gibbs free enthalpy expressed by G(T,P)=U-TS+PV.

Reference is also made to the thermodynamic potential at constant volume or Helmholtz free energy $F(T, V) = U - TS$.

In order to further characterize a phase transition, one very often uses so-called control parameters (e.g. temperature and pressure) that lead systems to instability when approaching their critical values, and the resulting changes in the corresponding order parameters (e.g. the density difference between a gas and a liquid) that describe the main physical changes in the system under investigation (equilibrium state) [93].
Each phase transition is characterized by an order parameter. The order parameter is a physical variable that gives a measure of the level of order in a system. In most cases, it describes many of the rearrangements of structure that occur due to the phase transition. It therefore allows a distinction to be made between the two phases involved in the transition. It can be a scalar quantity, a vector quantity or in certain cases a tensor quantity.

Important clarification, in this thesis we will deal with living systems, which unlike non-living systems exist in states far from thermodynamic equilibrium and phase transitions exist. Living systems are maintained by a flow of matter and energy between them and their surroundings, and an export of entropy to their surroundings to compensate for the creation and maintenance of structural order (entropy reduction) and functional organisation [94].

3.4 PHASE TRANSITIONS IN PHYSICS AND CELL BIOLOGY

Now, in order to apply the concepts just described to cancer, it is first necessary to determine what the relevant order and control parameters are [55]. In the case of a transition from normal to cancerous cells, the nature of the change taking place is that of a molecular and cellular reorganisation that leads to a drastic elimination of various cell cycle checkpoints and a simplification of the cell's functional programme that appears to be aimed exclusively at survival and proliferation, as already seen in section 2.2 [95].
In the case of the NTC phase transition, it is not difficult to identify macroscopically relevant physical changes; indeed, it is mainly from these changes that cancer is diagnosed [55]. These include gross alterations in the structure, function and organisation of cells and, to a certain extent, also of the surrounding tissue microenvironment [96].
With regard to control parameters, an obvious first choice might be temperature, as evidence [97] of reversible temperature-controlled NTC transitions in cells has been presented. However, biological systems are approximately isothermal, a necessary condition for many vital biochemical reactions to proceed normally, so temperature is likely to be a relevant control parameter for NTC phase transitions only in a limited number of heat-sensitive situations, such as testicular cancer [55]. The transition from oxidative phosphorylation

(normal cells) to glycolysis (tumour cells), hypothesised by Warburg as crucial for the emergence of the tumour phenotype, can be seen as a phase transition whose order parameter is the spatial distribution of metabolic enzymes [55]. Given the complexity of biological systems and the wide variety of cancer cell types, it is likely that there are multiple control and order parameters. Since cancer is not a single disease, but a collection of about 200 distinct pathological conditions, it is possible that there are different choices of order and control parameters for different types of cancer [55]. With this in mind, a list of candidate control parameters (in addition to temperature in some situations) includes chemical gradients, mechanical stress and pressure, therapeutic fluxes, electromagnetic radiation, ionising radiation, electric fields and concentration gradients of toxic carcinogens [55].

Thus, depending on the type of cancer, there may be different control parameters that take living cells from a normal to a cancerous state. These control parameters may involve external influences that destabilise the normal metabolism of the cell (reduced oxygen, increased concentration of carcinogens, reduced pH, increased harmful ionising radiation, etc.). On the other hand, the order parameter of the system defines the response to external perturbations [55]. In living systems, a clear example of an order parameter is the mitotic index (the fraction of the total cell population that undergoes mitosis) of cancer cells compared to the corresponding value of their normal counterparts for that organ. Furthermore, differences in the mechanical properties of cells and tissues can be identified, as tumour cells are generally softer mechanically, as described above, while the extracellular matrix (ECM) surrounding them is stiffer [98]. These properties can be used as secondary order parameters in addition to primary properties such as mitotic index, motility and metabolic rates.

The other key aspect concerns the energy of the system. It is noted that cancer progression must also involve a change at the population level, due to better adaptation of cancer cells to prevailing conditions and competition between the two coexisting phenotypes: normal and cancerous [55]. This change undeniably involves natural selection, which may favour the new phenotype, but natural selection does not cause the transition, it is a consequence of it [98]. Eventually, given the resources and time available, cancerous cells usually outnumber healthy ones in the organ or tissue in which they coexist, competing for space and resources. In Davies' work, used as the basis for this chapter, it is argued that this population shift in the direction of greater malignancy can be seen as a trend towards greater stability (and thus maximum entropy or minimum free energy) [55]. Static physical systems reach thermodynamic equilibrium by achieving minimum free energy within given physical constraints (e.g. constant temperature, volume or pressure), whereas living systems achieve dynamic stability (or robustness) as a competitive advantage over other species within the

externally imposed environment such as variable rate of nutrient supply, lower oxygen concentration or increased acidity [55].

3.5 THE MODEL OF DAVIES, DEMETRIUS AND TUSZYNSKI

Before moving on to the description of cancer propagation from an energy perspective, which will allow us to describe the spread of cancer cells as a form of instability. We will briefly describe the model proposed by Davies et al. , where he showed that indeed cancer propagation can be described through a first-order phase transition and a system energy that assumes a non-convexity profile.

The idea of the model is based on describing cancer in terms of a non-linear system, in which a living cell is represented as a stable attractor of a complex dynamic system [99]. In this article they present arguments that the transition from the stable 'normal state' attractor to the stable 'cancerous state' attractor can be described by a first-order (irreversible and discontinuous) phase transition. Under normal conditions, healthy cells represent a more stable state, whereas cancer is a metastable state (less stable but not necessarily unstable). With increasing cell damage caused by external events or internal dysregulation due to altered metabolism or accumulated genetic changes, the relative stability between these two types of states (normal and cancerous) shifts. This is similar to a first-order phase transition in physics, where two equilibrium states (ordered and disordered) reverse their roles (stable versus metastable) as the value of the control parameter changes. They are also characterised by extreme sensitivity to small perturbations and zero correlations throughout the system, which determine the global character of the phase transition. This property of phase transitions in the context of cellular transformations indicates a higher sensitivity and global nature of the NTC transition. For example, below 100°C liquid water is stable and water vapour is metastable, whereas above 100°C the situation is reversed. In the case of cells, the turning point between the two sets of conditions marks precisely the phase transition where the fate of the biological system hangs in the balance [55].

Based on the above, it is proposed in the work of Davies et al. that at this point in the NTC transition the situation is reversible, just as physical phase transitions can be reversed by a change in the value of control parameters. Once the system is pushed into one of the two stable phases, however, reversibility becomes much more difficult due to a potential energy barrier separating these states. This would also suggest that local interventions that do not change the global state of the organism, such as surgery and radiation, may not be entirely effective, or perhaps counterproductive, even in the absence of metastases and with perfect treatment plans.

Only the reversal of globally prevailing conditions, such as a shift in the values of the control parameters (analogous to lowering the temperature below a transition value or increasing the pH of the environment), can lead to the total eradication of cancer from the organism. Moreover, as first-order phase transitions are characterised by so-called hysteresis loops [93] that make them irreversible, the return to the original phase requires an 'overshoot' of the control parameter values, which is necessary to destabilise the new phase. Consequently, an NTC transition, depending on the control parameter that causes it, would require not only an intervention to reverse the change (e.g. reoxygenation and deacidification of the environment), but also overcompensation beyond what would be considered a normal set of parameters [55].

As already mentioned, NTC phase transitions can be considered analogous to changes in macroscopic equilibrium states in physical systems. This can be described mathematically as a dependence of the free energy F(x) as a function of the order parameter x parameterised by a control parameter a [55].
To illustrate the key ideas, Figure 3.4 shows the predictions of a toy model. For different values of the external control a, the stability 'landscape' described by F(x) changes character intuitively. Curve A shows a system with a single stable equilibrium phase; curve B illustrates the nucleation of a metastable state on the right; curve C presents two equally stable states; curve D shows how the previously metastable state has now acquired global stability and will thus be favoured by the system [55]. All these changes are achieved by manipulating a single (control) parameter a in the free energy function. These graphs schematically illustrate the concept of a typical phase transition. It can be shown that this quartic polynomial free energy function of the order parameter is a generic form describing a broad class of first-order phase transitions within the Landau approximation [93,100].

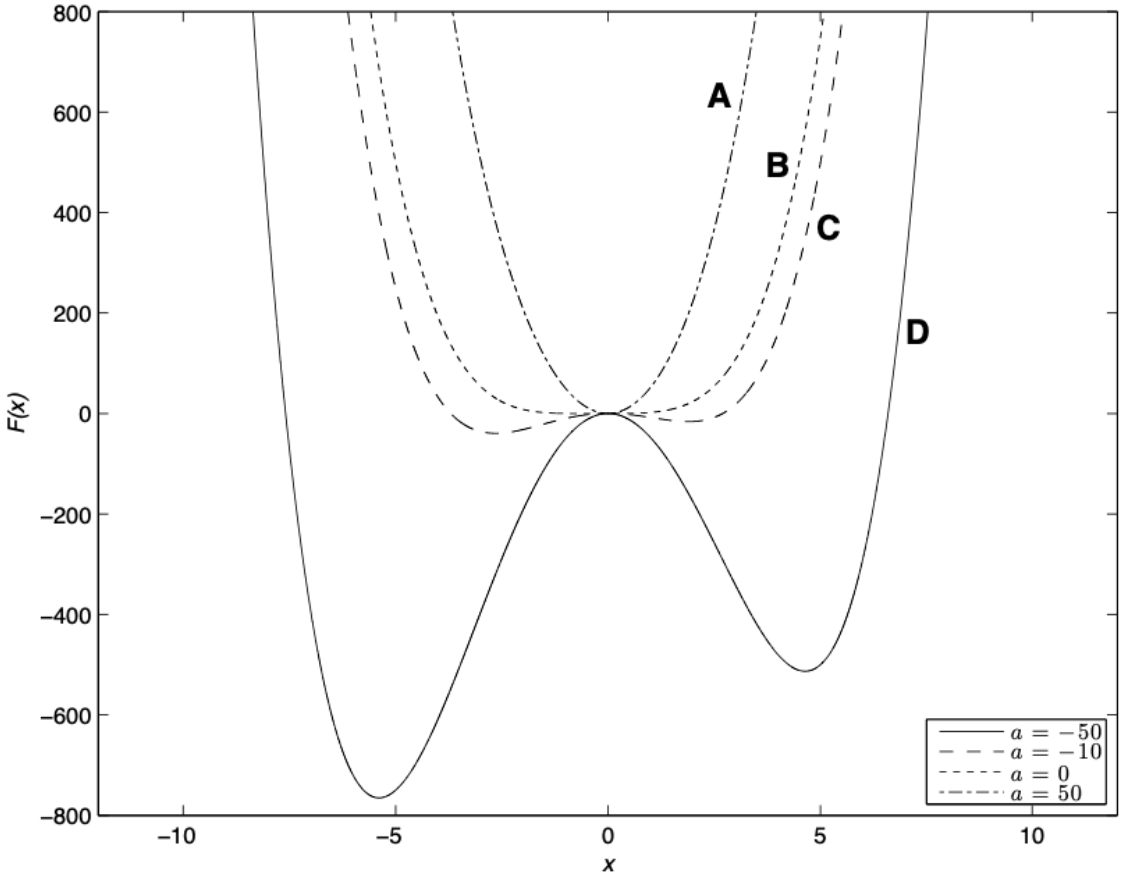

Figure 3.4 : Potential energy landscapes of the normal-to-cancer (NTC) phase transition model.
The free energy function $F(x) = x^4 + 3x^3 + ax^2$ of the order parameter x is plotted with different values for the control parameter a [55].

Translating the behaviour of this simple dynamic phase transition model into the language of cancer, curve A represents normal healthy cells, curve B the onset of the cancer phenotype (at a specific 'nucleation' site from which it spreads, like the growth of a vapour bubble in a liquid above boiling point), curve C a coexisting mixture of healthy and cancerous cells with comparable viability, and curve D the malignant state in which cancer predominates and is favoured. The panels graphically suggest that it becomes progressively more difficult to regress cancer with intervention, as the 'free energy landscape' changes depending on the control parameter. This is due to a potential energy barrier separating the two phenotypes [55].

4. CALCULUS OF VARIATIONS

4.1 EULER-LAGRANGE EQUATIONS

The fundamental problem of Calculus of Variations consists of finding the path or function that minimises a certain measure of cost or energy, and is named after the mathematicians Leonhard Euler and Joseph-Louis Lagrange, who independently developed the method used to solve the problem.

The Euler-Lagrange problem is formulated mathematically as follows: Given a functional, which is a function of several variables and their derivatives, find a function that minimises the functional under certain constraints. The function that minimises the functional is called the extremum of the functional.

The method used to solve the Euler-Lagrange problem is known as the Euler-Lagrange equation, which is a set of partial differential equations that must be satisfied by the extremum of the functional. The Euler-Lagrange equation is derived by taking the derivative of the functional with respect to the variables and setting it equal to zero.

The Euler-Lagrange problem has a wide range of applications in various fields such as physics, engineering and economics, where the goal is to find the optimal path or function that minimises a certain measure of cost or energy. In physics, for example, it is used to solve problems in mechanics, field theory and quantum mechanics. In engineering, it is used to optimise the shape of structures and the performance of systems. In economics, it is used to optimise the allocation of resources.

In summary, the Euler-Lagrange problem is a fundamental problem in the field of calculus of variations, which deals with finding the path or function that minimises a certain measure of cost or energy. The problem is named after mathematicians Leonhard Euler and Joseph-Louis Lagrange, who independently developed the method used to solve it, known as the Euler-Lagrange equation. The problem has a wide range of applications in various fields such as physics, engineering and economics, where the goal is to find the optimal path or function that minimises some measure of cost or energy.

4.2 BRACHISTOCHRONE CURVE

The Brachistochrone problem is a classic problem in physics and mathematics that deals with finding the fastest path of descent between two points. The problem was first posed by Johann Bernoulli in 1696, who challenged his contemporaries to find the shape of the curve that would allow a bead to slide from one point to another in the shortest possible time. The solution to the problem required the application of the principles of calculus and calculus of variations.

The Brachistochrone problem is an example of a calculus of variations problem. In this case, the objective is to find the path that minimises the descent time between two points. The solution to this problem requires the use of the principle of least action, which states that the path followed by a system between two points is the one that minimises the action, or the integral of the Lagrangian (the difference between kinetic and potential energy) along the path.
The solution to Brachistochrone's problem was first provided by Johann Bernoulli's brother, Jakob Bernoulli, who proved that the optimal path was a cycloid, a curve generated by tracing a point on the circumference of a rotating circle. Jakob Bernoulli's solution was later verified by mathematician and physicist Isaac Newton, who used the principles of calculus and the law of gravity to provide a theoretical proof of the solution.

Brachistochrone's problem had a significant impact on the development of the calculus of variations and physics. The solution of the problem not only provided an example of how the principles of calculus and the calculus of variations can be used to solve a problem, but also contributed to the development of the mathematical theory of mechanics and the study of the properties of cycloid curves. It also served as an inspiration for other problems such as tautochrone and catenary.
In summary, the Brachistochrone problem is a classical problem in physics and mathematics that deals with finding the fastest path of descent between two points. The solution of the problem requires the use of the principle of least action, which states that the path followed by a system between two points is the one that minimises the action. The solution to the problem was first provided by Jakob Bernoulli and later verified by Isaac Newton; the problem had a significant impact on the development of calculus of variations and physics.

4.3 WHAT IS CALCULUS OF VARIATIONS

The calculus of variations is a branch of mathematics that deals with the optimisation of functions, i.e. functions that take other functions as input. The objective of the calculus of variations is to find the function that minimises or maximises a given functional, subject to certain constraints.
One of the best known problems in the calculus of variations is to find the curve, or path, that minimises the distance between two points. This problem is known as the brachistochronous problem and was first posed by the physicist and mathematician Johann Bernoulli in 1696. The solution to this problem is a cycloid, a special type of curve generated by the motion of a point on the circumference of a rolling wheel.
Another important problem in the calculus of variations is to find the shape of a surface capable of containing a given amount of fluid with the smallest possible surface area. This

problem, known as the isoperimetric problem, was first formulated by the Greek mathematician Archimedes in the 3rd century BC. The solution to this problem is a sphere, which has the smallest surface area for a given volume of any geometric shape.
The calculus of variations also plays a fundamental role in the study of physics and engineering. In physics, it is used to study the behaviour of systems subject to various constraints, such as the motion of a particle under the influence of a force. In engineering, it is used to design optimal structures and systems in terms of cost, weight, strength and other factors.

One of the key concepts in the calculus of variations is the concept of the functional derivative, which is a generalisation of the concept of the derivative in calculus. The functional derivative of a functional is a function that describes how the functional changes as the input function changes. This concept is closely related to the Euler-Lagrange equation, which is a differential equation used to find the function that minimises or maximises a given functional.
To solve a calculus of variations problem, one must first define the functional to be optimised and any constraints. Then, the Euler-Lagrange equation must be used to find the function that minimises or maximises the functional, subject to the constraints. This process may involve the use of various mathematical techniques, such as partial differential equations and integral calculus.
The calculus of variations also plays an important role in control theory and optimisation, where it provides the mathematical basis for solving many problems related to dynamic systems. It is also used in quantum mechanics and quantum field theory. The principle of least action, which states that the path followed by a physical system between two points is that which minimises the integral of the action, is one of the fundamental principles of quantum mechanics and is an example of calculus of variations in physics.
In conclusion, the calculus of variations is a powerful and versatile branch of mathematics that plays a fundamental role in many areas of physics, engineering and mathematics. Its ability to optimise functions and its connection to many physical laws make it a useful tool for solving a wide range of problems and discovering new insights in various fields.

4.4 WHAT IS A PERTURBATION

In mathematics, physics and engineering, a perturbation is a small change in a system that causes a significant effect on its behaviour. It can refer to a change in a physical system, such as the introduction of a small disturbance in a stable equilibrium, or a change in a mathematical model or equation describing a system. Perturbation theory is a mathematical technique used to analyse the effects of perturbations on a system and is used in several fields, including quantum mechanics, control theory and electrical engineering.

4.5 FUNDAMENTAL LEMMA OF CALCULUS OF VARIATIONS

The idea of the perturbation may be introduced as follows. Here below, we denote by h(x) a perturbation of the function y(x)

$$\tilde{y}(x) = y(x) + \eta h(x) \tag{1}$$

Where:
y(x) is the extremizing function.
h(x) arbitrary function representing the variation from the extreme function.
y˜(x) is the perturbed function.

Let Ψ be a continuous function in $t_1 \leq x \leq t_2$ if the relation:

$$\int_{t_1}^{t_2} \Psi(x)h(x)\,dx = 0 \tag{2}$$

if that relation holds for all functions h(x) that vanish at $x = t_1$ and $x = t_2$ and are continuosly differentiable, then

$$\Psi(x) = 0 \tag{3}$$

4.6 HOW TO PROVE FUNDAMENTAL LEMMA OF CALCULUS OF VARIATIONS

We will prove this Lemma by contradiction i.e. will will assume that (3) is not true which will lead to a contradiction in (2).

Let Ψ be some value other than 0, we will assume it is positive at x=c and $t_1 \leq c \leq t_2$.

If this is true then according to continuity there is some small neighbourhood where $\Psi(x)$ is greater than 0.

Then according to continuity of $\Psi(x)$, there exists an interval

$$c - \delta \leq x \leq c + \delta$$

where $\Psi(x)$ is greater than 0. Since we can choose h(x) arbitrarily, choose h(x) greater than 0 on the ”same interval”

$$c - \delta \leq x \leq c + \delta$$

where $\Psi(x)$ and 0 everywhere else.

This choice of h(x) results in:

$$\int_{t_1}^{t_2} \Psi(x)h(x)\,dx > 0 \tag{4}$$

Therefore Ψ=0.

4.7 DERIVATION OF FIRST VARIATION

The first variation of calculus of variations is a way to find the change in a functional due to a small change in the curve or field being considered. To derive the first variation, we start with the definition of the functional:

$$J[y] = \int_{t_1}^{t_2} F(x, y, y')dx \tag{5}$$

where J[y] is the functional, F(x,y,y′) is the Lagrangian, t_1 and t_2 are the bounds of the integration, and x is a variable that varies over the interval $[t_1,t_2]$. The variable y represents the curve or field, and y′ is its derivative with respect to x. Now, let's consider a small change ∂y in the curve or field y. This will result in a corresponding change δJ in the functional J. We can express this change as:

$$\delta J = J[y + \delta y] - J[y] \tag{6}$$

Expanding the right-hand side of this equation using the definition of the functional, we get:

$$\begin{aligned} \delta J &= \int_{t_1}^{t_2} L(x, y + \delta y, y' + \delta y')dx - \int_{t_1}^{t_2} F(x, y, y')dx \\ &= \int_{t_1}^{t_2} L(x, y, y')dx + \int_{t_1}^{t_2} \frac{\partial F}{\partial y}\delta y dx + \int_{v}^{t_2} \frac{\partial F}{\partial y'}\delta y' dx - \int_{t_1}^{t_2} F(x, y, y')dx \\ &= \int_{t_1}^{t_2} \frac{\partial F}{\partial y}\delta y dx + \int_{t_1}^{t_2} \frac{\partial F}{\partial y'}\delta y' dx \end{aligned} \tag{7}$$

The terms ∂F/∂y and ∂F/∂y' are partial derivatives of the Lagrangian with respect to y and y′, respectively.

Now, we can use the chain rule to express $\delta y'$ in terms of δy:

$$\delta y' = \frac{d}{dx}(y + \delta y) - \frac{d}{dx}y \tag{8}$$
$$= \frac{d}{dx}\delta y$$

Substituting this expression for $\delta y'$ into the previous equation, we get:

$$\delta J = \int_{t_1}^{t_2} \frac{\partial F}{\partial y}\delta y dx + \int_{t_1}^{t_2} \frac{\partial F}{\partial y'}\frac{d}{dx}\delta y dx \tag{9}$$
$$= \int_{t_1}^{t_2} \left(\frac{\partial F}{\partial y} - \frac{d}{dx}\frac{\partial F}{\partial y'}\right)\delta y dx$$

4.8 DERIVATION OF THE EULER-LAGRANGE EQUATION

Assumptions: We have a function (Lagrangian), F (t, y(t), y′(t)), and known boundary values $y(t_1) = y_1$ and $y(t_2) = y_2$ of our function that we are looking for y(t). The function y(t) makes the following action functional J(y) stationary. That is, S(y) using y(t) will give us the value of the action that is either minimum, maximum or saddle point.

$$S(y) = \int_{t_1}^{t_2} F(t, y(t), y'(t))\, dt \tag{10}$$

Now we consider a small variation δy of y. So, $\delta y = \varepsilon\eta$, where ε is a very small real number and $\eta(t)$ is an arbitrary function. It must be defined and differentiable between t_1 and t_2 so we can differentiate w.r.t ε later on.

$\eta(t)$ must vanish at boundary points t_1 and t_2 as these points are fixed (unperturbed).
Therefore, $\eta(t_1) = \eta(t_2) = 0$

The variation of the action functional, when we plug in y(t) + εη is

$$J(y+\epsilon\eta) = \int_{t_1}^{t_2} F(t, y+\epsilon\eta, y'+\epsilon\eta')\, dt \tag{11}$$

A necessary condition for local extremum, (ie: min, max, saddle) of the action functional, is that the first derivative of J(y + εη) must vanish.
So,

$$\frac{\partial J(y+\epsilon\eta)}{\partial \epsilon} = 0 \tag{12}$$

Now we have the expansion of F(t, y+εη, y′ +εη′) around ε = 0 in (11).

$$J(y+\epsilon\eta) = \int_{t_1}^{t_2} (F + \epsilon\frac{dF}{d\epsilon} + O(\epsilon^2))\, dt \tag{13}$$

where $O(\varepsilon^2)$ is the ignored higher terms. Next, we compute ∂F/∂ε. For this we differentiate each argument in F (t, y + εη, y + εη)

$$\frac{\partial F}{\partial \epsilon} = \left(\frac{\partial F}{\partial y}.\frac{d(y+\epsilon\eta)}{d\epsilon} + \frac{\partial F}{\partial y'}.\frac{d(y'+\epsilon\eta')}{d\epsilon} + \frac{\partial F}{\partial t}.\frac{dt}{d\epsilon}\right) \tag{14}$$

So,

$$\frac{\partial F}{\partial \epsilon} = \left(\frac{\partial F}{\partial y}.\eta + \frac{\partial F}{\partial y'}.\eta' + 0\right) \tag{15}$$

We insert this back into the functional (13)

(16) $$J(y+\epsilon\eta) = \int_{t_1}^{t_2} (F + \epsilon\frac{\partial F}{\partial y}\eta + \epsilon\frac{\partial F}{\partial y'}\eta')\, dt$$

Now we use the previous necessary conditions for stationarity : Ψ=0. So, we differentiate the functional (16) with respect to ε and set it equal to zero.

(17) $$\frac{\partial J(y+\epsilon\eta)}{\partial\epsilon} = \frac{\partial}{\partial\epsilon}\int_{t_1}^{t_2} (F + \epsilon\frac{\partial F}{\partial y}\eta + \epsilon\frac{\partial F}{\partial y'}\eta')\, dt$$
$$\frac{\partial J(y+\epsilon\eta)}{\partial\epsilon} = \int_{t_1}^{t_2} (\frac{\partial F}{\partial\epsilon} + \frac{\partial\epsilon}{\partial\epsilon}.\frac{\partial F}{\partial y}\eta + \frac{\partial\epsilon}{\partial\epsilon}.\frac{\partial F}{\partial y'}\eta')\, dt$$
$$\frac{\partial J(y+\epsilon\eta)}{\partial\epsilon} = \int_{t_1}^{t_2} (\frac{\partial F}{\partial y}\eta + \frac{\partial F}{\partial y'}\eta')\, dt$$

So, $\partial J(y + \varepsilon\eta)/\partial\varepsilon=0$ when the integrand vanishes. To do this, we eliminate η and η' through integration by parts. Let us now impose that the first variation vanishes for an arbitrary perturbation η(x):

(18) $$\frac{\partial J(y+\epsilon\eta)}{\partial\epsilon} = \int_{t_1}^{t_2} (\frac{\partial F}{\partial y}\eta dt + \frac{\partial F}{\partial y'}\eta') \;= 0$$
$$\frac{\partial J(y+\epsilon\eta)}{\partial\epsilon} = \int_{t_1}^{t_2} \frac{\partial F}{\partial y}\eta dt + (0 - \int_{t_1}^{t_2} \eta\frac{d}{dt}(\frac{\partial F}{\partial y'})dt) \;= 0$$

$$\frac{\partial J(y+\epsilon\eta)}{\partial\epsilon} = \int_{t_1}^{t_2} \frac{\partial F}{\partial y}\eta dt - \int_{t_1}^{t_2} \eta\frac{d}{dt}(\frac{\partial F}{\partial y'})dt \;= 0$$

$$\frac{\partial J(y+\epsilon\eta)}{\partial\epsilon} = \int_{t_1}^{t_2} (\frac{\partial F}{\partial y} - \frac{d}{dt}(\frac{\partial F}{\partial y'}))\eta\, dt \;= 0$$

Since η is arbitrary, the expression in brackets must vanish so the integral is zero for all η. We now get the Euler Lagrange Equation (which we refer to as ”EL”):

$$\frac{\partial F}{\partial y} - \frac{d}{dt}(\frac{\partial F}{\partial y'}) = 0 \tag{19}$$

If satisfied for y(t), then J(y) becomes stationary.

5. ANALYTICAL STUDY

5.1 ENERGETIC FORMULATION OF THE PROBLEM

As previously stated, cancer, as assumed by Davies et al., can be described in terms of a non-linear dynamical system as a stable attractor.
Thus, we will end up with a system that has two attractors, healthy and cancer cells, and the transition from the stable attractor of the 'normal state' to the stable attractor of the 'cancerous state' can be described by a first-order phase transition. A first-order phase transition is one in which a discontinuity in the order parameter occurs. Of course, for this to occur, the Helmholtz free energy must assume a non-convexity profile, as shown in Figure 3.4 [55] or in Figure 5.1 below .

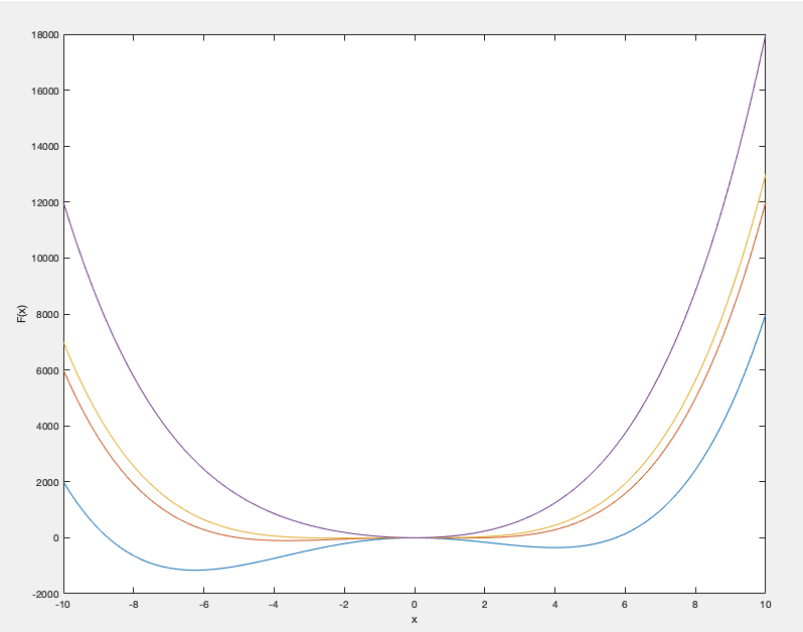

Fig. 5.1: Non-linear dynamical system that has two attractors, healthy and cancer cells. The free energy function $F(x) = x^4 + 3x^3 + ax^2$ of the order parameter x is plotted with different values for the control parameter a (temperature, pH).

For if the energy were convex, the only possible solution would be a fine mixture of healthy and cancerous cells, without localisation. The aim of the work will be to show that the forms of localisation observed in the Rizzoli experiments [88,89] are strongly connected with the study of non-convex energy minimisation problems. Cancer ultimately localises because there is an energy convenience. The non-convexity of the free energy, which is crucial to describe the occurrence of phase transitions, is driven by externally driven parameters like

temperature and pH. Here we are not interested at describing the energetics in detail, a topic that may be investigated in future studies.

To demonstrate this, we first consider the following energy defined on an interval (0, l), the interval representing a segment of healthy and cancerous cells in the system :

$$\mathcal{J}(u) = \int_0^\ell \left(\psi(u(x)) + \alpha \frac{u'^2(x)}{2} \right) dx$$

where ψ is a free-energy (Helmholtz) per unit of length, depending on the local value of u(x), later to be identified with an order parameter (that identifies healthy cells (say, for u=0) and cancerous cells (say, for u=1)). The term $\alpha u'^2/2$ (with $\alpha > 0$, mixture-dependent constitutive parameter) is the energetic cost to create a spatial variation of u; term is zero only if $u'(x) = 0$, that is, if u(x) is constant. This is the simplest way to describe a surface energy.

The main goal of this Chapter is to show that if the free energy is a convex function of the order parameter, then the only possible solutions are fine mixtures of healthy and cancerous cells. Instead, if the energy becomes non-convex, then there arises the possibility that two phase solutions can occur, representing for example the localisation of cancerous tissue that is evident in several manifestation of cancerous invasion in soft and hard tissues.

Now let us assume for simplicity that $\varrho_0 = dN/dx$ is the local cell density and assume that this is constant (ps: this is valid only is a short time-scale, since in general in a growing tissue N = N(t) will change with time; but let us assume here that the phenomenon of phase separation occurs faster than the process of cell division; time is also suppressed since we look at equilibrium). Then we set ϱ_0 =constant and we identify u(x) with the local concentration of healthy cells,

$$u(x) = \frac{dN_h(x)}{dN(x)}$$

so the total number of healthy cells in the tissue is

$$N_h = \varrho_0 \int_0^\ell u(x)\,dx \tag{1}$$

and clearly the number of cancerous cells is $N_c = \varrho_0 \int_0^\ell (1 - u(x))\,dx$.

Note that the total number of cells in the tissue is N = ϱ_0l. Note that if the total number of healthy cells is known, N_h, then since also the total number of cells N is known, we know the average concentration of healthy cells in the tissue,

$$\overline{u} = \frac{1}{\ell} \int_0^\ell u(x)\,dx = \frac{N_h}{\varrho_0 \ell} = \frac{N_h}{N}$$

5.2 APPROACH TO THE PROBLEM

We now pose the following Question: will the mixture of healthy and cancerous cells prefer to create a homogeneous (fine) mixture, or will there be an energetic preference to create "phases", where healthy and cancerous cells are inhomogeneously arranged in (0, l)? Inhomogeneity can be seen as a precursor to create localisations, often observed during the spread of metastasis. The study will be done in 1D for simplicity; however, as we will see, this case has already enough complexity. We are also neglecting mechanical aspects, but the underlying physics will be very similar. The problem will be approached through the classical methods of the Calculus of Variations.

We now consider the following problem: we want to find the unknown concentration u(x) that minimizes the following total potential energy functional (Hamilton's principle), composed of Strain energy and an incoming energy flow, which we will call chemical work, given by the chemical potentials from the nutrients in the system :

$$\mathcal{J}(u) = \underbrace{\int_0^\ell \left(\psi(u(x)) + \alpha\frac{u'^2(x)}{2}\right) dx}_{\text{total free energy of the system}} - \underbrace{(\mu_h N_h + \mu_c N_c)}_{\text{incoming energy}}$$

where μ_h and μ_c are prescribed chemical potentials of healthy and cancerous tissues, respectively. A concept introduced by Gibbs that is in direct analogy with electric potential, gravitational potential, thermal potential and mechanical potential, so we can say that the chemical potential of a chemical μ, is defined as the chemical energy (Uc) possessed by 1 mole of substance :

$$\mu = \frac{U_c}{N}$$

where N is the number of moles of the substance. That is, the chemical potential of a particular substance in a thermodynamic system is equal to the change in internal energy that the system would undergo if a small amount of that substance were added to it, at fixed entropy and volume, divided by the amount of substance added.

We can draw another analogy between chemical potential and thermal and electrical potentials. The application of an electric potential difference between two spatial positions or an electric potential gradient, also called an electric field, causes electrical conduction or transport of substances. The imposition of a temperature difference between two locations or a temperature gradient leads to the transfer of entropy or heat from high-temperature regions to low-temperature regions. In the same way a chemical potential difference between two locations, the chemical potential gradient is the driving force for the migration of the corresponding chemical species from regions of high chemical potential to regions of lower chemical potential. We say this because, if we were to consider the evolution aspect of the system and Fick's law, we could define $V = \mu_c - \mu_h$ as the speed of tumour propagation, establishing that if $\mu_c = \mu_h$ the tumour would not propagate. We will not look at the evolution aspect of the problem, but look at the problem at equilibrium. Our goal is to evaluate the behaviour of the system at equilibrium, so let us set this aspect aside for the moment.

If we assume that for a short time interval under consideration, the total number N of cells in the tissue remains constant, so that $N_c = N - N_h$, then the total potential energy above may be recast as (neglecting the arising constant):

$$\mathcal{J}(u) = \int_0^\ell \left(\psi(u(x)) + \alpha \frac{u'^2(x)}{2} \right) dx - (\mu_h - \mu_c) N_h$$

Then, by setting

$$\mu^* := \mu_h - \mu_c$$

and by using (1), the total potential energy functional can be expressed as

$$\mathcal{J}(u) = \int_0^\ell \underbrace{\left(\psi(u(x)) - \mu^* \varrho_0 u(x) + \alpha \frac{u'^2(x)}{2} \right)}_{:= F(u,u')} dx. \tag{2}$$

From Calculus of Variations we know that the function u(x) that minimizes the energy (2) solves the Euler-Lagrange equation:

$$\frac{\partial F}{\partial u} - \frac{d}{dx} \frac{\partial F}{\partial u'} = 0.$$

This equation is an ordinary differential equation for the unknown u(x). We will also consider the following boundary conditions in this problem,

$$u'(0) = u'(\ell) = 0,$$

as it will be derived in the following subsection. In the next steps, we will study the differential equation for u(x) and we will show under what conditions the problem admits homogeneous or inhomogeneous solutions.

5.3 EULER-LAGRANGE EQUATIONS

The first variation of the energy J(u) is achieved by perturbing the unknown u(x) with a perturbation η(x). So introduce a smallness parameter ε and the perturbed solution is

$$u_\varepsilon(x) = u(x) + \varepsilon\eta(x).$$

Then, the first variation of J must vanish for all arbitrary perturbations η,

$$\delta\mathcal{J} := \frac{d\mathcal{J}(u_e))}{d\varepsilon}\Big|_{\varepsilon=0} = 0.$$

Since η is totally arbitrary everywhere, we will obtain field equations and boundary conditions discussed in the previous section. Let us consider the total energy, calculated in the perturbed state:

$$\mathcal{J}(u_\varepsilon) = \int_0^\ell F(u_\varepsilon, u'_\varepsilon)\, dx$$

where $u_\varepsilon = u + \varepsilon\eta$, and where the perturbation η is completely free. Then, the condition of vanishing first variation of J writes (here $\eta' = d/dx$)

By using integration by parts, since

$$\delta\mathcal{J} = \int_0^\ell \left(\frac{\partial F}{\partial u} - \left(\frac{\partial F}{\partial u'} \right)' \right) \eta \, dx - \left[\frac{\partial F}{\partial u'} \eta \right]_0^\ell = 0 \tag{3}$$

for all perturbations η(x). At this point we make use of the Fundamental Lemma of Calculus of variations, that asserts that if it results (for any admissible function η(x), implies that η(0)=η(l)=0, then G(x) =0)

$$\int_0^\ell G(x)\eta(x)\, dx = 0$$

for all η(x), then it must result

$$G(x) = 0 \qquad \text{in} \qquad (0, \ell).$$

So, coming back to (3), since the perturbation η is free both in the interval (0, l) and at the boundary points, then we obtain the boundary value problem for the unknown concentration u(x):

$$\begin{cases} \dfrac{\partial F}{\partial u} - \left(\dfrac{\partial F}{\partial u'} \right)' = 0 \qquad (0, \ell) \\ \dfrac{\partial F}{\partial u'}\Big|_0 = 0\,, \; \dfrac{\partial F}{\partial u'}\Big|_\ell = 0. \end{cases}$$

If we take specifically F to coincide with the energy given in (2), we obtain:

$$\frac{\partial F}{\partial u} = \frac{\partial \psi}{\partial u} - \mu^* \varrho_0, \qquad \frac{\partial F}{\partial u'} = \alpha u', \qquad \left(\frac{\partial F}{\partial u'} \right)' = \alpha u''.$$

Thus the problem for the unknown concentration u(x) becomes, more explicitly:

$$(4) \qquad \begin{cases} \dfrac{\partial \psi}{\partial u} - \mu^* \varrho_0 - \alpha u'' = 0 \quad (0, \ell) \\ u'(0) = 0, \\ u'(\ell) = 0. \end{cases}$$

With the energy ψ(u) still unspecified, this problem cannot be solved explicitly. However, we can do considerations that will lead to the conclusion that the energy ψ(u) needs to be non-convex to observe phase separation, thus confirming the considerations done by Davies and others. This can be observed through the analysis discussed below.

5.4 PERTURBATIVE ANALYSIS

To find the possible existence of both homogeneous and inhomogeneous solutions, we need to study the ordinary differential equation (4). Since in general this equation will be non-linear in u (due to the non-convexity of the energy ψ(u)) we can still approach the problem through what is called a perturbative approach, which is illustrated in the following. Essentially, we look for solutions u(x) that are very close a homogeneous solution u_0=constant, but are inhomogeneous, that is

$$u(x) = u_o + \varepsilon v(x)$$

where v(x) is a inhomogeneous (non-constant) function, and ε is a small constant. The idea is that equation (4) must hold for all ε.

If we replace u(x) = u_0 + εv(x) in the problem (4), it becomes

$$(5) \qquad \begin{cases} \dfrac{\partial \psi(u_o + \varepsilon v)}{\partial u} - \mu^* \varrho_0 - \alpha (u_o + \varepsilon v)'' = 0 \quad \text{in} \quad (0, \ell) \\ u'(0) = 0 \\ u'(\ell) = 0. \end{cases}$$

If we expand in Taylor series the term

$$\frac{\partial \psi(u_o + \varepsilon v)}{\partial u} = \frac{\partial \psi(u)}{\partial u}\Big|_{u=u_o} + \varepsilon \frac{\partial^2 \psi(u)}{\partial u^2}\Big|_{u=u_o} v$$

then the problem above may be rewritten as

$$(6) \qquad \begin{cases} \left(\dfrac{\partial \psi(u)}{\partial u}\Big|_{u=u_o} - \mu^* \varrho_0 \right) + \varepsilon \left(\dfrac{\partial^2 \psi(u)}{\partial u^2}\Big|_{u=u_o} v - \alpha\, v'' \right) = 0 \quad \text{in} \quad (0, \ell) \\ \varepsilon v'(0) = 0 \\ \varepsilon v'(\ell) = 0. \end{cases}$$

Since the problem (7) must hold true for all ε, we effectively obtain two problems.
The first, for ε = 0, simply tells us what is the homogeneous solution:

$$\frac{\partial \psi(u)}{\partial u}\Big|_{u=u_o} - \mu^* \varrho_0 = 0$$

and this equation gives the homogeneous value of u_0, since μ* and ϱ_0 are prescribed (and ψ(u) is known, of course). Then, what is left in (7) delivers a problem for the unknown inhomogeneity field v(x)

$$(7) \qquad \begin{cases} v'' - \dfrac{1}{\alpha}\dfrac{\partial^2 \psi(u)}{\partial u^2}\Big|_{u=u_o} v = 0 \quad \text{in} \quad (0,\ell) \\ v'(0) = 0 \\ v'(\ell) = 0. \end{cases}$$

We now pose the following question: can v(x) be different than zero? We will now see that this is possible if and only if ψ is non-convex in u.

Bearing in mind that $\alpha > 0$, let us first consider the case where the energy is convex for all u,

$$\frac{\partial^2 \psi(u)}{\partial u^2} > 0,$$

and let us set

$$\beta^2 = \frac{1}{\alpha}\frac{\partial^2 \psi(u)}{\partial u^2}\Big|_{u=u_o}.$$

Then the equation for v(x) becomes

$$v'' - \beta^2 v = 0.$$

It is well known that in this case the solution is given by a linear combination of exponentials,

$$v(x) = c_1 e^{\beta x} + c_2 e^{-\beta x}.$$

Such solution has to satisfy the boundary conditions, and if we impose them we find that c_1=c_2 =0, meaning that if the energy is convex at the point u_o, it results v = 0, and henceforth the solution is homogeneous. Thus, if the energy is convex, the only possible solution is homogeneous (a fine mixture).

Let us now see what happens if instead there is some interval (u_1,u_2) where the energy is non-convex,

$$\frac{\partial^2 \psi(u)}{\partial u^2}\Big|_{u=u_o} < 0 \qquad \text{for} \qquad u \in (u_1, u_2).$$

just like the theories put froward by Davies and Desphande [55,91]. Now if we take $u_o \in (u_1, u_2)$ and if we set for simplicity

$$\beta^2 = -\frac{1}{\alpha}\frac{\partial^2 \psi(u)}{\partial u^2}\Big|_{u=u_o}$$

the equation for v(x) now becomes

$$v'' + \beta^2 v = 0.$$

It is well known that in this case, the solution is a linear combination of sine and cosine,

$$v(x) = c_1 \cos(\beta x) + c_2 \sin(\beta x).$$

Let us impose the boundary conditions, so since

$$v'(x) = -c_1 \beta \sin(\beta x) + c_2 \beta \cos(\beta x)$$

these result into the system

$$\begin{cases} v'(0) = c_2 \beta = 0 \\ v'(\ell) = -c_1 \beta \sin(\beta \ell) + c_2 \beta \cos(\beta \ell) = 0. \end{cases}$$

Since it must result $c_2 = 0$, then the only possibility for the second condition to be satisfied is

$$\sin(\beta\ell) = 0$$

and this means that it must result

$$\beta\ell = n\pi, \qquad n = 0, 1, 2, \ldots$$

Essentially, we thus find that there are infinite values of the parameter β for which the boundary condition is satisfied,

$$\beta_n = \frac{n\pi}{\ell}$$

(note that the first is trivial since $\beta_0 = 0$). So if inside the interval (u_1 , u_2) we can find u_o for which

$$\frac{\partial^2 \psi(u)}{\partial u^2}\Big|_{u=u_o} = -\alpha \frac{n^2\pi^2}{\ell^2}$$

then, there are non-trivial (non-zero) solutions of the inhomogeneous differential equation of the form

$$v(x) = c_n \cos\left(\frac{n\pi}{\ell}x\right),$$

where c_n are constants. Since actually each of these solutions satisfies the boundary conditions, the general solution may be taken as the linear combination

$$v(x) = \sum_{n=0}^{\infty} c_n \cos\left(\frac{n\pi}{\ell}x\right). \tag{8}$$

This function, that represents the general solution to (7), is called a Fourier series. A suitable choice of the coefficients c_n can represent both periodic oscillations, and localized solutions: see the examples represented below.

As illustrated in the figure below, if $\psi'' < 0$ in some region (meaning that this becomes non-convex) we obtain that the resulting solutions to the energy minimization problem become very rich, as to describe a number of phase separated configurations that may be associated both with fine mixtures of healthy and cancerous cells, or with separated phases representing healthy and cancer cells, or with the localization of cancer cells in a narrow region. All these features are observed in the experiments conducted by Rizzoli [88,89].
In reality, to describe the occurrence of inhomogeneous solutions, we should use a more involved approach, according to which if u_o falls inside a certain domain where $\psi'' < 0$, then there will be values u^*_1 and u^*_2 where $\psi'(u^*_1) = \psi'(u^*_2)$, such that

$$u_o = \chi u_1^* + (1 - \chi)u_2^*$$

where χ is the total fraction of cancer cells and $(1 - \chi)$ the total fraction of healthy cells, and whereas u^*_1 and u^*_2 are defined through the so-called Maxwell construction. This is a very challenging task for the study of cancer propagation but it is out of the interests of this Thesis.

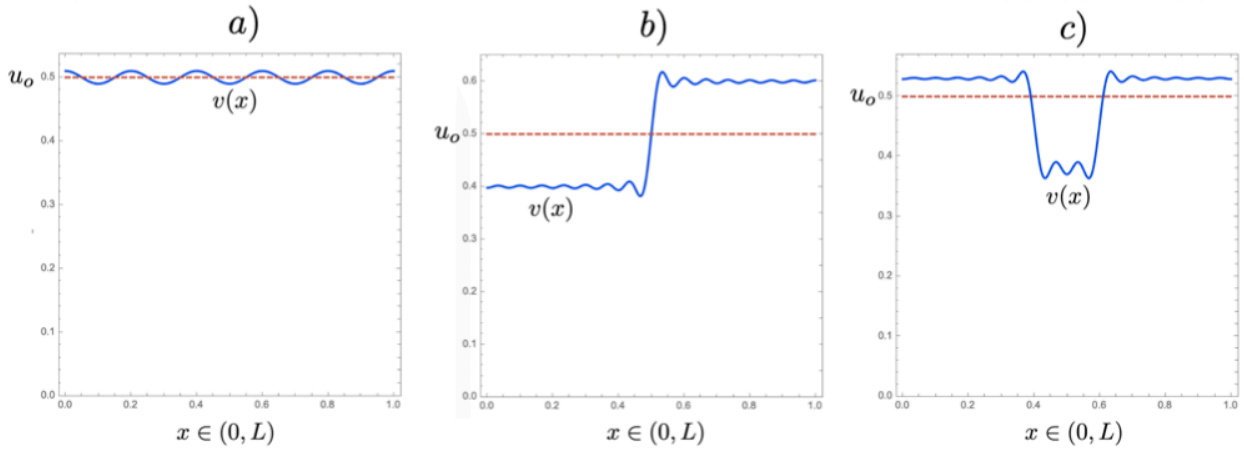

Figure 5.2: Graphical representation of some solutions represented by (8), that become possible as soon as the free energy becomes non-convex for a certain value of u_o. In these examples, we observe a periodic spatial distribution of healthy and cancer cells in (a), the segregation of healthy and cancer cells in two dis- tinct clusters in (b), and the localisation of cancer cells in a narrow region in (c).

In conclusion, in this Chapter we have shown that the description of cancer propagation according to an energetic perspective permits to describe the spread of cancer cells as a form of instability. The latter can take place if the free energy of the aggregate is chracterised by a non-convex energy with respect to an order parameter that describes regions occupied by healthy and cancer cells. This is consistent with well known results in the Theory of Phase Transitions.

It is clear that the description provided in this Chapter is overly simplified, as it neglects important aspects such as the rigorous definition of a free energy for the aggregate, the careful tuning of constitutive parameters, the rigorous study of stability of phase-separated regions, and furthermore, the study is confined to a 1D interval. However, this perspective provides an interesting approach to think of cancer spreading as a phase transition. The latter field has received significant attention in literature both for biological and non-biological materials, and can provide interesting insights in the understanding of the biophysical features of cancer propagation, both in soft and in hard biological materials. Future studies will address this idea in a more rigorous and systematic way, possibly relying upon available experimental data.

6. CONCLUSION

In this thesis, we have started with an in-depth study of bone tissue, highlighting the main mechanical, metabolic and haematopoietic functions. The structural organisation is described, starting with the macrostructure and highlighting the differences between cancellous and cortical bone, moving on to the microstructure to describe how the lamellae are arranged in the various osteons that form the bone matrix, and finally a look at the nanostructure where collagen fibres are the main element present in the matrix. In the second half of the chapter, we described the whole process of bone structure formation from skeletogenesis to bone maintenance and remodelling.

In concluding the analysis of the bone tissue, which was necessary to understand the rest of the work, we talked about secondary bone tumours, specifically tumours that originate in another part of the body and spread (metastasis) to the bone through the bloodstream or lymph nodes. In this thesis, we focused on secondary tumours and the process of metastasis, as bone is one of the most common sites of cancer spread.

Skeletal metastases are frequent complications of many cancers and cause bone complications (fractures, bone pain, disability) that negatively affect the quality and life expectancy of the patient. With the exception of some relatively rare malignancies, such as high-grade lymphomas or germ cell tumours affecting bone, metastatic bone disease is currently incurable. However, new drugs, such as tyrosine kinase inhibitors and immune checkpoint inhibitors, have significantly prolonged the control of primary disease in patients, leading to longer survival and, consequently, a sufficiently long lifespan for bone metastases to become clinically relevant. The epidemiology of bone metastases is therefore evolving. Consequently, the interest in studying the behaviour of bone metastases is increasing. This is a crucial step forward, because metastases are a complex, multistep process responsible for more than 90% of cancer-related deaths.

After this first preliminary study, we have then proposed a mechanistic model to study the gradual sequence of events involved in the colonisation of bone by cancer cells. This is, in reality, a rather complex process, divided in the following stages: the formation of a premetastatic niche in the bone marrow to attract circulating tumour cells (CTCs); then, primary tumour cells having to undergo an epithelial-mesenchymal transition (EMT) to invade the surrounding tissue and enter the microvasculature (intravasation) of the blood and/or lymphatic system; then, once in the bloodstream, the tumour cells can disseminate to distant organs, exit the blood vessels (extravasation) and establish themselves in the foreign

microenvironment, where they enter a state of quiescence or proliferate to later form macroscopic secondary tumours (metastasis).
We have seen that at all stages of the metastatic process, tumour cells migrate through very different microenvironments, including the stroma, the endothelium of blood vessels, the vascular system and the tissue of a secondary site. The ability to successfully traverse each of these stages and advance to secondary tumour formation and growth is dependent, in part, on the physical interactions and mechanical forces between the tumour cells and the microenvironment. For example, the physical interactions between a cell and the extracellular matrix, the collagen-rich scaffold on which it grows, play a key role in allowing cells to migrate from a tumour to nearby blood vessels. During intravasation and extravasation, cells undergo large elastic deformations to penetrate endothelial cell-cell junctions. In the vascular system, the interaction between cell speed and adhesion influences the binding of tumour cells to blood vessel walls and thus the location of sites where a secondary tumour can form and grow.We have found that the presence of metastasis will result in a number of changes in the cell.

In fact, normal, well-differentiated cells, which reproduce healthily, undergo apoptosis when damaged (or stimulated to do so by their internal clock) and adhere to each other to form regular tissues or organs, and tumour cells, poorly differentiated, which reproduce unfaithfully and sometimes unrestrictedly, escape apoptosis, colonise organs where they do not belong and associate in relatively disordered assemblages (tumours) rather than forming well-defined tissues and organs. If a normal cell undergoes a transition such that it escapes apoptosis due to the accumulation of genetic mutations or sometimes due to somatic damage (e.g. from ionising radiation or toxins), two classes of organisational changes are triggered: at the cellular level (cancer initiation) and at the population level (cancer progression).
The first category includes changes in cell metabolism, such as the switch from oxidative phosphorylation to glycolysis (the so-called Warburg effect), the epithelial-mesenchymal transition (EMT) characterised by changes in cell morphology and motility, and the triggering of a number of alterations in signalling and protein expression. These three classes of changes (physiological, morphological and molecular) are intimately related and probably result from epigenetic transformations. Changes at the population level involve the replacement of one group of cells, which adhere to each other to form a differentiated tissue, by another group of cells, which form a highly heterogeneous and more mobile aggregate: a tumour or neoplasm.
It is emphasised that although most investigations in the past have focused on biochemical and chemical carcinogens, it is becoming increasingly clear that living cells are also profoundly affected by physical forces, such as shear stress, surface attraction forces and

pressure. Moreover, different cell types respond differently to these stimuli. Understanding how normal and cancer cells respond to macroscopic chemical and physical variables is one of the most interesting developments, which promises not only to lead to a better understanding of the origin of cancer, but also to offer new diagnostic techniques and therapeutic recommendations.
We finally listed the main hallmarks of bone metastases and most cancers: self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limited replicative potential, sustained angiogenesis and avoidance of apoptosis.
The first part of this thesis work was aimed to understand in detail the main mechanisms involved in the process of bone metastasis. This study was necessary to understand the behaviour of tumour cells, the various genetic changes and the physical forces to which they are subjected.

In the third chapter, through the analysis of the results obtained from recent experiments conducted at the Rizzoli Orthopaedic Institute, we have developed the idea that the tumour propagation and spread can be described as a non-linear dynamic system with two attractors, where the transition from the stable attractor of the 'normal state' to the stable attractor of the 'cancerous state' can be described by a first-order phase transition, as shown in the work of Davies et al., taken as a reference for this chapter.
Under normal conditions, healthy cells represent a more stable state, whereas cancer is a metastable state (less stable but not necessarily unstable). With increasing cell damage caused by external events or internal dysregulation due to altered metabolism or accumulated genetic changes, the relative stability between these two types of states (normal and cancerous) shifts. This is similar to a first-order phase transition in physics, where two equilibrium states (ordered and disordered) reverse roles (stable versus metastable) as the value of the control parameter changes. It should also be noted that they are characterised by extreme sensitivity to small perturbations and zero correlations throughout the system, which determine the global character of the phase transition. This property of phase transitions in the context of cellular transformations indicates a higher sensitivity and global nature of the NTC transition.

Based on the above, it is proposed in the work of Davies et al. that at this point in the NTC transition the situation is reversible, just as physical phase transitions can be reversed by a change in the value of control parameters. Once the system is pushed into one of two stable phases, however, reversibility becomes much more difficult due to a potential energy barrier separating these states. This would also suggest that local interventions that do not change the global state of the organism, such as surgery and radiation, may not be entirely effective,

or perhaps counterproductive, even in the absence of metastases and with perfect treatment plans.

Only the reversal of globally prevailing conditions, such as a shift in control parameter values (analogous to lowering the temperature below a transition value or increasing the pH of the environment), can lead to the total eradication of cancer from the body. Moreover, since first-order phase transitions are characterised by so-called hysteresis loops that make them irreversible, a return to the original phase requires an 'overshoot' of the control parameter values, which is necessary to destabilise the new phase. Consequently, an NTC transition, depending on the control parameter causing it, would require not only an intervention to reverse the change (e.g. reoxygenation and deacidification of the environment), but also overcompensation beyond what would be considered a normal set of parameters.

As stated earlier, cancer, as hypothesised by Davies et al., can be described in terms of a non-linear dynamical system as a stable attractor. Thus, we end up with a system that has two attractors, healthy and cancer cells, and the transition from the stable attractor of the 'normal state' to the stable attractor of the 'cancerous state' can be described by a first-order phase transition. A first-order phase transition is one in which a discontinuity in the order parameter occurs. Of course, for this to occur, the Helmholtz free energy must assume a profile of non-convexity.

For if the energy were convex, the only possible solution would be a fine mixture of healthy and cancerous cells, without localisation. The aim of the work was to show that the localisation patterns observed in the Rizzoli experiments are strongly connected with the study of non-convex energy minimisation problems. Cancer eventually localises because there is an energetic convenience, The non-convexity of free energy, which is crucial to describe the occurrence of phase transitions, is driven by externally driven parameters such as temperature and pH. We are not interested here in describing the energetics in detail, a topic that could be investigated in future studies.

In conclusion, we have shown in this chapter that the description of cancer propagation from an energy perspective allows us to describe the spread of cancer cells as a form of instability. The latter can occur if the free energy of the aggregate is chromatised by a non-convex energy with respect to an order parameter describing the regions occupied by healthy and cancer cells. This is consistent with the well-known results of phase transition theory.

It is clear that the description provided in this chapter is oversimplified, as it neglects important aspects such as the rigorous definition of a free energy for the aggregate, the careful adjustment of the constitutive parameters, the rigorous study of the stability of the phase-separated regions, and, moreover, the study is limited to a 1D interval. Nevertheless, this perspective provides an interesting approach to thinking of cancer diffusion as a phase transition. The latter field has received significant attention in the literature for both biological and non-biological materials and may provide interesting insights into the biophysical characteristics of cancer propagation in both soft and hard biological materials. Future studies will address this idea in a more rigorous and systematic way, possibly building on the available experimental data.

The results from the experiments conducted by the Rizzoli team on bone cancer clearly show those signals, such as the presence of localisation, which suggest that the tumour may behave as a phase transition. So, in the final part of the thesis, we have illustrated how this general theory produces effects that compatible with the observations, although at this stage the analytical model is still very general. A later stage of this thesis work will be necessary for the derivation of a free energy in metastatic bone, including three-dimensional aspects, and also accounting for the fundamental biochemical and mechanical aspects that play a role in the process.

BIBLIOGRAPHY

1. Rinaldo Florencio-Silva, Gisela Rodrigues da Silva Sasso, Estela Sasso-Cerri, Manuel Jesus Simões, and Paulo Sérgio Cerri. Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells. Hindawi Publishing Corporation. BioMed Research International. http://dx.doi.org/10.1155/2015/421746

2. Amel HOUAOUI . Development of composite and hybrid materials based on bioactive glass for bone bioengineering

3. Culav EM, Clark CH, Merrilees MJ. Connective Tissues: Matrix Composition and Its Relevance to Physical Therapy. Phys Ther. 1999;79:308–319

4. Alyssa M. Weatherholt, Robyn K. Fuchs and Stuart J. Warden. Specialized connective tissue: bone, the structural framework of the upper extremity. doi: 10.1016/j.jht.2011.08.003

5. Tang, S.Y. Natural composites: The structure-function relationships of bone, cartilage, tendon/ligament, and the intervertebral disc. In Biomedical Composites; Elsevier, 2017; pp. 1–16 ISBN 978-0-08-100752-5.

6. Fuchs, R.K.; Thompson, W.R.; Warden, S.J. Bone biology. In Bone Repair Biomaterials; Elsevier, 2019; pp. 15–52 ISBN 978-0-08-102451-5.

7. Dorozhkin, S. Calcium Orthophosphates in Nature, Biology and Medicine. Materials 2009, 2, 399–498, doi:10.3390/ma2020399.

8. Gehron Robey, P. Noncollagenous Bone Matrix Proteins. In Principles of Bone Biology; Elsevier, 2008; pp. 335–349 ISBN 978-0-12-373884-4.

9. Rho, J.-Y.; Kuhn-Spearing, L.; Zioupos, P. Mechanical Properties and the Hierarchical Structure of Bone. Med. Eng. Phys. 1998, 20, 92–102, doi:10.1016/S1350-4533(98)00007-1.

10. Liu, X.; Wu, H.; Byrne, M.; Krane, S.; Jaenisch, R. Type III Collagen Is Crucial for Collagen I Fibrillogenesis and for Normal Cardiovascular Development. Proc. Natl. Acad. Sci. 1997, 94, 1852–1856, doi:10.1073/pnas.94.5.1852.

11. Saito, M.; Karakida, T.; Yamamoto, R.; Nagano, T.; Yamakoshi, Y.; Hayakawa, T.; Oida, S.; Gomi, K. Differentiation Potential of Osteoblast from Cultured C2C12 Cells on Zirconia Disk. Dental Materials Journal 2014, 33, 275–283, doi:10.4012/dmj.2013-321.

12. Henry JP, Bordoni B. Histology, Osteoblasts. [Updated 2022 May 8]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan.

13. Mitchell B. Schaffler and Oran D. Kennedy. Osteocyte Signaling in Bone. doi: 10.1007/s11914-012-0105-4

14. Brendan F. Boyce, Zhenqiang Yao, and Lianping Xing. Osteoclasts have Multiple Roles in Bone in Addition to Bone Resorption. doi: 10.1615/critreveukargeneexpr.v19.i3.10

15. Baig MA, Bacha D. Histology, Bone. [Updated 2022 May 8]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan.

16. Davide Carnelli, Pasquale Vena, Ming Dao, Christine Ortiz and Roberto Contro, 2013. Orientation and size-dependent mechanical modulation within individual secondary osteons in cortical bone tissue. http://dx.doi.org/10.1098/rsif.2012.0953

17. Encyclopedia britannica, 16 april 2015. cancellous bone.

18. Eiki Koyama, Yoshihiro Shibukawa, Motohiko Nagayama, Hiroki Sugito, Blanche Young, Takahito Yuasa , Takahiro Okabe, 2008. A distinct cohort of progenitor cells participates in synovial joint and articular cartilage formation during mouse limb skeletogenesis. doi:10.1016/j.ydbio.2008.01.012

19. Peter Dy , Patrick Smits , Amber Silvester , Alfredo Penzo-Méndez , Bogdan Dumitriu , Yu Han , Carol A. de la Motte , David M. Kingsley , Véronique Lefebvre , 2010. Synovial joint morphogenesis requires the chondrogenic action of Sox5 and Sox6 in growth plate and articular cartilage. doi:10.1016/j.ydbio.2010.02.024

20. Véronique Lefebvre and Pallavi Bhattaram, 2010. Vertebrate skeletogenesis. doi:10.1016/S0070-2153(10)90008-2.

21. B.K. Hall and T. Miyake, 1992. The membranous skeleton: the role of cell condensationsin vertebrate skeletogenesis.

22. Tatjana Sauka-Spengler and Marianne Bronner-Fraser, 2008. A gene regulatory network orchestrates neural crest formation. doi:10.1038/nrm2428.

23. Ayuko Kimura, Eri Sakaguchi, Masaru Nonaka, 2009. Multi-component complement system of Cnidaria: C3, Bf, and MASP genes expressed in the endodermal tissues of a sea anemone, Nematostella vectensis. doi:10.1016/j.imbio.2009.01.003.

24. Radwan Abu-Issa and Margaret L. Kirby, 2007. Heart Field: From Mesoderm to Heart Tube. doi: 10.1146/annurev.cellbio.23.090506.123331.

25. Akter F, Ibanez J. Bone and cartilage tissue engineering. In: Akter F, editor. Tissue Engineering Made Easy [Internet]. 1st ed. New York: Elsevier Inc.; 2016. pp. 77-98. DOI: 10.1016/B978-0-12-805361-4.00008-4

26. Provot S, Schipani E, Wu JY, Kronenberg H. Development of the skeleton. In: Marcus R, Dempster D, Cauley J, Feldman D, editors. Osteoporosis [Internet]. 4th ed. New York: Elsevier; 2013. pp. 97-126. DOI: 10.1016/B978-0-12-415853-5.00006-6

27. Wojnar R. Bone and Cartilage–Its Structure and Physical Properties.Weinheim: Wiley-VCH Verlag GmbH & Co.; 2010

28. Cashman KD, Ginty F. Bone. New York: Elsevier; 2003. pp. 1106-1112

29. Clarke B. Normal bone anatomy and physiology. Clinical Journal of the American Society of Nephrology. 2008;3:131-139

30. Muscolino JE. Kinesiology the Skelatal System and Muscle Function. 2nd ed. New York: Elsevier Inc.; 2011

31. Provot S, Schipani E, Wu JY, Kronenberg H. Development of the skeleton. In: Marcus R, Dempster D, Cauley J, Feldman D, editors. Osteoporosis [Internet]. 4th ed. New York: Elsevier; 2013. pp. 97-126. DOI: 10.1016/B978-0-12-415853-5.00006-6

32. Bone Development and Growth WRITTEN BY Rosy Setiawati and Paulus Rahardjo Submitted: September 12th, 2018 Reviewed: November 8th, 2018 Published: December 14th, 2018. DOI: 10.5772/intechopen.82452

33. OpenStax College. Anatomy & Physiology. Texas: Rice University; 2013. pp. 203-231

34. Dimitrios J Hadjidakis, Ioannis I Androulakis, 2006. Bone Remodeling. doi: 10.1196/annals.1365.035

35. Erik Fink Eriksen, 2010. Cellular mechanisms of bone remodeling. DOI 10.1007/s11154-010-9153-1.

36. National Cancer Institute, 2020. Metastatic Cancer Research.

37. Croucher PI, McDonald MM, Martin TJ. Bone metastasis: the importance of the neighbourhood. Nat Rev Cancer 16: 373–386, 2016. doi:10.1038/nrc.2016.44.

38. Mundy GR. Metastasis to bone: causes, consequences and thera- peutic opportunities. Nat Rev Cancer 2: 584–593, 2002. DOI: doi:10.1038/nrc867.

39. Philippe Clezardin, Rob Coleman, Margherita Puppo, Penelope Ottewell, Edith Bonnelye, Frederic Paycha, Cyrille B. Confavreux, Ingunn Holen, 2021. BONE METASTASIS: MECHANISMS, THERAPIES, AND BIOMARKERS. https://doi.org/10.1152/physrev.00012.2019

40. Weilbaecher KN, Guise TA, McCauley LK. Cancer to bone: a fatal attraction. Nat Rev Cancer 11: 411–425, 2011. doi:10.1038/nrc3055.

41. Hofbauer LC, Rachner TD, Coleman RE, Jakob F. Endocrine aspects of bone metastases. Lancet Diabetes Endocrinol 2: 500–512, 2014. doi:10.1016/ S2213-8587(13)70203-1.

42. Coleman RE, Rubens RD. The clinical course of bone metastases from breast cancer. Br J Cancer 55: 61–66, 1987. doi:10.1038/ bjc.1987.13.

43. Galasko CS. Monitoring of bone metastases. Schweiz Med Wochenschr 111: 1873–1875, 1981.

44. Coleman RE. Clinical features of metastatic bone disease and risk of skeletal morbidity. Clin Cancer Res 12: 6243s–6249s, 2006. doi:10.1158/1078-0432.CCR-06-0931.

45. Turpin A, Duterque-Coquillaud M, Vieillard MH. Bone metastasis: current state of play. Transl Oncol 13: 308–320, 2019. doi:10.1016/j. tranon.2019.10.012.

46. Costa L, Badia X, Chow E, Lipton A, Wardley A. Impact of skeletal complications on patients' quality of life, mobility, and functional independence. Support Care Cancer 16: 879–889, 2008. doi:10. 1007/s00520-008-0418-0.

47. Molecular Biology of the Cell. 3 edition. New York: Garland Pub; 1994.

48. Weinberg RA: The Biology of Cancer New York: Garland Science; 2007.

49. Fearon ER, Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 1990, 61:759-767.

50. Nunney L: Lineage selection and the evolution of multistage carcinogenesis. Proc Biol Sci 1999, 266:493-498.

51. Warburg O, Posener E, Negelein E: Uber den Stoffwechsel der Carcinomzelle. Biochem Z 1924, 152:309-344.

52. Vander Heiden MG, Cantley LC, Thompson CB: Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 2009, 324:1029-1033.

53. Fuhrmann A, Staunton JR, Nandakumar V, Banyai N, Davies PCW, Ros R: AFM stiffness nanotomography of normal, metaplastic and dysplastic human esophageal cells. Phys Biol 2011, 8:015007.

54. Kumar S, Weaver VM: Mechanics, malignancy, and metastasis: the force journey of a tumor cell. Cancer Metastasis. Rev 2009, 28:113-127.

55. Paul CW Davies , Lloyd Demetrius and Jack A Tuszynski, 2011. Cancer as a dynamical phase transition. doi:10.1186/1742-4682-8-30.

56. Denis Wirtz, Konstantinos Konstantopoulos and Peter C. Searson, 2011. The physics of cancer: the role of physical interactions and mechanical forces in metastasis. doi:10.1038/nrc3080

57. Chambers, A. F., Groom, A. C. & MacDonald, I. C. Dissemination and growth of cancer cells in metastatic sites. Nature Rev. Cancer 2, 563–572 (2002).

58. Steeg, P. S. Tumor metastasis: mechanistic insights and clinical challenges. Nature Med. 12, 895–904 (2006).

59. Yang Liu, Xuetao Cao, 2016. Characteristics and Significance of the Pre-metastatic Niche. DOI:https://doi.org/10.1016/j.ccell.2016.09.011.

60. Héctor Peinado, Simon Lavotshkin, David Lyden, 2011. The secreted factors responsible for pre-metastatic niche formation: old sayings and new thoughts. DOI: 10.1016/j.semcancer.2011.01.002.

61. Kalluri, R. & Weinberg, R. A. The basics of epithelial- mesenchymal transition. J. Clin. Invest. 119, 1420–1428 (2009).

62. Chaffer, C. L. & Weinberg, R. A. A perspective on cancer cell metastasis. Science 331, 1559–1564 (2011).

63. Polyak, K. & Weinberg, R. A. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nature Rev. Cancer 9, 265–273 (2009).

64. Thiery, J. P. & Sleeman, J. P. Complex networks orchestrate epithelial-mesenchymal transitions. Nature Rev. Mol. Cell Biol. 7, 131–142 (2006).

65. Hotary, K., Li, X. Y., Allen, E., Stevens, S. L. & Weiss, S. J. A cancer cell metalloprotease triad regulates the basement membrane transmigration program. Genes Dev. 20, 2673–2686 (2006).

66. De Wever, O., Demetter, P., Mareel, M. & Bracke, M. Stromal myofibroblasts are drivers of invasive cancer growth. Int. J. Cancer 123, 2229–2238 (2008).

67. Provenzano, P. P., Inman, D. R., Eliceiri, K. W. & Keely, P. J. Matrix density-induced mechanoregulation of breast cell phenotype, signaling and gene expression through a FAK-ERK linkage. Oncogene 28, 4326–4343 (2009).

68. Wirtz, D. Particle-tracking microrheology of living cells: principles and applications. Annu. Rev. Biophys. 38, 301–326 (2009).

69. Friedl, P., Wolf, K. & Lammerding, J. Nuclear mechanics during cell migration. Curr. Opin. Cell Biol. 23, 1–10 (2010).

70. Dahl, K. N., Kahn, S. M., Wilson, K. L. & Discher, D. E. The nuclear envelope lamina network has elasticity and a compressibility limit suggestive of a molecular

71. Gerlitz, G. & Bustin, M. Efficient cell migration requires global chromatin condensation. J. Cell Sci. 123, 2207–2217 (2010).

72. Crisp, M. et al. Coupling of the nucleus and cytoplasm: role of the LINC complex. J. Cell Biol. 172, 41–53 (2006).

73. Lammerding, J. et al. Lamin A/C deficiency causes defective nuclear mechanics and mechanotransduction. J. Clin. Invest. 113, 370–378 (2004).

74. Cross, S. E., Jin, Y. S., Rao, J. & Gimzewski, J. K. Nanomechanical analysis of cells from cancer patients. Nature Nanotech. 2, 780–783 (2007).

75. Turitto, V. T. Blood viscosity, mass transport, and thrombogenesis. Prog. Hemost. Thromb. 6, 139–177 (1982).

76. Zhu, C., Yago, T., Lou, J. Z., Zarnitsyna, V. I. & McEver, R. P. Mechanisms for flow-enhanced cell adhesion. Ann. Biomed. Eng. 36, 604–621 (2008).

77. Chang, K. C. & Hammer, D. A. The forward rate of binding of surface-tethered reactants: effect of relative motion between two surfaces. Biophys. J. 76, 1280–1292 (1999).

78. Hynes, R. O. Integrins: bidirectional, allosteric signaling machines. Cell 110, 673–687 (2002).

79. Lorger, M., Krueger, J. S., O'Neal, M., Staflin, K. & Felding-Habermann, B. Activation of tumor cell integrin $\alpha_v\beta_3$ controls angiogenesis and metastatic growth in the brain. Proc. Natl Acad. Sci. USA 106, 10666–10671 (2009).

80. Nash, G., Turner, L., Scully, M. & Kakkar, A. Platelets and cancer. Lancet Oncol. 3, 425–430 (2002).

81. Crissman, J. D., Hatfield, J., Schaldenbrand, M., Sloane, B. F. & Honn, K. V. Arrest and extravasation of B16 amelanotic melanoma in murine lungs. A light and electron microscopic study. Lab. Invest. 53, 470–478 (1985).

82. Weiss, L. Patterns of metastasis. Cancer Metastasis Rev. 19, 281–301 (2000).

83. Weiss, L. Comments on hematogenous metastatic patterns in humans as revealed by autopsy. Clin. Exp. Metastasis 10, 191–199 (1992).

84. Trepel, M., Arap, W. & Pasqualini, R. In vivo phage display and vascular heterogeneity: implications for targeted medicine. Curr. Opin. Chem. Biol. 6, 399–404 (2002).

85. Burdick, M. M. & Konstantopoulos, K. Platelet- induced enhancement of LS174T colon carcinoma and THP-1 monocytoid cell adhesion to vascular endothelium under flow. Am. J. Physiol. Cell Physiol. 287, C539–C547 (2004).

86. Chang, S. F. et al. Tumor cell cycle arrest induced by shear stress: roles of integrins and Smad. Proc. Natl Acad. Sci. USA 105, 3927–3932 (2008).

87. Hanahan D, Weinberg RA: The hallmarks of cancer. Cell 2000, 100:57-70.

88. Francesca Salamanna, Veronica Borsari, Silvia Brogini, Gianluca Giavaresi, Annapaola Parrilli, Simona Cepollaro, Matteo Cadossi, Lucia Martini, Antonio Mazzotti, Milena Fini, 2016. An in vitro 3D bone metastasis model by using a human bone tissue culture and human sex-related cancer cells.

89. FRANCESCA SALAMANNA, VERONICA BORSARI, SILVIA BROGINI, PAOLA TORRICELLI, SIMONA CEPOLLARO, MATTEO CADOSSI, AND MILENA FINI , 2017. A Human 3D In Vitro Model to Assess the Relationship Between Osteoporosis and Dissemination to Bone of Breast Cancer Tumor Cells.

90. Hirao M, Hashimoto J, Yamasaki N, Ando W, Tsuboi H, Myoui A, Yoshikawa H. Oxygen tension is an important mediator of the transformation of osteoblasts to osteocytes. J Bone Miner Metab. 2007; 25:266–76.

91. Alberto Ippolito, Antonio DeSimone, Vikram S. Deshpande. Contact guidance as a consequence of coupled morphological evolution and motility of adherent cells. doi.org/10.1007/s10237-022-01570-9

92. Phase Transitions. A Multinational Journal. 2023

93. Landau LD, Landau LD: Statistical Physics London: Pergamon Press; 1958.

94. Prigogine I: From Being to Becoming: Time and Complexity in the Physical Sciences San Francisco: W. H. Freeman; 1980.

95. Weinberg RA: The Biology of Cancer New York: Garland Science; 2007.

96. Kumar S, Weaver VM: Mechanics, malignancy, and metastasis: the force journey of a tumor cell. Cancer Metastasis Rev 2009, 28:113-127

97. Coffey J, Birtle AJ, Cogill G, Christmas TJ, Rapley EA, Huddart RA: A discussion of the biology of testicular cancer and current concepts in the management of stage I and bilateral disease. Clin Oncol (R Coll Radiol) 2005, 17:441-447.

98. Kauffman SA: The Origins of Order: Self-Organization and Selection in Evolution New York: Oxford University Press; 1993.

99. Ao P, Galas D, Hood L, Zhu X: Cancer as robust intrinsic state of endogenous molecular-cellular network shaped by evolution. Med Hypotheses 2008, 70:678-684.

100. Stanley HE: Introduction to Phase Transitions and Critical Phenomena New York: Oxford University Press; 1971.

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