Multimodality Approach To Study The Fractal Physiology Of Tumor Angiogenesis

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List of Abbreviations and Variables

\(\Delta \chi\)  Susceptibility difference
\(\eta_0\)  Water diffusion coefficient \([m^2 \cdot s^{-1}]\)
\(\gamma\)  Gyro-magnetic ratio \([s^{-1} \cdot T^{-1}]\)
\(\Lambda(r)\)  Lacunarity
\(\omega\)  Larmor frequency \([Hz]\)
\(\rho\)  Proton spin density
\(a_t\)  Specific activity \([Bq \cdot ml^{-1}]\)
\(C_{AIF}\)  Arterial Input Function \([\mu g \cdot ml^{-1}]\)
\(C_{norm}\)  Normalized Compactness
\(C_{v(t)}\)  Tracer Concentration of the venous output \([\mu g \cdot ml^{-1}]\)
\(C_{VOI}\)  Tracer concentration in the Volume Of Interest \([\mu g \cdot ml^{-1}]\)
\(D_{inj}\)  Injected dose \([Bq]\)
\(k_{trans}\)  Transfer constant \([s^{-1}]\)
\(R_1\)  Longitudinal relaxation rate \([s^{-1}]\)
\(R_2\)  Transversal relaxation rate \([s^{-1}]\)
\(v_e\)  Volume of EES \([m^3]\)
bFGF  basic Fibroblast Growth Factor
BMI  Body Mass Index \([kg \cdot m^{-2}]\)
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<tr>
<td>BSA</td>
<td>Body Surface Area [m²]</td>
</tr>
<tr>
<td>C</td>
<td>Compactness</td>
</tr>
<tr>
<td>CD31</td>
<td>Cluster of Differentiation 31 - PECAM-1</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer Stem Cell</td>
</tr>
<tr>
<td>DCE</td>
<td>Dynamic Contrast Enhancement</td>
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<tr>
<td>ECM</td>
<td>Extra Cellular Matrix</td>
</tr>
<tr>
<td>EES</td>
<td>Extravascular Extracellular Space</td>
</tr>
<tr>
<td>FA</td>
<td>Flip Angle [deg]</td>
</tr>
<tr>
<td>FD</td>
<td>Fractal Dimension</td>
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<tr>
<td>FDG</td>
<td>Fluorodeoxyglucose</td>
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<td>FID</td>
<td>Free Induction Decay</td>
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<td>Fluoromisonidazolo</td>
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<tr>
<td>GE</td>
<td>Gradient Echo</td>
</tr>
<tr>
<td>h(t)</td>
<td>Transport function</td>
</tr>
<tr>
<td>Hct</td>
<td>Hematocrit</td>
</tr>
<tr>
<td>MRA</td>
<td>Magnetic Resonance Angiography</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MTT</td>
<td>Mean Transit Time [s]</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission tomography</td>
</tr>
<tr>
<td>Q</td>
<td>Flow rate [mm³ · s⁻¹]</td>
</tr>
<tr>
<td>R(t)</td>
<td>Residual function</td>
</tr>
<tr>
<td>ROI</td>
<td>Region Of Interest</td>
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<tr>
<td>S</td>
<td>Signature</td>
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<tr>
<td>S</td>
<td>Surface [m²]</td>
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<tr>
<td>SE</td>
<td>Spin Echo</td>
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SRµCT  Synchrotron Radiation Based µComputer Tomography
SUV    Standardized Uptake Value
T_E    Echo Time [ms]
T_R    Repetition Time [ms]
TBF    Tumor Blood Flow [µl · g⁻¹ · s⁻¹]
TBV    Tumor Blood Volume [µl · g⁻¹]
TNTR   Tumor to Normal Tissue Ratio
TOF    Time Of Life
V      Volume [m³]
VEGF   Vascular Endothelial Growth Factoe
VOI    Volume Of Interest
VSI    Vessel Size Index
LIST OF FIGURES
Acknowledgements

A the end of this thesis, I would like to express my gratitude to all the people that, in one way or another, contributed and supported me in these four years. Without their help this work would not have been possible.

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• and the Nature, that makes everything possible!
Summary

Angiogenesis is one fundamental element of tumor progression, since it is the process through which tumor develops its own vessel network and uses it to recruit nutrients from the host tissue. It is composed of several sub-processes that happen at different scales. Different mechanisms work first at a molecular and cellular level to promote the formation of the smaller capillaries, then others enables the maturation of the vessels and the organizations in a network structure. The link with the vascular network of the host tissue and the delivery of blood and nutrients complete the process. But, because the physiological effects acts as feedback, usually positive, at a molecular level, angiogenesis can be consider as a never-ending process.

A multimodality approach has been chosen to study the different process at a different scale: magnetic resonance imaging to study the vascular architecture and physiology non-invasively, positron emission tomography to analyze the molecular processes involving glucose and oxygen. In addition to these in-vivo approaches, ex-vivo technique as synchrotron radiation-based \( \mu \) computer tomography is needed to visualize vessel at a capillary level as well as immuno-histochemistry to study the physiological process at cellular level.

The aim of this thesis was to apply the concept of fractal physiology together with a multi-modality image approach to study the tumor angiogenesis in mouse model.

The project has been structures in two parts: in the first part, the experimental setup together with the protocols for different image modalities was developed: magnetic resonance imaging MRI, synchrotron radiation based micro-computer tomography SR\( \mu \)CT, positron emission tomography PET and immune histochemistry IHC. The second part focused on developing a framework to perform the analysis of the acquired images which takes into account the fractal physiology concepts.

After testing different lines of tumor cells either in-vitro or in-vivo, C51-
colon cancer cell line was chosen and used in all experiments that were performed with balb/c nude mice. After the s.c. inoculation (day 0), experiments were carried out at day 6, 8, 10 and 12 to follow the evolution of angiogenesis. The following physiological parameters were measured *in-vivo*: hypoxia and glucose metabolism (PET); tumor haemodynamic, vessel permeability, vessel size index and anatomy (MRI). At the last stage of the tumor, day 12, tumor were explanted and prepared for the *ex-vivo* measurements: angiography (SRµCT); endothelial cells distribution and perfusion (IHC). Because of the repeated measurements in a short period of time, it was impossible to put animal under anaesthesia for more than 3 hours, including animal preparation, for each experiments. Due to this limitations, five different protocols had been implemented to combine the different image techniques.

Data have been analysed using either pattern analysis and histogram analysis techniques by means of *in-house* developed code in C++ and Matlab languages.

The result section is organized in four parts that corresponds to the four levels of the tumor hierarchy: molecular level, vascular network structure, physiological effects and interface with the host tissue.

**a) Molecular level:** tumor hypoxia and glucose metabolism have been studied by means F-MISO-PET and FDG-PET respectively. In all subjects, tumor were found to be hypoxic. At day 6, a great portion of tumor was hypoxic while, during tumor growth, we observed an heterogeneous distribution of the radiotracer with the formation of hypoxic and non-hypoxic regions that coexist together. Similarly, FDG study showed a more homogeneous glucose distribution at day 6 compared to day 12. Because the presence of glucose is an indicator of cellular activity, it is reasonable suppose that, during tumor growth, only few parts of the tumor remains metabolically active because of the formation of the vascular network.

In-vivo findings were confirmed by the IHC experiments, analyzing the relative distribution of endothelial cells, perfused cells and hypoxic cells. Metabolically active tumor cells were observed in regions with endothelial cells but not in hypoxic regions.

**b) Vessel network architecture** has been investigated by means of SRµCT. Two different acquisition modes had been used: absorption contrast and phase contrast. The acquired 3D data set clearly showed a chaotic structure of the tumor vessel. Fractal analysis yielded a Fractal Dimension
FD of $2.78 \pm 0.03$ (N=6) versus $2.23 \pm 0.02$ measured in brain sample. The smaller FD means a more simple and organized geometrical structure, which is a property of the healthy organs. On the contrary, higher value of FD are synonymous of complex system, and in case of tumor not hierarchical structure.

The absence of hierarchy in the organization of tumor vessel is also proved by the Vessel Size Index VSI readouts, measured by MRI. VSI, defined as the average vessel diameter on the vessels in the voxel, allows to measure the distribution of the vessel diameters distribution during tumor growth. Longitudinal measurements showed very similar distribution with a prevalency of capillaries (5-20µm). The number of such capillaries appreciably increases with the tumor growth, which means the formation of new angiogenic capillaries. Despite the increase of the capillaries, a reduced number of arterioles or venules (20-100µm) are formed by angiogenic process. Bigger vessel as arteries or veins (100-200µm) are not present in any stage of the tumor growth. This lack of organization of tumor vessels is an explanation of the heterogeneous growth of tumor tissue. A non uniform delivery of blood, and consequently of oxygen and glucose, means a non uniform availability of nutrients. Only regions perfused by blood have the possibility to replicate cells and therefore to growth. Other regions can remain in a dormant state, develop angiogenesis or become necrotic according to the environmental conditions.

c) Physiological effects. Such anatomical features have important implications on the tumor physiology: first, tumor haemodynamic will be determined by the vascular anatomy. The measurement of Tumor Blood Volume TBV by means of MRI showed an heterogeneous distribution of these parameters. High values of TBV in different regions of the tumor during its growth, indicate the formation of new vessel and therefore that the angiogenic process is taking place. To better quantify the heterogeneity, FD has been computed over the TBV maps. Longitudinal results indicates change in the FD values which, from a physiological point of view, means a change in the TBV distribution and therefore a change in the structure of the vessel network.

Second, the functionality of the vessels has been evaluated measuring their permeability by means of MRI. It defines the velocity and quantity of the blood that extravasates through the capillaries. Also in this case, different regions with different permeability values has been detected. The spatial quantification of the extravasated maps has been done evaluating of lacunarity which describes the gap between the distribution of the extravasated volume patterns, the higher the lacunarity, the higher the variability of its gaps and therefore the higher the heterogeneity of blood distribution. We
observed oscillating trend that we interpreted as a continuous rearrangement of the vascular network during angiogenic process. Such re-arrangement, together with the continuous formation of capillaries prevents their maturation to bigger vessels to form a functional network.

A consequence of this abnormal anatomy is that the delivery of the therapeutic drugs is not efficient, therefore non vascularized regions of the tumor will not receive the appropriated amount of drugs. Recent treatment strategies propose, first, to stabilize the vessel network by a pro-angiogenic drug and then to administer the tumor-toxic drug. We therefore evaluated the effects of a pro-angiogenic treatment on the stabilization/maturation of tumor vasculature. Animals were treated with DMOG, which leads to an activation of hypoxia signalling and hence can be considered pro-angiogenic. Analyzing TBV maps no difference has been observed using conventional analysis methods as histogram analysis; however significant differences have been observed by using shape and texture quantifications. This results indicate that tumor heterogeneity needs to be accounted for the evaluation of therapy response.

Angiogenesis leads to the formation of a chaotic tumor vascular architecture with important physiological consequences. Longitudinal studies demonstrates that tumor does not growth in a homeostatic behaviour, but rather in a dynamic continuous rearrangements. Tumor treatment, and anti-angiogenic treatments in particular, are directly influenced by the angiogenic properties. The leakiness of the vessels and its chaotic structure does not allow a uniform drug distribution. In this regards, measurements of spatial distribution, as fractal dimension and lacunarity, allow to quantify the homogeneity or heterogeneity of the drug delivery. This allows either to guess about the success of the treatment or to monitor individually its efficacy.

One of the of the great advantages of this research framework is the immediate possibility to implement it into existing clinic environment at a relatively low cost. MRI and PET are established techniques used daily in the hospitals as well as the histological examinations performed after tumor surgery. The implementation of pattern analysis to evaluate shapes and texture of tumor images is a straightforward process based on software tools which can be easily added to other on computer analyses constantly already performed in hospital medical imaging.

Last but not the least, this work is an example that research in oncology could profit extensively from the interaction, and the sharing of the knowledge of experts from different and complementary fields, working for a common task.
Sommario

L’angiogenesi, cioè la formazione di nuovi vasi sanguigni, è un passo fondamentale nello sviluppo tumorale. Mediante tali vasi, infatti, il tessuto tumorale è in grado di reperire i nutrienti necessari alla sua crescita dai tessuti limitrofi.

Il processo di angiogenesi è composto da molteplici processi secondari che hanno luogo a diversi livelli: alcuni meccanismi promuovono la formazione di piccoli capillari a livello molecolare e cellulare, successivamente altri ne permettono la maturazione e l’organizzazione. Il passo finale è costituito dall’unione tra questa rete vascolare e quella dei tessuti limitrofi permettendo al tumore di assorbire nutrienti dal tessuto che li ospita. L’interazione tra i due sistemi vascolari genera tuttavia dei feedback, solitamente positivi, a livello molecolare influenzandone i processi che hanno originato i capillari e rendendo l’angiogenesi tumorale un processo senza fine.

In questo lavoro, è stato scelto un approccio multimodale per studiare i processi a differenti livelli: mediante risonanza magnetica (MRI) si sono investigate le proprietà della struttura vascolare, con tomografia ad emissione di positroni (PET) si sono analizzati i processi molecolari legati al metabolismo dell’ossigeno e del glucosio. In aggiunta a queste tecniche in-vivo, altre metodiche ex-vivo con maggiore risoluzione spaziale come la tomografia computerizzata basata sulla radiazione di sincrotrone (SRμCT) ed esami istologici (IHC) condotti al microscopio fluorescente sono stati necessari per la visualizzazione dei singoli capillari e per lo studio dei processi a livello cellulare.

Lo scopo di questa tesi è lo studio dell’angiogenesi tumorale in modelli animali mediante l’uso di un approccio multi-modale e l’applicazione dei concetti della fisiologia frattale.

Il progetto è stato strutturato in due parti. Nella prima parte è stato sviluppato il setup sperimentale e i vari protocolli per le diverse metodiche utilizzate: risonanza magnetica, tomografia computerizzata basata sulla radi-
azione di sincrotrone, tomografia ad emissione di positrone e esami istologici. La seconda parte è invece stata focalizzata sullo sviluppo di un framework che implementasse i concetti della fisiologia frattale per l’analisi dei dati acquisiti.

In seguito a test in-vitro e in-vivo di differenti linee cellulari, la linea C51 corrispondente al carcinoma del colon è stata scelta ed usata in tutti gli esperimenti in combinazione con topi nudi di tipo balb/c. Le cellule tumorali sono state iniettate sotto cute all’inizio di ogni esperimento (giorno 0) e le misure sono state eseguite nei giorni 6, 8, 10 e 12 per seguire l’evoluzione dell’angiogenesi. I seguenti parametri fisiologici sono stati misurati in-vivo: ipossia e distribuzione del glucosio (PET), emodinamica del tessuto tumorale, permeabilità dei vasi, indice di dimensione vascolare e struttura anatomica (MRI). Al giorno 12, corrispondente all’ultimo stadio dello sviluppo tumorale, i tumori sono stati espiantati e preparati per gli esperimenti ex-vivo: SRµCT per la visualizzazione della rete vascolare e IHC per determinare la distribuzione di cellule endoteliali e di cellule perfuse. A causa dei ripetuti esperimenti in brevi periodi di tempo, non è stato possibile sottoporre gli animali per più di 3 ore per ogni sessione di misura. A causa di questa limitazione cinque differenti protocolli sono stati studiati per combinare insieme i differenti metodi di misura.

I dati sono stati analizzati utilizzando sia tecniche di pattern analysis che sistemi di histogram analysis per mezzo di codici sviluppati in linguaggio C++ e Matlab.

I risultati sono stati organizzati in quattro parti che corrispondono ai quattro livelli del processo di angiogenesi: effetti a livello molecolare, struttura della rete vascolare, effetti fisiologici e interazione con i tessuti circostanti.

a) Effetti a livello molecolare. L’ipossia tumorale e il metabolismo del glucosio sono stati studiati rispettivamente per mezzo di F-MISO-PET e FDG-PET. In tutti i soggetti, i tumori sono risultati ipossici. Nella prima fase dello sviluppo, corrispondente al giorno 6, una predominante porzione del tumore era ipossica, mentre durante le fasi di crescita è stata osservata una eterogenea distribuzione del tracciante corrispondente a una continua formazione di zone ipossiche e non-ipossiche. Similmente, lo studio della distribuzione di FDG ha mostrato una distribuzione più omogenea al giorno 6 che al giorno 12. Siccome la presenza di glucosio è un indice di attività cellulare, è ragionevole supporre che, durante la crescita tumorale, solo alcune regioni del tumore rimangono metabolicamente attive a causa della formazione del nuovo sistema vascolare. I risultati ottenuti in-vivo sono stati confermati dagli esami istologici, anal-
izzando le distribuzioni relative di cellule endoteliali, di cellule perfuse e di cellule ipossiche. Cellule perfuse metabolicamente attive sono state trovate nelle vicinanze di cellule endoteliali ma non in corrispondenza di cellule ipossiche.

b) **Struttura della rete vascolare.** La struttura della rete vascolare è stata studiata in prima istanza per mezzo di SRμCT usando sia la tecnica a contrasto di fase che quella a contrasto di assorbimento. Le immagini 3D acquisite mostrano chiaramente una struttura caotica dei vasi. La dimensione frattale FD è risultata essere $2.78 \pm 0.03$ (N=6) contro $2.23 \pm 0.02$ misurata in campioni cerebrali. Una FD più piccola significa una più semplice struttura geometrica che è propria degli organi sani. Al contrario, valori più elevati di FD sono sinonimi di sistemi più complessi e, nel caso dei tumori, di una struttura non gerarchica.

L'assenza di una struttura gerarchica nell’organizzazione della rete vascolare tumorale è stata dimostrata anche per mezzo dell’indice di dimensione vascolare VSI misurato per mezzo della MRI. L’indice VSI, che è definito come il diametro medio dei vasi presenti in un voxel, permette la misura della distribuzione del diametro dei vasi durante la crescita tumorale. Gli studi longitudinali mostrano una distribuzione molto simile con una prevalenza di capillari (5-20\(\mu\)m). Il numero di tali capillari cresce sensibilmente durante lo sviluppo del tumore e ciò implica la formazione di una densa rete di capillari. Nonostante questo, solo un piccolo numero di arteriole o venule (20-100\(\mu\)m) sono state osservate durante il processo di angiogenesi. Vasi più grandi come arterie o vene (100-200\(\mu\)m) non sono state osservate in nessun stadio della crescita tumorale.

Questa mancanza di organizzazione del del sistema vascolare può spiegare la crescita eterogenea del tumore. Una non uniforme irrorazione sanguigna del tessuto tumorale comporta una distribuzione non uniforme di ossigeno e nutrienti. Solo alcune regioni irrorate dal sangue avranno la disponibilità di garantire il metabolismo cellulare e di conseguenza la replicazione cellulare. Le altre regioni possono rimanere in uno stato dormiente, dare origine al processo di angiogenesi o diventare necrotiche a seconda delle condizioni ambientale.

c) **Effetti fisiologici.** Le caratteristiche anatomiche precedentemente descritte hanno importanti implicazioni nella fisiologia dei tumori: ad esempio l’intera emodinamica sarà determinata dalla struttura anatomica della rete vascolare. La distribuzione del volume di sangue tumorale TBV, misurato per mezzo di MRI, è risultata essere eterogenea. Elevati valori di TBV in differenti regioni del tumore durante la sua crescita, indicano la presenza di
nuovi vasi e quindi che l’angiogenesi è in atto. Per quantificare l’eterogeneità, è stata calcolata la dimensione frattale FD della mappa TBV. Misure longitudinali hanno mostrato variazioni di FD durante lo sviluppo del tumore che, da un punto di vista fisiologico significa un cambiamento della distribuzione del volum di sangue TBV e quindi della struttura vascolare.

La funzionalità dei vasi è stata studiata misurando la loro permeabilità mediante MRI. La permeabilità definisce la velocità e la quantità di sangue che esce dai capillari. Anche in questo caso sono stati osservati differenti regioni con differenti livelli di permeabilità. La quantificazione spaziale delle mappe di permeabilità è stata effettuata valutandone la lacunarità che descrive la distribuzione dei gap tra i vari pattern presenti nella mappa di permeabilità. Alti valori di lacunarità corrispondono a grandi variazioni dei gap e conseguentemente a un elevato livello di eterogeneità della distribuzione. È stato osservato un andamento oscillatorio del valore di lacunarità che può essere interpretato come un continuo rimodellamento della rete vascolare durante il processo di angiogenesi. Tale rimodellamento, insieme alla continua formazione di nuovi capillari, ne impedisce la loro naturazione e quindi la trasformazione in vasi più grandi rendendo l’intera struttura poco funzionale. Questa mancanza di funzionalità del sistema vascolare del tumore ha ripercussioni negative anche nella sua cura. Tale anomalia rende infatti inefficiente il rilascio dei farmaci tumorali in quanto, solo alcune parti del tumore saranno adeguatamente perfuse dal farmaco. Per ovviare a tale problema, recenti strategie propongono prima di amministrare un farmaco pro-angiogenico che stabilizzi la funzionalità dei vasi e successivamente il farmaco citotossico. Per testare questa possibilità, abbiamo valutato i cambiamenti indotti da un farmaco pro-angiogenico, chiamato DMOG, che induce un attivazione dei segnali cellulari legati allo stato di ipossia ed è quindi considerato un trigger dell’angiogenesi. Analizzando le mappe TBV mediante tecniche convenzionali come la histogram analysis non è stata trovata alcuna differenza tra il gruppo trattato e quello di controllo. Invece, differenze significative sono emerse usando la pattern analysis che permette la valutazione dei parametri di forma e di texture. Questi risultati indicano che l’eterogenità del tessuto deve essere presa in considerazione per la valutazione dell’efficacia del trattamento terapico.

L’angiogenesi tumorale porta alla formazione di una struttura vascolare caotica con importanti conseguenze fisiologiche. Gli studi longitudinali dimostrano che il tumore non cresce in un modo omeostatico, piuttosto è soggetto a continui rimodellamenti.

Le terapie, ed in particolare le terapie anti-angiogeniche sono direttamente influenzate dal processo di angiogenesi. La porosità dei vasi e la loro struttura
caotica non consento una distribuzione omogenea dei farmaci citotossici. Per questo, la valutazione di parametri come la dimensione frattale o la lacunarità permettono di quantificare l’omogeneità o l’eterogeneità della distribuzione del farmaco, permettendo da un lato di valutarne l’efficacia e, dall’altro di monitorare individualmente ogni singolo paziente. Uno dei grandi vantaggi di questo innovativo metodo di analisi è la semplice implementazione in ambiente clinico a basso costo. MRI e PET e esami istologici sono metodi di imaging che vengono usati giornalmente per la stadiazione e il follow-up di pazienti L’implementazione di tecniche di pattern analysis consiste semplicemente nell’installazione di codici software sulle normali workstation usate per l’analisi delle immagini.
SOMMARIO
Chapter 1

Introduction

The tumor disease is known since ancient Hippocrates described malignant tumors in the fourth century BC naming them for the first time carcinomas. The number of physicians and scientists who, for centuries, searched for the cure of cancer is incalculable. In recent years the systematic studies in many disciplines have produced important results, succeeding in prolonging live expectancy of the people diagnosed with several tumors that were regarded as untreatable few years ago (so to considered certain malignancies curable) and enhancing quality of life of the patients. Nevertheless, no definitive cure has been found for cancer that today is, worldwide, the third cause of death following cardiovascular diseases and infection and parasite diseases [143].

There are two reasons that explain the partial failure in finding a tumor cure [151]. On one side, there is the intrinsic complexity in focusing the research field not only because of the hundreds of different human tumors identified but also because the same cancer types vary from case to case, showing for example different phenotypes in different person. On the other side, the tools used to investigate human cancer have proved weak and sometimes inadequate. For instance, data collected in vitro on cell cultures or in vivo in animals has shown to be, in many cases, unsuitable for the direct clinical use.

The need to study all aspects related to tumor, from carcinogenesis to cure, in more details, had pushed recent research to develop more and more sophisticated methodologies and consider tumor with a multidisciplinary approach [15]. The work of scientists from different fields as medicine, biology, chemistry, physics, mathematics, physiology contributed to create broader outlook and a clearer general picture.

In this framework the work of this thesis focuses on one key element, tumor angiogenesis. Historically, among the several aspects of tumor development, a great effort had been made to study angiogenesis. Angiogenesis is one fun-
damental element of tumor progression, since it is the process through which tumor develops its own vessel network and uses it to recruit nutrients from the host tissue. Understanding this process has a primary importance in a cancer treatment as anti-angiogenic therapy: the rationale of this therapy is to interfere with angiogenesis keeping the tumor without nutrients and therefore impeding its development [84]. Anti-angiogenic therapy has been under development in the last decades. Despite the eort of several scientists working in dierent elds, the angiogenic process in tumor tissue is not completely established and it is evident that conventional methods of investigation can only partially disclose the complexity of the formation of vases in tumor, that are so different from those of healthy tissue.

Looking from a wider prospective than conventional physiology, tumor angiogenesis must be studied using both, a technical apparatus which exploits advanced imaging techniques and an unconventional theoretical approach based in particular on fractal objects, dynamics of non-linear systems, complexity of living organisms and their possible representation with chaotic behaviour.

To better describe the theoretical foundation of the thesis, a brief description of the concept of fractal is necessary. Fractal objects, that are the main ingredient of the fractal theory developed by B. Mandelbrot [83], are geometrical shapes that preserve their structure at different scale levels. Such self-similarity is shown by many organs and systems in the living organism as vascular network, lungs and kidneys structure, heart and brain physiology, present fractal structures. On the other hand, this self similarity is tightly connected with the complexity of the organization of living systems proposed by J.G. Miller [90]. He proposed eight nested hierarchical levels of living organism: cell, organ, organism, group, organization, community, society and supranational system. Focusing on the first three subjects, we can see that the structure and the physiological behaviour of each levels is quite similar, for example the structure of the organism composed by several organs, each of them with a specific task, is very similar to the one of the organ composed by several sub-units specialized to achieve a deinite objective. 

The vascular network is a clear example of this concept: the organism has a macro system of arteries and veins that deliver blood to the organs, organs have a vascular system with similar structure which deliver blood to the sub-units, the sub-units in turn will have a system to deliver blood to the single tissues and so on.

The key point is that the interaction between different levels is dynamic, that means that the actual status will determine the next one, and it is non-linear, i.e. the output is not linearly proportional to the input. Moreover, a process
is the sum of several sub-processes, all of them connected by positive and negative feedback [128].

All these aspects together with the fractional calculus [153], are the main issues of the fractal physiology, a new concept introduced by Bruce J. West [149] [152] in the last two decades. This recent theory is focused to the complexity of the physiological systems and characterization of such complexity through the fractal measure of their dynamics, with fractal dynamics being described by the fractional calculus. In other word, West unified the concepts of fractal geometry, non-linear systems, chaotic behaviour of organs and its physiology in a framework which founded a natural and ideal application to the study of living organism.

In this contest, the study of tumor development, and angiogenesis in particular, benefits of this multidisciplinary view that allows to take into account different aspects of such extremely complex process. Angiogenesis, in fact, is composed of several sub-processes that happens at different scales. Different mechanisms work first at a molecular and cellular level to promote the formation of the smaller capillaries, then others enables the maturation of the vessels and the organizations in a network structure. The link with the vascular network of the host tissue and the delivery of blood and nutrients complete the process. But, because the physiological effects acts as feedback, usually positive, at a molecular level, angiogenesis can be consider as a never-ending process.

The only possibility to study the different process at a different scales is to use the most appropriate technique to that specific scale and process, or in other words, a multi-modality approach. Nowadays, different kind of in-vivo and ex-vivo image methods allow to study different aspects of organs and tissue development. Magnetic resonance imaging is particular suitable to differentiate anatomical details in soft tissues as tumors, it also provided established methods to monitor physiological processes related to blood dynamics. On the other hand, positron emission tomography allows studying the metabolic behaviour of several metabolites, in our case oxygen and glucose. Nevertheless, to achieve microscopic resolution, ex-vivo techniques as synchrotron radiation-based micro computer tomography and microscopy are needed.

Single parts of the fractal physiology framework have been already implemented in several works that aim to investigate or model either physiological processes or pathological situation. Fractal dimension or lacunarity, for example, have been used to model the heartbeat [28], to study the perfusion of lungs [134] and breathing [105], as well as the structure of the brain [158] and its dynamics [69]. Regarding the application to oncology, a consistent
number of works relate to the evaluation of the fractal dimension of the tumor shape or to the lacunarity of its texture as reviewed by J.W. Baish [7]. Despite the increasing number of application of the concept of fractal physiology, they are limited to a specific process or pathological condition and usually use only one image technique. This means in other words, that it is possible to investigate only one level of the multi-scale processes.

The originality of the work described in this thesis consists in using these two approaches together: on one side the implementation of fractal physiology to consider the angiogenic process in its entirety and, on the other one, the use of a multimodality approach to measure all the physiological parameters at different levels.

Objectives

The objective of the thesis was to develop experimental imaging strategies and image analysis tools for characterizing neoangiogenesis in murine tumor models. Specific objectives were:

1. To develop multimodal imaging approaches for characterizing the architecture and function of tumor neovasculature.
2. To develop a mathematical framework using concepts of pattern analysis and fractal physiology for analyzing imaging results by explicitly considering tumor heterogeneity.
3. To demonstrate the potential of the multimodal imaging in combination with pattern analysis for assessing treatment response in experimental tumor models.

Outline

This PhD thesis is organized according to the different aspects relevant for tumor angiogenesis. Following three technical chapters on the theoretical background and the experimental setup, chapter five to eight discuss:

- the molecular process underlying angiogenesis
- the vascular architecture
- the physiological consequence of vascular anatomy
- and some aspects of the tumor host interface.

A brief description of each chapters is given below.

Chapter two focuses on the **theoretical knowledge** that constitutes the background of the work. This chapter is divided in three parts that constitute three of the four milestones of this work. The first deals with the concept of fractal geometry, a geometrical concept that has several application in physics and life science. The second concept describes the non-linear dynamic of the tumor evolution. The third part put these two concepts together to demonstrate that angiogenesis is a multi-scale non linear process and that the geometrical structure of the vessel network shows fractal properties. These aspects, that constitutes the main issues of fractal physiology, change drastically the way to approach tumor studies.

Chapter three presents the **techniques** used to perform *in-vivo* and *ex-vivo* experiments. The animal models and the **experimental setup** are described as well as the different image modalities used to acquire anatomical and physiological images of angiogenesis during tumor development: magnetic resonance imaging, positron emission tomography, synchrotron radiation-based $\mu$CT and immune-histochemistry. A complete description of the protocols is also provided.

The fourth chapter introduces the fourth milestone of this thesis: **pattern analysis**. Its purpose is to define two classes of estimators that quantify the shape of the tumor and its texture. The quantification of the texture, which include some concepts of the fractal physiology, plays a fundamental role because it allows the evaluation of the spatial distribution of physiological process.

The next four chapters present the **results achieved in this project**. Each chapter deals with a specific aspect of the angiogenic process. The environmental conditions that trigger the angiogenesis at a **molecular level** are presented in chapter five. *In-vivo* measurement of hypoxia and glucose metabolism are discussed together with *ex-vivo* histological examination of the tumor section.

The aim of the sixth chapter is to study the **geometrical properties of the vessel network**. In particular, the results presented in this chapter try to quantify the complexity and the chaos that angiogenic network presents. The evaluation of fractal dimension and average vessel diameter are used for
this purpose. The possibility to distinguish between veins and arteries using SRµCT will be also discussed.

Chapter seven aims to investigate the **physiological effects** produced by the angiogenic vessel. To accomplish this task, MRI techniques are the most useful methods since they allow monitoring such effects *in-vivo*. First, results about the haemodynamic and blood volume are discussed in details. Then, the functionality of the vessel are investigated measuring the vascular permeability. Finally, the effects produced by a *pro-angiogenic* drug over the angiogenic process will be presented.

The eighth chapter describes the study of the **interface between tumor and host tissue**. Such interface plays an important role either in the tumor development or in the formation of metastases because all the nutrients, blood, and eventually potential metastatic cells pass through it. *In-vivo* results from MRI and *ex-vivo* results from histological examinations will be presented.

**Conclusions and outlooks**

The ninth chapter deals with a comprehensive **discussion** of the achieved results together with the possible impacts of such approach to study tumor angiogenesis in human beings.

**Appendix**

Finally, three appendices describe in details some specific concepts used in this project and placed at the end to keep fluent the reading of the thesis. Appendix A describes the **biology of tumor** development through the definition of the different steps that tumor cells have to overcome to became tumorigenic. Appendix B focuses on the **angiogenesis** and its evolution during tumor growth, it is a more detailed explanation of the concepts already presented in second chapter. The last appendix, C, presents the theoretical background of **non linear models**, described in the second chapter, by means of the explanation of the Lotka-Volterra model applied to tumor growth. Such simple tumor model is quite a good example of non-linear system, with a complex and chaotic behaviour to study the theoretical implications of its dynamics.
Chapter 2

Theoretical background

The concept of fractal physiology has been introduced and developed by Bruce J. West in the last two decades [149] [150]. He has suggested a perspective to look at physiological process combining together different notions from different fields as fractal geometry, non-linear dynamic behaviour of living systems and complexity. While fractal geometry refers to a particular geometrical feature of the a living structure, the complexity is related to its dynamics which is an expression of the non linear behaviour of such systems. Such concepts that are tightly correlated, offer the possibility to approach the tumor growth from another sight compared to the standard approaches. In particular the use of mathematical tools as fractal dimension, introduced by Benoît Mandelbrot [83], together with lacunarity, or shape and texture analysis enable to evaluate specific features of the tumor geometry and dynamics otherwise impossible to estimate.

In the following sections we will explain in detail the concept of fractal and how it is related with the physiology living systems as animal or human beings, then we will consider their implications on tumor angiogenesis. Finally in such framework, the tools used for the quantification of the physiological parameters related to tumor angiogenesis will be introduced..

2.1 Fractal physiology

Geometrical curve with fractal properties had been studied since the the 17th century when Karl Weierstrass gave examples of functions everywhere continuous but nowhere differentiable. At the beginning of 20th, Helge von Koch and Waclaw Sierpinsky gave two examples, the snow-flake and and the
Sierpinsky’s triangle respectively, with self similarity properties. Some years later, Housdorff [59] [82] introduced the notion of fractal dimension as generalization of the euclidean dimension and Kolmogorov and Tihomirov [72] developed it in the middle of the century. But it was Benoît Mandelbrot who put all the concepts together and developed the concept of fractal geometry explained in his most famous book *The Fractal Geometry of Nature* in 1982 [83].

After this publication, the concept of fractal became very popular and several applications in different scientific and sociological fields started to proliferate. Among them, the applications to the anatomy and physiology of living organism (animals, plants, insects, reptile and of course human beings) are surprising and their development is just at the beginning. The basic notion of fractals applied first to healthy human physiology, and subsequently to pathological conditions as cancer are reported.

### 2.1.1 What fractal means

According to the the definition of Mandelbrot [83], a fractal is a rough or fragmented geometric shape that can be split into parts, each of which is (at least approximately) a reduced-size copy of the whole. This property is called self-similarity,

![Figure 2.1: Steps to generate the snow flake starting from an equilateral triangle. Image adapted from http://fractalenlightenment.com/](http://fractalenlightenment.com/)
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As example, let us consider one of the first fractal object: the snow flake\(^1\) described by the Swedish mathematician Helge von Koch in 1904 without knowing the concept of fractals. It can be constructed by starting with an equilateral triangle as depicted in Fig. 2.1 then recursively altering each line segment as follows:

1. divide the line segment into three segments of equal length
2. draw an equilateral triangle that has the middle segment from step 1 as its base and points outward.
3. remove the line segment that is the base of the triangle from step 2

An object generated in this way, shows some specific properties that differ a lot from the conventional surface or object we are used to:

- it has a finite area but infinite perimeter
- the curve is continuous everywhere but differentiable nowhere

As a matter of fact, if we think to a normal plane as the surface of a piece of paper, we can evaluate a finite area, a finite perimeter and differentiate it in most of the points of the delimitation curve.

Another fundamental property concerns its dimension. If we think at the objects of everyday life, we know that objects have dimension equal to 3, planes as piece of paper equal to 2 and lines have dimension equal to 1. This common notion of dimension is called topological dimension\(^2\). Roughly speaking, it is the minimum number of coordinates \(n\), with \(n\) integer and non-negative needed to specify each point within it. Due to the previous properties, for fractal objects it is possible to extend the concept of topological dimensions defining the fractal dimension\(^3\) as follow:

\[
FD = \frac{\log N(l)}{\log(l)}
\]  

\(^1\)also known as Koch curve

\(^2\)The covering dimension of a topological space \(X\) is defined to be the minimum value of \(n\), such that every finite open cover of \(X\) has a finite open refinement in which no point is included in more than \(n+1\) elements. If no such minimal \(n\) exists, the space is said to be of infinite covering dimension. The \(n\)-dimensional Euclidean space \(\mathbb{R}^n\) has covering dimension \(n\), with \(n\) integer and non-negative.

\(^3\)For a rigorous mathematical definition, fractal dimension can be derived from the Hausdorff dimension in case of \(n\) fractional but non-negative.
where \( N(l) \) is the number of self-similarity objects and \( l \) is the reducing factor.

In the previous example of the snowflake substituting \( N(l) = 4 \) and \( l = 3 \) in the eq 2.1 we obtain a fractal dimension FD equal to 1.2619. As we can see such fractal dimension is a fractional number, it is positive as the topological dimension but it is not integer. The origin of word *fractal*, coined by Mandelbrot in 1975, refers to such fractional number.

A complex theory had been defined for fractal geometry after Mandelbrot definition and it is applied in several fields of science. We can summarize all the applications in three main topics:

- **geometrical forms** to study the properties of the external shape of the objects and quantify the texture of the pattern inside

- **dynamical process** to investigate the solutions of non-linear dynamic system otherwise impossible to 'predict'

- **statistical process** to monitor or predict the evolution of a process by investigate a part of its time evolution

In the following chapters we will investigate in details the first two points that constitutes one of the milestone of this project.

### 2.1.2 Fractals in Nature

Fractals objects are ubiquitous in Nature. It seems that a huge number of objects or living beings followed the *self-similarity* property to growth. In botanic, for example, the structure of a tree is a fractal. In fact, the pattern of ramification is statistically constant from the beginning of the trunk till the end of the smallest branch. The same consideration can be done for the roots of any kind of vegetables or plants. The reason of this structure in plants is clear: it’s the best way to incorporate water and nutrients from the ground.

Animals, including human beings, are much more complex and do not have a fractal shape. Anyway, the shape and the functionality of the organs show fractal properties. The ramification of the bronchial trees down to alveolar structures in lungs [48], the pattern of the activation of brain regions, the trabecular structure of the bones [110] and the structure of the vessel network are just some examples.

The same arguments can be applied to non-living objects. The growth of crystals [20], the shape of the coastline [65], the river networks, the mountain
2.1. FRAC TAL PHYSIOLOGY

Figure 2.2: Example of fractal structure in nature; a) branching of the trunk of a tree and its root; b) ramification of a river, from http://ex perimentalized.blogspot.com; c) structure of a marine shell, from http://pakway.wordpress.com/; d) romanesco cauliflower, from http://dels-old.nas.edu/

range, craters [22], and many others, follow the principle of self-similarity. Fractal patterns have been also found in the paintings of American artist Jackson Pollock [129] but, in this case, Pollock did not thought at the fractal dimension when he painted his masterpieces!

The only difference of all of these examples compared to the mathematical definition of fractal objects, like the snow flake, is the iteration number: in the real objects it is finite. This means that there is a break of the self-similarity properties at a microscopic level and the definition of the eq.2.1 has to be adapted to the real cases as described in ch.4.
For our purposes we will focus our attention on the fractal structure of the vessel network as described in the next section.

Figure 2.3: Example of fractal structure: a) ramification of the bronchial trees in humans, from http://www.medscape.com; b) artery (red) and veins (blue) network of the human kidneys, adapted from [117]; d) fractal image derived from Mandelbrot set; d) Lavander Mist, Jackson Pollock, 1950 National Gallery of Art, Washington, D.C., from http://www.abstract-art.com

### 2.1.3 Dendrites and fractal structures

The formation of the fractal structures presented before is one of the challenge in this field. Several studies can be find in literature about the modelling of fractal structure in life science. Nevertheless, it is extremely hard to build such kind of models because of the huge amount of non-independent parameters to control.

In these regards, dendrites are quite helpful to understand the basic mecha-
nism of fractal growth. In geology, for example, a dendrite is defined as a crystals with characteristic tree-like structure where atoms bind along the energetically favourable crystallographic directions in a non-equilibrium state. This kind of structure shows fractal properties and can be studied by means of fractal geometry. Up to now the basic growth modes known for fractals have been diffusion-limited aggregation (DLA), the dielectric breakdown model (DBM) and cluster-diffusion-limited aggregation (CDLA) [29].

DLA, which is the basis also for the other two modes, is the process whereby particles undergoing a random walk due to Brownian motion cluster together to form aggregates of such particles. This theory, proposed by Witten and Sander [155] is applicable to aggregation in any system where diffusion is the primary means of transport in the system. This model is extensively studied with several computer simulations in different cases [29] [92] [112].

Similarly to crystal dendrites, plants, roots and vascular system presents the same tree-like structure. It reasonable suppose that this kind of structure at a molecular level is the most favourable in terms of energy consumption and, at a macroscopic level, it is the most suitable for the life adaptation (water supplying, air exchange,...). These aspects are described quite in details in the next chapters.

Despite the dendrites and physiological fractals share the same basic formation mechanisms, the translation of theoretical models as DLA is not so straightforward. The huge amount of parameters that play a central role in the physiological process, together with the external conditions (as temperature, pressure, humidity, pH,...) which influence it are not easy to manipulate in a model. The fact that these are dynamic processes in a non-equilibrium state, and sometimes in a non-linear regime, make the modelling not an easy task.

### 2.1.4 Fractals structure of vascular system

Vascular system in animals and humans plays a fundamental role in all the physiological process. As suggested by A. Van Heuvelen [61] we can think at the vascular system as ”a system which continuously supply nutrients to and remove waste from the doors of ten trillion homes, about ten thousand times the homes on the Earth. Moreover, the nutrients at some homes vary from one minute to the next, by a factor $10^{29} \div 50$, while at the same time other homes require a constant supply. Finally, the system has to be efficient for about 80 years, hopefully longer!”

A human body consists in about $10^{13}$ cells that need a continuous and vari-
able supply of nutrients such as oxygen and glucose. During physical exercise, the oxygen consumption rate by muscle cells increases by a factor 10 or even 20. An increase in temperature of 10 K double the rate of biochemical processes and the corresponding need for oxygen. Fingers, because of altered temperature and muscular demand, may in extreme cases have an oxygen consumption rate that varies by a factor 50. At the same time, the need of oxygen in brain cells remains more or less constant.

No person is able to build a system like that and Nature took ages to optimize such vascular system. At the actual stage of mammals evolution it is composed by three main parts:

- the heart: the central pump of the system
- the lungs that are the oxygen supply and the carbon dioxide disposal depot
- the system of vessels that carries supply (arteries) and remove waste from the cells (veins)

The heart has four chambers and operates like two simple two-stroke pumps. The working principle, as depicted in Fig.2.4, is quite simple: the left heart pumps the blood with nutrients into the arteries, when the blood has reached the cells it released the nutrients and pick up the wasting compound as carbon dioxide. Then, through the veins it reaches the right pump of the heart and, from here, it is pumped to the lungs where exchanges carbon dioxide with fresh oxygen. From lungs it goes again to the left part of the heart to restart the cycle.

Because the heart is a pulsed pump, the blood going out of the heart has a pulsed shape with correspondent high velocity. By contrast, the flow at a cellular level has to be constant and the velocity very slow to allow the chemical reactions. Therefore, the system of vessel has two defined tasks:

- delivery blood according to the need of each organ
- transform the blood flow from pulsed to continuous

To accomplish the first task the aorta, the first artery going out of the heart, as to ramified in smaller arteries as showed in Fig.2.4. Defining the flow rate as the volume of blood that flows into the aorta per time unit:

\[
Q_A = S \cdot v = \left[ \frac{mm^3}{s} \right]
\] (2.2)
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Figure 2.4: a) Scheme of aorta ramifications: $S$ represents the section of the vessel and $v$ the blood velocity. b) A schematic representation of the human circulatory system. The percentage indicate the fraction of the total flow going to different body parts at rest. The number in parentheses indicate the factor by which flow changes during physical exercise. *Image adapted from [61]*

where $S$ is the cross-section of the aorta and $v$ the blood velocity. We can also define the flow rate for each branch $i$ as:

$$Q_i = S_i \cdot v_i$$  \hspace{1cm} (2.3)

Because the total flow rate has to be constant for the mass conservation law and Bernoulli’s principle, the flow rate in the aorta has to be equal to the sum of the flow rates in the branches:

$$Q_A = \sum_i Q_i = \sum_i S_i \cdot v_i = \sum_i \pi \left( \frac{\phi_i}{2} \right)^2 \cdot v_i$$  \hspace{1cm} (2.4)

From the last equation we can see that the flow rate in each branch depends of its diameter or, in other words, flow rate is modulated by vessel diameter. Therefore aorta is ramified in smaller arteries, one for each organ.
(or sub-unit of the organ). At the input of each organ, the correspondent artery will be ramified in others smaller arteries with section defined accordingly to the needed flow rate.

A further step has to be done do accomplish the second task. As told before, the flow in the main arteries is pulsed because of the pulse of the heart and because the contraction/release movement of the arteries wall that help the blood to flow. The progressive ramification in smaller vessels continues till the diameter of about $5 \mu m^4$. So small vessels are called capillaries and constitute the interface between the vessel system and the cells. Their task is to delivery the blood with nutrients. The number of these capillaries is very high because they usually fully perfuse the organ tissue. According to eq.2.4 and supposing the velocity constant in each capillary, we can write:

$$Q_A = \sum_c q_c = \sum_c S_c \cdot v = S_{TOT} \cdot v$$

(2.5)

where $S_{TOT}$ is the total area of the capillaries. As we can qualitatively see in Fig.2.5 its value is much higher compared the bigger arteries that means that, because $Q_A$ has to be constant, the capillary velocity $v$ has to be really small. Moreover, the combined effect of the several ramifications and the reduction of velocity has the effect to soften the pulsed shape of the blood flow till reducing it to a constant flow.

Once the cells has metabolized the nutrients and produced wasting compounds as carbon dioxide, they have to be removed. This task is performed by another class of capillaries on the veins side. Those venous capillaries combine themselves in backward ramifications of increasing sections up to bigger veins that collect blood with carbon dioxide from the different organs. Some consideration has still to be done regarding the structure of the vessels. They are constituted by several layers that bestow them specific features. Arteries incorporate in the middle layer many elastine fibers and smooth muscle fibers, allowing them to withstand high pulsed pressure. Veins, that operates under low pressure have one-way valves to prevent back-flow of blood. Such layers structure, in addition, allows the regulation of blood flow changing vessel diameter according to the physiological need. At a capillary level this regulation is even more important. Capillaries incorporate at their beginning a sphincter, a muscle that contracts or relaxes

$^4$This value is more or less constant for a lot of mammals including mice and human beings. It is enough large to allow all the molecules involved in the cellular metabolism to flow out.
causing the closure or the opening of the capillaries. Such fine-tuning allows the local modulation of the blood flow in response to changes in temperature, pH, oxygen and carbon dioxide concentrations.

The vascular system described above is typical example of fractal structure. The number of ramifications, the length of the branches and its sections are not random but follow a defined scheme. For each organ this scheme is repeated from the big arteries and veins down to the capillaries. This fractal
structure is the only one that allows to delivery the blood from one starting point (the heart) to several points, with the minimum expenditure of energy, conserving the mass and modulating the velocity and the flow rate.

2.1.5 Fractals and tumor

Several reviews about the use of fractal concepts in pathology have appeared in literature in the last decades [151] [49] [33] [7] [130]. The first objective which all these studies have in common is the identification of the pathological state by comparison with an healthy situation. The second goal is the quantification of the grade of the pathology. As we will see in the next chapters, fractal geometry allows the evaluation of parameters, as fractal dimension or lacunarity, that describe the structure of the object to study. Let us consider, for example, a brain tumor. We know from 2.1.4 that the vessel network, and therefore the brain vessel network, has a fractal geometry. Also the tumor vessel network shows fractal geometry, but we know that it is more irregular, it is chaotic, often leaky and haemorrhagic [11] [97]. This means that brain vessels and tumor vessel structures are different, and the degree of difference can correspond to the degree of pathology (aggressiveness, stage,...).

The application of the fractal concepts to pathology and, in particular, to cancer can be summarized in four areas: the vessel architecture, the border of tumor, the parenchima structure, the cellular and nuclear morphology.

**Vessel architecture.** As already mentioned, tumor vasculature are more chaotic in appearance than the normal vasculature [7] as showed in 2.4. The reason for the lack of regularity in the vessel formation is the non uniform growth of the tumor tissue. The absence of control in replication originates different regions with different cell density and consequently different need of nutrients, therefore vessels will develop accordingly to these needs following a chaotic pattern. Some study relates this level of non-uniformity with the aggressiveness of the tumor. This subject will be develop in details in the following chapters.

**Border of the tumor.** The border of the tumor differs a lot from the border of normal organs. Usually organs are well delimited and have a round shape, they work together and they interact each other by defined rules. These features are lost in tumors: the lack of control in cellular replications, the chaotic vessel development and the absence of negative feedback gives the tumor intrinsic instability and unregulated growth. This means that the tumor will try to growth without any control infiltrating the host tissue. Such
infiltration will originate a shape that is characteristic of the combination tumor-host tissue and shows specific fractal behaviour. The investigation of the tumor border plays an important role in the breast cancer [119] [109] and melanoma [89] [23] detection. In the first case it allows to distinguish between neoplastic mass and benign micro-calcification, in the second one between melanoma and skin blemish.

**Parenchimal structure.** Similar consideration about the tumor growth, already cited for the tumor border, can be done about the parenchimal structure. The unregulated growth originates a non-homogeneous structure composed by several contiguous regions with completely different behaviour. The quantification of such inhomogeneity allows to define specific patterns that occur in similar kind of tumor. This particular topic is relatively new and few studies are present in the literature.

**Cellular and nuclear morphology.** Cellular morphology plays an important role in the behaviour of the cells as reported by Smith et al. [125]. Different morphology suggests different ways to interact with the neighbour cells either in physiological or in pathological case. Even more important is the case of cancer studies in which biologists looks for similar features common to tumor cells in order to distinguish them from the healthy ones. An interesting case has been reported for oral squamous carcinoma by Goutzannis et al. [51]. In this study the nuclear fractal dimension was evaluated for human specimens from patients with oral cavity carcinoma in order to assess its potential value as prognostic factor. The results presented higher mean values for carcinoma cells compared to the normal mucosa and a significant correlation with the nuclear size. Patients with fractal dimension lower than the median value of the sample had statistically significant higher survival rates. The conclusion was that, within the sample of patients studied, fractal dimension was proven to be an independent prognostic factor of survival in oral cancer patients.

### 2.2 Fractal physiology and tumor angiogenesis

In the previous sections we have described the geometrical concept of fractals and how this is related to the anatomy and physiology of the living organs and tumors. We have seen that one of the main features of fractal structure
is the self-similarity. To better understand the evolution of tumor angiogenesis, we have to consider the self-similarity property in a wider framework. A living organism, in fact, is composed of several organs and all of them interact and change informations together. Each organs, in turn, is made by sub-regions or functional units that interacts and exchange informations in a similar way of the organs. The living organism can be therefore consider as a complex object, composed of several organs, composed by sub units, composed by tissues, and so on. In this context tumor has to be considered as an organ which grows in an abnormal way, made of abnormal subunits and not an isolated entity.

Such complexity has important implications: first, tumor growth and angiogenesis are multi-scale processes. Second, the dynamic of such process is determined either by the interactions between internal subunits or external organs, thus the results will be a complex network of feedback in a non linear regime. Third, the scale used to investigate the process plays an important role: looking at tumor angiogenesis at a macroscopic level (arteries and veins) will be different but similar than at microscopic level (capillary) or at molecular scale. Last, everything happens at a particular level influences the processes at the other levels.

All these aspects as complexity, non linear dynamics and multi-scale properties are described in the following sections.

2.2.1 Complexity of the anatomical systems

We can consider a living organism, as human or animal beings as proposed by J.G.Miller [90] in his Living System Theory. By his definition, ”living systems are open, self-organizing systems that have the peculiar characteristic of life and interacts with their environment. This take places by means of information, matter and energy exchange”. Miller proposed eight nested hierarchical levels: cell, organ, organism, group, organization, community, society and supranational system. Each level comprises twenty critical sub-systems that perform an essential life process and, in turn, exchange matter, energy and informations. All the processes between the different levels and subunits determine the complexity of the systems. Fig.2.6 shows the hierarchical organization of the living systems as proposed by J.G.Miller.

In this framework, since the last five levels are the topics of social science, we will focus our attention only the first three levels cell, organ, organism. Among them, as proposed by Grizzi et al. [52], we introduce sub-cellular entities, tissues and apparatus as well.

Tumor growth, and angiogenesis, have to be placed in this general view of
the organism. They are the result of the abnormal physiological interactions between the different levels and, eventually, with some external factors. Following the theory of living systems, Grizzi and Chiriva-Internati [52] introduced the concept of dynamics of anatomical forms which studies their transformations in relation to the causes of the changes. Similar to the first principle of kinematics\footnote{Newton’s first laws of motion: every body remains in a state of rest or constant velocity unless acted upon by an external unbalanced force.}, they assume that an anatomical form in a state of morphological stability tends to preserve its shape in the surrounding space. However if we apply any perturbation \( P(x,y,z,t) \) (internal or external) it abandons its state of rest and enters the phase of modifications. The word state denotes the pattern configurations of the system at a particular instant, which is specified by a large number of variables. In particular, we can better define the state variables \( V \) as the composition of:

- shape \( S(x,y,z,t) \) of the anatomical structure we are considering which will be a function of the spatial coordinates \( x,y,z \) and will evolve during time \( t \)

- texture \( T(x,y,z,t) \) which is the content of the shape or, in other words, the spatial distribution of the state variables inside the shape
The temporal behaviour of the anatomical form can be therefore expressed with the general formula:

\[
\frac{dV}{dt} = f(S, T, P, t) \tag{2.6}
\]

In absence of perturbation \( P \) from the previous definition, we can deduce that measuring the shape \( S \) and the texture \( T \) of an anatomical form at a definite instant \( t \) we can have a direct information of its state. The presence of an unknown perturbation \( P \neq 0 \) produces a change in the state \( V \). If we are able to measure the states before and after the perturbation, we will able to get information about the perturbation. Furthermore, if we measure them at different time points, we will have information about the dynamics of the system and, in some way, we should be able to guess its evolution\(^6\).

How to evaluate the shape and the texture of tumor during angiogenic process is the main goal of this work. Next sections will describe the links between this theoretical formulation and the physiological processes involved in tumor angiogenesis.

### 2.2.2 Tumor angiogenesis

In this sections we are briefly describing the basics physiological process underlying tumor angiogenesis. For a detailed overview of this subject refer to App. and Descritto meglio in appendice. A cancer cell has to progressed through a series of mutations to became tumorigenic [97]: self sufficient in growth signalling, insensitive to antigrowth signals, unresponsive to apoptotic signals, capable of limitless replications and tumorigenic. Current evidence indicates that these neoplastic properties may be necessary but not sufficient for a cancer cell to transform into a tumor. To achieve this last capability, the avascular neoplastic mass has to recruit and sustain its own blood supply forming a new vascular network connected with the oe of the host tissue. Such process is called angiogenesis [84] [57] and the subprocess which triggers the formation of new vessels in the avascular neoplastic mass is called angiogenic switch.

\(^6\)This is the aim of any theory which tries to model a dynamical system, from weather forecast to stock trend, from economy evolution to sports betting. In our case its even more important because the knowledge of tumor development allows us to look for the most appropriated treatment and make a correct prognosis.
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The main reason for the neoplastic mass to development new vessel is its highly dependence from the microenvironment to recruit oxygen and nutrients. The diffusion limit of oxygen is about 100µm, that means that all the cells has to be located within 100-200µm from the nearest vessel [42]. The limit size for this vessel cooption is less than 1mm, such neoplastic mass can remain in this state of dormancy for long time without causing clinical symptoms and without possibility to be detected.

The lack of oxygen, i.e. the hypoxia level, is therefore the starting point which triggers the angiogenic process. Cells respond to the reduced oxygen level through *hypoxia-inducible factor 1 HIF-1* [58]. It is composed of two subunits HIF-1α and HIF-1β. In the hypoxic condition, HIF-1 binds to hypoxia-response elements (HREs), thereby activating the expression of numerous hypoxia-response genes, such as the pro-angiogenic growth factor vascular endothelial growth factor (VEGF). In the presence of oxygen, HIF-1α is bound to the tumor suppressor Von HippelLindau (VHL) protein.

For this reason the hypoxia level in tumor plays a fundamental role in tumor angiogenesis. Hypoxia level is also a crucial point in tumor treatment, it is reported, in fact, to be associated with the resistance to chemotherapy, immunotherapy, and radiotherapy [19][160]. The following steps that driven the switch to the angiogenic state are the: [97]:

- increased expression by tumor cells of angiogenic proteins, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF);

- increased expression of angiogenic proteins by stromal cells (i.e., stromal fibroblasts), a process induced by the tumor itself

- decreased expression of endogenous angiogenesis inhibitors (i.e., thrombospondin-1, TSP1) by tumor cells and by stromal fibroblasts

- recruitment of bone marrow-derived endothelial precursors (only in specific tumors)

Tumor blood vessels are architecturally different from their normal counterparts [11]: they are irregularly shaped, dilated, tortuous and can have dead ends. They are not organized into definitive venules, arterioles and capillaries like their normal counter parts, but rather share chaotic features of all of them. The vascular network that forms in tumors is often leaky and hemorrhagic, partly due to the overproduction of VEGF. Tumor vessels have also been reported to have cancer cells integrated into the vessel wall [84][43].
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Blood flows irregularly in tumor vessels, moving more slowly and sometimes even oscillating. Tumors can be quite heterogeneous in their vascular patterns, and are able to overproduce their capillary networks. In normal tissues, by contrast, vessel density is dynamically controlled by the metabolic needs of oxygen and nutrients [11].

These characteristics of tumor vessels are well known, both in patient and animal models. Despite that, their quantification is not straightforward due to experimental and analytical issues. One of the aim of this project is to develop a method to give a quantification of these features.

Coming back to the evolution of dynamic form defined in eq.2.2.1 we have now the possibility to make the link with the theoretical formulation. We can associate the dynamic form to the tumor. It will be described by a shape $S(x,y,z,t)$ and a texture $T(x,y,z,t)$ that are the geometrical boundary and its content respectively. The texture is composed by different kind of cells that follow specific processes to form the tumor, such processes constitutes the perturbations $P(x,y,z,t)$ that allow the tumors to evolve. An example is the angiogenic switch that transform the tumor from the avascular state to the vascularized state promoting the angiogenesis. The measurement of the physiological parameters such as oxygen concentrations, glucose metabolism, vessel density, blood perfusion, and vessel permeability allow to define the state $S$ of the tumor during the different step of the growing process.

2.2.3 Angiogenesis is a multi scale process

At a molecular level, angiogenesis can be modelled as a balance between pro-angiogenic and anti-angiogenic factors that respectively promote and inhibits the formation of the new vessels. Nevertheless, angiogenesis is composed of several steps that take place at different scales. Once the process has started, the endothelial cells are recruited and the formation of the smaller capillaries starts. We are in the range of 5-20$\mu m$. The next step is the formation of bigger vessels as arterioles and venules, 20-200$\mu m$ and further arteries and veins 200-500$\mu m$ This vascular system grows like a network and the connection between the different parts will determine the perfusion of the tissues. The better is the structure of the network, the better will be the delivery of the blood. Afterwards, the link between the tumor network and the vascular system of the surrounding tissue will be tightly established and tumor can be consider as an organ which interacts with other organs but in abnormal conditions. Newly formed vessels are now able to produce important physiological effects as draining blood from the vascular system,
 variate the local level of pH and hypoxia, mechanically compress the host tissue, introduce tumor cells in the bloodstream or in the lymphatic system, and many others. All these steps are summarized in Fig. 2.7.

Figure 2.7: Angiogenesis is an ongoing process composed of several subprocesses that take place at different levels.

What is important to notice is that all these physiological effects produce a feedback at molecular level. The variation of hypoxia, for example will influence the angiogenesis; similarly the availability of glucose will allow the cells replications. All these feedbacks influence the whole process, making it an ongoing process and, in the case of tumor endless.

To study all these different processes at different scales from molecular to macroscopic level, a single image technique is not enough to cover such a wide range. A multimodality approach, with imaging modalities appropriated to each specific level, is therefore needed as it is described in ch. 2.3.1.

2.2.4 Angiogenesis is a non-linear process

A linear system is defined as the system in which the effect is proportional to the force that has generated it: for example the elongation of the spring is proportional to applied force. In a non linear system this relation of proportionality between cause and effect is not longer valid. Only in a restricted range it will be sometimes possible approximate the problem to the linear
In living systems, most of the processes follow a non-linear dynamics due to the nature of the processes themselves \[151\]. Tumor growth, for example, follows an exponential growth, metabolic rate is proportional not to the mass of the body but to the $mass^{3/4}$, similarly the breath rate is proportional to the $mass^{1/3}$ \[154\], and so on. The reason of this non-linearity is twofold: first, physiological systems often underlie a power-law relation as

\[ y = Ax^b \]  

between variables $y$ and $x$, in the previous example metabolic rate and body mass, respectively. The factor $b$ is the **power index**. Second, what we are considering is not a single process but it the sum of several elementary sub-processes that we are not able to distinguish. In any case, in physiological studies is practically impossible to isolate a single process and study it separately from the others.

Angiogenesis is the result of several processes, each of them non-linear, that work together to the formation of the new vessel network. Moreover, tumor angiogenesis follow rules completely uncorrelated with the ones of the normal angiogenesis in healthy tissue. Thus, the problem has to be approached in a non-conventional way, using, for instance, non-linear tools such as the concepts of fractal geometry. Appendix C will describe in detail all the issues regarding the non-linearity of the tumor growth.

A fundamental issue that arises in modelling of non-linear physiological systems is the control of feedback. Because physiological process is a network of complex non-linear sub-processes, the systems have to analyse and balance all the feedback from each single process to ensure the proper operation. Two basic mechanisms are known up to now: homeostatic control and allometric control mechanism.

**Homoeostasis**, according to the definition of Cannon \[21\], is the property of a system, either open or closed, that regulates its internal environment and tends to maintain a stable condition. This is possible by means of multiple dynamic equilibrium adjustments and regulation mechanisms. Homoeostasis seems to be the evolutionary strategy selected to enable the human body to maintain an internal balance \[152\]. The homeostatic control of physiological networks classifies the dynamics as **negative feedback**, because such homeo-
static networks respond in ways to dampen environmental disturbances including fluctuations. However, the control of certain networks has the opposite behavior, they have a positive feedback, because the networks respond in ways to amplify perturbations [151]. Systems that use this kind of mechanism, called allometric control, are the heart which increase the heartbeat during stress, or the immune system which amplifies its response in the presence of virus or bacteria. Allometric controls is a relatively new concept that take into account the long-time memory and long range interaction [148], [152]. They are strictly related with the fractal characters of physiological system and can be described by the fractional calculus developed by B.J. West [153]. We can conclude observing that in healthy systems while the homoeostatic controls tend to avoid any system variability while the allometric ones tries to control the variability adapting the system behaviour to the new situation. In a pathological situation as tumors, these consideration are still valid but the way to control the variability does not work properly, or follows other rules.

2.3 Quantification of physiological parameters

The quantification of variables related to physiological processes is a crucial and non trivial point in physiology. In oncology it is even more complicated for the chaotic features of tumor tissues quite far from the regularity showed by the healthy organs. In this context of tissue heterogeneity, multi scale and non-linear processes, the standard approaches does not work properly. In particular a multi modality approach is needed to be able to investigate the different levels. Moreover, a specific analysis which takes into account the state of the tumor, i.e. the shape and its content, is needed as well. Particularly important is the evaluation of the fractal dimension, tightly connected with the fractal structure of the angiogenic network and the lacunarity of the blood perfusion as described below.

2.3.1 Multimodality approach

As we described in the ch.2.2.3, angiogenesis is a multi scale process involving objects that vary in a very large range: from molecular dimensions up to 500 µm. A single image technique is not enough to cover such a wide range. A multimodality approach, with imaging modality appropriated to a specific range or a specific physiological process, is therefore needed.
CHAPTER 2. THEORETICAL BACKGROUND

In this work Magnetic Resonance Imaging MRI, Positron Emission Tomography PET, Synchrotron Radiation-based micro Computer Tomography SRµCT and Immune-HistoChemistry IHC have been used.

At a molecular and cellular level, while PET allows to monitor the metabolism of oxygen and glucose, IHC allows to visualize the distribution of the different kinds of cells allowing to distinguish between endothelial, tumor and muscle cells for example. IHC enable also the study of the extracellular environment as hypoxia or pH level. Despite a lower spatial resolution compared to IHC, PET technique has the great advantage perform experiment in-vivo and therefore to follow the alteration of tumor metabolism during its growth. On the other side, IHC allows in principle to evaluate any kind of cellular parameters or conditions with a extremly high spatial resolution but only ex-vivo measurement are possible.

In the range of capillaries dimension, 5-20µm, no non-invasive in-vivo techniques are available at the moment. Nevertheless SRµCT is the only technique which offers the possibility to visualize the capillary structure of a tumor sample together with the full vascular system with micrometric resolution. Absorption contrast and phase contrast mode are both usable according to the sample dimension.

MRI results to be quite useful image technique to obtain information about the physiological effects induced by angiogenesis together with anatomical images. By means of MRI, in fact, it is possible to evaluate the perfusion of the tumor, the permeability and the functionality of the vessels, the quantity of blood extravasated into the extracellular compartment and the average diameter of the newly formed vessel. The great advantage of MRI is that it is possible to perform non-invasively in-vivo experiments, this means that is possible to monitor such physiological effects during the entire period of the tumor growth.

An important issue in the multimodality approach is how to combine the data together. Several kind of co-registration algorithms, which are based on elastic or non-elastic transformations, allow the fusion of the 3D dataset from different methodology. In this way it is possible compare the different information about a specific region of the tumor, or even about each voxel.

---

8The spatial resolution varies in a range of 30-100 µm according to the used acquisition mode to the available equipment.
9The spatial resolution is of the order of few mm
10The spatial resolution is of the order of µm
11The spatial resolution varies according to the technique and the equipment available: from 0.1 nm (electronic microscope) to 0.1 µm (optical microscope)
Despite the procedure of co-registration, from the geometric side, is almost established, it is still an issue considering living organism as animals or humane beings. Soft tissues, as tumors, change their shape with the position of the subjects, therefore a system which ensure the correct position reproducibility in each experiments is absolute necessary. Anyway, where this is not always possible, non-elastic co-registration algorithms can help, but only in case of small deformation of the tumor shape.

### 2.3.2 Fractal dimension

Fractal Dimension FD, defined in ch. 2.1.1 by the eq 2.1 is a statistical quantity that gives an indication about the complexity of an object, in our case the angiogenic vessel network or any other structure of the tumor. The most used algorithms to evaluate FD are essentially two: the box-counting and the differential box counting method. While the first allows the evaluation of FD over a binary data set, the second one allows to take into account also the grey level values of the images.

FD as measurement of morphometry is particularly attractive because of its invariance to shifts, rotations and scalings. Moreover it is independent from the imaging method we are using.

Over the evaluation of the vascular network, FD can be also used to quantify the roughness of the tumor-parenchymal border and the cellular-nuclear morphometry. In this work we have used FD to evaluate the complexity of the angiogenic network and the perfusion map distribution of the tumor tissue.

Fractal dimension has also been reported to be one of the most promising tool for monitoring the effectiveness of anti-angiogenic therapies in various clinical contexts. As a matter of fact, FD is proposed as a prognostic factor for some tumors as laryngeal carcinoma, endometrial carcinoma and ovarian cancer.

Last but not the least, FD can be used to model the growth of tumor vasculature as proposed by Tsafnat et al. The development of a model which is able to mimic all the aspects concerning the formation of the vessels in tumor is, in the end, the ultimate goal of all the angiogenic researches.

### 2.3.3 Lacunarity

Lacunarity was originally introduce by Mandelbrot as a second method to evaluate texture pattern together with fractal dimension. Some fractal
data set, in fact, can have similar fractal dimension but different texture \[74\].

The name comes from Latin *lacunae* and stands for lack, gap or hole. Roughly, we can say that lacunarity is a measure of the gaps distribution in a texture. Low lacunarity indicates the homogeneity and translational invariance due to similar gap sizes, whereas high lacunarity indicates heterogeneity or a wide range of gap sizes of the texture in question \[74\]. The possibility to quantify the texture of an anatomical images or a physiological maps provides two opportunities: on one side it allows to have a number which quantify the information embedded in the image. On the other hand, it is possible to individuate regions in the image that share the same lacunarity value, or equivalently, that have the same texture. This last property is widely used in the pattern recognition techniques, largely used in the past in military strategy and luckily, nowadays also in medicine \[86\] \[1\].

A number of algorithms have been proposed to evaluate lacunarity. In this work we have adopted the Gliding-Box algorithm, developed by Allain and Cloitre \[4\] and described in ch. 4.2.5. Such algorithm was introduced first for ecological studies \[108\] and then used as the basis for a more general approach to the study of the spatial distribution \[107\].

Lacunarity and fractal dimension, even if they refer to different features of a texture, are closely related and, in principle, can be evaluated from the same gliding-box algorithm as proposed by Borys \[16\].
Chapter 3

Materials and Methods

Several imaging techniques are available to study vascular systems in healthy organs, and in principle the same methods can be applied to the study of vascular networks in tumor tissue either in-vivo or ex-vivo. In-vivo technique as MRI allows to perform experiments to investigate both the anatomical structure and the physiological effects of newly formed vessel. PET enables the study of the glucose and oxygen metabolism that are directly correlated with the angiogenic process. Despite the great advantage of performing in-vivo experiments, and consequently the possibility to perform longitudinal studies, these two methods that cannot reach the spatial resolution needed to study capillaries. To achieve such goal, ex-vivo techniques as SRµCT are needed. IHC techniques are then useful to visualize cellular mechanism and molecular effects with microscopic resolution.

This chapter aims to introduce all these image modalities that have been used in this work together with the description of the protocols and the experimental setup established to perform experiments.

3.1 Magnetic Resonance Imaging

MRI is an imaging technique primarily used in medical settings to produce high quality images inside the human body, in particular of soft tissue [62]. It is based on the principles of nuclear magnetic resonance (NMR) [55] that is a physical phenomenon of the magnetic property of nuclei, which have a positive nuclear spin quantum number. Under the influence of an external static magnetic field these nuclei will precess about the direction of the magnetic field with an angular frequency (Larmor frequency). Applying a radiofrequency RF, such nuclei are able to absorb its energy and release a
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modified RF depending on the interaction between them and the nuclei of the tissue. Thus, combining sequences of RF pulses, gradients to select different portion (slices) of the tissue and a static magnetic field is it possible to obtain a tomographic image of the examined sample.

Compared to the other established tomographic techniques that is the Computer Tomography CT, MRI has the advantage not to use ionizing radiation, and therefore not to release radiation dose to the body. On the other side, the acquisition time is usually longer and the spatial resolution worse.

Several kinds of techniques have been developed to investigate anatomical structures or physiological processes of the human or animal body [12]. In this section we are focusing our attention on the methods related to the evaluation of the angiogenesis such as MRI angiography to visualize the vessel network, dynamic contrast enhancement DCE-MRI to study the blood perfusion and the vessel permeability and finally the vessel size index VSI methods to measure the average vessel diameter.

All the MRI experiments have been performed at the Institute for Biomedical Engineering (Animal Imaging Center, ETHZ Zürich).

3.1.1 Angiography

Magnetic Resonance Angiography MRA is a special magnetic resonance technique which is able to visualize blood vessels, or better, the blood flowing in the vessels. While blood flow effects and the resulting tissue motion are responsible for a number of artifacts which degraded the quality of the image, MRA uses advantageously such blood flow to image the vascular anatomy. MRA techniques can be classified in two categories:

- Time-Of-Flight TOF-MRA
- Contrast Enhanced CE-MRA

A third type of MRA is called Phase Contrast (PC-MRA) but it was not used in this work.

Time-of-flight MRA

The basic idea of Time-of-flight TOF angiography, also known as inflow angiography, is demonstrated in Fig. 3.1 [17]. The blood flow is assumed to be perpendicular to the imaging plane (or volume in the case of a 3D study). For repetition times $T_R$ shorter than the longitudinal relaxation time $T_1$ of the stationary spins within the slice, the signal will be reduced due to partial saturation effects. Blood flow in the
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\[ \Delta s = v T_R \]

Figure 3.1: Schematic representation of *Time-of-flight* principle valid for \( T_R << T_1 \)

vessel will move spins from outside the slice which have not been subjected to the spatially selective radio-frequency pulses into the imaging slice. These unsaturated or fully relaxed spins have full equilibrium magnetization and, therefore, upon entering the slice will produce a much stronger signal than stationary spins assuming that a gradient echo sequence is applied. This effect is referred to as *inflow enhancement*.

The amount of inflow enhancement depends on several factors, including tissue specific parameters like \( T_1 \), sequence specific parameters (flip angle, and \( T_R \)), and geometrical parameters like slice thickness and orientation, or blood flow velocity.

TOF angiography are classified into three groups as depicted in Fig.3.2: sequential 2D technique which provides multiple thin sections of vessels, 3D single slab where the whole volume is excited simultaneously, and will then be subdivided into thin partitions, or slices by using an additional phase encoding scheme in the slice select direction. The third group is a combination of the previous two and consists in the sequential acquisition of several slabs. Typically, 3D techniques are applicable in combination with fast flow situations, while 2D techniques may be applied for the visualization of slower flow as in the case of tumor.

**Contrast enhanced MRA**

It takes the advantage of injecting contrast agents during imaging acquisitions to increase the contrast. A Gadolinium-based contrast agent is injected as a bolus directly into a vein, and images are acquired during the first pass of the agent through the arteries. Arteries are detectable by the enhancement of the MR signal. During the following passes the concentration is quickly
reduced because of the fast clearance and the signal decays. The half life of such kind of compounds is of the order of minutes. Another possibility is to use a different type of contrast agent, as superparamagnetic iron oxide nanoparticles, that remains in the circulation for a longer time. Such agents, usually called blood-pool agents, have an half life of hours and have the capacity to label the blood. Because they produce a dephasing and consequently a degradation of the signal, vessels can be individuated by a suppression of the signal. Since longer time is available for acquisition, higher resolution imaging in principle is possible. On the other hand in this case is not possible to distinguish between arteries and veins.

### 3.1.2 Tumor Hemodynamic

For a given 3D-tumor, its tumor hemodynamic can be described by the following parameters:\footnote{These definitions have been adopted from [9]}

\begin{align}
TBV(x,y,z) & := \text{Tumor Blood Volume in the } (x,y,z)-\text{voxel is defined as the volume of blood in a voxel divided by the mass of the voxel.} \\

TBV(x,y,z) & = \frac{\text{volume of blood in } (x,y,z)\text{-voxel}}{\text{mass of the } (x,y,z)\text{-voxel}} = \left[ \frac{\mu l}{g} \right] \tag{3.1} \\

\text{The TBV can be split into two components: the plasma volume (PV) and the red cell volume (RCV):} \\

TBV(x,y,z) & = PV + RCV \tag{3.2}
\end{align}

Figure 3.2: TOF-MRA techniques: a) multislice 2D; b) volume 3D (single slab); d) multi-slab 3D. Figure adapted from [77]
3.1. MAGNETIC RESONANCE IMAGING

TBF(x,y,z): the Tumor Blood Flow in the (x, y, z)—voxel is defined as the net blood flow through the voxel divided by the mass of the voxel

\[ TBF(x,y,z) = \frac{\text{net blood flow through the } (x,y,z)\text{-voxel}}{\text{mass of the } (x,y,z)\text{-voxel}} = \left[ \frac{\mu l}{g \cdot s} \right] \] (3.3)

MTT(x,y,z): the Mean Transit Time of the (x, y, z)—voxel is defined as the ratio between TBV and TBF:

\[ MTT(x, y, z) = \frac{TBV(x, y, z)}{TBF(x, y, z)} = [s] \] (3.4)

It described the average amount of time it takes any molecules to pass through the voxel

Hct(x,y,z): the hematocrit, defined as the percentage of the red cell in the blood:

\[ Hct(\%) = 100 \frac{RCV}{TBV} \] (3.5)

Hct in the capillaries \( (H_{cap}) \) is smaller than in the large vessel \( (H_v) \).  

The dilution theory [88], [162], [161], [123] that describes the tracer kinetics in a volume of interest (VOI) can be applied to assess the tumor hemodynamic with dynamic MR imaging. The basic hypothesis of such theory are the following:

- the tracer is non-diffusible
- there is no recirculation of the tracer
- there is no tracer leakage through the wall of the vessel
- the system is considered to be in the steady state during the experiment
- the dose of the tracer is small enough not to perturb the system

Under these conditions, consider a VOI with a feeding artery and a venous output as depicted in Fig 3.3.

A bolus of tracer is administered i.v. at time \( t = 0 \) and the concentration that reaches the feeding artery is called arterial input function \( C_{AIF}(t) \). The

\[^2\text{In humans } H_{cap}=0.25\% \text{ and } H_v=0.45\% \] [121]. For modelling vascular network it is commonly used \( H_{cap}=0.30\% \) and \( H_v=0.40\% \) [9].

\[^3\text{either in a voxel or in a region of the tumor}\]

\[^4\text{This assumption is not completely true in the tumor vessel as discussed in ch.9}\]
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![Diagram](image)

Figure 3.3: Schematic representation of a VOI with a feeding artery and a venous output.

Individual particles of the tracer follow different paths through the VOI and their transit time thus have a distribution characteristic of the flow and of the vascular structure [102]. The probability density function of these transit times is called transport function $h(t)$. Hence, the concentration of the venous output $C_v(t)$ is done by the convolution between $C_{AIF}(t)$ and $h(t)$:

$$C_v(t) = C_{AIF}(t) \otimes h(t) = \int_0^t C_{AIF}(\tau) h(t - \tau) d\tau$$  \hspace{1cm} (3.6)

The function $h(t)$ has the dimension $s^{-1}$ and it is normalized to 1

$$\int_0^\infty h(\tau) d\tau = 1$$  \hspace{1cm} (3.7)

From $h(t)$ we can define a residual function $R(t)$

$$R(t) = 1 - \int_0^t h(\tau) d\tau$$  \hspace{1cm} (3.8)

which represents the tracer fraction present at a time $t$ after the injection of the bolus. At time $t = 0$, $R(0) = 1$ that means the total bolus of the tracer is ideally present in the VOI. Then, $R(t)$ decreases according to eq. 3.8.

Because $h(t)$ is a distribution it is possible to calculate the mean transit time (MTT) required for any particle of tracer to pass through the VOI:

$$MTT = \frac{\int_0^\infty \tau h(\tau) d\tau}{\int_0^\infty h(\tau) d\tau}$$  \hspace{1cm} (3.9)

Applying the central volume theorem of indicator dilution theory [88], MTT can also be evaluated as the ratio between TBV and TBF

$$MTT = \frac{TBV}{TBF}$$  \hspace{1cm} (3.10)
according to eq. 3.3 TBV can be evaluated as \[121\]:

\[
TBV = \frac{K_H}{\rho} \int_0^\infty C_{VOI}(\tau)d\tau - \int_0^\infty C_{AIF}(\tau)d\tau \tag{3.11}
\]

where \(C_{VOI}(t)\) is the tracer concentration in the VOI, \(\rho\) is the density of the tissue and \(K_H\) is correction factor \[121\] which takes into account the different hematocrit levels in capillaries and normal vessel

\[
k_H = \frac{1 - H_v}{1 - H_{cap}} \tag{3.12}
\]

From eq. 3.8, 3.9, 3.10 and 3.11 we obtain the equation relative to TBF

\[
C_{VOI}(t) = \frac{\rho}{K_H} TBF (C_{AIF}(t) \otimes R(t)) \tag{3.13}
\]

Eq. 3.11 and 3.13 are the central equations to determine TBV and TBF. The concentration \(C_{VOI}(t)\) and \(C_{AIF}(t)\) are measurable by DCE-MRI as follow. The MR signal in \(T_2\)-weighted gradient echo is:

\[
S(t) = S(0)e^{-TE R_2^*} \tag{3.14}
\]

Assuming \(R_2^*\) as tissue the relaxation rate the signal prior the injection of the tracer (contrast agent) is:

\[
S_{PRE}(t) = S_{PRE}(0)e^{-TE R_2^*} \tag{3.15}
\]

after the injection of the tracer and considering a linear relationship between the tracer concentration \(C(t)\) and the change in \(R_2^*\) we get

\[
R_2^* = R_2^{*0} + r C(t) \tag{3.16}
\]

where \(r\) is the intrinsic relaxation rate of the tracer. Therefore, the signal after tracer administration will be:

\[
S_{POST}(t) = S_{POST}(0)e^{-TE (R_2^{*0} + r C(t))} \tag{3.17}
\]

Combining eq 3.15 and 3.17 we can evaluate the tracer concentration \(C(t)\) in a region:

\[
C(t) = -\frac{K}{T_E} \ln \frac{S_{POST}(t)}{S_{PRE}(t)} \tag{3.18}
\]
Finally, substituting the value of $C(t)$ of the VOI and of the feeding arteria (AIF) in eq. 3.13 we can get the $TBV$.

$$TBV = \frac{K_{VOI} K_H}{K_{AIF} \rho} \int_0^\infty \ln \left( \frac{S_{VOI}^{POST}(\tau)}{S_{VOI}^{PRE}(\tau)} \right) d\tau$$

The proportionality factors $K_{VOI}$ and $K_{AIF}$ are unknown in principle, but their ratio is commonly considered equal to 1 as proposed by [121]. To evaluate TBF a deconvolution have to be performed in eq. 3.13 between $C_{VOI}(t)$ and $C_{AIF}(t)$. Because $R(0) = 1$, $R(t)$ can be easily evaluated from $R_p(t)$. Knowing TBV and TBF, MTT can be calculated from eq. 3.10

### 3.1.3 Vessel Permeability

Capillary vessels like arterioles and venous are permeable to the substances present in the blood to deliver nutrients to the tissue and pick up the metabolites produced by the tissue itself. This process is possible because of the structure of the capillaries presents several pores. In the bigger vessels these pores are not present and allow the blood to flow inside. Tumor vessels, however, present several of these pores not only at a capillary level but also in the bigger vessels. The quantification of the permeability of the vessels is possible by the measurement of $k_{trans}$ (transfer constant) and $v_e$ (extravascular volume) as described below.

The most common approach is to use a small-sized contrast agent like Gd-DOTA (gadolinium-tetraazacyclododecane-tetraacetic) which is able to leak in the extracellular space during DCE-MRI acquisition. The presence of contrast agent in the extracellular space produced a signal enhancement that depends on $k_{trans}$ and $v_e$. The measured MRI signal can be derived from the Ernst equation:

$$S[R_1(t)] = S[\rho, R_2^*(t)] \cdot sin(\alpha) \cdot \frac{1 - e^{-TR_{R1}(t)}}{1 + (1 - e^{-TR_{R1}(t)}) \cos(\alpha)}$$

where $\rho$ represent the proton spin density, $R_1(t)$ and $R_2(t)$ the longitudinal and the FID decay, $\alpha$ the flip angle and $T_R$ the repetition time. For short $T_R$ we have $T_R R_1 << 1$ and therefore we can use the first order approximation from previous equation:

$$\approx S[\rho, R_2^*(t)] \cdot sin(\alpha) \cdot T_R R_1(t)$$
We assume that the change in $R_1(t)$ is proportional to the concentration of the contrast agent $C_t(t)$, i.e.

$$R_1(t) = R_{10} + r_{1,GdDOTA} \cdot C_t(t) \quad (3.22)$$

where $R_{10}$ is the relaxation rate of the tissue without contrast agent and $r_{1,GdDOTA}$ is the relaxation rate of the Gd-DOTA. From eq. 3.21 and 3.22 we can define the signal enhancement

$$E[R_1(t)] = \left( \frac{S[R_1(t)]}{S[R_{10}]} - 1 \right) = \frac{r_{1,GdDOTA}}{R_{10}} \cdot C_t(t) \quad (3.23)$$

and finally obtain the concentration

$$C_t(t) = \left( \frac{S[R_1(t)]}{S[R_{10}]} - 1 \right) \cdot \frac{R_{10}}{r_{1,GdDOTA}} \quad (3.24)$$

Three different models have been developed by Tofts [133], Larsson [76] and Brix [18] to parametrize the curve from eq. 3.24 and therefore the pharmacokinetic Gd-DOTA. We have adapted to tumors the general model proposed by Tofts [131], [132] and [133] for the brain.

This model consists in three compartments as shown in Fig 3.4: the plasma volume is connected with the EES (Extravascular Extracellular Space) and the kidneys that drain tracer from plasma. Because the velocity of the kidneys compartment is much slower compared to the extracellular space, we can neglect it. The Gd-DOTA is directly injected as a bolus into the plasma compartment. Three principle parameters are defined:
$k_{trans}$ is the transfer constant expressed in $[s^{-1}]$ that is the volume transfer constant between blood plasma and EES. It is a measure of the vessel permeability and indicates how fast the blood flows out of the vessels.

$v_e$ is the volume of EES per unit volume of tissue, i.e. the volume fraction of EES. It indicates the volume of blood which flows out of the vessels.

$k_{ep}$ is the rate constant between EES and plasma expressed in $[s^{-1}]$ they are related by the following expression:

$$K_{ep} = \frac{K_{trans}}{v_e}$$ (3.25)

The differential equation for the tissue in the EES compartment is:

$$\frac{d}{dt} (C_t) = K_{trans}(C_p - \frac{C_t}{v_e})$$ (3.26)

and its solution [132] is:

$$C_t(t) = K_{trans} \int C_p(\tau) \cdot e^{-K_{ep}(t-\tau)} \cdot d\tau$$ (3.27)

where $C_p(\tau)$ is the concentration of Gd-DOTA in the plasma, i.e. the arterial input function which is characterized by the body distribution and kidney elimination. Because we are interested in model the initial tracer uptake, and not the entire uptake kinetics, we can approximately describe $C_p(\tau)$ as a step function

$$C_p(\tau) = \begin{cases} 
0 & \text{if } \tau < \tau_0 \\
C_p0 = \frac{D_{Gd}}{v_p} & \text{if } \tau \geq \tau_0
\end{cases}$$ (3.28)

where $D_{Gd}$ is the injected dose of Gd-DOTA and $v_p$ is the plasma volume. In this case the eq. [3.29] becomes:

$$C_t(t) = v_e C_{p0} \cdot \left(1 - e^{-\frac{K_{trans}}{v_e} \cdot t}\right)$$ (3.29)

At this point we can estimate $k_{trans}$ as the first derivate in the point $t = 0$

$$\left.\frac{d}{dt} (C_t)\right|_{t=0} = K_{trans} \cdot C_{p0}$$ (3.30)

and $v_e$ from the limit

$$\lim_{t \to \infty} C_t(t) = v_e \cdot C_{p0}$$ (3.31)
Alternatively a bi-exponential function can be used as a model to fit the $C_t(t)$ from the data:

$$C_t(t) = a \left(1 - e^{-k_a(t-t_a)}\right) + b \left(1 - e^{-k_b(t-t_b)}\right) \quad (3.32)$$

where $a, b$ are the amplitudes, $k_a, k_b$ the rates and $t_a, t_b$ the start values ($t_a < t_b$). Combining eqs. 3.32, 3.30 and 3.31 we obtain:

$$k_{\text{trans}} = \frac{a \cdot k_a}{C_{p0}} \quad (3.33)$$

$$v_e = \frac{a + b}{C_{p0}} \quad (3.34)$$

### 3.1.4 Vessel Size Index

The dimension of the vessels is a very important index in the study of the angiogenesis. On one hand the vessel diameter is strictly related with the blood volume delivered to the tumor, on the other hand it is an index of the organization of the vessel network. If a consistent number of vessel has large diameter, it will mean that the network is organized in arteries veins and capillaries, therefore it will be more functional.

The distribution of the diameter of the vessel in a region is evaluated by means of the Vessel Size Index (VSI). Its evaluation is based on the simultaneous measurements of the changes $\Delta R_2$ and $\Delta R_2^*$ induced by the injection of an intravascular superparamagnetic contrast agent [137, 136]. $\Delta R_2$ and $\Delta R_2^*$ are evaluated using SE (Spin Echo) and GE (Gradient Echo) sequences respectively.

In the Gradient Echo experiments the signal without contrast agent is

$$S_{\text{GE}}(t) = S_0 \cdot e^{-R_2^* T_E} \cdot F(T_E) \quad (3.35)$$

where $T_E$ is the echo time and $R_2^*$ is the FID relaxation rate constant, $F(T_E)$ modelizes the macroscopic field inhomogeneities that for high-resolution images is assumed to be very small $F(T_E) \sim 1$. A paramagnetic (or super-paramagnetic) contrast agent introduced in the vascular system creates an additional susceptibility difference $\Delta \chi$ between blood vessel and surrounding tissue\textsuperscript{5}. It causes magnetic field distortion near the vessels, resulting in increase of $R_2^*$. For vessels modelled as infinite cylinders, the spread of Larmor frequency due to $\Delta \chi$ is given by [70, 156]:

$$\Delta \omega = 2\pi\gamma \Delta \chi B_0 \quad (3.36)$$

\textsuperscript{5}It is assumed that the contrast agent does not extravasate in the tissue.
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where \( \gamma \) is the gyro-magnetic ratio and \( B_0 \) the main magnetic field. In presence of the contrast agent, the GE signal becomes \([135, 156]\):

\[
S_{GE}(t) = (1 - \xi_0)e^{-\frac{2}{3}\delta \omega \xi_0 T_E + \xi_0} \cdot e^{-R_2^* T_E}
\]

(3.37)

where \( \xi_0 \) is the blood volume fraction. For small \( \xi_0 \), the (3.35) can be simplified

\[
S_{GE}(t) = e^{-\frac{2}{3}\delta \omega \xi_0 T_E} \cdot e^{-R_2^* T_E}
\]

(3.38)

From (3.38) and (3.35) we can obtain

\[
\Delta R_2^* = \frac{2}{3} \delta \omega \xi_0
\]

(3.39)

In the Spin Echo experiments the signal without the presence of contrast agent is:

\[
S_{SE}(t) = S_0 \cdot e^{-R_2 T_E}
\]

(3.40)

which in presence of contrast agent becomes \([135, 70]\)

\[
S_{SE}(t) = S_0 \cdot (1 - \xi_0)e^{-0.694\delta \omega \frac{2}{3} D \eta_0 T_E + \xi_0} \cdot e^{-R_2 T_E}
\]

(3.41)

In the above formula \( D \) is the water diffusion coefficient and \( \eta_0 \) is

\[
\eta_0 = \xi_0 R^{-\frac{2}{3}}
\]

(3.42)

The VSI is defined as the average value of \( R^{-\frac{2}{3}} \) considering \( f(R) \) the distribution function of \( R \)

\[
R^{-\frac{2}{3}} = \frac{\int_{0}^{\infty} R^{-\frac{2}{3}} f(R_v) dR_v}{\int_{0}^{\infty} f(R_v) dR_v}
\]

(3.43)

For small \( \xi_0 \) eq. (3.41) reduces to:

\[
S_{SE}(t) = S_0 \cdot e^{-0.694\delta \omega \frac{2}{3} D \eta_0 T_E}
\]

(3.44)

From eq. (3.40) and (3.44) we obtain:

\[
\Delta R_2 = 0.694\delta \omega \frac{2}{3} D \frac{1}{2} \eta_0
\]

(3.45)

combining eq. (3.39), (3.45), (3.42) and (3.36) we obtain the vessel size index VSI

\[
VSI = 0.425 \left( \frac{D}{\gamma \Delta \chi B_0} \right)^{\frac{2}{3}} \left( \frac{\Delta R_2^*}{\Delta R^2} \right)^{\frac{2}{3}}
\]

(3.46)

and the blood fraction \( \xi_0 \):

\[
\xi_0 = \frac{3}{4\pi} \frac{\Delta R_2^*}{\gamma \Delta \chi B_0}
\]

(3.47)
3.2 Synchrotron Radiation-based microComputed Tomography

X-ray tomography using conventional x-ray tubes, usually called Computed Tomography CT is an established methodology routinely used in clinics and animal research. Despite the small dimension of CT scanner, they allow to achieve a spatial resolution of about 50µm that is not sufficient to resolve the capillary structure of the vessel network. This limitation is mainly due to the weak flux produced by the x-ray tube.

A better x-ray radiation for tomographic imaging can be produced by a synchrotron. Synchrotron radiation refers to the electromagnetic radiation emitted by relativistic charged particles (energies of several GeV), circulating in storage rings, at those parts of the rings where they are accelerated by a magnetic field [30]. Such kind of radiation offers a high photon flux over a large range of X-ray energies, high brilliance, small angular beam divergence, high level of polarization and coherence, low emittance and the possibility for monochromatization. Owing to these advantages, synchrotrons can principally achieve qualitatively better and faster measurements than X-ray tubes with an overall similar experimental set-up [38].

Synchrotron radiation-based micro-computed tomography (SRµCT) requires an experimental setup that consists of a high-precision sample stage (rotation table combined with translation tables perpendicular to the beam), a sample holder, a scintillator and subsequent light optics with a charge-coupled device (CCD) camera. The mechanical stability of the sample movement (translation and rotation) and also the quality of the scintillator material and the number of recorded angular projections are key experimental conditions to achieve tomographic images, with a resolution of the order of few µm [94].

Tomographic images are reconstructed from the projection images by means of filtered back-projection algorithm which is a simplified version of the reconstruction algorithm used in conventional CT. Since the beam used by SRµCT is a parallel beam, and not a cone beam, there is no overlap of the image projections and, therefore, the reconstruction can be done slice by slice.

Two kinds of acquisitions can be used: absorption contrast and local phase contrast. While the first allows to visualize the structure of the object evaluating its absorption coefficients, phase contrast allow the measure of the real part of the refractive index as described in the next two sections.

Experiments have been performed in three different facilities:

- SLS (Swiss Light Source, PSI, Villigen, Switzerland) at the beamline
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TOMCAT, in collaboration with Sabrina Lang, Prof. Marco Stampa-panoni, and Prof. Bert Müller

- ESRF (European Synchrotron Radiation Facility, Grenoble, France) at the beamline ID 19, in collaboration with Sabrina Lang, Dr. Timm Weitkamp, and Prof. Bert Müller

- HASYLAB (Hamburger Synchrotronstrahlungsabor, DESY, Hamburg, Germany), beamline BW 2 at the tomography operated by the Helmholtz-Zentrum Geesthacht HZG (Dr. Felix Bechmann) in collaboration with Sabrina Lang and Prof. Bert Müller

3.2.1 Absorption Contrast

The objective of absorption contrast mode CT is to obtain 3D anatomical information of an object by the reconstruction of the attenuation coefficients \( \mu(x, y, z) \) \[121\]. The sample is placed on a support, able to rotate of 360° and translate along x, y, z axis, between the x-ray source (synchrotron radiation) and the detector (CCD camera).

When an X-ray beam with intensity \( I_0 \) penetrates the matter, it interacts with the electrons, Such interaction causes the attenuation of the beam according to the following law:

\[
I = I_0 e^{-\mu d}
\]

where \( d \) is the thickness of the homogeneous medium. The absorption coefficient depends on two factors: the composition of the object including density and the energy of the beam. The composition of the object is what we want investigate: while regions of tissue with a small value of \( \mu \) weakly attenuate the beam, regions with higher value will strongly attenuate it. In particular, for a 3D object, which is characterized by a distribution of attenuation coefficient \( \mu(x, y, z) \), the total attenuation \( a \) for the projection along the direction \( \vec{r} \) is:

\[
a_r(E) = \int r \mu(x, y, z, E) dr
\]

The dependence on the energy needs some further explanations. According to its energy, x-ray beam interacts with the electrons of the matter in three possible ways: photoelectric effect (pe, \( E < 0.1 \text{MeV} \)), Compton effect (Ce, \( 0.1 \text{MeV} < E < 10 \text{MeV} \)) and pairs production (PP, \( E > 10 \text{MeV} \)). The absorption coefficient is the sum of this three contributes:
3.2. SYNCHROTRON RADIATION-BASED MICROCOMPUTED TOMOGRAPHY

\[ \mu = \mu_{pe} + \mu_{Ce} + \mu_{pp} \]  

(3.50)

For the energy of the diagnostic x-ray beam we are considering, the cross section for the pair production is zero. Photoelectric effect causes the total absorption of the x-ray quanta and the consequent emission of an electron which is immediately absorbed due to the high cross section. Compton effect causes a partial absorption of the x-ray energy and its scattering with a lower energy, the remaining energy is absorbed by the electron which is ejected from the atom.

From the image point of view, the scattering due to the Compton effects is a source of artifacts and noise because the scattered photon reaches the detector in a position which is different from the point of interaction in the matter. Such problem can be overcome using a beam energy in the range of the dominant photoelectric effect.

A second source of noise arises from the width of the x-ray spectrum: if the incident photons have different energies, we will have different attenuation coefficients\(^6\) that means different informations for each interaction point in the matter. This inconvenient can be solved using a monochromatic beam or selecting a specific energy with a monocromator.

These two specific features of the x-ray beam cannot be achieved with the conventional tubes used in the CT scanner, but can be obtained by the x-ray radiation produced by the synchrotron accelerator. The dimension of such machine (diameter from 100 m up to few kilometres!), the extremely complex experimental setup and the high level of radiation dose released to the sample restrict the use in clinical routine. Nevertheless absorption contrast SRµCT is a powerful method to obtain 3D images of ex-vivo samples with micrometric resolution \(^{93}\).

**Sample preparation and experimental setup**

Animals were usually sacrificed on day 12 after tumor inoculation. First \(350\mu l\) of Ketamine/xylazine were administered i.p. to deeply anaesthetized the mouse and then, 5min later, the whole body was perfused with 25ml heparin to avoid thrombosis and 20ml of PFA (4%) via the cannulation of the aorta at a rate of 4ml/min. Afterwards the body was perfused alternatively with one of the following methods:

- **contrast agent**: the medium used was a suspension of barium sulfate (BaSO\(_4\)) and physiological solution with a concentration of 80 g/l.

\(^6\)Because it is not possible to discriminate between the different components of the energy spectrum, we will measure an average value of the different attenuation coefficients.
suspension was filtered (pore size 40 \( \mu m \), BD Falcon, USA) to obtain particles with dimensions comparable to the diameters of micro-vessels. Before injection, the suspension was made homogeneous using the ultrasonic bath (Sonorex Digital 10P, Bandelin) at the temperature of 37 °C for a period of 10 min. Finally, 10 ml barium sulfate suspension was injected via the aorta applying the peristaltic pump (Watson Marlow 101 U/R). At the end of the procedure, the tumor was explanted and fixed in PFA (4%).

- Corrosion casting\textsuperscript{[75]}: Immediately following heparin and PFA perfusion, the polyurethane PU4ii (vasQtec, Switzerland), were infused at the same rate. Tumor sample were therefore explanted and after resin curing (12 days at room temperature), soft tissue was macerated in 7.5% KOH, followed by decalcification with 5% formic acid, each for 24 h at 50 °C. Casts were washed with water and freeze-dried.

Absorption contrast experiments have been performed at SLS, beamline TOMCAT (Villigen Switzerland) and at HASYLAB, beamline BW2 (Hamburg, Germany). The experiments at TOMCAT were carried out in local absorption contrast mode using the photon energy of 18 keV and the exposure time of 350 ms/projection. The sample were rotated between 0° and 180° in steps of 0.12° producing 1500 projections. Filtered back-projections algorithm was used to reconstruct the images with an isotropic voxel size of 3.7 \( \mu m \).

To avoid the formation of bubble in the liquid around the sample, the shutter was closed during CCD readout.

Measurement at BW 2 (operated by the Helmholtz-Zentrum Geesthacht HZG \textsuperscript{[10]}) were performed using a standard setup for absorption contrast tomography with a photon energy of 10 keV. A number of 720 projections were acquired rotating the sample in steps of 0.25° from 0° to 180°. Filtered back-projections was used for the image reconstruction with a voxel resolution of 2.1 \( \mu m \) and 4.25 \( \mu m \) (binning factor 2).

### 3.2.2 Phase Contrast

The usage of the conventional absorption contrast technique can be limited for small biological samples that show very weak attenuation contrast difference in which is impossible the use of contrast medium. However those sample can produce significant phase shifts of the X-ray beam. The interaction cross section of the X-ray phase shift can be as much as three orders
of magnitude larger than that of absorption, and thus the use of the phase signal can provide substantially increased contrast [57].

An added advantage is that phase signals are produced with much lower dose deposition than absorption, which can be very important when radiation damage becomes an issue. Various phase-contrast X-ray imaging methods have been developed, including interferometric methods, propagation methods and techniques using a crystal analyser.

The phase-contrast techniques used in this project was a Differential Phase-Contrast (DPC) technique with a grating interferometer. The operation principle of such method is well described by Weitkamp et al. [147].

A silicon phase grating \( G_1 \) divides the incident X-ray beam into essentially the first two diffraction orders, which, through the Talbot effect [53], form a periodic interference pattern in the plane of the gold analyser or absorption grating \( G_2 \). A phase object placed in the incident beam will cause slight refraction and therefore modifications of the original wavefront profile. These variations result in changes of the locally transmitted intensity through the analyser. This detected signal contains quantitative information on the phase gradient of the object.

To separate the phase information from other contributions, a phase-stepping approach is used. The phase grating is displaced transversely to the incident beam, along \( x_g \), over one grating period whilst acquiring projections for at least four steps to approximate a sinusoidal curve. The intensity signal in each pixel in the detector plane oscillates as a function of \( x_g \). The phase \( \varphi \) of this intensity oscillation in each pixel is related to the wavefront phase profile \( \Phi \) and to the decrement of the real part of the object’s refractive index \( \delta \) by

\[
\varphi = \frac{\lambda d}{g_2} \cdot \frac{\partial \Phi}{\partial x} = \frac{2\pi d}{g_2} \int_{-\infty}^{+\infty} \frac{\partial \delta}{\partial x} \partial z
\]  

(3.51)

where \( g_2 \) is the pitch of the absorption grating, \( \lambda \) is the X-ray wavelength and \( d \) is the distance between the two gratings (Talbot distance).

Tomographic reconstruction of \((x, y)\) taken for a sufficient number of different viewing angles of the sample yields the three-dimensional distribution of the X-ray refractive index \( n(x, y, z) \) of the object. Given the small deviation of \( n \) from unity, the refractive index is generally expressed in terms of its difference to water \( \Delta \delta = \delta - \delta_{H2O} \)

**Sample preparation and experimental setup**

Tumor samples were explanted at the last stage of tumor growth, usually at day 12 after tumor cells injection. In some cases where the growth was
really fast, the termination criteria imposed to sacrifice the animals on day 10. Tumoral mass was explanted after the i.p. administration of 350 µl of ketamine/xylazine to deeply anaesthetize the mouse, fixed in PFA and fixed in an Eppendorf tube to avoid motion during the acquisition. No further preparation was required for this technique.

Phase contrast tomography data were acquired at the beamline ID 19 (ESRF, Grenoble) using the described grating interferometer technique. Two different setup have been used with a photon energy of 26 keV. In the first we used an exposure time of 1 s/projection, each projection consists of 8 images over 2 grating periods. The specimen was rotated in steps of 0.36° between 0° and 180°. In the second case the exposure time was reduced to 0.5 s/projection with 4 images per grating period. The rotation steps were changed to 0.36° from 0° to 180°. The Talbot distance between the gratings was set to 376 mm for both experiments.

### 3.3 Positron Emission Tomography

Positron Emission Tomography (PET) is a nuclear medicine imaging technique which produces 3D images of functional processes in the body. The system is able to detect gamma rays produced by the annihilation of a positron emitted by a β⁺ radionuclide, which is introduced into the body on a biologically active molecule.

The radionuclide used in PET are typical isotopes with short half lives such as $^{11}$C ($T_{1/2} \approx 20$ min), $^{13}$N ($T_{1/2} \approx 10$ min), $^{15}$O ($T_{1/2} \approx 2$ min), $^{18}$F ($T_{1/2} \approx 110$ min) [146] [124]. A short half life allows to keep the dose released to the patient as low as possible in order not to produce side effects. This kind of radioactive nuclei are instable due to the excessive number of protons. They stabilize by decay of a proton into a neutron, a positron and a neutrino:

$$ p^+ \rightarrow n^0 + e^+ + \nu $$ (3.52)

The positron, as antimatter particle, has a very short half time and its free mean path in tissue is about 1 mm. The positron is scattered by the electron clouds of neighbouring atoms, dissipates energy, and is captured by an electron. The annihilation of these positron-electron pair produces two γ-quanta. For the conservation of angular momentum and energy, the two quanta travel in opposite directions ($\alpha \approx 180°$) and their energy is 511 keV:

$$ e^+ + e^- \rightarrow \gamma + \gamma $$ (3.53)
3.3. POSITRON EMISSION TOMOGRAPHY

The gamma rays are detected by a ring array of detectors, usually scintillators coupled with photomultiplier or avalanche photo-diodes. The raw data consist in a list of coincidence events representing the simultaneous detection (in a window of few nanoseconds) of the annihilation quanta from a pair of opposite detectors. Coincidence events are grouped into projection images called sinograms, and sorted by the angle of each view. The final image is produced from such sinograms using filtered back projection or mutual information algorithms [146].

To image the physiological processes in the body, radionuclides are binding to a specific molecule which is metabolite in a specific organ or takes part in a specific physiological process. The complex of radionuclide and carrying molecule is called radio-tracer. Several kinds of radio-tracers had been developed either for clinical or research studies. Routine applications are established in the field of oncology, cardiology, neurology and psychiatry.

In oncology, PET is used for diagnosis, staging and monitoring treatment of cancer [15]. The most common used radio-tracer is $^{18}$F-FDG (fluorodeoxyglucose), a glucose analogue which is used to investigate the metabolic activity and to detect tumor metastases. Another useful radio-tracer is $^{18}$F-MISO (Fluoromisonidazole) which allows to study the cellular oxygen metabolism and therefore to visualize the hypoxic regions of the tumor. Others compound had been recently developed to monitor tumor proliferation ($^{18}$F-FLT), tissue perfusion ($^{15}$O-H$_2$O) and cellular apoptosis (Annexin V) [7].

In this work both $^{18}$F-FDG and $^{18}$F-FMISO had been used and they are described in detail below.

PET experiments and autoradiography have been performed at the Institute for Pharmaceutical Science (ETH Zürich) in collaboration with Dr. Steffi Lehmann and Dr. Michael Honer.

3.3.1 $^{18}$F-MISO

$^{18}$F-FMISO (Fluoromisonidazolo) can be considered as a marker of hypoxic cells.

Hypoxia plays an important role in tumor angiogenesis, and together with pH, has a strong impact on therapeutic outcome [4]. As the oxygen tension is reduced below 10-15 mmHg cells become increasingly resistant to radia-

\[7\] only for animal studies.
tion damage induced by radiotherapy. Anoxic cells are approximately three times more resistant to radiation than those irradiated under normoxic conditions.

Tumor uptake is normally quantified in term of Standardized Uptake Value $SUV$ or Tumor to Normal Tissue Ratio $TNTR$, usually blood or muscle. Typically, a $TMR > 1.2$ has been used as a threshold between normoxia and hypoxia [6]. First proposed in 1986, its mechanism of hypoxia selectivity has been well defined and it is now proposed as the standard clinical PET hypoxia marker.

![FMISO molecule and retention diagram](image)

Figure 3.5: FMISO molecule (left). The retention of FMISO is inversely related to the intracellular partial pressure of oxygen (right). Picture adapted from [103].

When $^{18}$F-FMISO diffuses into cells, it is first reduced by nitroreductase enzymes to a radical form. Under aerobic conditions, the radical compound will be reoxidized and diffuse out of the cells in a clearance process. Under hypoxic conditions, these radicals will bind to intracellular macro-molecules and accumulate inside the cell [146]. Almost all nitromidazole derivative compounds share similar mechanisms, of retention and accumulation in hypoxic tissue.

### 3.3.2 $^{18}$F-FDG

$^{18}$F-FDG (2-deoxy-2-$^{18}$fluoro-D-glucose), a glucose analogue, is certainly the most used PET radiotracer and the first one approved by regulatory authorities for clinical use [124]. The 2-deoxyglucose method has originally
been developed by Skoloff et al. [126] for radiographic studied using $^{14}$C labelled2-DG. The FDG kinetics, described in 1978 by Ido et al. [141] is illustrated in Fig.3.6. FDG enters the cell by the same carrier as glucose, but at a much higher rate. It is then phosphorylated by hexokinase generating a large amount of FDG-6-phosphate. This substance is trapped and accumulated in the cell, because it does not enter the standard metabolic pathways, and because it can leave the cell only slowly by the action of glucose-6-phosphatase.

Figure 3.6: FDG and glucose metabolism in the cell. Picture adapted from [141].

As reported already by Warburg et al [144] in 1931, cancer cells showed an enhanced glucose metabolism compared to the healthy cells. Tumor, in fact, have an increased cellular uptake of glucose and a higher rate of glycolysis than the normal tissue. This features make FDG almost ideal to individuate tumor cells through the body and for this reason is widely use in clinical routines. However it is not fully tumor specific. Various forms of infection, inflammation, granulomatous disease and many other physiological or pathological conditions shows high uptake of FDG originating false-positive results [79].

3.3.3 Radio-tracer quantification

In many PET studies the objectives is not just to visualize a specific activity, correspondent to the number of photons detected, but to quantify the inves-
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tigated physiological parameter. The following quantities can be evaluated in PET studies.

**Standardized Uptake Value (SUV).**

\[
SUV = \frac{a_t \cdot v_t}{D_{inj} \cdot \frac{M}{m_t}} \quad (3.54)
\]

where \(a_t\) is the specific activity [Bq/ml] in the region of interest (ROI) and \(v_t\) the correspondent volume [ml], \(m_t\) is the mass of the ROI [g], \(D_{inj}\) the injected dose activity [Bq] and \(M\) the mass of the body [g]. The quantity:

\[
\frac{a_t \cdot v_t}{m_t} = c_t \quad (3.55)
\]

correspond to the tissue concentration [Bq/g].

This definition of SUV is independent of the body weight. However, it has been shown that FDG distributes in fat at a lower concentration than in other tissues. This produce a sensible error in the SUV evaluation in obese patients. This dependence is eliminated normalizing to the body mass index (BMI) instead of body weight:

\[
SUV = \frac{a_t \cdot v_t}{D_{inj} \cdot \frac{BMI}{m_t}} \quad (3.56)
\]

An alternative definition of SUV has been proposed normalizing to the body surface area (BSA) \[146\]

\[
SUV = \frac{a_t \cdot v_t}{D_{inj} \cdot \frac{BSA}{m_t}} \quad (3.57)
\]

The major rational to use BSA is that many metabolic activities, particularly energy metabolism, are proportional to the surface of the body and not to its weight.

**Tumor to Normal Tissue Ratio (TNTR).**

It is a simple but robust quantification of the tumor uptake compared to a normal tissue in which we expected no alteration in FDG metabolism. The normal tissue is usually a muscle but blood can be used as well.

\[
TNTR = \frac{T_t}{M_T} \quad (3.58)
\]

where \(T_t\) and \(M_T\) are the radio-tracer uptake in the tumor and in the muscle respectively. TNTR is independent from administered dose, body weight and
therefore from any error in their evaluation. Nevertheless, the individuation of the threshold which distinguish between physiological and pathological situation is not trivial.

3.4 Immunohistochemistry

Immunohistochemistry (IHC) refers to the process of detecting antigens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in ex-vivo biological tissues [116]. IHC takes its name from the roots "immuno," in reference to antibodies, and "histo," meaning tissue. IHC staining is an established methodology, widely used in the diagnosis of abnormal cells such as those found in cancerous tumors. IHC is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue. For example, specific molecular markers are characteristic of particular cellular events such as proliferation or apoptosis. Visualising an antibody-antigen interaction can be accomplished in two ways: immunoperoxidase staining and immunofluorescence. In the first one, an antibody is conjugated to an enzyme, such as peroxidase, that can catalyse a colour-producing reaction. In the other one antibody can also be tagged to a fluorophore, such as fluorescein or rhodamine.

The next sections are describing the three staining procedure used in this work that are CD31, Pimonidazole and Hoechst staining used to mark endothelial cells, hypoxic and perfused cells, respectively.

IHC experiments have been performed at the Institute of Pharmacology and Toxicology (UZH, Zürich) in collaboration with Dr. Steffi Lehmann and Ruth Keist.

3.4.1 Markers

Three markers have been used to investigate angiogenesis: CD31, Pimonidazole and Hoechst (Sigma-Aldrich, USA).

CD31

Cluster of Differentiation 31 (CD31), also known as Platelet endothelial cell adhesion molecule (PECAM-1), is a glycoprotein produced by the immunoglobulin gene “superfamily”, and it is normally expressed by endothelia and selected hemapoietic element [36]. CD31 is found on the surface of platelets, monocytes, neutrophils, and some types of T-cells, and makes up a large
portion of endothelial cell intercellular junctions. The encoded protein is a member of the immunoglobulin superfamily and is likely involved in leukocyte migration, angiogenesis, and integrin activation. CD31 is used primarily to demonstrate the presence of endothelial cells in histological tissue sections. The presence of endothelial cells in a tumor tissue is an indicator of tumor vessels and it is consider one of the first steps of angiogenesis as reported in App. [A and B]

**Pimonidazole**

Pimonidazole is a 2-nitroimidazole bioreductive chemical probe with an immuno recognizable side chain and a pharmacokinetic quite similar to \(^{18}\)F-MISO radiotracer described in ch.3.3.1. Nitroimidazoles undergo a hypoxia-dependent one-electron reduction catalyzed by cellular reductases, resulting in reactive intermediates that form adducts with cellular components at pO\(_2\) tensions of typically 10 mm Hg or less. Because of its high water solubility, chemical stability, low toxicity, and efficient tumor uptake Pimonidazole is one of the ideal compound to assess hypoxic measurements in ex-vivo sample tissue.

**Hoechst**

The Hoechst stains are part of a family of fluorescent stains for labelling DNA in fluorescence microscopy. Because these fluorescent stains label DNA, they are also commonly used to visualize nuclei and mitochondria. Two types are commonly used: Hoechst 33258 and Hoechst 33342. Both dyes are excited by ultraviolet light at around 350 nm, and both emit blue/cyan fluorescence light around an emission maximum at 461 nm. The Hoechst stains may be used on living or fixed cells, and are often used as a substitute for another nucleic acid stain, DAPI. The key difference between them is that the additional ethyl group of Hoechst 33342 renders it more lipophilic, and thus more likely to cross-interact with cell membranes. In some applications, Hoechst 33258 is significantly less permeant. Because the Hoechst stains bind to DNA, they can disrupt DNA replication during cell division. Consequently they are potentially mutagenic and carcinogenic therefore care should be taken in their handling and disposal. In this work, Hoechst 33342 has used as a marker of perfused cells because its ability to visualize living nuclei as proposed by [78].

**3.4.2 Staining procedure**

A volume of 100 µl of Pimonidazole (concentration 12 µg/µl) has been administered via lateral tail vein to each animal 60 min before euthanization.
Subsequently, 2 min before euthanization 100 µl of Hoechst (concentration 5 µg/µl) has been administered with same method. Following animal death, tumor sample have been immediately explanted and fixed in cryo-medium gel and stored at -80°C. Sample have been sliced in sections of 20 µm thickness, the distance between sections was set to 500 µm) in order to have sections from the different regions of the tumor. After the last staining with CD31 antibody, fluorescence images have been acquired by means of fluorescence microscope (AxioImager.Z1 Zeiss, with apotome option).

3.5 Experimental set-up

3.5.1 Animal Model

The following tumor cell lines has been tested either in-vitro or in-vivo to choose the ideal to use in the longitudinal experiment:

- U87 human glioblastoma
- BT479 human breast carcinoma
- B16 murine melanoma
- C51 murine colon carcinoma
- HCRG human hepatocarcinoma

The conditions required for the selection where: good growth in-vitro and homogeneous and fast growth in-vivo with high angiogenic proliferation.

The C51 was the one who best satisfied such conditions and it was used for all the experiments. Balb/c nude mice, weighting 22-27 g and obtained from Charles River Laboratories France were used for all experiments considering the establishment of the setup and the longitudinal studies. Groups of about 10 animals have ben used for each set of experiments.

A suspension of 10⁶ C51 tumor cells (murine colon carcinoma) was injected sub-cutaneously on the right flank of each mouse. Animals were monitored daily to check their health condition and tumor growth. After 6 days from injection, the tumor reached an average diameter of about 5 mm. The termination criteria, according to the Swiss Federal Law for Animal Protection, imposed to euthanized the animals when the tumor reached a volume of 2 cm³, usually between day 12 and 15.
3.5.2 Experimental protocols

Because of the repeated measurements in a short period of time, it was impossible to put animal under anaesthesia for more than 3 hours, including animal preparation, for each experiment. Due to this limitation, five different protocols had been implemented to combine the different image techniques.

**Longitudinal MRI + IHC.**

The goal of this protocol was to measure the evolution of the physiological parameters related to angiogenesis during tumor growth. For each experiment (Fig. 3.7), ten mice had been injected on day 0 sub-cutaneously on the right flank with a suspension of $10^6$ C51-cells. MRI acquisition to evaluate vessel permeability, hemodynamic, VSI and angiography were performed on days 6, 8, 10, 12.

MRI experiments have been performed using a PharmaScanner (*Bruker Biospin*, Ettlingen, Germany) equipped with a 4.7T magnet. A volume resonator operating in quadrature mode and a 4-elements (2x2) coil array operating at 200 MHz, were used for transmission and signal reception, respectively. MRI data acquisition are reported in Tab. 3.1, 3.2, 3.3 and 3.4.

On day 12, mice were perfused with Pimonidazole and Hoechst and sacrificed. Afterwards, tumor was explanted and frozen a cryo-gel medium at -80°C. Fluorescence microscopic images have been acquired after the preparation of sample slices according to the procedure described in ch. 3.4

**Longitudinal MRI + SRμCT.**
The goal of this protocol was to measure the evolution of the physiological parameters related to angiogenesis during tumor growth. For each experiment (Fig. 3.8), ten mice had been injected on day 0 sub-cutaneously on the right flank with a suspension of $10^6$ C51-cells. MRI acquisition to evaluate vessel permeability, hemodynamic, VSI and angiography were performed on days 6, 8, 10, 12.

MRI experiments have been performed using a PharmaScanner (Bruker Biospin, Ettlingen, Germany) equipped with a 4.7T magnet. A volume resonator operating in quadrature mode and a 4-elements (2x2) coil array operating at 200 MHz, were used for transmission and signal reception, respectively. MRI data acquisition are reported in Tab. 3.1, 3.2, 3.3 and 3.4.

On day 12, mice were sacrificed and the tumor was prepared for the SRμCT measurements. According to the procedure described in ch. 3.2, tumor were perfused with BaSO$_4$ or casting polymer for the absorption contrast SRμCT mode or simply fixed in PFA (4%) for phase-contrast acquisitions. MRI data acquisition are reported in Tab. 3.1, 3.2, 3.3 and 3.4.

**Longitudinal PET**

The aim of this protocol was to investigate the glucose metabolism and the hypoxia distribution during tumor growth. Twenty four mice, divided in two groups (A and B) had been injected on day 0 sub-cutaneously on the right flank with a suspension of $10^6$ C51-cells (Fig. 3.9). Groups A and B was further divided in two sub-groups, A1-A2 and B1-B2 respectively. F-MISO experiments have been performed at days 6, 8, 10 and 12 on group
A1 and at days 6 and 12 for group A2.
FDG experiments have been performed at days 6, 8, 10 and 12 on group B1
and at days 6 and 12 for group B2.
PET experiments were performed on the 16-module variant of the quad-
HIDAC tomograph (Oxford Positron Systems) using alternatively $^{18}$F-MISO
and $^{18}$F-FDG radiotracers. Animals were lightly restrained and injected with
520 MBq of the radiotracer (100–120 µl per injection via a lateral tail vein.
Animals were anesthetized with isoflurane in an air/oxygen mixture 80 min
after injection. PET data were acquired in list-mode from 90 to 120 min
after injection and reconstructed in a single time frame with a voxel size of
1 mm$^3$ and a matrix size of 120x120x200 voxel.

Pro-angiogenic drug MRI.
Its purpose is to evaluate the effect of a pro-angiogenic drug, as DMOG,
during tumor growth. Twelve mice where used for each experiment and in-
jected sub-cutaneously on the right flank, at day 0 with a suspension of $10^6$
C51-cells. On day 7 (Fig. 3.10), the mice had been divided into 2 groups of
6 animals. The first group had been treated with the DMOG drug and the
second one with NaCl solution (placebo). The treatment had been repeated
on day 9 and 11. MRI measurement to assess the hemodynamic, permeability,
VSI and angiography of the tumor had performed before (day 6) and after
(day 12) the treatment.
MRI experiments have been performed using a PharmaScanner (*Bruker Biospin*, Ettlingen, Germany) equipped with a 4.7T magnet. A volume resonator operating in quadrature mode and a 4-elements (2x2) coil array operating at 200 MHz, were used for transmission and signal reception, respectively. MRI data acquisition are reported in Tab. 3.1, 3.2, 3.3 and 3.4.

Table 3.1: Sequence parameters used for MRI high resolution anatomical images T1 and T2-weighted.

<table>
<thead>
<tr>
<th>sequence parameter</th>
<th>anatomy T1</th>
<th>anatomy T2</th>
</tr>
</thead>
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<tr>
<td>sequence type</td>
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<td>T2 weighted</td>
</tr>
<tr>
<td>Contrast agent</td>
<td>Gd-Dotarem</td>
<td>Endorem</td>
</tr>
<tr>
<td>Contrast agent dose [µl]</td>
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<td>70 µl</td>
</tr>
<tr>
<td>FOV (inplane) [pixel²]</td>
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<td>20x20</td>
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<tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>acquisition time [min]</td>
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### Table 3.2: Sequence parameters used for Time-Of-Flight MRI angiography

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<tr>
<td>Contrast agent</td>
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<td>Contrast agent dose [µl]</td>
<td>——</td>
</tr>
<tr>
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<td>echo time TE [ms]</td>
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<td>acquisition time [min]</td>
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### Table 3.3: Sequence parameters used for Dynamic Contrast Enhancement DCE-MRI T1 and T2-weighted

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<td>sequence</td>
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<td>DCE - T1</td>
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<tr>
<td>type</td>
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</tr>
<tr>
<td>Contrast agent dose [µl]</td>
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<td>100</td>
</tr>
<tr>
<td>FOV (inplane) [pixel^2]</td>
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<td>20x20</td>
</tr>
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<td>resolution (inplane) [mm^2]</td>
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Table 3.4: Sequence parameters used to evaluate MRI T2 and T2* maps for Vessel Size Index VSI quantification

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<th>VSI - T2* map</th>
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</thead>
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<td>Endorem</td>
<td>Endorem</td>
</tr>
<tr>
<td>Contrast agent dose [$\mu l$]</td>
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<td>70</td>
</tr>
<tr>
<td>FOV (inplane) [pixel$^2$]</td>
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<td>slice thickness [mm]</td>
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Chapter 4

Pattern analysis

The study of the morphology of the tumor mass was probably the first approach made by scientists to study such disease. With the term morphology we mean the structure and the content of the object tumor, or better its shape and its parenchyma. Their changes are the expression of the insight mechanisms that regulates tumor growth and, therefore, their measurement gives an indication of the internal processes that allow the tumor to develop. Although it is well known that the shape and parenchyma structure of a tumor is strictly correlated with its grade of malignancy, its quantification is not straightforward. One way to address this problem is given by pattern analysis which aims to quantify the morphometry of the tumor, or any other geometrical object, by means of two series of statistical estimators: shape and texture estimators. Their definitions and uses are the topic of this chapter.

4.1 Shape analysis

The descriptors of the shape should be able to quantify with numbers or with functions the shape of the volume we are considering. They have to satisfy the following essential basic properties [120]:

- invariance to shift
- invariance to rotation
- invariance to isotropic scaling

Invariance to reflection may also be desirable in some application where the visualized image is specular to its original.
The shape factors described below meet the previous criteria and, in principle, they are applicable to any kind of volume.

4.1.1 Volume and Surface

Let us consider a 3D image composed of \( N \) voxels, its volume is defined as:

\[
V = \sum_{x,y,z} v(x, y, z)
\]  

(4.1)

where \( v(x, y, z) \) is the volume of the voxel with \((x, y, z)\)-coordinates. Its surface is defined as the sum of the external faces of the boundary voxel, in practice it is the sum of the area of the faces of each voxel not connected with its neighbour.

\[
V = \sum_{x,y,z} s(x, y, z)_{\text{boundary}}
\]  

(4.2)

4.1.2 Compactness

Compactness \( C \) is defined \[17\] as:

\[
C = \frac{A^3}{V^2}
\]  

(4.3)

where \( V \) is the volume of the tumor and \( A \) its surface area. \( C \) is dimensionless, the smaller it is the more compact is the volume. The minimum value of compactness corresponds to the sphere which is equal to \( 36\pi \). Normalizing eq. (4.3) to this value we obtain:

\[
C_{\text{norm}} = 1 - \frac{36\pi}{C}
\]  

(4.4)

where \( C_{\text{norm}} \) varies in the range \([0, 1]\), 0 in the case of the sphere. Compactness is a simple but robust measure of the shape of an object. It is evident that it is invariant to shift, rotation, and isotropic rescaling.

4.1.3 Signature

Signature measure is a more sophisticated method to quantify the shape of the contour or surface for 2D and 3D images respectively. For a 3D tumor volume, its center of mass is defined by the three coordinates \( \tilde{x}, \tilde{y}, \tilde{z} \), as follow:
4.1. SHAPE ANALYSIS

\[
\tilde{x} = \frac{1}{N} \sum_{n=0}^{N-1} x(n) \quad \tilde{y} = \frac{1}{N} \sum_{n=0}^{N-1} y(n) \quad \tilde{z} = \frac{1}{N} \sum_{n=0}^{N-1} z(n) \quad (4.5)
\]

where \(N\) is the number of voxel, and \(x(n), y(n), z(n)\) their coordinates. It should be noted that the center of mass of concave regions could lie outside of the region.

The signature \(d(n)\) is defined as the distance between each \(n\)-voxel of the surface and the center of mass:

\[
d(n) = \sqrt{|x(n) - \tilde{x}|^2 + |y(n) - \tilde{y}|^2 + |z(n) - \tilde{z}|^2} \quad (4.6)
\]

Plotting \(d(n)\) as a function of \(n\) we can easily have a quantification of the roughness of the surface. Fig. 4.1 and 4.2 [118] show two different situations in case of breast lesions. In the first one, a benign lesion which correspond to microcalcification of the breast tissue, has smooth boundaries, almost oval or even circular. In this case, the signature profile will be regular without spikes. The situation is different in the case of malignant lesion where the mass, a breast carcinoma, has a star-like shape due to its several infiltrating branches. The signature profile is irregular and presents several spikes correspondent to the infiltrating part of the tumor.

![Figure 4.1: 2D projection of a benign breast lesion (microcalcification) and its signature. Pictures adapted from [118].](image)

Other different way to evaluate signature have been proposed [120] including chain coding and skeletonization.
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4.2 Texture analysis

Similar to shape factors, the descriptors of the texture should satisfy the criteria of invariance to shift, rotation and scaling. Furthermore, because they have also to be invariant to grey level scale of the image, they must be applied to the analysis of the raw data only. Several class of texture quantification related to image analysis are described in the literature [139]. Here we present the standard histogram analysis methods which is routinely used in clinics and research, and other two new methods, fractal dimension and lacunarity, coming from chaos theory and fractal geometry.

4.2.1 Histogram analysis

Simple measurements of texture may be derived from the investigation of grey-level distribution (or histogram) of the given data set [120]. Consider the normalized grey-level distribution $p(l)$, the 1st moment, called mean can be defined as follow:

$$m_k = \sum_{l=0}^{L-1} lp(l)$$  \hspace{1cm} (4.7)

where $l = 0, 1, 2, ..., L - 1$ are the grey levels. It represents the mean grey level of the data set. Any other $k^{th}$ moments can be derived from:

$$m_k = \sum_{l=0}^{L-1} (l - \mu)^k p(l)$$  \hspace{1cm} (4.8)
4.2. TEXTURE ANALYSIS

The $2^{nd}$ moment, called variance represents the dispersion of the grey-values around the mean value

$$m_2 = \sum_{l=0}^{L-1} (l - \mu)^2 p(l)$$  \hspace{1cm} (4.9)

The $3^{rd}$ and $4^{th}$ moments, called skewness and kurtosis, indicate the asymmetry and uniformity of the distribution respectively.

$$\text{skewness} = \frac{m_3}{m_2^{3/2}} \quad \text{kurtosis} = \frac{m_4}{m_2^2}$$  \hspace{1cm} (4.10)

We have to note that with histogram analysis any information concerning the spatial distribution of the grey-level value is completely lost.

4.2.2 Fractal Dimension

Fractal Dimension FD, is a statistical quantity that gives an indication of how complex a texture appears. Among the different geometrical definitions of fractal dimension, the box-counting or Hausdorff definitions are usually considered for 2D and 3D analysis.

As we have seen in ch.2.1.1 and 2.3.2, fractal dimension is a quantification of the geometrical structure of the fractal objects that are quite common in nature. The higher is the complexity of the object, the higher is the corresponding fractal dimension. For example, consider the four images depicted in Fig.4.3 in order of complexity: the circle, the snow-flake, the Sierpinsky triangle, and the Sierpinski carpet. We can easily observe that the most simple and regular object is the circle and the most complex texture is shown by the Sierpinski carpet. The values of the fractal dimension are respectively: 1.000 which coincide with a line, and 1.892 that is closed to the plane value (2.000).

Different algorithms have been developed to measure the fractal dimension of 2D and 3D data set. In this work we have implemented the Box-Counting algorithm, described below, that is the simplest but robust for large data set.

4.2.3 Box-counting Algorithm

Fractal dimension was evaluated according to the box-counting method originally proposed by Mandelbrot [83]. Consider the binary map $TBV_{bin}$ and an overlapped lattice as depicted in Fig. 4.4. For a given size $s$ of the grid it is possible to count the number of boxes $N(s)$ needed to cover the image. Iterating this procedure to smaller box size, we can get different value of $N(s)$
Figure 4.3: Circle (FD=1.000), snow-flake (FD=1.262), Sierpinsky triangle (FD=1.585) and Sierpinsky carpet (FD=1.892) show different levels of complexity.

The fractal dimension $F_D$ is mathematically defined as:

$$F_D = -\lim_{s \to 0} \frac{\log[N(s)]}{\log(s)}$$  \hspace{1cm} (4.11)

which corresponds to the slope of the function $N(s)$ for small value of box size $s$. To satisfy the strict mathematical definition, the scaling should be applied to the infinitesimal scale, but in real image data set the limit is forced by the voxel dimension.

Figure 4.4: Scheme of box-counting algorithm: $r\text{TBV}_{bin}$ map overlapped by a lattice with step $s$. 

4.2. TEXTURE ANALYSIS

4.2.4 Lacunarity

The concept of lacunarity was established and developed from the scientific need in different fields to analyse multiscaling texture patterns images. In medical images, for example, spatial patterns can be associated to a particular disease. Another application is the estimation of the population density by texture analysis of urban spaces registering by satellite images.

Lacunarity can be defined as a complementary measure of fractal dimension. It permits to distinguish spatial patterns through the analysis of their gap distribution at different scales [107]. Gaps in an image can be understood as pixels with a specific value (e.g. foreground pixel in a binary image) or a certain interval of values (in grey scale images).

The higher the lacunarity of a spatial patterns, the higher will be the variability of its gaps, and the more heterogeneous will be its texture.

There are many algorithms to calculate lacunarity of an image. Among them, two algorithms have been commonly used: Gliding-Box and Differential Box-Counting.

4.2.5 Gliding-Box Algorithm

The Gliding-Box Algorithm [4] was proposed by Allain and Cloitre in 1991. It allows the study of lacunarity of a random or deterministic fractal data set by analyzing the fluctuation of the mass distribution function.

Let us consider a binary 2D image as depicted in 4.5, with the background value 0 (orange) and the signal value 1 (blue). A lattice with a mesh size equal to the pixel size \(a\) is put over the image. Now consider a box \(r\) which glides on every nodes of this lattice.

For a gliding box of size \(r\) we define the mass \(M(r)\) of the box as the sum of the pixel with value equal to 1

\[
M(r) = \sum_{\text{pixel}} \text{pixel}_{\text{value}=1}
\]

and the mass distribution in the collection of gliding boxes \(n(M,r)\) as the number of the gliding box with size \(r\) and mass \(M\).

Dividing \(n(M,r)\) by the total number of boxes \(B(r)\) we obtain the probability function

\[
Q(M,r) = \frac{n(M,r)}{B(r)}
\]

\[1\]In principle the box can be any geometrical figure, for example a circle with diameter equal to \(r\) [4].
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Figure 4.5: Illustration of the gliding-box method over a binary image: signal voxel equal to 1 (orange), background voxel equal to 0 (blue). The mesh size corresponds to the pixel dimension $a$. The box of size $r$ can glide over the image for each nodes of the mesh.

For an homogeneous lattice that is transitional invariant, the mass embed-
ded in a gliding box is independent of its positions, and $Q(M,r)$ is a Dirac function. For a self-similarity set, $Q(M,r)$ depends on the box size $r$ and on the image size $l$.

It must be noted that the true statistical behaviour of $Q(M,r)$ is reached only for $r/L<<1$, i.e. when the set is larger respect to the box size.

According to the properties of the statistical distributions we can define its statistical moments $Z_q^{(G)}$

$$Z_q^{(G)} = \sum_M M^G Q(M,r)$$  \hspace{1cm} (4.14)

In general, a complete knowledge of all these different moments is necessary to achieve a proper physical characterization of the image. Anyway, the first (mean-square deviation) and the second moments (mean value) are enough to define the lacunarity $\Lambda$ at scale $r$:

$$\Lambda(r) = \frac{Z_q^{(2)}(r)}{[Z_q^{(1)}(r)]^2}$$  \hspace{1cm} (4.15)

In the case of an homogeneous translational invariant lattice, $Q(M,r)$ is a Dirac function, $Z_q^{(2)} = [Z_q^{(1)}]^2$, and the lacunarity $\Lambda$ is independent of $r$ and equal to 1. Non-transationally invariant lattice has lacunarity larger than unity.
Figure 4.6: Three different binary images with the same number of signal voxel (25) and lacunae (231) but placed at indifferent positions. Using histogram analysis these images are identical (same histogram analysis) while using lacunarity estimator they results to be different ($\lambda_1 > \lambda_2 > \lambda_3$).

Image with a big amount of "void“ pixels are expected to be very lacunar with lacunarity much larger than 1. By the contrary, image with a small amount of void pixels has a small lacunarity close to unity.

The definition [4.15] is general since it can be applied to any image which is not necessarily fractal at an arbitrary scale $r$. 
Chapter 5

Molecular and cellular level

Several processes that happen at a molecular level drive the angiogenic pathway. Environmental conditions as hypoxia level or glucose availability are fundamental to promote or inhibit angiogenesis. While hypoxia is a sort of trigger of the angiogenic process, glucose metabolism is an indicator of the cells activity. Both of them play an important role in cancer growth and are an indication of the aggressiveness of the tumor. Moreover, the availability of the endothelial cells that are the main components of the vessel wall, is essential to promote the formation of the vessel network. The simultaneous presence of endothelial cells and perfused tissue cells is a proof of the functionality of the vessels.

In this chapter, we are presenting the results achieved about two different studies. The first refers to the oxygen and glucose metabolism, the second one to the distribution of the endothelial cells together with the hypoxic and perfused cells distribution.

5.1 Hypoxia

Hypoxic regions of the tumor have been investigated in-vivo by means of PET using FMISO radiotracer, which is a marker of the hypoxic cells as described in ch. 3.3.1. Experiments had been performed at days 6, 8, 10 and 12 after tumor injection according to the PET longitudinal protocol (ch. 3.5.2). Autoradiography on sections obtained from tumor samples previously imaged with FMISO-PET have been also acquired sacrificing the mouse immediately after the measurement.

In all subjects, tumor showed a uniform distribution of FMISO only at the early stage of the tumor growth (Fig. 5.1). At this stage, that corresponds to day 6 after injection, a great portion of the tumor can be consider
hypoxic. During tumor growth, we observed a heterogeneous FMISO distribution with the formation of hypoxic and non-hypoxic regions that coexist together. On day 12 different kind of hypoxic distribution had been observed: some tumors showed a big hypoxic region while others few smaller ones.

Figure 5.2: Autoradiography after FMISO-PET at days 6, 8, 10 and 12 for one different mouse every day

Autoradiography images that allow achieving a much better spatial resolution, but only on a single section without the possibility of a three dimensional data-set, are showed in Fig. 5.2. We observe that the tumors present
an homogeneous hypoxic distribution only at days 6, while they show an heterogeneous distribution during the other days. This means that after day 6 the angiogenic process took place and some regions of the tumor has been supplied by blood.

Hypoxia level has been quantified evaluating the Tumor to Normal Tissue Ratio (TNTR). In this case, femoral muscle has been chosen as normal tissue. Longitudinal results are plotted in Fig. 5.3. Assuming a value of TNTR=1.2 as threshold between normoxia and hypoxia as proposed by Astner [6], we see that the tumor is constantly in the hypoxic state. We also observe an increase of the value during the tumor growth, meaning a higher uptake of FMISO. If we compare Fig. 5.3 and Fig. 5.2, we can conclude that during its development, tumor increase the level of hypoxia but this is confined in specific regions. The other part of the tumor does not show FMISO uptake and we can therefore conclude either the tumor is not hypoxic or that there are no vessels to deliver the radiotracer. This last situation is compatible with a necrotic state especially at the last stage of the tumor growth.

Figure 5.3: Tumor to muscle ratio evaluated on FMISO-PET at days 6, 8, 10 and 12 after tumor injection. The threshold between normoxia and hypoxia is assumed to be 1.2 as proposed by [6]. The green line has been drawn just to show the trend of the data and it does not represent any model or fit.
5.2 Glucose metabolism

Glucose metabolism experiments have been performed \textit{in-vivo} by means of PET using FDG radiotracer (ch. 3.6). FDG is a glucose analogue but does not complete the standard metabolic pathway and remains trapped into the cell. In this experiment it is used as a marker of the metabolic activity of the tumor cells.

Experiments has been performed on day 6 and 12 after tumor inoculation. Similar to FMISO results, the distribution of FDG at day 6 resulted to be much more homogeneous than the one at day 12. This means that during tumors growth only few parts of it remain metabolically active while the majority does not. This behaviour is explained by the fact that the vessel architecture is chaotic and not fully functional (ch. 6) therefore, the blood will be delivered non-uniformly to the tumor tissue. In other words, only the regions perfused by functional vessel will have blood delivery, and therefore glucose availability needed for the replication.

If we do a step backward we can correlate the hypoxic readout with glucose readout and discriminate four possible scenarios:

- hypoxic region with no metabolic activity: in this situation there is an hypoxic state, meaning the absence of vessel and therefore no glucose is available. In this situation tumor tissue has to promote angiogenesis to proliferate and the low oxygen concentration will trigger the angiogenic switch. The formation of the new vessel allows the delivery of blood and glucose and the metabolic activity will start.

- non hypoxic region with metabolic activity: in such case the vessels are already formed, the blood is suppying oxygen and glucose and all the replicative processes are working.

- non hypoxic region with no metabolic activity: in this scenario the vessel network is already formed as it is able to deliver oxygen. In principle also glucose is delivered but the replication does not take place and the glucose is not metabolized. This state is also compatible with a necrotic state in which there are no living tissue and vessel to deliver FMISO (therefore no signal as in the case of non hypoxic cells) and FDG.

- hypoxic region with metabolic activity: it is impossible because the aerobic metabolic process required oxygen.

To quantify the level of metabolic activity, the average value of normalized activity due to FDG uptake has been evaluated on day 6 and 12. Re-
Figure 5.4: Normalized activity evaluated over PET-FDG data at days 6 and 12 after tumor inoculation. The orange line has been drawn just to show the trend of the data and it does not represent any model or fit.

Results, plotted on Fig. 5.4, showed a global decrease of FDG uptake (3 times smaller). Because of the non-uniform FDG uptake, this does not mean that the tumor cells have decreased their replicative potential, but that only a small portion of tumor is active.

Unfortunately, because the use of the same radioactive nucleus $^{18}\text{F}$ in both radiotracers, it is impossible to measure simultaneously FMISO and FDG uptake and make a correlation voxel by voxel the hypoxia level and the metabolic activation.

5.3 Endothelial cells distribution

The endothelial cells distribution has been evaluated over sections from explanted tumors stained with CD31, a common endothelial cell marker. Endothelial cells are the main component of the vessel walls and they are recruited during angiogenesis to form the new network.

A non-uniform distribution of endothelial cells has been found in all the examined sections with some areas of high concentration.

To verify the simultaneous presence of hypoxic and perfused cells, the same sections had been stained with Pimonidazole$^{[1]}$ and Hoechst, respectively.

$^{[1]}$Pimonidazole is a hypoxia marker with kinetics very similar to FMISO.
Figure 5.5: Histological tumor section stained for: a) hypoxia, b) endothelial cells, c) perfused cells. Image d) shows the fusion of a), b) and c).

A typical situation is shown in Fig. 5.5 where the same section has been stained with CD31 (red), Pimonidazole (green) and Hoechst (blue). Fig. 5.5d clearly shows the coexistence of endothelial and perfused cells, meaning that vessels are formed and are able to deliver blood. On the contrary, the hypoxic region does not show the presence of any endothelial cells, meaning that vessels are not formed and therefore oxygen is not present. A possible evolution in this region is the expansion of the angiogenic vessel or the death of all the hypoxic cells forming a necrotic area.
Chapter 6

Vessel network architecture

The vessel network is a fundamental part of a living organ or tissue. It is, in fact, the system which delivers blood and the nutrients needed for the metabolic processes and which, at the same time, removes the waste products produced by them. The formation of the tissues and the organogenesis can take place only if there is an adequate and constant blood supply that is determined by the fractal architecture of the vessel network as describe in ch. 2.1.4.

Healthy organs, at a macroscopic level, present a defined vessel structure that is substantially similar in any individuals; the brain, for example, present the same fractal network of vessels, with almost the same number of bifurcations, same number of arteries and veins in any person and therefore similar fractal dimension, the same for the other organs. This is because the formation of the vessel system inside the organo-genesis follows univocal and settled steps. In tumors the situation is different. As reported in the Appendix A, tumor breaks most of the biological rules that govern the formation of the organs producing abnormal tissue with physiological behaviour quite far from the healthy tissue. The architecture of the vessel network, in particular, results to be more chaotic, not fully functional and with no hierarchical organization. If we compare the same kind of tumor in different subjects, at a first sight, we will notice very few similarities in the angiogenic vessels network. Nevertheless, as presented below, it is possible to observe some geometrical characteristics shared by the same tumors in any individuals. The quantification of the chaotic structure of the tumor vessel network by means of fractal dimension analysis and VSI (vessel size index) is the goal of this chapter.
6.1 Chaotic structure of vessel network

The tumor vessel network has been successfully visualized by means of SR\(\mu\)CT. Two different acquisition methods had been used: absorption contrast and phase contrast following the protocols described in ch. 3.2.1 and 3.2.2.

Samples for absorption contrast has been previously perfused alternatively with two kind of contrast mediums: a suspension of BaSO\(_4\) nanoparticles and PU4ii, a polyurethane-based casting resin, covered with Osmium to increase the X-ray absorption coefficient of the vessels.

Samples for phase contrast have been explanted and embedded in PFA (4\%).

Fig. 6.1 shows a first example of absorption contrast SR\(\mu\)CT of a tumor and a portion of surrounded tissue perfused by BaSO\(_4\) nanoparticles. We can clearly see a more chaotic structure of the vessel in the tumor part compared to the muscle one. An extravasation of the contrast media is also visible around the vessels demonstrated their leakiness. The leakiness of the vessel is a crucial property of such newly formed vessels and it will be detailed discuss in ch. 7.2.

Fig. 6.2 is another example of absorption contrast SR\(\mu\)CT of a tumor casted with PU4ii resin. It shows again a chaotic structure and a predominance of capillaries (segmented and visualized in blue), the absence of bigger vessels proves that the hierarchical organization of arteries, arterioles and capillaries is not present as expected. A second rounded shape (segmented in red) resulted from a uniform extravasation of the casting medium is also present. It is reasonable to assume that it corresponds to the tumor regions perfused by those capillaries. A similar situation, founded in another tumor sample, is shown in Fig. 6.3 where vessel have been segmented using skeletonization process.

Fractal dimension FD is a shape estimator to quantify such chaotic structure of the capillary network described above. FD has been evaluated over the 3D data sets after the data binarization and using the Box-counting algorithm described in ch. 4.2.2. The average value and the standard deviation of the fractal dimension for a group of 6 samples is 2.78±0.03. This value should be compared with the fractal dimension of murine healthy colon, organ from which the tumor model used (C-51 cell line) derives. Unfortunately, the experimental setup to visualize its vascular structure and measure its fractal dimension is planned to be operational next year and no data are available at the moment. Moreover, no similar data exists in literature for the murine
6.1. CHAOTIC STRUCTURE OF VESSEL NETWORK

Fig. 6.1: Absorption contrast SRµCT of a tumor sample and a portion of muscle perfused with BaSO₄ solution. A complex and leaky vessel network is present at the tumor side, while the muscle region presents a simpler network.

To overcome this problem, a SRµCT experiment of the brain has been performed in order to have the full vessel structure of the brain. The left hemisphere is showed in Fig. 6.4. FD has been evaluated in six different regions of the brain and reported an average value of 2.23 ± 0.02, considerably smaller than the tumor value. The smaller FD means a more simple and organized geometrical structure which is a property of the healthy organs. On the contrary, higher value of FD are synonymous of complex systems, and in case of tumor of the lack of a hierarchical structure (ch. 4.2.2).

These results are supported by FD values measured in other organs for different kind of illness reported in literature and summarized in Tab. 6.1. Despite the different combination of tissues and diseases (vessel architecture, breast tissue geometry, skin lesions, lung structure and brain tissue), FD is always smaller in the healthy state than in pathological one. The biological explanation of this behaviour has to be investigated in each specific situation, but...
globally, we can assume that the organs change their anatomical structure as a consequence of the disease and that FD can measure these changes.

We have to note that BaSO$_4$ nanoparticles needs particularly attention in the preparation of the solution. As most of the nanoparticles, they tend to aggregate in microstructures of around 1-20µm structure, considerably bigger than capillaries diameter, and therefore able to occlude the vessel making impossible the perfusion. To prevent such phenomena, the solution need to be first treats with sonication and then quickly injected into the heart left ventricle.

The correct planning is a crucial point in this kind of experiments. First, only few installation in Europe allow to perform SRµCT experiments and the application proposal for beam time need to be approved by a committee. Furthermore, the acquisition time needed to achieve a good spatial resolution and sensitivity to visualize tumor capillaries varies from 2 to 6 hours accordingly to sample dimensions and spatial resolution. These restriction
6.1. CHAOTIC STRUCTURE OF VESSEL NETWORK

forces us to measure only few tumor samples from each group of animals.

The vessel network structure can be visualized in principle either by \textit{in-vivo} MRI angiography. Several trials had been performed to visualize vessel with MRI either using time-of-flight or contrast enhanced techniques. Different T1 contrast agents have been also used to increase the contrast between vessel and tissue but no significative results has been achieved. The explanation for this is twofold: on one hand the spatial resolution of MRI angiography, around 50-70\textmu m in the best case, is insufficient to visualize the vessel tumor structure (5-60\textmu m ). On the other hand the quantity of the blood flowing in tumor vessel, as well as its velocity, is smaller compared to the healthy organs. This produce a global decrease of the sensitivity either for contrast enhanced or time-of-flight techniques.

Figure 6.3: Skeletonization of an absorption contrast \textit{SR\mu CT} of a tumor sample casted with PU4ii resin. Capillaries in the range of 5-20\textmu m are depicted in violet, capillaries in the range of 20-30\textmu m in red. The yellow rounded shape correspond to a portion of the tumor perfused by capillaries.
6.2 Arterioles and venules

Up to now we have discussed about generic vessels, without specifying if they were arteries or veins. This distinction is quite important in the study of angiogenesis because of the different purposes of the two kind of vessels has to satisfy. Arteries and arterial capillaries have to supply blood to the tumor, while venous capillaries have to remove waste products of the metabolic process from the tumor. This means that the blood flows in opposite direction. Another important characteristic concerns the structure: while arteries incorporate a layer of muscle fibres in their wall to allow the synchronized contraction with the heartbeat, veins do not present such layer. Because of this different wall structure, we expect different refractive indices, with real and imaginary part, which is the physical parameter measured by phase contrast SR$\mu$CT. This characteristic, in principle, should allow to discriminate artery from veins.

A first trial to proof such idea has been done on a tumor sample embedded in PFA and measured with phase contrast SR$\mu$CT. Data were segmented with two thresholds correspondent to the two peaks in the image histogram. The
### 6.2. ARTERIOLES AND VENULES

<table>
<thead>
<tr>
<th>organ</th>
<th>Fractal Dimension</th>
<th>Fractal Dimension</th>
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Table 6.1: Fractal Dimension in some healthy and pathological situations. Data are expressed as mean±SD

Result is showed in fig[6.5]. Vessels depicted in light blue are obtained from the first threshold while the green ones from the second threshold. At the moment we can claim that these vessels are different because of the different refractive index, but we cannot guess which of the two are arterial or venous capillary. A second experiment, planned for next year, aims to measure the refractive index of the abdominal aorta and vena cava. The goal is to use such values for the segmentation and discriminate between arteries and veins. It is important to note, that despite the smaller spatial resolution of the phase contrast techniques compared to the absorption contrast, it is the only one that allows the possibility to visualize the venous tree. As a matter of fact, the injection of the contrast medium through the left heart ventricle during body perfusion, allow to fill only of the arterial tree. When the medium reach the capillaries it stops, because usually its viscosity is to high to flow in the extracellular marix. In case of contrast agent with very low viscosity, or in case of liquid suspension as BaSO₄ nanoparticles, they can leak out of the capillary and partially perfuse the region. Nevertheless, because the nega-
tive pressure of the venous tree is no longer present, the probability for the venous capillaries to drain liquids from the extracellular matrix is very low. The only possibility is to increase the pressure of the injection into the left ventricle in order to win the resistance of the extracellular matrix, but in this case the high pressure will destroy both the arterial and venous capillaries. The bigger vessel will remain intact but all the capillary structure, which is fundamental in angiogenic studies, will be lost.

Figure 6.5: Artery and venous tree of a tumor sample acquired with phase contrast SRμCT. The two colours correspond two a different peaks used for the segmentation of the image.

6.3 Diameter distribution during tumor growth

The diameter of the angiogenic vessel had been evaluated from the Vessel Size Index (VSI) maps obtained from MRI T₂ and T₂* maps, according to

\[1\] the right atrium is opened during perfusion procedure to let the blood flow out of the body.
the procedure described in ch.3.1.4. The value of VSI for each voxel had been calculated from eq.6.3 using the following parameters: homogeneous diffusion coefficient $D = 10^{-9} \text{m}^2\text{s}^{-1}$, giro-magnetic ratio $\gamma = 42.58 \cdot 10^6$ and susceptibility difference $\Delta \chi = 1 \cdot 10^{-7}$. VSI, which is defined as the average diameter of the vessels in a voxel, is particular attractive because it allows to estimate in-vivo the vessel diameters distribution during tumor growth. Such diameters distribution play an important role in the study of angiogenesis because it is an indicator of the type of newly formed vessel: a very small VSI means the formation of capillaries, higher values of VSI imply the formation of bigger vessels.

MRI-VSI acquisitions ($T_2$ and $T^*_2$) have been performed during tumor growth on day 6, 8, 10 and 12 after cells inoculation for a group of 10 mice. Afterwards, VSI maps and the relative histograms have been evaluated according to ch.3.1.4.

Histograms showing the diameter distribution at day 6, 8 and 10 are plotted in Fig.6.6. As first consideration we can observe that the shape of the distribution remains more or less constant during the growth of the tumor. If we look carefully at the values, we see that for each day we can see a predominance of the capillary ($5-20\mu\text{m}$) and very few bigger vessels. The number of such capillaries appreciably increases at day 10, which means the formation of new angiogenic capillaries.

Despite the increase of the capillaries, no arterioles or venules ($20-100\mu\text{m}$) are formed by angiogenic process. Bigger vessel as arteries or veins ($100-200\mu\text{m}$) are not present in any stage of the tumor growth. This results, which is found more or less identical in all the animals, suggests that the network of tumor angiogenic vessel is a basic network composed only of capillaries, the absence of bigger vessels imply that the hierarchical organization in arterioles-capillaries and veins-venules-capillaries is not present at all in the tumor.

Same result has been founded also using ex-vivo SR$\mu$CT for the last stage of the tumor as described in the previous chapter. Combining the two results together we can make some important conclusions. First, angiogenic vessel network is composed mainly by capillaries and does not show a hierarchical organization. Second, the network structure is complex and chaotic. Last, such structure is basically the same during the whole tumor growth.

This kind of vessel architecture, makes the blood flow irregular and its delivery not homogeneous. In particular, the chaotic placement of the capillaries with several shunts cause flow-back and recirculation that does not allow the blood to flow continuously as it should be. These aspects will pro-
Figure 6.6: Longitudinal 3D evaluation of VSI during tumor growth for one mouse: anatomical MR T2-weighted image with tumor delineated in red (first line), VSI map (second line) and VSI histogram distribution (third line). The average diameter is represented on the x-axis while the number of voxel on the y-axis.

duce abnormal physiological effects that makes tumor behaviour completely different from healthy organ. These issues will be described in the next chapters.
Chapter 7

Physiological effects

The results showed in the previous two chapters demonstrates that tumor, first, is not a homogeneous tissue and its metabolic activity is confined in some regions of the tumor. Second, the angiogenic vessel network, triggered by the hypoxic level, is chaotic and composed of capillaries. Such anatomical features have important implications in the physiology of the tumor and in the interaction between tumor itself and the surrounded organs.

Due to the chaotic architecture, blood is not flow uniformly in the vessel and this will produce a non uniform perfusion of the tumor tissue. Moreover, the continuous formation of capillaries prevents their maturation in bigger vessels to form a functional network. As a matter of fact, such network will be quite leaky, and blood will able to flow out of the vessel even before the capillary termination originating in some case micro haemorrhages. Those issues will be widely described in this chapter.

Angiogenesis is also an important issue in the tumor treatment. Because of drugs are delivered to the tumor by the vessel network, its functionality is fundamental for the success of the treatment. If the vessel network does not work properly, the drugs will not reach all the tumor cells. On the other hand, tumor vessels itself constitute a target of the treatment: destroying the vessel it will be impossible for the tumor receive nutrients and therefore tumor cells will die. This is the principle of anto-angiogenic therapy and, several efforts has been made in the last two decades in this direction.

The strategy is complex and it does not aim an immediate destruction of the vessel since would make the delivery of further doses of drugs impossible, allowing the tumor cells to survive in a hypoxic state that will trigger new angiogenesis. Recent approaches propose to follow an opposite direction: administer a drug that stabilize and make functional the vessel network before the administration of the chemotherapeutic drugs. In this framework, we have studied the effects produced by a pro-angiogenic drug during tumor
7.1 Haemodynamic response of tumor vessel

The first physiological effect induced by angiogenesis is the circulation of blood in the tumor that can be estimated by the evaluation of Tumor Blood Volume (TBV). The presence of TBV indicate the presence of new vessels and therefore that the angiogenic process is taking place.

TBV has been evaluated according to the procedure described in ch.3.1.2 by means of Dynamic Contrast Enhancement (DCE) MRI using an iron-oxide nanoparticles based contrast agent (Endorem). Concentration curves (eq.3.18) for a group of ten mice, at day 6 and 10 after tumor inoculation, are depicted in Fig.7.1 where each time point represent the concentration value averaged over the whole tumor. Despite no spatial information is keeping into account in this kind of experiment, comparing the different curves we can observe different uptake levels that means a different haemodynamic for each tumor sample.

Fig.7.2 shows the TBV maps distributions for a single mouse at day 6, 8 and 10 after tumor injection. Different regions with different level of TBV are present, changing day by day. This means high level of heterogeneity common to each stage of the tumor growth.

To quantify such heterogeneity in a simple way, a threshold T, correspondent to 20% of the maximum, has been applied to TBV maps. The perfused and non-perfused volume correspondent to $TBV > T$ and $TBV < T$ respectively, have been evaluated for a group of 10 mice and plotted in Fig.7.3. We can see that the perfused volume constitutes the main part of the tumor but a non-negligible portion of it remains non-perfused during growth. Furthermore, at the last stage the difference was minimum and in many case 50% of the tumor was perfused and the other 50% not. A 3D reconstruction of perfused and non-perfused volumes for a single mouse, during tumor development, is shown in Fig.7.4.

To better quantify the heterogeneity level, fractal dimension which is an indicator of the geometrical order/disorder has been computed over the TBV maps. The average results for a group of 10 mice, at day 6, 8, 10 and 12, are showed in Fig.7.5. From the curve we can individuate two aspects: first, there is a decrease of fractal dimension at day 8 and 10, while the value at day 6 and 12 is more or less similar. This means that the order, or in other words
Figure 7.1: T2 weighted DCE-MRI using iron-oxyde nanoparticles based contrast agent (Endorem) at day 6 (top) and day 10 (bottom) after tumor inoculation. Concentration curves are derived from the uptake curves according to eq. 3.18. Data refer to a group of 10 mice, 2 of them died during the experiment.

the level of heterogeneity, at the early and last stage is similar but something happened in between. From a geometrical point of view this means that the reciprocal positions and dimensions of the regions changed during tumor development. From a physiological point of view, such rearrangements indicate changes in the positions and the volumes of perfused and non-perfused regions. Such changes are induced by the angiogenic process to satisfy the need of oxygen and nutrients of the tumor.

The second aspect we can observe is the small standard deviation at each time point. This means that, despite the huge biological variability common to these kind of experiments, fractal dimension is a good indicator of the level of TBV heterogeneity during tumor growth.
Figure 7.2: TBV maps distributions for a single mouse at days 6, 8 and 10 after tumor injection.

Figure 7.3: Average value and standard deviation of TBV at days 6, 8, 10 and 12 after tumor injection (top). Perfused and non-perfused volume expressed as % of the total volume (bottom). Data relates to a sample of 10 mice.

The haemodynamic of the vascular network is tightly correlated with its geometrical structure. We have seen from ch. 6.3 that the angiogenic network
is composed mainly by capillaries and does not show a hierarchical organization. Moreover such vessels are quite leaky and haemorrhagic. Nevertheless, we have observed that the region of the tumor with high density of capillaries (according to VSI results) show an increment of the TBV compared to the other regions. On one hand this result is reasonable since we expect an increase of the blood volume in conjunction with the number of the vessel. On the other hand we cannot distinguish between the blood flowing in the vessel (real blood volume) from the one leaking out of the vessels due to their high permeability as described in the following chapter. Placing all these aspect together we can conclude that the haemodynamic response of the tumor, due to the need of oxygen and nutrients of its tissue, is not homogeneous and not efficient.

7.2 Permeability of the vessel

The second physiological effects of the angiogenic process is to allow the blood to extravasate from the capillaries and perfuse the tissue. Such properties, called vessel permeability, is common to all kind of vessel systems but the type of molecules able to leave the blood stream and their quantity vary from organ to organ. The study of the vessel permeability in tumor plays an important role because it is an indicator of the functionality of the vessel. As described in ch.6 and tumor vessels differ from healthy ones because they are often leaky
and haemorrhagic. This lack of functionality is translated in a non uniform delivery of blood to the tissue and therefore in a non-homogeneous blood supply. Such aspect is well explained by Fig. 7.6 in which the permeability map for a single tumor is showed.

We can see that three distinct regions show three different curves correspondent to the Dynamic Contrast Enhancement \( DCE \) of the MR signal during the administration of Gd-Dotarem as contrast agent. The first curve shows a steep slope followed by a plateau which means a fast extravasation of the contrast agent and a rapid accumulation in the tissue or, equivalently, that the vessel in that regions are highly permeable. The other two curves show a constant smoother slope without plateau. This behaviour correspond to a slow but constant extravasation of the contrast agent into the tissue and therefore a smaller permeability.

As described in 3.1.3 the steepness of the slope correspond to the transfer constant \( k_{\text{trans}} \) between the plasma and the extracellular compartment, and
7.2. PERMEABILITY OF THE VESSEL

The plateau level to the extravasated volume $v_e$.

Figure 7.6: DCE-MRI and signal enhancement curve for three regions with different permeability value.

The values of $k_{trans}$ and $v_e$ have been evaluated for a group of 10 mice at day 6, 8, 10 and 12 after the tumor inoculation following the protocol described in ch.3.5.2 and depicted in Fig.7.7. The $k_{trans}$ and $v_e$ results are plotted in Fig.7.8. We initially observe an increase of $k_{trans}$ at day 8, while the value at day 6 and 12 is similar. An increase of the permeability in tumor environment is compatible with the rearrangements of the vessel during angiogenic process. In fact, newly formed vessel are not mature and the stabilization in a structured network takes time, in case of tumor we can say that at such stage the vessel are more leaky than permeable. During tumor development, there is time for the stabilization of the vessel and this explain the decrease of the permeability at day 12.

Such interpretation is also confirmed by the trend of $v_e$. The extravasated volume shows a peak at day 8, the same day of the maximum $k_{trans}$, which means that a huge amount of contrast agent extravasates through the vessel into the tissue. In other word, at this stage, vessel are very leaky, maybe even haemorrhagic, and a lot of blood can drizzle the tissue.

As described before, tumor presents areas with different level of permeability and extravasated volume. Such aspect plays an important role in the formation of the tumor itself. A non uniform distribution of the blood
Figure 7.7: T1 weighted DCE-MRI using Gd-based contrast agent (Gd-Dotarem) at day 6 (top) and day 12 (bottom) after tumor inoculation. Signal enhancement curves are derived from the uptake curves according to eq.3.23. Data refer to a group of 10 mice, 2 of them died during the experiment.

correspond to a non uniform distribution of nutrients for the cells. While in the healthy organs this is a physiological situation in which different part of the organ needs different amount of nutrients, in tumor such behaviour does not reflect any physiological need. In any case, tumor will growth where nutrients will be available, therefore where ther is the presence of blood.

The quantification of the distribution of the blood patterns had been performed evaluating the lacunarity over the extravasated maps. In Fig7.5 are plotted the average lacunarity values and their standard deviations for a group of 10 mice, at day 6, 8, 10 and 12 after tumor injection. In this case, lacunarity describes the gap between the distribution of the extravasated volume patterns, the higher the lacunarity, the higher the variability of its gaps and therefore the higher the heterogeneity of blood distribution. From the
7.2. PERMEABILITY OF THE VESSEL

Figure 7.8: Average value and standard deviation of transfer constant $k_{\text{trans}}$ (top) and extravasated volume $v_e$ (bottom) at days 6,8,10 and 12 after tumor injection. Data relates to a sample of 10 mice.

plot we observe that the level of lacunarity is smaller at day 12 compared to day 6. We also observe a local minimum and a local maximum at day 8 and 12 respectively, but considering the non-negligible standard deviation we cannot do any supposition about them. The decrease of lacunarity during tumor development implies a loss in the heterogeneity, i.e. at the extravasated volume distribution becomes more homogeneous. This result can be explained again considering the maturation of the vessel network during tumor development. At the last stage the vessel network will be more stable than at the early stage ensuring a more homogeneous delivery of blood. We also observed that such stabilization does not cover the entire tumor volume but it is confined only in some regions of it. We also observe a local minimum and a local maximum at day 8 and 12 respectively, but considering the non-negligible standard deviation we cannot do any supposition about them.

Comparing the results of permeability with the ones from TBV we can
observed a strong correlation between them. In other words, most of the regions which show high level of TBV, show also high values of $k_{\text{trans}}$ and $v_e$. This means that if there is an increase of the demand of blood by tumor tissue, this is accomplished by the increase of blood volume. Nevertheless, we have to observe that the circulation of such blood is highly inefficient because of the high permeability ($k_{\text{trans}}$) of the vascular network that allows the blood to leak out quite easily. This is supported but the high value of $v_e$ which is a measure of the quantity of blood extravasated through the vessels to the tissue.

7.3 Pro-angiogenic drug effects

The last two chapters have demonstrated the abnormal and inefficient structure of the angiogenic vessels. A new concept in tumor treatment propose first the administration of drugs that stabilizes the vessel network in order to make it more functional and afterwards the administration of chemotherapy. The aim of this part of the work to evaluate the effects of a pro-angiogenic drug, during tumor growth.

Two groups of ten mice were used in this experiment: the first one was treated with DMOG, a drug that inhibiting prolylhydroxylase domain proteins which leads to the induction of hypoxia signalling through the stabilization of hypoxia-inducible factor 1α HIF-1α [78] and, therefore, it is supposed to promote and stabilize newly formed vessel. The second group was treated with NaCl solution as a placebo. Experiments to assess the haemodynamics and vascular permeability have been performed at day 6 and 12 after the tumor inoculation following the protocol described in ch. 3.5.2.

All the animals developed a detectable tumor within approximately five days following the inoculation. Typically, the termination criterion for the experiment (tumor volume $2 \text{ cm}^3$) was reached within about three weeks. Tumors in both the DMOG and NaCl group showed a very similar shape: Tumors developed into compact oblate shapes embedded between skin and muscle. Volume and compactness measurements showed no significant difference between the two groups either at day 6 or day 12 as reported in Tab. 7.1.

DMOG and NaCl treatments did not lead to a reduction in tumor volume or tumor growth rate compared to the longitudinal study results. Similar values of volumes and compactness during tumor growth for both the DMOG and NaCl treated group indicating that the drug did not affect overall tumor growth. The shape of the tumor appears strongly influenced by biomechan-
7.3. PRO-ANGIOGENIC DRUG EFFECTS

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Table 7.1: Volume and compactness values at day 6 and 12 for DMOG and NaCl treated group. Compactness values have been evaluated according to eq.4.4.

Physical constraints of the implantation site: the tumor cells are enclosed in a compartment between the skin and muscle responsible for preferential growth in two dimensions leading to an oblate tumor shape.

Standard histogram analysis has been performed by evaluating the 1st (mean) and the 2nd (standard deviation) moment of the TBV distribution of the tumor at day 6 and day 12. There was no statistically significant difference between DMOG and NaCl treated groups at any test phase as shown in Fig. 7.9 and Tab. 7.2. In fact, mean TBV values seem to remain constant for each group between days 6 and 12 despite significant tumor growth and we might conclude that DMOG did not affect tumor vasculature.

![Figure 7.9: Average TBV box-plot at day 6 and at day 12 of DMOG and NaCl groups. The two groups are not significant different at day 12 (p >> 5%).](image-url)
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<td>Lacunarity</td>
<td>6.81±0.37</td>
<td>3.22±1.06</td>
<td>yes</td>
</tr>
</tbody>
</table>

Table 7.2: Results from standard histogram analysis (average TBV) and from texture analysis (Fractal Dimension and Lacunarity). As we expected, at day 6 the two groups are not significant different. On day 12, where we should find the effect of DMOG drug, standard histogram analysis is not able to discriminate between the two groups, while texture analysis reveals a significant difference.

In contrast, texture analysis showed different results. The evaluation of lacunarity showed difference in the spatial distribution of the perfused regions in DMOG treated tumor compared to the NaCl group as depicted in Fig.7.11 and Tab.7.2. At day 6 (prior to drug treatment) we did not observe any difference in lacunarity values between the two treatment groups. Interestingly there was a significant decrease of the lacunarity values in the NaCl group between day 6 and 12, while the value remained unchanged in the DMOG treated group. Based on the definition of lacunarity this indicates a reorganization of the vasculature in the NaCl treated group: the originally disperse perfusion pattern seems to collapse to a few highly vascularized regions as the tumors grow. In the DMOG, tumor vascularization pattern remains more homogeneous even for large tumors. This situation is well reflected by Fig. 7.10 showing a heterogenous distribution of perfused regions in DMOG treated animals as compared to essentially one large non-perfused regions in NaCl treated mouse.

Similarly, the evaluation of the fractal dimension of the TBV map revealed significant differences between the two groups (Fig.7.12). Again, the fractal dimension of the TBV maps prior to treatment (at day 6) was identical. Following one week treatment with DMOG a significant increase of the fractal dimension was observed, which was not the case for the NaCl group, for which the values remained on the pretreatment level. Hence pattern analysis revealed significant effect of DMOG treatment both with regard to lacunarity and fractal dimension: While in the first case DMOG inhibited
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Figure 7.10: TBV maps at day 12 of a DMOG (left) and NaCl (right) treated mouse.

A decrease in lacunarity values upon tumor growth, its led to a significant increase in fractal dimension.

Figure 7.11: Lacunarity box-plot at day 6 and at day 12 of DMOG and NaCl groups. The two groups are significant different at day 12 ($p << 5\%$).

Texture analysis provides a quantitative readout of the qualitative finding. Lacunarity and fractal dimension analysis captures statistically significant differences between the two treatment groups after 6 days of treatment, while the two readouts were found to be identical for the two groups at baseline (day 6 following inoculation). In NaCl group the lacunarity decreased over time, indicating of the fusion of many small unperfused and perfused tumor regions into few large ones. This situation is characteristic for developing solid tumors, with large and increasing domains becoming necrotic.
Figure 7.12: Fractal dimension box-plot at day 6 and at day 12 of DMOG and NaCl groups. The two groups are significant different at day 12 ($p << 5\%$) with confined tumor regions still exhibiting proliferating growth. In DMOG treated animals we did not observe a change in lacunarity during the treatment period. This may be interpreted by a vessel stabilizing effects of the drug: by improving vessel function may prevent the formation of large hypoxic and ultimately necrotic domains. This interpretation is supported by a recent study. It has been shown that decreased PHD levels in host endothelial cells did not affect growth and vessel density in tumor xenografts but led to improved tumor vessel maturation likely linked to improved tumor perfusion and oxygenation (Mazzone et al). Since systemically administered DMOG also affects PHD in host vessels, this mechanism might also account for the maintenance of the vascular structure in DMOG treated animals in our study and explain The post-treatment finding of the formation of several small perfused islets surrounded by non-perfused small regions, which translates into a higher lacunarity value as compared to placebo group.

Combining the information from the analysis of lacunarity and fractal dimension we may conclude that DMOG promoted the formation of new vessels and stabilized the existed ones. Despite high chaotic nature of this neovasculature, it appears that blood supply is sufficient to warrant small perfused region throughout the tumor.
Chapter 8

Aspects of tumor-host tissue interface

Solid tumors are not simply clones of cancer cells which form a foreign body but they can be considered as abnormal organ composed of multiple cell types and extracellular matrix. Tumors have to interact with the host tissue to growth and immuno-system and has to contrast its expansion. Most of these interaction take place at the interface between tumor an host tissue. With the term ”tumor-host interface”, we refer to the portion of tissue that it is not clearly a part of the tumor mass but neither part of the muscle. This region, usually well visualized with magnetic resonance imaging and visible during tumor excision, present several features that will be described in this chapter.

From the angiogenic point of view, the interface plays an extremely important role: the newly vessel crossing it are the links between the tumor and body, in such vessels the nutrients are delivered to the tumor and the growing or anti-growing factors present in this regions determines if the angiogenic process will going on or not.

The interface micro-environment is also fundamental for the expression of tumor phenotype: it will determine if the tumor will be aggressive or not and if it will infiltrate the surrounded tissue or will growth in a compact shape.

Metastatic process is also tightly related with the interface structure. In fact, if a tumor cell has the possibility to leave the tumor mass to metastasize elsewhere, it has to follow the blood stream or it has to be drained by the lymphatic system. Both lymphatic and venous capillaries are located at such interface.

In the next sections we are presenting the results of the experiments performed to study the tumor-host tissue interface achieved either with in-vivo
CHAPTER 8. ASPECTS OF TUMOR-HOST TISSUE INTERFACE

or *ex-vivo* techniques.

### 8.1 In-vivo results

The two phenotype that indicates the aggressiveness of a tumor are the capabilities to infiltrate host tissue and the possibility to metastasise in other organs of tissues [15]. The infiltration potential can be macroscopically measured by the shape of the tumor. A ramified shape, for example, means that the tumor is proliferating in several directions and is infiltrating the host tissue. Usually this kind of tumor has a bad prognosis By the contrary, a rounded shape implies that the growing is homogeneous and confined, the tumor cells do not show ability to infiltrate the tissue and, usually, they are associated with good prognosis.

The value of compactness $C_{\text{norm}}$, during tumor growth is shown in Fig. 8.1. As we can see the value is always around 0.9, quite close to the sphere value which is 0. This results that the shape of this kind of sub-cutaneous tumor is almost rounded and that it is not infiltrating.

![Figure 8.1: Average value and its standard deviation of normalized compactness $C_{\text{norm}}$. at day 6, 8, 10 and 12 after tumor inoculation. Data are referred to a sample of 10 animals.](image)

The metastatic process, i.e. the possibility for a tumor to growth in other tissues far from the original place, is a complex process not fully understood. One of the known model is that a cell has to leave the tumor, cross the interface and reach the other organ. To accomplish this task there are only two possibility [145]: follow the blood stream or the lymph draind by the
lymphatic system. The first way is extremely hard to study *in-vivo* with conventional imaging techniques. The second one, instead, is well documented by T1-weighted MRI showed in Fig. 8.2. In this series of high-resolution images we can clearly see the tumor on left, the lymph-node on the right and the lymphatic vessel in between. Because we injected Gd-Dotarem, which is an extracellular contrast agent able to extravasate through the vessels in the tumor, we can assume that it has been drained by the lymphatic vessel and carried to the lymph-node as demonstrated by the contrast enhancement in the pictures. This means that there is a direct connection between tumor and lymph-node. Moreover, because such lymph-node present abnormal dimension, much larger than normal, we can assume that tumor cells has metastasised the first lymph-node.

![Tumor, Connection, Lymph Node](image)

**Figure 8.2:** Stack of T1-weighted hres-MRI images after the administration of Gd-Dotarem as contrast agent. Tumor mass is visible on the left side of the images, lymph-node on the left and the lymphatic vessel, which connects them, in the middle.

As said before, the metastatic invasion that follow the blood stream pathway is not easy to demonstrates *in-vivo* because the vessel connection between tumor and metastases is not direct but follow the whole circulatory system. Despite that, it is possible to study the permeability of the vessel in the interface. If this region presents permeable vessel, the probability for a cell on the tumor surface to leave the mass and 'jump' in a venous capillary will be higher than the case of non-permeable vessel.

DCE-MRI images for a group of 10 animals at different stage during tumor growth to evaluate the permeability of the interface region. A typical situation founded in most of the tumor examined is shown in Fig. 8.3 where the signal enhancement curves of the interface and tumor regions are plotted. Tumor presents a larger value of transfer constant $k_{\text{trans}}$, because of the higher steepness, but a smaller value of extravasated volume $v_e$ compared to the interface region. This means that in tumor a smaller amount of
blood extravasates rapidly, while in the interface a consistent volume of blood extravasates continuously. The higher amount of exchanged blood between capillary and extracellular space in the interface regions increases the probability that a tumor cell follow blood flow to metastasise.

Figure 8.3: T1-weighted DCE-MRI (Cd-Dotarem). Anatomical image (left) showing the tumor and the interface delineated with red and blue respectively.

8.2 Ex-vivo results

At a cellular level, the interface between tumor and the surrounded tissue consists of a mixture of extracellular matrix molecules, tumor cells, endothelial cells, fibroblasts and immuno cells. The extracellular matrix which produce promoters, inhibitors factors and endothelial cells are key-points in the angiogenic process as described in App[A]. Study of these molecular interactions were beyond the scope of the thesis, we therefore focused only on the aspects related to the formation of new vessels in the interface regions. Tumor with surrounded tissue sample explanted at the last stage of tumor development (day 12) have been stained with different procedure to investigate the cells type, their perfusion and the hypoxia distribution. Fig[8.4] shows a section of tumor and muscle tissue stained with hematoxylin and cosin (HE). The left picture clearly shows the tumor cells (brown dots) on the left side and the muscle fibres (red stripes) on the right. In the middle there is a region in which both tumor cells and muscle fibres coexists. This is the tumor-host tissue interface produced by the infiltration of the tumor cells in the muscle. The right picture of Fig[8.4] shows a magnification of this
8.2. EX-VIVO RESULTS

infiltration where a branch of tumor cells is expanding in the muscle. In such branch to vessels, the two browner rounded structures made of endothelial cells, are also visible and constitute a connection between the circulatory systems of the muscle and of the tumor. In this case the tumor is really aggressive and the colonization of the muscle implies its loss of functionality.

Figure 8.4: Section of tumor samples stained with hematoxylin-eosin (HE) showing the tumor-muscle interface delineated in blue(left). A tumor infiltration with two vessel (right)

The presence of endothelial cells has also been investigate with CD31 staining together with Hoechst and Pimonidazole stains to mark hypoxic and perfused cells respectively. In first picture of Fig.8.5 it is visible a section of tumor (top) with surrounded tissue (bottom), in this case fat tissue that recognizable by the shape of the structures. We can see a small amount of endothelial cells (red stripes) that means a primitive vessel network and an hypoxic environment (green) demonstrated by CD31 and Pimonidazole stains respectively. Perfused cells (blue) are also present in the tumor and in the fat near the endothelial cells even if a vessel network is not present.

The second picture of Fig.8.5 show a rounded structure founded in a tumor sample surrounded by a consistent layer of fat. Because of the lack of endothelial cells (red) meaning the absence of any kind of vessel, supported by a uniform hypoxic condition (green) and small amount of perfused cell (blue), we presume this is a colony of tumor cell in the dormant state in which the angiogenic switch is off. This colony, in principle, can be a metastases produced by one or more tumor cells detached from the surface of the tumor.
Figure 8.5: Portion of a section containing tumor and surrounded fat (left). Possible metastases founded in the interface region (right). Both have been stained with CD31 (red, endothelial cells, Pimonidazole (green, hypoxic cells) and Hoechst (blue, perfused cells)
Chapter 9
Conclusions and Outlooks

The present thesis aimed to apply the concept of fractal physiology together with a multi-modality image approach to study the tumor angiogenesis in mice model. The first part of the work was devoted to develop the experimental setup to perform experiments in different modalities, the second part was focused to establish a framework to perform the analysis of the acquired images which takes into account the fractal physiology concepts. The results, already presented and discussed in the previous chapters, are here summarized and discuss with a global view of the outcomes.

Fractal physiology and tumor angiogenesis

Fractal physiology is related in different ways to tumor angiogenesis. First fractal geometry can be applied to determine chaotic structure of the angiogenic vessel network. The biological background behind is the abnormal and non uniform distribution of the tumor mass that need different quantity of nutrients in different regions as demonstrated by F-MISO PET and FDG-PET which measure the oxygen and glucose metabolism respectively [79]. It also reasonable to assume that the presence of vessels implies the presence of nutrients that constitutes a positive feedback for the growing process, in other word the presence of nutrients stimulate the cell replication but the increased number of cells determines a larger need of nutrients and therefore stimulate the vessel growth. The fast vessel growth, due to the positive feedback, has two implications: on one side it prevents the formation of bigger vessels, on the other side it prevents the maturation of the vessel structure keeping them leaky and sometimes haemorrhagic.
From a geometrical point of view all these aspects can be quantified pattern
analysis. In particular, with fractal dimension it is possible to quantify the complexity of the vessel network, otherwise impossible to evaluate. Moreover, the tumor blood volume distribution which indicates the quantity of blood flowing in the vessels, can be spatially measured by means of lacunarity. It is important to stress the fact that conventional methods, as histogram analysis, are not able to provide any information on the spatial distribution but only average values over the whole volume [120] [151].

Secondly, fractal physiology can interpret the break of homeostasis in tumor. As defined by Cannon [21] and reported in ch.2.2.4, homeostasis is the property of a system that regulates its internal environment and tends to maintain stable conditions. Although homeostasis does not guarantees the perfect stability also in the healthy organs as demonstrated by West [150], it seems to be completely absent in tumors. The non-homogeneous distribution of several physiological parameters as oxygen concentration, glucose concentration and blood volume distribution support this statement. Longitudinal in-vivo studies reported in ch.7 demonstrates that the tumor does not growth in a homeostatic manner, in fact it does not try to maintain stable and homogeneous conditions throughout the whole tumor tissue. By the contrary it develops different regions with with different phenotype: some part of the tumor can be more vascularized, consequently with high replication ratio and therefore more aggressive for the host tissue, other regions are not vascularized and therefore in an hypoxic, or even in a necrotic state.

The third aspect, directly connected to the break of the homeostasis, is the dynamic rearrangements of the different regions of the tumor. In other word, because of the interactions between environmental conditions and tumor physiological effects, the angiogenic network and tumor tissue continuously adapt their shapes and function but without defined and constant rules. This apparent chaotic behaviour, that is not random, is the results of the combination of all the non linear dynamic sub-processes that work together to promote angiogenesis and tumor growth.

Even more important are the consequences in the modelling of the drug pharmacokinetics or in the tumor regression model after radiation treatment. It is well know that tumor cells reacts in a different way to the drug or radiation depending of the local conditions [15]. High level of oxygen concentration during radiation treatment, for example, has been shown to increase the number of un-repairable damage of cell DNA favouring the death of oxygenated cells [127]. Because, as demonstrated in ch.5.1 the oxygen distribution is not uniform, the response of the tumor will not be uniform:
oxygenated regions will regress because of the radiation but the hypoxic regions will not respond to the treatment.

Similar consideration can be made about the pharmacokinetics of the drug, and in particular for anti-angiogenic treatments that have the tumor vascular system as target in order to prevent the nutrients supply \[84\]. The idea is to proceed in two steps, first to reinforce the vessel network and then to deliver the treatment. This original strategy is based on a simple consideration that, if the vessels are destroyed, there is no possibility to delivery further drugs and the survived cells can fall again in an hypoxic state and restart the angiogenic process from the beginning. Furthermore, the absence of vessel makes impossible the delivery of contrast agents for in-vivo MRI or CT acquisition in order to monitor the tumor regression. This is still an open problem in anti-angiogenic therapy that could be partially overcome considering the spatial distribution of the vessel network, or in other word the distribution of the tumor regions perfused by blood. As we have seen in this work, these parameters can be quantified in terms of lacunarity and fractal dimension assessing on one side the regression of the tumor and therefore the efficacy of the treatment, on the other side the kinetic distribution of the anti-angiogenic drug.

The last thing to consider is the metastatic potential of the tumor which is directly connected to the angiogenic vessel network \[157\]. There are two possibility for a cell, or group of cells, to leave the tumor mass: follow the blood stream or the lymphatic system as showed in ch.8.1. In this regards, the interface between tumor and host-tissue plays a fundamental role. In fact, the interaction between the tumor vascular network and the one of the surrounded tissue determines the probability of metastatization. Such interaction can be modelled in the context of fractal geometry between the two tissues.

The quantification of metastatic potential is determinant in tumor prognosis because metastasis are the real cause of death of cancer \[15\]. Usually, the primary tumor can be treated by surgery or local therapy and does not constitutes a severe risk. Unfortunately the dissemination of metastasis through the whole body is a fast and exponential process in most cases impossible to control. Because it is unfeasible to treat individually each of the tens possible metastasis when detected, systemic approaches as chemotherapy are needed. All these considerations are evidences that the tumor must be considered as a part of the human body rather than an foreign entity. In fact, as the healthy tissue, tumor associates itself to the vascular systems, develops a vessel network, rearranges dynamically its structure, and uses the vessel system
to interact long-term and long-distance with the rest of the body\cite{152}. The peculiar thing is that the tumor makes all this actions differently and fractal physiology is a unique strategy to interpret these differences.

**Pattern analysis and tumor angiogenesis**

The heterogeneity and the intrinsic biological variability of tumor tissues make the use of tools as pattern analysis necessary. The analysis of the shape and the texture of the tumors allows the quantification of the spatial distribution of physiological parameters that is otherwise impossible to evaluate as demonstrated in the pro-angiogenic study described in ch.\textsuperscript{7.3}. The possibility to evaluate the complexity of a texture, that means assign a number to the spatial distribution of specific parameter, is probably the main advantage of this approach. Its applications in research and clinics are manifold. One of the most important is the possibility to **monitor individually the efficacy of the treatment**. Usually, in clinics, radiologists and oncologists determine if the treatment is succeeding by measuring the tumor regression or average values throughout the entire volume. As we have seen, tumor shows quite heterogeneity in all of its phenotypes and this averaging suppresses all the local effects. Unfortunately these local effects will determine the prognosis: if the treatment is not able to operate uniformly and destroy all the tumor tissue, local colonies of cells may survive and the probability of tumor recurrence becomes extremely high. The analysis of the texture from CT and MRI images by means of the evaluation of fractal dimension and lacunarity allows to account for spatial heterogeneity of tumor treatment response and, consequently, to optimize the continuation of the treatment individually for each patient.

In this context, standard treatment indicators as perfusion, permeability, hypoxia, and so on, that are already considered as prognostic factors in tumor regression, may become more reliable predictors. The spatially resolved assessment of therapy effects allows on one side to optimize the treatment strategy and on the other side to study the interaction with the surrounded tissue which is crucial to determine the malignant potential of the tumor.

Textures estimators as measure of complexity has also an application in the development of tumor modelling. Several kind of models have been developed and proposed in literature\cite{32,27} aiming to model different phases of the tumor development. What we can conclude from reviewing these models, is the absolutely necessity to consider the heterogeneity and the spatial distribution of tumor tissue on one hand, and the interaction between tumor
and healthy tissue on the other one. In this regard, the evaluation of the fractal dimension of the two vascular networks, the one relative to tumor and the one relative to host tissue, can help to model and quantify the interaction between them. Same consideration can be done for the quantification of the texture for tumor and host-tissue.

The analysis of the **tumor shapes** is an important issue in tumor detection and classification. During diagnostic examination, the difference in shape is one of the factors that determine if a neoplastic mass is benign or malignant, with drastic implications regarding the prognosis and the treatment. Rounded shapes (Fig.4.1) are usually associated with benign lesions while ramified ones (Fig.4.2) are associated with malignant lesions due to the infiltrations of the tumor branches in the healthy tissue. For example, the investigation of the tumor shape plays an important role in the breast cancer [119] [109] and melanoma [89] [23] detection. In the first case it allows to distinguish between neoplastic mass and benign micro-calcification, in the second one between melanoma and skin blemish.

**Outlook**

Multimodality approach, together with fractal physiology concepts have been shown to be complementary tools for the analysis of tumor evolution. Although not all the methods described in this work are suitable for in-vivo imaging, the possibility to measure physiological parameters during tumor growth and some specific anatomical or histological details post-mortem with very high resolution, makes this approach ideal for studying tumor angiogenesis, and also gives valuable insight in the evolution of the tumor growth in general.

One of the great advantages of this research framework is the possibility to implement it into the existing clinic environment. MRI (though not all the contrast agents used in this work are approved for clinical use) and PET are established techniques used daily in the hospitals as well as the histological examinations performed after tumor surgery. SRCT is still a research method but significant progresses have done in the last years and a increasing use of this technique for medical applications is envisaged in the near future. Moreover, the implementation of pattern analysis to evaluate shapes and texture of tumor images is a straightforward process based on software tools which can be easily added to workstations that already
performed image analysis in hospital medical imaging department. The outcomes of the implementation of this method can be described, in temporal sequence, as follows: first they could become a tool to study the angiogenesis evolution directly on patients without passing through a semi-physiological animal model. Second, and probably most importantly, they should allow monitoring and quantifying the efficacy of the treatment based on subsequent screenings using imaging biomarkers. Third, with the increasing patient population undertaking this screening, an analysis of subject specifies response to the therapies (chemo and radiation) can be performed, i.e. the approach may be used to stratify patients groups.

The further important aspects concerns the **cost of the clinical implementation**. As already mentioned, the image techniques are well established in hospitals and allow to achieve today data set with sufficient spatial and temporal resolution to perform the analysis. Certainly, improvements of the design of detectors can be done to increase the sensitivity of each method but no radical changes are necessary. Yet, multimodal MRI and PET in combination with advanced data analysis does not need a novel hardware design and therefore does not add major costs. The implementation of the software to perform the analysis need some considerations. First, a more structured and user friendly code needs to be developed for a daily use in clinic-research environment. Furthermore, if this methodology will have the potentiality to become a standard medical procedure, complex procedure of certification according to national and international protocols will be required. This process would need the contribution from medical, technical, and legal experts.

How to combine all the information from different image modalities is not an easy task. Although several kinds of algorithms allow the co-registration of 3D data set, either with rigid or elastic transformations, the fusion between images of different origin without anatomical markers is still an open issue. For in-vivo studies, this problem can be solved using the same patient positioning systems in the different modalities to guarantee a good reproducibility. Instead, specific novel techniques has to be developed to co-register ex-vivo with in-vivo data. After the explantation, in fact, tumor change its shape because it does not experience the mechanical constraints of the surrounded tissues; also all the manipulations contribute to these changes. The implementation of **robust co-registration methods** between in-vivo and post-mortem techniques either in research or clinic routine, is still an area which needs further thorough investigation.

Looking from a broad prospective, this methodology, together with new findings in the molecular biology, shows the potentialities to contribute to a
patient specific cure based on *personalized treatment* adapted to the regression of the tumor. This kind of approach has a twofold benefits: maximize the effects of the treatment and reduce the side effects. Significant contributions from research in cancer stem cells and system medicine should help in this direction.

Last but not the least, this work is an example that research in oncology could profit extensively from the interaction, and the *sharing of the knowledge* of experts from different and complementary fields, working for a common task.
Tumor biology

Tumor is the result of a series of specific molecular events that alter the normal properties of tissue cells. Although cancer comprises at least 100 different types of diseases, all cancer cells share one important feature: the processes which regulate the normal cell division are disrupted and produce a limitless cell replication. Such abnormal function arises from inherited mutation or it is induced by environmental factors as UV or X-ray radiation, toxic chemicals, viruses, pollution, etc... Several studies suggest that most cancers are not the result of one single event or factor, rather, four to seven events are usually required for a normal cell to evolve into malignant tumor [15].

Several lines of evidence indicate that tumorigenesis in humans is a multistep process and that these steps reflect genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives [57]. This development proceeds via process formally analogous to Darwinian evolution, in which a succession of genetic changes, each conferring a specific type of growth advantage, leads to the progressive conversion of normal cells into cancer cells.

The steps involved in carcinogenesis can be divided into three distinct stages: initiation, promotion and progression [85]. Initiation constitutes an irreversible genetic change, usually a mutation in one or more genes. Promotion is generally associated with an increased proliferation of initiated cells increasing their population. Progression is the accumulation of more genetic mutations that lead to the acquisition of the malignant or invasive phenotype.

In this appendix we present the different aspects of the tumor growth in order to have a comprehensive picture of the whole process. The first section briefly gives a basic classification of tumor types. In the subsequent section we delineate the basic genetic mechanism that lead to cancer. In the third
section we described the biological and physiological expressions of these genetic transformations showing the different steps of tumor growth. Finally, parallel to these widely accepted interpretation of cancer development, an innovative theory involving cancer stem cell is presented.

A.1 Tumor classification

As mentioned before, tumor growths in a series of steps. From a cellular point of view four different steps can be individuated. The first step, called **hyperplasia** consists of an uncontrolled cell division originating the formation a colony with too many cells. These cells appear normal, but they have lost any control of growth. The second step is **dysplasia** resulting from further growth, accompanied by abnormal changes to the cells. The third step requires additional genetic changes that give the cells the ability to spread over a wider area of tissue. The cell loose their original function and form an **anaplastic** mass. At this stage, because of the tumor is still confined within its original tissue, it is non invasive and it is not consider malignant. The last step occurs when the tumor cells **metastasize**. This means that tumor cells have acquired the ability to invade the surrounding tissues, including bloodstream and lymphatic system, and spread in other locations. At this point the tumor is considered invasive and therefore malignant.

The classifications of tumor is done according to the cell type that was originally altered. Commonly, five types of tumor are classified:

- **Carcinomas** result from alteration of epithelial cells which cover the surface of skin and the surface of internal organs. Most of the tumor are carcinomas. Some examples are: breast carcinoma, prostate carcinoma, lung carcinoma and ovarian carcinoma, ...

- **Sarcomas** result from changes in muscle, bone, fat, or connective tissue.

- **Leukemia** results from malignant white blood cells.

- **Lymphoma** is a tumor of the lymphatic system cells that derive from bone marrow.

- **Myelomas** are tumors of specialized white blood cells that makes antibodies.
Only a small number of the approximately 35,000 genes in the human genome have been associated with cancer. Alterations in the same gene often are associated with different forms of cancer. These malfunctioning genes can be broadly classified into three groups [37]:

- **Proto-oncogenes** produce proteins that normally enhance cell division or inhibit normal cell death. The mutated form of these genes are called **oncogenes**.

- **Tumor-suppressors genes** make proteins that normally prevent cell division or cause cell death.

- **DNA-repair genes** help to repair mutations that lead to cancer

Mutations that produce oncogenes accelerate the growth, while those that affect tumor-suppressor prevent the normal inhibition of the growth. In either case, uncontrolled cell growth occurs.

### A.2.1 Oncogenes

In normal cells, proto-oncogenes code for proteins that send signal to the nucleus to stimulate cell division. The pathway includes a membrane receptor for the signal molecule, intermediary proteins that carry the signal through the cytoplasm, and transcription factors in the nucleus that activate the genes for cell division as depicted in Fig. [A.1] for the RAS pathway. Oncogenes are altered version of such proto-oncogenes that code for these signaling molecules. The oncogenes activate the signalling cascade continuously, resulting in an increased production of factors that stimulates growth.

Two proto-oncogenes that play an important role in cancer development are **MYC** and **RAS**.

**MYC** is a proto-oncogenes which codes for a transcription factor. Mutation in **MYC** convert it into an oncogenes associated with 70% of cancers. **RAS** normally function as "on-off" switch in the signal cascade. Mutations in **RAS** cause the signal pathway to remain "on", leading to uncontrolled cell growth. About 30% of tumors, including lung, colon, thyroid and pancreatic carcinoma, have a mutation in **RAS**.

One copy of an oncogene is sufficient to cause an alteration in the cell growth. The presence of an oncogene in a germ line cell (egg or sperm) results in an

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[37] This section is adapted from *Cell Biology and Cancer* [37]
Figure A.1: Signal transduction pathway for RAS proto-oncogenes. A signal binds to tyrosine kinase receptors on the cell surface. This activates the membrane protein which activates proteins, such as kinases, in the cytoplasm. Several other proteins may be involved in the cascade, ultimately activating one or more transcription factors. The activated transcription factors enter the nucleus where stimulate the expression of the correspondent genes, in this case the cell division.

inherited predisposition for the offspring. However, a single oncogene is not usually sufficient to cause cancer, so inheritance of an oncogene does not
A.2. GENETIC OF CANCER

necessarily result in cancer.

A.2.2 Tumor-suppressors genes

The proteins coded by tumor suppressor genes normally inhibit cell growth, preventing tumor formation. Mutation in these genes results in cells that no longer show physiological inhibition of cell growth and division. The products of tumor suppressor genes may act at the cell membrane, in the cytoplasm or in the nucleus. Mutation in tumor suppressor genes results in a loss of function and they are usually recessive. This means that the trait is not expressed unless both copies of the normal genes are mutated. In some cases, the first mutation is already present in a germ line cell, thus all the cells in the individual inherit it. Because the mutation is recessive, the trait is not expressed. Later, a mutation occurs in the second copy of the gene in a somatic cell. In that cell both copy of the gene are mutated and the cell develops uncontrolled growth.

An example of this kind of tumor is the hereditary retinoblastoma. When one parent carries a mutation in one copy of the $RB$ suppressor gene, it is transmitted to the offspring with a 50% probability. If the offspring, who has received the one mutated $RB$ gene from a parent, has a mutation in the second copy of the $RB$ gene, he will develop retinoblastoma.

Other cancer associated with defects in tumor suppressor genes are familial adenomatous polyposis of colon which results from mutations of both copies of the APC gene; hereditary breast cancer from mutation of $BRCA2$ gene; and hereditary breast and ovarian cancer from $BRCA1$ gene. Although these examples suggest a clear connection between hereditary and cancer, only few types of cancer show such connections. Furthermore, mutations in both copies of a tumor suppressor gene can occur in a somatic cell, so these cancers are not always hereditary.

A.2.3 DNA-repair genes

They are involved in DNA repair and maintenance of the chromosome structure. Environmental factors, such as ionizing radiation, UV light, toxic chemicals and many other factors can damage DNA in different ways. Errors in DNA replication can also lead to mutations. Proteins coded by DNA-repair genes repair damage to chromosomes and minimizing mutations in the cell. When a DNA-repair gene is mutated its product is no longer made preventing DNA repair and allowing further mutations to accumulate in the cell. These drastically increase the probability to develop cancer.
XP genes (Xeroderma Pigmentosum) for example, is a family of seven genes, whose products are responsible to remove DNA damage caused by UV light and other carcinogenesis. Individuals that present damage in XP genes are very sensible to UV light and have very high probability to develop skin cancer.

Another example is the BLM gene which maintain stable the structure of the chromosome. A BLM defects is associated with the Bloom syndrome, an inherited disorder that leads to increased risk of, cancer, lung disease and diabetes.

### A.2.4 Tumor growth and cell cycle

Normal cells grow and divide in ordered steps in accordance with the cell cycle. Mutations in proto-oncogenes or in tumor suppressor-genes allow a tumor cell to growth and divide without the normal controls imposed by the cell cycle. There are checkpoints in the cycle at the end of G1 and G2 that can prevent the cell from entering the S or M phases as showed in Fig. A.2

![Cell cycle process for a normal cells. Checkpoints are present at the end of G1 and G2 and can prevent the cell from entering the S or M phases](image.png)
Several proteins **control** the **timing** of the events in the cell cycle, which is tightly regulated to ensure that cells divide only when necessary. Major control switches of the cell cycle are cyclin-dependent kinases that constitutes the mentioned checkpoints. Each cyclin-dependent kinase forms a complex with a particular cyclin, a protein that binds and activates the cyclin-dependent kinase. The kinase part of the complex is an enzyme that adds a phosphate to various proteins required for progression of a cell through the cycle. These added phosphates alter the structure of the protein and can activate or inactivate the protein, depending on its function.

There are specific cyclin-dependent kinase/cyclin complexes at the entry points into the G1, S, and M phases of the cell cycle, as well as additional factors that help prepare the cell to enter S phase and M phase.

In terms of cell division, normal cells differ from cancer cells in at least four ways:

- Normal cells require external growth factors to divide. When synthesis of these growth factors is inhibited by normal cell regulation, the cells stop dividing. Cancer cells have lost the need for positive growth factors, so they divide whether or not these factors are present. Consequently, they do not behave as part of the tissue, they have become independent cells.

- Normal cells show contact inhibition. They respond to contact with other cells by ceasing cell division. Therefore, cells can divide to fill in a gap, but they stop dividing as soon as there are enough cells to fill the gap. This characteristic is lost in cancer cells, which continue to grow after they touch other cells, causing a large mass of cells to form.

- Normal cells age and die, and are replaced in a controlled and orderly manner by new cells. Apoptosis is the normal, programmed death of cells. Normal cells can divide only about fifty times before they die. This is related to their ability to replicate DNA only a limited number of times. Each time the chromosome replicates, the ends (telomeres) shorten. In growing cells, the enzyme telomerase replaces these lost ends. Adult cells lack telomerase, limiting the number of times the cell can divide. However, telomerase is activated in cancer cells, allowing an unlimited number of cell divisions.

- Normal cells cease to divide and die when there is DNA damage or when cell division is abnormal. Cancer cells continue to divide, even when there is a large amount of damage to DNA or when the cells are abnormal. These progeny cancer cells contain the abnormal DNA;
so, as the cancer cells continue to divide they accumulate even more damaged DNA.

One important protein in the cell cycle is p53, a transcription factor that binds to DNA, activating transcription of a protein called p21. p21 blocks the activity of a cyclin-dependent kinase required for progression through G1. This block allows time for the cell to repair the DNA before it is replicated. If the DNA damage is so extensive that it cannot be repaired, p53 triggers the cell to apoptosis. A mutation in the gene Tp53 which codes P53 protein is common to several types of cancer. Other proteins that stop the cell cycle by inhibiting cyclin-dependent kinases are p16 and RB. All of these proteins, including p53, are tumor suppressors.

A.3 Tumor growth

As already mentioned, it is widely accepted that tumor growth is a multi-step process and that these steps reflect the genetic alterations that drive a normal cell into a cancer cell. As proposed by Hanahan and Weinberg [57], tumor progression can be divided in six phases, each of one corresponds to a specific alteration in the normal cell physiology or, in other words, a novel capability acquired by the tumorigenic cell:

1. Self-sufficiency in growth signal
2. Insensitivity to growth inhibitor signal
3. Evasion of programmed cell death
4. Limitless replicative potential
5. Sustained angiogenesis
6. Tissue invasion and metastasis

A.3.1 Step 1: Self-sufficiency in growth signal

Normal cells require mitogenic growth signals GS before they can move from a quiescent state into an active proliferative state. These signals are transmitted into the cell by trans-membrane receptors that bind distinctive classes

\[\text{This section is adapted from The Hallmarks of Cancer [57], The Biology of Cancer [145] and from Rediscovering Biology [37].}\]
A.3. TUMOR GROWTH

of signaling molecules: diffusible growth factors, extracellular matrix components, and cell-to-cell adhesion/interaction molecules. No type of normal cell can proliferate in the absence of such stimulatory signals. Many of the oncogenes in the cancer act by mimicking normal growth signaling in one way or another. The conclusion is that tumor cells generate many of their own growth signals, thereby reducing their dependence on stimulation from their normal tissue microenvironment.

Three common molecular strategies for achieving autonomy are known, involving alteration of extracellular growth signals, of transcellular transducers of those signals, or of intracellular circuits that translate those signals into action. While most soluble mitogenic growth factors (GFs) are made by a cell type in order to stimulate the proliferation of another one, many cancer cells acquire the ability to synthesize GFs themselves, creating a positive feedback signalling loop often termed autocrine stimulation.

The cell surface receptors that transduce growth-stimulatory signals into the cell interior are themselves targets of deregulation during tumor pathogenesis. GF receptors, often carrying tyrosine kinase activities in their cytoplasmic domains, are overexpressed in many cancers. Receptor overexpression may enable the cancer cell to become hyperresponsive to ambient levels of GF that normally would not trigger proliferation.

Cancer cells can also switch the types of extracellular matrix receptors (integrins) they express, favoring ones that transmit progrowth signals. These bifunctional, heterodimeric cell surface receptors physically link cells to extracellular matrix (ECM). Successful binding to specific moieties of the ECM enables the integrin receptors to transduce signals into the cytoplasm that influence cell behavior, ranging from quiescence in normal tissue to motility, resistance to apoptosis, and entrance into the active cell cycle. Conversely, the failure of integrins to forge these extracellular links can impair cell motility, induce apoptosis, or cause cell cycle arrest.

The most complex mechanisms of acquired GS autonomy derive from alterations in components of the downstream cytoplasmic circuitry that receives and processes the signals emitted by ligand-activated GF receptors and integrins.

Following on of the described strategies, successful tumor cells are those that have acquired the ability to co-opt their normal neighbours by inducing them to release abundant fluxes of growth-stimulating signals

**A.3.2 Step 2: Insensitivity to growth inhibitor signal**

Within a normal tissue, multiple antiproliferative signals operate to maintain cellular quiescence and tissue homeostasis; these signals include both soluble
growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the surfaces of nearby cells. These growth-inhibitory signals, like their positively acting counterparts, are received by trans-membrane cell surface receptors coupled to intracellular signaling circuits.

**Antigrowth signals** can block proliferation by two distinct mechanisms. Cells may be forced out of the active proliferative cycle into the quiescent state $G_0$ from which they may reemerge on some future occasion when extracellular signals permit. Alternatively, cells may be induced to permanently relinquish their proliferative potential by being induced to enter into postmitotic states, usually associated with acquisition of specific differentiation-associated traits.

Incipient cancer cells must evade these antiproliferative signals if they are...
to prosper. Much of the circuitry that enables normal cells to respond to antigrowth signals is associated with the cell cycle clock, specifically the components governing the transit of the cell through the G1 phase of its growth cycle. Cells monitor their external environment during this period and, on the basis of sensed signals, decide whether to proliferate, to be quiescent, or to enter into a postmitotic state.

While the components and interconnections between the various antigrowth and differentiation-inducing signals and the core cell cycle machinery are still being delineated, the existence of an antigrowth signaling circuitry is clear, as is the necessity for its circumvention by developing cancers.

A.3.3 Step 3: Evasion of programmed cell death

The ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of cell attrition. Programmed cell death, called apoptosis, represents a major source of this attrition.

Observations accumulated over the past decade indicate that the apoptotic program is present in latent form in virtually all cell types throughout the body. Once triggered by a variety of physiologic signals, this program unfolds in a precisely choreographed series of steps. Cellular membranes are disrupted, the cytoplasmic and nuclear skeletons are broken down, the cytosol is extruded, the chromosomes are degraded, and the nucleus is fragmented, all in a span of 30-120 min. In the end, the shriveled cell corpse is engulfed by nearby cells in a tissue and disappears, typically within 24 hours.

The apoptotic machinery can be broadly divided into two classes of components: sensors and effectors. The sensors are responsible for monitoring the extracellular and intracellular environment for conditions of normality or abnormality that influence whether a cell should live or die. These signals regulate the second class of components, which function as effectors of apoptotic death. The sentinels include cell surface receptors that bind survival or death factors.

Many of the signals that elicit apoptosis converge on the mitochondria, which respond to proapoptotic signals by releasing cytochrome C, a potent catalyst of apoptosis. The p53 tumor suppressor protein can elicit apoptosis by upregulating expression of proapoptotic Bax in response to sensing DNA damage; Bax in turn stimulates mitochondria to release cytochrome C.

Resistance to apoptosis can be acquired by cancer cells through a variety of strategies. Surely, the most commonly occurring loss of a proapoptotic regulator through mutation involves the p53 tumor suppressor gene. The resulting functional inactivation of its product, the p53 protein, is seen in
greater than 50% of human cancers and results in the removal of a key com-
ponent of the DNA damage sensor that can induce the apoptotic effector
cascade. Signals evoked by other abnormalities, including hypoxia and onco-
gene hyperexpression, are also funneled in part via p53 to the apoptotic
machinery; these too are impaired at eliciting apoptosis when p53 function
is lost.

A.3.4 Step 4: Limitless replicative potential

Three acquired capabilities, growth signal autonomy, insensitivity to anti-
growth signals, and resistance to apoptosis, all lead to an uncoupling of a
cell’s growth program from signals in its environment. In principle, the result-
ing deregulated proliferation program should suffice to enable the generation
of the vast cell populations that constitute macroscopic tumors. However,
research performed over the past 30 years indicates that this acquired dis-
ruption of cell to cell signaling, on its own, does not ensure expansive tumor
growth. Many and perhaps all types of mammalian cells carry an intrin-
sic, cell-autonomous program that limits their multiplication. This program
appears to operate independently of the cell-to-cell signaling pathways de-
scribed above. It too must be disrupted in order for a clone of cells to expand
to a size that constitutes a macroscopic, life-threatening tumor.

Cells in culture have a finite replicative potential. Once such cell pop-
ulations have progressed through a certain number of doublings, they stop
growing (senescence). The senescence of cultured human fibroblasts can be
circumvented by disabling their pRb and p53 tumor suppressor proteins,
enabling these cells to continue multiplying for additional generations until
they enter into a second state termed crisis. The crisis state is character-
ized by massive cell death, karyotypic disarray associated with end-to-end
fusion of chromosomes, and the occasional emergence of a variant cell that
has acquired the ability to multiply without limit, the trait termed immor-
talization.

The phenomenon of senescence was originally observed as a delayed response
of primary cells to extended propagation in vitro and has thus been associ-
ated with mechanisms of divisional counting. More recently, the senescent
state has been observed to be inducible in certain cultured cells in response
to high level expression of genes such as the activated ras oncogene.

A.3.5 Step 5: Sustained angiogenesis

The oxygen and nutrients supplied by the vasculature are crucial for cell
function and survival, obligating virtually all cells in a tissue to reside within
A.3. TUMOR GROWTH

100\(\mu\)m of a capillary blood vessel. During organogenesis, this closeness is ensured by coordinated growth of vessels and parenchyma. Once a tissue is formed, the growth of new blood vessels is transitory and carefully regulated. Because of this dependence on nearby capillaries, it would seem plausible that proliferating cells within a tissue would have an intrinsic ability to encourage blood vessel growth. But the evidence is otherwise. The cells within aberrant proliferative lesions initially lack angiogenic ability, curtailing their capability for expansion. In order to progress to a larger size, incipient neoplasias must develop angiogenic ability. Counterbalancing positive and negative signals encourage or block angiogenesis. One class of these signals is conveyed by soluble factors and their receptors, the latter displayed on the surface of endothelial cells; integrins and adhesion molecules mediating cell-matrix and cell-cell association also play critical roles. The angiogenesis-initiating signals are exemplified by vascular endothelial growth factor VEGF and acidic and basic fibroblast growth factors FGF1/2. Each binds to transmembrane tyrosine kinase receptors displayed by endothelial cells. A prototypical angiogenesis inhibitor is thrombospondin-1, which binds to CD36, a transmembrane receptor on endothelial cells coupled to intracellular Src-like tyrosine kinases. There are currently more than two dozen angiogenic inducer factors known and a similar number of endogenous inhibitor proteins.

Integrin signaling also contributes to this regulatory balance. Quiescent vessels express one class of integrins, whereas sprouting capillaries express another. Interference with signaling from the latter class of integrins can inhibit angiogenesis, underscoring the important contribution of cell adhesion to the angiogenic program. Extracellular proteases are physically and functionally connected with proangiogenic integrins, and both help dictate the invasive capability of angiogenic endothelial cells.

The ability to induce and sustain angiogenesis seems to be acquired in a discrete step (or steps) during tumor development, via an angiogenic switch from vascular quiescence. When three transgenic mouse models were analyzed throughout multistep tumorigenesis, in each case angiogenesis was found to be activated in midstage lesions, prior to the appearance of fullblown tumors. Similarly, angiogenesis can be discerned in premalignant lesions of the human cervix, breast, and skin (melanocytes). Tumors appear to activate the angiogenic switch by changing the balance of angiogenesis inducers and countervailing inhibitors. One common strategy for shifting the balance involves altered gene transcription. Many tumors evidence increased expression of VEGF and/or FGFs compared to their normal tissue counterparts. In others, expression of endogenous inhibitors such as thrombospondin-1 or \(\beta\)-interferon is downregulated. Moreover, both transi-
tions may occur, and indeed be linked, in some tumors. The mechanisms underlying the full angiogenic process, and in particular the angiogenic switch, presents specific features according to the different types of tumors and it is only partially understood.

A.3.6 Step 6: Tissue invasion and metastasis

Sooner or later during the development of most types of human cancer, primary tumor masses spawn pioneer cells that move out, invade adjacent tissues, and thence travel to distant sites where they may succeed in founding new colonies. These distant settlements of tumor cells, called metastases, are the cause of 90% of human cancer deaths. The capability for invasion and metastasis enables cancer cells to escape the primary tumor mass and colonize new terrain in the body where, at least initially, nutrients and space are not limiting. The newly formed metastases arise as amalgams of cancer cells and normal supporting cells conscripted from the host tissue. Like the formation of the primary tumor mass, successful invasion and metastasis depend upon all of the other five acquired hallmark capabilities.

Invasion and metastasis are exceedingly complex processes, and their genetic and biochemical determinants remain incompletely understood. At the mechanistic level, they are closely allied processes, which justifies their association with one another as one general capability of cancer cells. Both utilize similar operational strategies, involving changes in the physical coupling of cells to their microenvironment and activation of extracellular proteases. Several classes of proteins involved in the tethering of cells to their surroundings in a tissue are altered in cells possessing invasive or metastatic capabilities. The affected proteins include cell-cell adhesion molecules (CAMs) and integrins, which link cells to extracellular matrix substrates. Notably, all of these adherence interactions convey regulatory signals to the cell. The most widely observed alteration in cell to environment interactions in cancer involves E-cadherin, a homotypic cell to cell interaction molecule ubiquitously expressed on epithelial cells. Coupling between adjacent cells by E-cadherin bridges results in the transmission of antigrowth and other signals via cytoplasmic contacts with catenin to intracellular signaling circuits that include the Lef/Tcf transcription factor. E-cadherin function is apparently lost in a majority of epithelial cancers, by mechanisms that include mutational inactivation of the E-cadherin or catenin genes, transcriptional repression, or proteolysis of the extracellular cadherin domain. Changes in integrin expression are also evident in invasive and metastatic cells. Invading and metastasizing cancer cells experience changing tissue microenvironments during their journeys, which can present novel matrix
components. Accordingly, successful colonization of these new sites (both local and distant) demands adaptation, which is achieved through shifts in the spectrum of integrin or subunits displayed by the migrating cells.

The second general parameter of the invasive and metastatic capability involves **extracellular proteases**. Protease genes are upregulated, protease inhibitor genes are downregulated, and inactive zymogen forms of proteases are converted into active enzymes. Matrix-degrading proteases are characteristically associated with the cell surface, by synthesis with a transmembrane domain, binding to specific protease receptors, or association with integrins. A further dimension of complexity derives from the multiple cell types involved in protease expression and display. In many types of carcinomas, matrix-degrading proteases are produced not by the epithelial cancer cells but rather by conscripted stromal and inflammatory cells; once released by these cells, they may be wielded by the carcinoma cells.

The activation of extracellular proteases and the altered binding specificities of cadherins, CAMs, and integrins are clearly central to the acquisition of invasiveness and metastatic ability. But the regulatory circuits and molecular mechanisms that govern these shifts remain elusive and, at present, seem to differ from one tissue environment to another. The acquired capability for invasion and metastasis represents the last great frontier for exploratory cancer research.

The paths that cells take on their way to becoming malignant are highly variable. Within a given cancer type, mutation of particular target genes such as \textit{ras} or \textit{p53} may be found in only a subset of otherwise histologically identical tumors. Further, mutations in certain oncogenes and tumor suppressor genes can occur early in some tumor progression pathways and late in others. As a consequence, the acquisition of biological capabilities such as resistance to apoptosis, sustained angiogenesis, and unlimited replicative potential can appear at different times during these various progressions. Accordingly, the particular sequence in which capabilities are acquired can vary widely, both among tumors of the same type and certainly between tumors of different types Fig.[A.3]. Furthermore, in certain tumors, a specific genetic event may, on its own, contribute only partially to the acquisition of a single capability, while in others, this event may aid in the simultaneous acquisition of several distinct capabilities.

### A.4 Cancer stem cells

Normal stem cells are defined as cells that have the ability to perpetuate themselves through self-renewal and to generate mature cells of a particu-
lar tissue through differentiation [122]. A normal stem cell must possess two qualities to perform its natural function: self-renewal and differentiation [81]. Self-renewal is a special cell division that enables a stem cell to produce another stem cell with essentially the same development and replication potential. Differentiation is the second function of a stem cell and involves the production of daughter cells that become tissue-specific specialized cells. A cancer stem cell (CSCs) is defined as a cancer cell, that possesses characteristics associated with normal stem cells, specifically the ability to give rise to all cell types found in a particular cancer [13]. The interesting in stem cells in cancer research origins from the fact that most cancer contain tumor cells that display stem cell-like characteristics, several reports have suggested that they are 25% of the total number of cells within certain tumors [54] [114]. In particular CSCs have been isolated in some type of leukemia [63], in breast [2] and brain tumor [113]. According to the most recent knowledge, there are two possible ways in which cancer stem cells can arise. Originally it was postulated that they origin from a mutation of normal stem cells [41], more recently it has been proposed that they also be derived from a progenitor cell that gives rise to self-renewing cancer stem cells [122].

Two important observations led to the hypothesis that cancer stem cells may be responsible for growing and maintaining tumors [81]. First, most tumors arise from a single cell, but not all the cells within a tumor are identical. The second observation upon which the cancer stem cell theory was built came from studies that demonstrated that a large number of cancer cells were required to grow a tumor. These observations were seemingly at odds with the traditional stochastic model of cancer development. The stochastic model predicts that every cancer cell has the potential to form a new tumor, but entry into the cell cycle is a stochastic event that occurs with low probability. Under the assumptions that all cancer cells have similar potential to grow tumors and that tumors are usually clonal in origin, one would expect that even a few cancer cells would be able to grow new tumors. There are two possible explanations for these observations. First, the tumors could contain a cell hierarchy in which only a mi- nority population of tumor stem cells could self-renew and thus was capable solely of re- generating a tumor. The other cancer cells may have had only limited capacity to replicate and thus contribute to tumor bulk but not to tumor maintenance [81].

The hypothesis that the growth of solid cancer could be driven by cancer stem cells has profound implications for cancer therapy. Because of small numbers of disseminated cancer cells can be detected at sites distant
A.4. CANCER STEM CELLS

from primary tumours in patients that never manifest metastatic disease, it is possible that they lack the ability to form a new tumour such that only the dissemination of rare cancer stem cells can lead to metastatic disease. If so, the goal of therapy must be to identify and kill this cancer stem cell population instead of the other tumor cells [122].

The previous hypothesis could also explain the failure to develop therapies that eradicate completely solid tumors. Although currently available drugs or radiotherapy can shrink metastatic tumours, these effects are usually transient and often do not appreciably extend the life of patients. One reason for the failure of these treatments is the acquisition of drug resistance by the cancer cells as they evolve; another possibility is that existing therapies fail to kill cancer stem cells effectively.

Future challenges in this field will be the isolation of CSCs from different type of solid tumor in order to develop diagnostic markers able to detect them, as well efficient drugs capable to target CSCs.
Appendix B

Tumor angiogenesis

An association between cancer and blood vessels has been observed for more than a century since E. Goldmann conducted one of the first studies on the influence of the vascular system on the growth of malignant diseases in man and animals [50]. The aim of his work was to answer the following questions: 1) how far is the vascular system responsible for the dissemination of malignant growths), 2) what are the general conditions of circulation in these growths? and 3) what purpose does the multiplication of blood-vessels in malignant growths serve merely that of nutrition or also that of defence? Since then, generations of scientists tried to deal with Goldmann’s questions. A fundamental step was done in 1971 by J. Folkman [44] who hypothesized that the tumor growth and its eventual metastases are angiogenic dependent, and therefore, the tumor development should be blocked by blocking angiogenesis. Such intuition opened the current era of research in antiangiogenic therapy to treat cancer that, still nowadays, it is one of the most challenging field in cancer research.

In the following appendix we presents the biological background of tumor angiogenesis. We are focusing, in particular, on the influence that angiogenesis has on tumor growth and on the difference between vascular system in tumor and healthy tissue. The last part deals with the mechanisms exploited in angiogenic therapy.

B.1 Angiogenesis in healthy tissue

Angiogenesis is a normal and physiological process in growth and development, as well as in wound healing and in granulation tissue. The formation of the new vessels is a multi-step process that starts with
the differentiation of endothelial cells and end with the formation of the main vessels and their organization in a functional network. Commonly, four steps in the formation of new vessels are considered [24]: development of an endothelium-line vasculature, vasculogenesis, angiogenesis and arteriogenesis.

B.1.1 Development of endothelium-line vasculature

The first step in the formation of new vessels is the recruitment of endothelial cells (ECs) that constitutes the bricks of the vessel wall. While in the embryo they differentiate from angioblasts, in the adult they differentiate from endothelial progenitor cells (EPCs), mesoangioblasts and multipotent adult progenitor cells [25]. EPCs can also contribute to the vessel growth releasing angiogenic growth factors. Subsequently, the sprouting endothelial cells are assembled into a vascular labyrinth. Although endothelial cells are the main constituent of the vessels, they alone can initiate, but not complete, angiogenesis; periendothelial cells are essential for vascular maturation. During vascular myogenesis, mural cells stabilize nascent vessels by inhibiting endothelial proliferation and migration, and by stimulating production of extracellular matrix. They thereby provide hemostatic control and protect new endothelium-lined vessels against rupture or regression [24].

B.1.2 Vasculogenesis

Vasculogenesis refers to the formation of small blood vessels that constitute the primary network by the endothelial progenitors. This is a process strictly related with embryonal development. Endothelial and hematopoietic cells share a common progenitor that are the hemangioblasts. In the yolk sac, hemangioblasts form aggregates in which the inner cells develop into hematopoietic precursors and the outer population into endothelial cells (Fig. B.1). Angioblasts may migrate extensively before in-situ differentiation and plexus formation. Vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR) 2 and basic fibroblast growth factor (bFGF) influence angioblast differentiation [40], whereas VEGFR 1 suppresses hemangioblast commitment. Molecules mediating interactions between endothelial cells and matrix macromolecules, fibronectin or matrix receptors (α5 integrin), also affect vasculogenesis.
B.1.3 Angiogenesis

Following the formation of primary vessels, the vascular bed expands and matures into a **system of stable vessels**. As showed in Fig. B.1 from the original mother vessel, three ways to generate daughter vessels are possible: sprouting, bridging in which connection between the opposite wall of the capillary are established, sectioning the lumen, and intussusception where the capillary wall extends into the lumen to split a single vessel in two.

One of the first stimuli for the vessel expansion is the **hypoxia level** [25]. Initially, cells are oxygenated by simple diffusion of oxygen, but when tissues grow beyond the limit of oxygen diffusion, hypoxia triggers vessel growth by signaling through hypoxia-inducible transcription factors HIFs [112] which upregulate many angiogenic genes.

Following the hypoxia trigger, several processes mediate the remodelling of the lumen and maturation of the vessel. Vasodilatation which cause an increase in vascular permeability in response to VEGF initiate the process. This increase in permeability is mediated by the formation of fenestrations, vesiculovacuolar organelles and the redistribution of platelet endothelial cell adhesion molecule (PECAM)-1. The resulting effects is the extravasation of plasma proteins that lay down a provisional scaffold for migrating endothelial cells.
The migration is necessary to increase the length and the dimension of the existing vessels or to form new vessels from the preexisting ones. For endothelial cells to emigrate from their resident site, they need to loosen interendothelial cell contacts and to relieve periendothelial cell support. Once assembled in new vessels, endothelial cells have to acquire specialized characteristics to accommodate local physiological requirements and become quiescent and survive for years.

B.1.4 Arteriogenesis

During arteriogenesis smooth muscle cells (SMCs) are incorporated in the vessel wall. SMCs have a complex origing depending on their locations. For example, SMCs surrounding some thoracic vessels are derived from neural crest, whereas coronary SMCs are derived from epicardium, and other SMCs arise from mesenchyme. Once mural cells have been recruited, they further muscularize the nascent vasculature by sprouting or by migrating alongside pre-existing vessels, using these as guiding cues, such as in the retina or in the heart where smooth muscle coverage proceeds in an epicardial to endocardial direction. In mesenchyme-rich tissues, such as in the lung, in situ differentiation of mesenchymal cells contributes to muscularization.

Mural cells, according to the specific region of vessels, acquire special characteristics, including contractile components. In the case of the large thoracic vessels as aorta, vena cava, pulmonary arteries and veins, they undergo considerable remodelling during development to increase their section and wall thickness.

B.2 Angiogenesis in tumor

B.2.1 Tumor dormancy

The formation of new vessel is one of the crucial step in tumor development. It is well established that the initial avascular neoplastic mass cannot grow beyond the size of 1-2 mm. Because the absence of vessel able to supply oxygen, neoplastic cells are highly dependent from their microenvironment. The diffusion limit of oxygen is approximately 100µm requiring that all the mammalian cells be located within 100-200µm of blood vessel. Because non-angiogenic tumors fail to recruit new blood vessels, but require

1This is the reason why mature vessels need to become destabilized
B.2. ANGIOGENESIS IN TUMOR

oxygen and nutrients for their long-term survival, they remain limited in size to less than 1-2 mm in diameter \[97\]. This microscopic lesion, which usually does not produce clinical symptoms and in most of the case is not detectable, is associated with a resting state of the tumor called tumor dormancy \[5\]. Studies, performed over subjects died in automobile accident or other trauma, have documented the presence of in-situ (breast, prostate and thyroid) small neoplastic mass without any previous diagnosis of cancer \[14] \[91\].

B.2.2 Angiogenic switch

The process which drives the tumor from the avascular dormant state to the angiogenic one is called angiogenic switch. This change of phenotype, due to the need of oxygen and nutrients to expand is mediated by various signals that trigger the angiogenic process. These include metabolic stress as low pO2, low pH or hypoglycaemia, mechanical stress as pressure generated by proliferating cells, immune or inflammatory response and genetic mutations \[67] \[26\].

![Figure B.2: Angiogenic balance. Figure adapted from \[11\]](image)

The angiogenic switch can occur at different stages of the tumour progression, depending on the tumour type and the environment \[11\]. Classically, it is modelled as a balance as depicted in Fig. B.2: on one plate there are the
activators that promote angiogenesis, on the other one the inhibitors. The reciprocal interaction between them determine if the angiogenesis can take place or not according to the following steps [97]:

- increased expression by tumor cells of angiogenic proteins, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), plateled derived growth factor (PDGF) and angiogenic oncogenes such as Ras [100]

- increased expression of angiogenic proteins by stromal cells as stromal fibroblasts, a process induced by the tumor itself

- decreased expression of endogenous angiogenesis inhibitors as thrombospondin 1 TSP1 and by many others anti-angiogenic peptides, hormone metabolities and apoptosis modulator as p53 and PTEN [100].

- in some tumors recruitment of bone marrow-derived endothelial precursors

The development of vessels cause a rapid expansion of tumor mass which can mechanically interfere with the organ function as intestine occlusion, or release molecules as cytokines that alter the normal physiology causind clotting and bleeding.

B.2.3 Role of microenvironment

The tumor microenvironment is a mixture of extracellular matrix molecules, tumor cells, endothelial cells, fibroblasts and immune cells [100] [84] that strongly influence the formation of the new vessels and, more in general, the tumor development.

The extracellular matrix ECM is a three-dimensional structure of heterogeneous macromolecules. In addition to providing structural support to cells and tissues, it supports adhesion of cells, transmits signals through adhesion receptors, and binds, stores and presents growth factors and other biologically active molecules.

Basement membranes (BM) are specialized sheet-like extracellular matrix structures that are closely attached to cells. Blood vessel endothelial cells are supported by vascular basement membranes [66]. The abnormalities of the basement membrane in tumor vessels make the vessels dynamic. Although the tumor vessels are almost completely covered by basement membranes,
The extracellular matrix functions as a storage place for stimulators and inhibitors of angiogenesis that are liberated by proteases. Particularly, matrix metalloproteases (MMPs) are involved in several steps of angiogenesis and cancer progression. Not only they degrade basement membranes and other physical barriers, enabling tumor endothelial cells to migrate and metastatic cells to spread, but they also affect cellular and immune processes. MMPs generate growth-promoting signals. They can release precursors of growth factors, and through their effects on the extracellular matrix composition, as well as indirectly, they regulate proliferative signals through integrins. VEGF, the most potent and best studied angiogenesis promoter, gets activated and liberated from the ECM by MMPs. The ECM is not just a passive storage and sequestering place for vascular growth factors; the ECM components as such play an important role in tumor angiogenesis. Many of them, including collagen I, III, XV, fibronectin, fibulin-1, perlecan, laminin-1 and -8 promote angiogenesis, and stabilize blood vessels during angiogenesis. Particularly fibronectin seems to play an important role in these processes.

Another important components of the microenvironment are the fibroblasts. Their role is to synthesize ECM and BM components, regulate ECM degradation, epithelial differentiation and behavior, inflammation, wound healing and tissue fibrosis as well as are involved in cancer progression. Fibroblasts and myofibroblasts often represent the majority of the stromal cells within various types of carcinomas, yet the specific contributions of these cells to tumor growth are poorly understood.

Aside tumor cells and endothelial cells, immune cells plays an important role in tumor growth. Inflammation is a crucial function of the immune system that protects against pathogens and initiates specific immunity. Chronic inflammation is associated with most human cancers. The immune cells seem to have a dual role in cancer progression. On one hand, the immune system is capable of recognizing and attacking cancer cells. The tumor cells need to develop ways to escape the immune surveillance. On the other hand, strong evidence suggests that cancer associated inflammation promotes tumor growth and progression. Chronic inflammation increases the risk of certain cancers; this is best characterized in cases of chronic ulcerative colitis and Crohns disease that clearly associate with colorectal cancers. Further-
more, many cancers arise at the sites of chronic inflammation, inflammatory cells are abundantly found in cancers and cancer cells produce inflammation regulators.

### B.3 Tumor vessels and normal vessels

Tumor vessels presents substantial differences if compared to healthy vessels. First of all the mechanisms of formation in tumor are altered version of the physiological ones (sprouting, bridging and intussusception). Moreover, they can form new vessels by co-opting exixting vessel [26], or by incorporate bone marrow-derived endothelial precursors.

Several molecules are involved in tumor [84] as in physiological angiogenesis but the temporal and spatial expression of these regulators is not well coordinated in tumours. In addition, tumour vessels lack protective mechanisms that normal vessels acquire during growth. For example, they may lack functional perivascular cells, which are needed to protect vessels against changes in oxygen or hormonal balance, provide them necessary vaso-active control to accommodate metabolic needs, and induce vascular quiescence.

The imbalance of angiogenic regulators (Fig. B.2) causes the abnormal formation of the network vessel that is, in most of the case, disorganized, tortuous and can present shunt. These structure is responsible of the non physiological blood flow, and consequently of the non uniform delivery of nutrients to the tumor. Moreover, the vessel wall is not always formed by a homogeneous layer of endothelial cells. Instead, it may be lined with only cancer cells or a mosaic of cancer and endothelial cells [43] that drastically increase the permeability of the vessel wall making it leaky and haemorrhagic. Finally, another important aspect in tumor angiogenesis is that it is an ongoing and endless process. Because of the continuous and unbalanced secretion of pro-angiogenic factors, vessels has no time to mature and stabilize their structure. On the contrary in healthy angiogenesis, vessels became stable and quiescent for long time. after the formation of the vascular network,

### B.4 Anti-angiogenic therapy

As previously mentioned, it was J. Folkman [44] in 1971 who first hypothesized the treatment of the tumor by blocking its angiogenesis. Currently, investigation into mechanisms of angiogenesis inhibition is an important and
promising area of research and two complementary approaches are under development: inhibition of the angiogenic process and normalization of the tumor vasculature.

B.4. ANTI-ANGIOGENIC THERAPY

The first and the more studied approach consists in inhibiting the angiogenic process to deprive tumor of nutrients and, therefore, blocking its development. The possible targets for this kind of therapy are all the subjects involved in the formation of the new vessels as [47] [99]:

- Inhibitors and activators of angiogenesis. The studies to inactivate the VEGF protein to prevent the expression of VEGF family go in this direction. Two independent approaches are used to inactivate the VEGF protein in newborn mice: inducible, CreloxP-mediated gene targeting; or administration of mFlt(1-3)IgG, a soluble VEGF receptor chimeric protein

- Endothelial cell functions as the signalling and cross-talk among vascular cells which govern the assembling the vascular structure during maturation and remodelling

- Vascular endothelium. In this case the target is the already formed vasculature as in the case of advanced stage of cancer.

In recent years, new therapies have been tested and approved by the FDA as Bevacizumab and Sunitinib, several others compounds are currently being tested in phase III [67]. Bevacizumab, (trade name Avastin, Genentech/Roche) is a humanized monoclonal antibody that specifically targets and neutralizes to VEGF-A [84]. It was the first anti-angiogenic drug developed and approved for human cancer. Sunitinib (trade name Sutent, Pfizer) is an oral, small-molecule, multi-targeted receptor tyrosine kinase (RTK) inhibitor that was approved by the FDA for the treatment of renal cell carcinoma and gastrointestinal stromal tumor. Despite the positive control on tumor growth, they are not without side effects: hypertension, risk of bleeding, gastrointestinal perforation, hands-foot syndrome are reported.

A common problem for this kind of therapy is the intrinsic and acquired resistance to anti-angiogenic drugs developed during the treatment. Acquired resistance to specific antibodies can be caused by a redundancy of angiogenesis stimulators [67]. An example is the up-regulation of the angiogenesis stimulator basic fibroblast growth factor (bFGF) within the tumor after treatment with antiVEGFR-2 antibody therapy; this effect is probably
caused by the elevated levels of hypoxia induced by the drug treatment.

B.4.2 Normalization of the vasculature

An alternative approach which goes in the opposite direction, was proposed by R.K. Jain [64]. It consists in the transient normalization of the abnormal structure and function of tumor vasculature to make it more efficient for oxygen and drug delivery. Drugs that induce vascular normalization can alleviate hypoxia and increase the efficacy of conventional therapies if both are carefully scheduled [64].

The starting point of this theory is a paradox underlying tumor treatment. One would expect that destroying the vasculature would severely compromise the delivery of oxygen and therapeutics to the solid tumor, producing hypoxia that would render many chemotherapeutics, as well as radiation, less effective. Indeed, some studies show that antiangiogenic therapy can compromise the delivery of drugs to tumors, as well as antagonize the outcome of radiation therapy. To resolve this paradox, R.K. Jain proposed to administer antiangiogenic agents, in order to normalize the abnormal tumor vasculature, resulting in more efficient delivery of drugs and oxygen to the targeted cancer cells. This concept is summarized in Fig. B.3.
B.4. ANTI-ANGIOGENIC THERAPY

Figure B.3:  
A) Tumor vasculature is structurally and functionally abnormal. It is proposed that antiangiogenic therapies initially improve both the structure and the function of tumor vessels. However, sustained or aggressive antiangiogenic regimens may eventually prune away these vessels, resulting in a vasculature that is both resistant to further treatment and inadequate for the delivery of drugs or oxygen.  
B) Dynamics of vascular normalization induced by VEGFR2 blockade. On the left is a two-photon image showing normal blood vessels in skeletal muscle; subsequent images show human colon carcinoma vasculature in mice at day 0, day 3, and day 5 after administration of VEGFR2-specific antibody.  
C) Diagram depicting the concomitant changes in pericyte (red) and basement membrane (blue) coverage during vascular normalization.  
D) These phenotypic changes in the vasculature may reflect changes in the balance of pro and antiangiogenic factors in the tissue.  
Adapted from [64]
Appendix C

Lotka-Volterra model adapted to tumor growth

Lotka-Volterra equations are widely used in ecology and biology to describe the evolution of two competitive populations [45]. Despite their simple formulation, they well model the dynamic of any kind of populations that are in competition for something. A well known example is the predator-prey model in which predators and preys populations compete together to survive. The evolution of such kind of dynamic model is usual complex due to the non-linearity behaviour of the systems and to the strong dependence of the boundary conditions. Some of the possible states, as extinction, stable co-exist, oscillating growth, chaos bifurcation and chaotic behaviour can rapidly change even if a small perturbation is applied.

In the following section, we are discussing in details the Lotka-Volterra equations applied to tumor growth together with the implications regarding angiogenesis.

C.1 Lotka-volterra equations

Malignant tumor growth is the result of multiple genetic and epigenetic changes, where each one is insufficient, by itself, to transform the cell but lead to cancer when summed by accumulation. At a cellular level, each genetic change has the potential to produce a sub-population with physiologic properties distinctly different from its predecessors. If a sequential set of conditions take place, the transformed cells can change their behaviour into tumor behaviour, ranging from benign, limited growth to gradations of aggressiveness and lethality to the host (App. A). The physiological interaction
between host tissue and neoplastic cells will determine the evolution of the
tumor. Lotka-Volterra equations provide a perfect frame to model this kind of in-
teractions, in which tumor and healthy cells compete together for space and
nutrients in a small volume of tissue within an organ. Assuming \( N_T \) and
\( N_H \) the number of cells in the tumor and host tissue population respectively,
Lotka-Volterra equations are defined as follow:

\[
\frac{dN_T}{dt} = r_T N_T \left( \frac{K_T - N_T - \alpha_{TH} N_H}{K_T} \right) \quad (C.1)
\]

\[
\frac{dN_H}{dt} = r_H N_H \left( \frac{K_H - N_H - \alpha_{HT} N_T}{K_H} \right) \quad (C.2)
\]

where:

- \( r_T, r_H \) are the intrinsic rate of growth for each population
- \( K_T, K_H \) are the maximum number of cells allowed according to the
  space and nutrients availability in absence of competing population
- \( \alpha_{HT} \) is the competition coefficient measuring the effects on \( N_H \) caused
  by the presence of tumor cells \( N_T \)
- \( \alpha_{TH} \) is the competition coefficient measuring the effects on \( N_T \) caused
  by the presence of healthy cells \( N_H \). This interaction is the result of
  other two effects: the growth inhibition \( \alpha_{THi} \) and the growth stimula-
  tion \( \alpha_{THs} \)

\[
\alpha_{TH} = \alpha_{THi} - \alpha_{THs} \quad (C.3)
\]

### C.1.1 Fixed points

We can non-dimensionalize the model substituting

\[
u_1 = \frac{N_1}{K_1} \quad \quad u_2 = \frac{N_2}{K_2}
\]

\[
a_{12} = b_{12} \cdot \frac{K_2}{K_1} \quad \quad a_{21} = b_{12} \cdot \frac{K_1}{K_2}
\]

\[
\tau = r_1 t \quad \quad \rho = \frac{r_2}{r_1}
\]

in eqs. (C.1) and (C.2)

\[
\frac{du_1}{d\tau} = u_1 (1 - u_1 - a_{12} u_2) = f_1(u_1, u_2) \quad (C.7)
\]
C.1. LOTKA-VOLTERRA EQUATIONS

\[
\frac{du_2}{d\tau} = \rho u_2 (1 - u_2 - a_{21} u_1) = f_2(u_1, u_2) \quad (C.8)
\]

We can evaluated the fixed points \(u_1^*, u_2^*\), i.e. the steady states and the phase plane singularities, as the solutions of the equations:

\[
f_1(u_1, u_2) = 0; \quad (C.9)
\]

\[
f_2(u_1, u_2) = 0; \quad (C.10)
\]

Four different points \(u_1^*, u_2^*\) are obtained from previous equations:

1. \(u_1^* = 0, u_2^* = 0\)
2. \(u_1^* = 1, u_2^* = 0\)
3. \(u_1^* = 0, u_2^* = 1\)
4. \(u_1^* = \frac{1-a_{12}}{1-a_{12}a_{21}}, u_2^* = \frac{1-a_{21}}{1-a_{12}a_{21}} \) valid for \(a_{12}a_{21} \neq 1\)

C.1.2 Stability analysis

To evaluate the stability of these points, the characteristic equation

\[
det(A - \lambda I)_{u_1^*, u_2^*} = 0 \quad (C.11)
\]

has to be solved to get the eigenvalues \(\lambda\). \(I\) represents the identity matrix and \(A\) is defined as:

\[
A = \begin{bmatrix}
\frac{\partial f_1}{\partial u_1} & \frac{\partial f_1}{\partial u_2} \\
\frac{\partial f_2}{\partial u_1} & \frac{\partial f_2}{\partial u_2}
\end{bmatrix}
\quad (C.12)
\]

According to the value of \(\lambda\) the fixed points can be classified as:

- **attractors**: if \(\lambda_1, \lambda_2 < 0;\)
- **repellers**: if \(\lambda_1, \lambda_2 > 0;\)
- **saddles**: if \(\lambda_1 < 0, \lambda_2 > 0;\)
Figure C.1: Attractors points in the phase space and correspondent eigenvalues. Figure adopted from [80].

Figure C.2: Repellers points in the phase space and correspondent eigenvalues. Figure adopted from [80].

Attractors are considered **stable** while repellers and saddles are **unstable**.
For the evaluated four fixed points we can analyse their stability as follow:

1. Fixed point: \( u_1^* = 0, \ u_2^* = 0 \)

\[
\text{det}(A - \lambda I)_{0,0} = \begin{vmatrix} 1 - \lambda & 0 \\ 0 & \rho - \lambda \end{vmatrix} = 0 \Rightarrow \begin{cases} \lambda_1 = 1 \\ \lambda_2 = \rho \end{cases} \quad (C.13)
\]

Because of both eigenvalues are positive, it is an unstable repeller.

2. Fixed point: \( u_1^* = 1, \ u_2^* = 0 \)

\[
\text{det}(A - \lambda I)_{1,0} = \begin{vmatrix} -1 - \lambda & -a_{1,2} \\ 0 & \rho(1 - a_{2,1}) - \lambda \end{vmatrix}_{1,0} = 0 \Rightarrow \begin{cases} \lambda_1 = -1 \\ \lambda_2 = \rho(1 - a_{2,1}) \end{cases} \quad (C.14)
\]

It can be:

\( \text{if } a_{2,1} > 1 \Rightarrow \text{stable} \)
\( \text{if } a_{2,1} < 1 \Rightarrow \text{unstable saddle} \) \quad (C.15)

3. Fixed point: \( u_1^* = 0, \ u_2^* = 1 \)

\[
\text{det}(A - \lambda I)_{0,1} = \begin{vmatrix} 1 - a_{1,2} - \lambda & -a_{1,2} \\ -\rho a_{2,1} & -\rho - \lambda \end{vmatrix}_{0,1} = 0 \Rightarrow \begin{cases} \lambda_1 = -\rho \\ \lambda_2 = 1 - a_{1,2} \end{cases} \quad (C.16)
\]

It can be:

\( \text{if } a_{12} > 1 \Rightarrow \text{stable} \)
\( \text{if } a_{12} < 1 \Rightarrow \text{unstable saddle} \) \quad (C.17)

\(^1\text{for a rigorous classification we should consider the real part of } \lambda, \text{ since the eigenvalues can be complex.}\)
4. Fixed point: $u_1^* = \frac{1-a_{12}}{1-a_{12}a_{21}}$, $u_2^* = \frac{1-a_{21}}{1-a_{12}a_{21}}$

$$\det(A - \lambda I)_{u_1^*, u_2^*} = A(1 - a_{12}a_{21})^{-1} \begin{vmatrix} a_{12} - 1 - \lambda & a_{12}(a_{12} - 1) \\ \rho(a_{21}(a_{21} - 1) & \rho(a_{21} - 1) - \lambda \end{vmatrix}_{0,1} = 0$$  \hspace{1cm} (C.18)

$$\Rightarrow \lambda_1, \lambda_1 = \frac{1}{2s} \cdot [m + \rho p \pm [(m + \rho p)^2 - 4\rho sm p]^{\frac{1}{2}}]$$  \hspace{1cm} (C.19)

where $m = (a_{12} - 1)$, $p = (a_{21} - 1)$ and $s = (1 - a_{12}a_{21})$.

### C.2 Physiological considerations

According to the model definitions in eq. C.1, C.2 and C.3, the possibility for tumor population to invade the host tissue depends on three parameters: the carrying capacity for tumor $K_T$ and healthy population $K_H$, and the competitive coefficient $\alpha_{HT}$ measuring the effects on $N_H$ caused by the presence of tumor cells $N_T$.

Mathematically, tumor population has three strategies to vary these parameters for unlimited growth [45].

The first one consist in increasing its own carrying capacity $K_T$. This can be accomplished through the induction of angiogenesis which increases the nutrients and oxygen availability making cells replication and survival of the newly formed cells possible. The results is an explosive growth of tumor population. This is consistent with studies of several tumor models that have shown that non-angiogenic growth is limited but a switch to angiogenic phenotype results in rapid, unrestricted growth [84] [11]. An alternative strategy to increase $K_T$ is to produce autocrine growth factors. In this case, tumor cells produce growth factors that provide environmental support for further increases in the tumor population [45].

The second strategy maximizes $\alpha_{HT}$, which represents the deprivation of resources in one population caused by the presence of the other population. Therefore, the presence of tumor cells may cause a decline in the normal cell populations by acquiring substrate ordinarily available to the normal cells. Some studies, in fact, have observed that tumor cells generally acquire glucose and other substrates more avidly than normal cells [3].

The last strategy available to the tumor is the reduction of the environmental carrying capacity $K_H$ for normal cells. This corresponds to findings in some tumor models in which the tumor invades by breaking down the extracellular matrix in adjacent normal tissue through metalloproteinase,
which the tumor produces directly or induces in adjacent fibroblasts. The increased interstitial pressure compresses the adjacent normal tissue resulting in reduced blood flow and increased mechanical stress on the normal cells, both of which will decrease $K_H$.

### C.3 Bifurcation to chaos, delay, lattice

The model of tumor growth, described up to now with the Lotka-Volterra equations, describes the evolution of two populations, tumor and hosting tissue, in ideal conditions. Despite such simple formulation that allows to model some aspects of tumor growth, it has some limitations. It does not take into account any spatial information and it consider only two populations. In the real case, as we have widely described in this thesis, spatial informations are essential and the many kinds of cells are involved in tumor growth. Furthermore, the population does not interact immediately, but delays can occurs during the transmission of the signals between cells as in the case of immune response.

A more general formulation of the eq. C.1, C.2, which consider more populations, is the following:

$$\frac{dN_i}{dt} = r_i N_i (1 - \sum_{j=1}^{N} \alpha_{ij} N_j(t))$$

(C.20)

It represents the reciprocal interaction between $P$ population $x_i (i = 1...P)$, with the growth rate $r_i$. The stability analysis of this system indicates that it presents several kinds of complex dynamics, including chaotic behaviour in narrow region of the parameter space.

With the term chaotic behaviour, or simply chaos, we do not mean a state of randomness or disordered behaviour. Chaos, in fact, is defined as a state of the dynamical systems in which the following properties are satisfied: it is sensitive to initial conditions, it is topologically mixed and presents dense periodic orbits. The sensitivity to initial conditions has the major biological implications: it means that a small perturbation of the initial state may lead to significantly different final behaviour. In other words for tumor growth, a small change in the expressions of the balance between growth and inhibitor factors determines a strong difference in the tumor development.

If we consider also the delay that can occur in the interactions between
APPENDIX C. LOTKA-VOLTERA MODEL ADAPTED TO TUMOR GROWTH

populations, ex C.20 changes in the following\(^2\)

\[
\frac{dN_i}{dt} = r_i N_i (1 - \sum_{j=1}^{N} \alpha_{ij} N_j (t - \tau_{ij}))
\]  
(C.21)

where \(\tau_{ij}\) is the time delay for the interaction between populations \(i\) and \(j\).

The analysis of the stability of the fixed points of this system presents several theoretical difficulties and usually is made performing numerical simulations.

The last parameters to introduce in order to have a global simulation of the real tumor growth is the spatial distributions. This task results to be very complicated because of the heterogeneity of tumor tissues. A possibility consists first, in sectioning the tumor in a 3D lattice composed of a number \(V\) of voxel \(v(x,y,z)\) and then write for each voxel the eq. C.23.

\[
\frac{dN_i}{dt}_{|_{v_{x,y,z}}} = \left[ r_i N_i \cdot (1 - \sum_{j=1}^{N} \alpha_{ij} N_j (t - \tau_{ij})) \right]_{v_{x,y,z}}
\]

Because each voxel interacts with the other part of the tumor, two kind of interaction has to be considered: short range interactions with the neighbours voxel and long range with the other part of the tumor.

\[
\frac{dN_i}{dt}_{|_{v_{x,y,z}}} = \left[ r_i N_i \cdot (1 - \sum_{j=1}^{N} \alpha_{ij} N_j (t - \tau_{ij})) \right]_{v_{x,y,z}} + \gamma_i N_i_{|_{v_{x,y,z}}} + \delta_i N_i_{|_{v_{x,y,z}}}
\]

where \(\gamma_i\) and \(\delta_i\) are the factors for short- and long-term interactions respectively. The study of this system is extremely complicated and is beyond the scope of this thesis, but its analysis is an interesting area for further research.

---

\(^2\)For a detailed discussion of delayed Lotka-Volterra systems refer to \[96\], \[104\] and \[159\].
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Curriculum Vitae

I was born on March 2nd 1975 in Torino (Italy) as son of Armando Dominietto and Maria Maddalena Bresciano. I grew up in Torino and Alpette and in summer 1994, I graduated from the Industrial Technical Institute ”E. Agnelli”.

In November 1994, I started my studies in Physics at the University of Torino. In May 1999, I joined the group of Prof. Giuseppe Scielzo at the Institute of Cancer Research and Treatment (Candiolo, Italy) where I carried out my master thesis entitled Implementation and dosimetric characterization of a multi-leaf collimator for medical use in 2000.

From October 2000 to November 2004, I attended the School of Specialization in Medical Physics, which I successfully completed with the thesis CT-MR-SPECT image fusion in the radiotherapy of brain tumour: validation and clinical implementation of an entropy based algorithm. During this period I worked at Novara Hospital (Radiotherapy, Nuclear Medicine, Radiology and Medical Physics department) as specializing medical physicist under the supervision of Prof. Marco Bambilla. I have also achieved the qualification of Expert in Radio-protection (grade I) at the Italian Welfare Minister.

In 2005, I moved to Geneva where I started the collaboration with the group of Prof. Ugo Amladi at TERA Foundation (CERN, Geneva) where I was involved in two projects: the study of the medical uses of radioisotopes produced by a 30 MeV cyclotron and the development of a 3D dose calculation code for proton pencil beam irradiation in radiotherapy treatment.

In May 2007, I joined the group of Prof. Markus Rudin (Institute for Biomedical Engineering, ETH Zürich) where I started my PhD project. The work focused on the study of tumor angiogenesis in mice model by means of innovative multi-modality approach and novel analysis techniques.