

École polytechnique de Louvain

Contrast-enhanced micro-CT for 3D X-ray based histology to assess the influence of antiangiogenic drugs on tumor vascularization and necrosis

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Abstract

Cancer treatment faces numerous challenges, prompting ongoing research to explore new strategies. One such strategy involves targeted therapies, particularly the use of antiangiogenic drugs. The aim of this type of drug is to inhibit the formation of new blood vessels, which are crucial for tumor growth and metastasis. This thesis focuses on a specific antiangiogenic drug which is pazopanib, a tyrosine kinase inhibitor, to evaluate its efficacy in treating tumors. For this purpose, this research focuses on a specific category of tumors, soft tissue sarcoma, and more specifically on two dedifferentiated liposarcomas. These liposarcomas tumors, harvested from patients, were transplanted into immunodeficient mice. Visualisation of tumor blood vessels was achieved by staining tumors with Hf-POM and conducting ex-vivo computed tomography scans. Moreover, histopathological evaluation using H&E and CD31 stains was also performed.

The general aim of this thesis is to assess the complementarity and added value of contrast-enhanced microfocus X-ray computed tomography (CECT) compared to classical 2D histology for ex-vivo tumor visualization. Classical 2D histology, while the reference method for examining tumors, offers limited visualization of blood vessels due to its 2D representation. Moreover, this technique relies on the subjective analysis of pathologists and the specific sections selected, which can lead to slice-dependent conclusions given to heterogeneous nature of tumors.

The first objective is to compare tumor visualization using classical 2D histology and CECT to identify blood vessels, necrosis, and adipocytes. Despite the advantages of CECT, issues with the uniform distribution of the contrast agent persist, as twelve tumors showed areas without contrast agent. Secondly, improved visualization of tumor vasculature is studied by exploring and refining blood vessels segmentation in CECT images. Although the methods tested were satisfactory, challenges remain in accurately segmenting complex blood vessel structures, ensuring connectivity, eliminating hemorrhagic zones, and detecting small, centrally located blood vessels. Finally, the effectiveness of ex-vivo CECT in measuring impact of pazopanib on liposarcomas is investigated. No significant differences were

found between control and pazopanib tumors in terms of volume, blood vessels density, and average blood vessels thickness. However, control tumors had larger blood vessels at the edges, while pazopanib tumors had smaller, more centrally located blood vessels.

Contents

1	Introduction	1
2	Literature review	3
2.1	Tumor ethiology	3
2.2	Tumor growth mechanism	4
2.3	Tumor types	5
2.3.1	Soft tissue sarcomas	5
2.3.2	Metastasis	5
2.4	Tumor micro-structure	6
2.4.1	Angiogenesis	7
2.4.2	Necrosis	9
2.5	Diagnosis	11
2.5.1	Symptoms review	11
2.5.2	<i>In-vivo</i> imaging	11
2.5.3	Markers screening	12
2.5.4	Biopsies	12
2.5.5	<i>Ex-vivo</i> imaging	14
2.6	Treatment	25
3	Problem statement and objectives	31
4	Visual comparison of tumors imaged by CECT and classical 2D histology	33
4.1	Introduction	33
4.2	Materials and methods	33
4.2.1	Preclinical models	33
4.2.2	Sample	34
4.2.3	CECT image acquisition and reconstruction	35
4.2.4	Classical 2D histological assessment	35
4.3	Results and discussion	36
4.4	Conclusion	42

5	Segmentation of tumor vascularization	44
5.1	Introduction	44
5.2	Materials and methods	44
5.2.1	Sample	44
5.2.2	CECT image preprocessing	44
5.2.3	CECT image segmentation	45
5.3	Results and discussion	50
5.3.1	Closing	52
5.3.2	Comparison between the three proposed methods	57
5.3.3	Remaining segmentation problems	61
5.3.4	Other methods	62
5.4	Conclusion	64
6	Influence of antiangiogenic drugs on tumor vascularization	66
6.1	Introduction	66
6.2	Materials and methods	66
6.2.1	Sample	66
6.2.2	CECT image preprocessing	67
6.2.3	CECT image segmentation	67
6.2.4	Volume fraction calculation	67
6.2.5	Characterization of blood vessel thickness	70
6.2.6	Classical 2D histology	71
6.2.7	Statistical analysis	71
6.3	Results and discussion	72
6.3.1	Tumor volume	72
6.3.2	3D visualisation of blood vessels	74
6.3.3	Blood vessels quantification	75
6.3.4	Blood vessels distribution	79
6.3.5	Blood vessels characterization	83
6.3.6	Correlation between CECT and histology blood vessel quantification	86
6.4	Conclusion	89
7	Conclusion and future perspectives	91
A	<i>In-vivo</i> imaging	96
A.1	Ultrasonography	96
A.2	Magnetic resonance imaging (MRI)	97
A.3	Positron emission tomography (PET)	99
A.4	X-ray and computed tomography (CT)	100
A.5	PET/CT	102

B	More information on immunohistochemistry (IHC) of sarcomas	104
C	Phase contrast imaging techniques	105
D	Radiotherapy : fractionating process, types of treatments and side effects	107
E	Systemic treatment : types of treatments and side effects	109
F	Ranges and thresholds used for segmentation	111
G	Reliability evaluation of tumors	112

List of Figures

2.1	Tumor growth mechanism with tumor initiation as first step with the mutation of one cell. The mutated cell then proliferates. Finally, a variant cell appears due to progression process and natural selection causes the variant cell to invade everything because it reproduces faster. [1]	4
2.2	Details of the tumor structure containing the tumor core (dark grey) surrounded by the stroma (light grey). The tumor core is made up of tumor cells, while the stroma is the transition zone between tumor cells and normal tissue. There is the presence of cancer-associated fibroblast (yellow) as well as blood vessels (red) and immune cells (blue) in both regions : tumor core and stroma. The role of these different elements is nutritional support, growth and waste elimination for both tumor and healthy tissue. [2]	7
2.3	Angiogenesis in tumors showing early stage of tumor development (1) Tumor growth with a blood vessels (BV) network growing into the tumor region. (2) Tumor is growing and new BV network appears supplying the tumor with nutrients and oxygen (3) A massive tumor with fully penetrating BV network supplying the tumor but also leading to tumor cells traveling to the body with the blood upstream [3]	8
2.4	Mechanism of tumors angiogenesis. First, hypoxia will induce the expression of vascular endothelial growth factor (VEGF). Then, VEGF will bind to receptor and trigger cascade of signalling pathway. This pathway will result in the degradation of the basement membrane. After that, there is the endothelial cells proliferation and migration and finally, the maturation of the blood vessel. [4]	9
2.5	Development of necrosis. The first part represents a normal cell. Then, there is the swelling of the cells due to imbalance ionic concentrations. Finally, the explosion of these cells is shown and this explosion leads to the releasing its entire contents which trigger inflammation.[5]	10
2.6	Different types of biopsy. (A) Needle biopsy, (B) Endoscopic biopsy precisely a bronchoscopy to get tissue from inside the lungs, (C) Skin biopsy and (D) Bone marrow biopsy [6]	14

2.7	Different categories of cancerous cell shape on histological analysis with H&E staining. (A) Leiomyosarcoma representing characteristic spindle cell morphology with fascicle organization. (B) Epithelioid angiosarcoma forming abundant polygon-shaped cytoplasm. (C) Ewing sarcoma with round cell morphology (D) Undifferentiated pleomorphic sarcomas exhibit extreme nuclear pleomorphism. [7]	16
2.8	Dedifferentiated liposarcoma with different staining. (A) Hematoxylin and eosin, magnification 100x. (B) Mouse double minute 2 (MDM2) shows nuclear overexpression, magnification 200x. (C) Cyclin-dependent kinase 4 (CDK4) show nuclear overexpression, magnification 200x. [8]	17
2.9	Imaging results of cancerous liver sample. (A) Phase-contrast CT (PCCT) with the red line delimiting the tumor present in the lower left corner from the healthy tissue above it. (B) Classical 2D histology (C) Absorption CT and (D) MRI. It can be seen that PCCT (A) and MRI (D) provide the best visualization of the morphological change associated with tumor pathology. In fact, the difference in color between tumor tissue and healthy tissue is more pronounced, enabling the edges of the tumor to be clearly seen. In addition, PCCT offers improved visualization of anatomical morphology such as soft-tissue and vascular structures. [9]	21
2.10	Microfil combined with μ CT (A) Microfil-perfused tumor with Microfil polymer in yellow. This polymer allows a better visualisation of blood vessels. (B) 3D computational reconstruction showing surface rendering of vasculature (top left), thickness of the vessels demonstrated by a heat map (top right) and vessel separation demonstrated with a sphere-filling model of the full tumor (bottom left) and of a cross-section through centre of tumor (bottom right). [10]	23
2.11	Comparison of 3 different staining agents for SR μ CT of mouse lungs. Mouse lungs was embedded in paraffin and scanned with SR μ CT scans with voxel size equal to 2.6 μ m. (a) Phosphotungstic acid (PTA) offers a very good contrast of the cartilage of the trachea as well as good visualization of the bronchi (<i>b</i>) and blood vessels (<i>v</i>). (b) Iodine (I_2) staining provided a strong contrast between blood vessels and bronchi. In fact, the clear staining of the red blood cells enabled the blood vessels to be distinguished from the respiratory system. (c) Chrome alum allows very little distinction of bronchi and lightly enhances contrast in blood vessels. [11]	24

2.12	Comparison between unstained and stained CT of <i>ex-vivo</i> lungs sample. (A) Unstained CT and (B) iodine in ethanol (I_2E) stained μ CT. The comparison between A and B demonstrates the usefulness of contrast agents for visualizing soft tissue in the lung. In image A, the gray values of the different lung tissues hardly change, which makes it difficult to visualize lung structure. However, in the image with the staining agent, high-contrast were achieved. I_2E enabled the identification of more detailed lung structures, such as connective tissue and vascular walls. The staining significantly improved the images contrast and the visualisation of the different tissues of the lungs such as lobe bronchi and small airways indicated by white arrows.[12]	25
2.13	Three conventional categories of treatment for sarcoma : radiation therapy, surgery and chemotherapy with example for each type. In this image, systemic treatments are represented only by chemotherapy, but it is important to note that there are also targeted therapy, immunotherapy and hormonal therapy. [13]	26
4.1	Use of patient-derived xenograft in cancer therapy. First, the tumors are achieved from surgical resection in human patients. Then, these tumors are cut into pieces and grafted into immunodeficient mice. These mice will undergo several tests and the tumors will then be collected to be transplanted again into other mice. [14]	34
4.2	Visualization of control tumors of Experience 113 which present large dark areas in CECT that correspond to pinker areas on classical 2D histology. A. Classical 2D histology of CARLL tumor. It can be seen that the centre of the tumor is pinker in color than the edges of the tumor, which are bluish or purplish. B. CECT of of CARLL tumor. The same central area as in A. is visualised as completely black on CECT with dark grey margins. The edges of the tumor, in contrast, are light grey with white dots which may be blood vessels. C. Classical 2D histology of CALLL1 tumor. D. CECT of CALLL1 tumor. There is also a correspondence between the pinker areas of the tumor on histology and the darker areas on CECT.	37
4.3	Classical 2D histology of CARLL tumors (A.) and CALLL1 (B.) from Experience 113. The right side shows an enlargement of the area in the red box located on the tumor on the left side. The areas observed correspond to the change from a pinkish to a bluish-purple color. It can be seen that apart from the difference in color, there is no morphological difference between the two areas. In fact, there is no change in terms of cell density or cell nucleus morphology that could, for example, indicate the presence of necrosis.	38

4.4	Tumors CALLL1 (A.) and CBLLRLL (B.) from Experience 113. From left to right: CECT of the tumors zoomed in on the lighter areas that appear in the centre of dark areas; then identification of these areas on classical 2D histology; enlargement of the area in the red box and finally enlargement of the area in the green box. It can be seen that the brightest areas on CECT correspond to the areas where cell density is lowest, or even absent on histology.	39
4.5	Tumor CARLR from Experience 113. Left: CECT of the tumor, showing blood vessels in white. Middle: location of the area visualised (framed in red). Right: Classical 2D histology of the tumor on which blood vessels appear orange/brown. The white dots on the CECT can easily be matched with the white dots surrounded by brown/orange on the histology as shown for the blood vessels circled in green. However, on the histology image, there are numerous orange/brown lines and dots on the inner side of the tumor which do not correspond distinctly to CECT features as can be seen in the area circled in blue.	40
4.6	Tumor CBLLRLL from Experience 113. Left: CECT of the tumor, showing adipocytes as small black dots. Middle 1: location of the area visualised (framed in red). Middle 2: Classical 2D histology of the tumor on which adipocytes appear as small white dots. Right : Enlargement of the area of the classic 2D histology image where adipocytes are present. This shows that the very dark spots on the CECT correspond to the white spots on the histology, which are easily identifiable as adipocytes.	41
4.7	A. and B. Identification of adipocytes on CALLR tumor of Experience 114. A. CECT slice with adipocytes present in the upper left of the tumor. B. Application of a threshold to segment the adipocytes visible in blue. The image shows the same region as in A. C. and D. Identification of adipocytes in CBLULLR tumor of Experience 114. Segmentation of the adipocytes visible in blue allows their location to be visualised throughout the whole tumor.	42
5.1	Images of tumor C189 – 2015. A. No filter applied, image is heavily pixelated. B. The Median filter in 3D with Neighborhood = 26 and Iterations = 3 has been applied, resulting in a smoother image.	45

5.2	Erosion of tumor CBLULLL. A. No erosion has yet been applied to the tumor. Only <i>Interactive thresholding</i> has been applied with a threshold equal to 175. It can be seen that many features have been selected at the edges of the tumor. These features reflect both the interface of the tumor with the paraffin and the hemorrhagic zones. It is relatively difficult to distinguish the blood vessels at the edge, and those on the inside are barely visible. B. Erosion of 2% of the length of the smallest axis. Blood vessels are seen more clearly and distinctly. However, some plaques are still too present at the edge of the tumor. C. Erosion of 4% of the length of the smallest axis. The vascular tree is much more visible and blood vessels can be clearly distinguished. A few small plaques are still present, but these are located inside the tumor, so it is not possible to remove them with erosion without losing part of the tumor and therefore the blood vessels.	47
5.3	Application of the <i>Remove island</i> option from Avizo software on CALLL tumor (Experience 114). A. Elements smaller than 10 voxels are removed B. Zoom in on the central part of A. C. Elements smaller than 15 voxels are removed D. Zoom in on the central part of A. The blue circles frame the small blood vessels that are unfortunately removed when <i>Remove island</i> is applied. However, if the removal of 10 voxels is applied, the number of blood vessels lost is reduced. E. In red, blood vessel segmentation after <i>Remove island</i> has been applied to the tumor and what has been removed in green. F. Zoom in on the central part of E.	48
5.4	Various step of blood vessels segmentation shown on CALUR tumor (Experience 114). A. Erosion of 1% of the length of the smallest axis. Some plaques are still present at the edge of the tumor. B. Erosion of 2% of the length of the smallest axis. An improvement in plaque reduction is visible while preserving blood vessels. C. Final result with removal of selected volumes that were smaller than 10 pixels. D. Difference between the final segmentation result visible in blue and the initial segmentation obtained simply by applying a threshold value of 152 with ' <i>Interactive thresholding</i> ' option visible in red.	49
5.5	Illustration of the various stages of blood vessels selection when applying <i>Magic Wand</i> strategy (<i>Method 3</i>) on tumor CARLL (Experience 113). A. First step with the <i>Magic Wand</i> tool used with a <i>Masking</i> of 195-255. The largest blood vessels have been selected. B. Second stage: this time the <i>Masking</i> is 180-255. Most of the holes from the previous stage are filled in, and new connections have been created between blood vessels, mainly in the center of the tumor. C. Last and third step with the addition of small inner blood vessels (163-254) with bigger range and big outer blood vessels with smaller range (217-254).	50

5.6	Illustration of the presence of cuts in blood vessel segmentation using Method 1 as defined in Section 5.2.3. A. Pazopanib CBRURLR tumor (Experience 114). B. CARUL control tumor (Experience 114). Visualisation of whole tumors on the left and zoom on the central part on the right. The green circles surround the different parts of a blood vessel that appear detached on segmentation when they should form a single vessel.	52
5.7	Illustration of the effects of different structural elements that can be used for closing. A. Sub-volume of the CALLR tumor (Experience 114) to which a threshold was applied using <i>Interactive thresholding</i> . Next, a cleaning step was performed. First, an erosion followed by the removal of all elements smaller than 10 voxels (segmentation method used in the chapter 6) B. A closing using ball of 20 pixels was performed on the sub-volume presented in A. 6) C. A closing using balls of 10 pixels was performed on the sub-volume presented in A. D. A closing using cubes of 10 pixels was performed on the sub-volume presented in A. E. A closing using disk of 10 pixels at angles $0^\circ - 0^\circ$ was performed on the sub-volume presented in A. F. A closing using lines of 10 pixels at angles $0^\circ-0^\circ$ was performed on the sub-volume presented in A.	53
5.8	Enlargement of the CALLLR tumor sub-volume to which closing with different morphological elements has been applied. A. Closing using balls of 10 pixels B. Closing using cubes of 10 pixels C. Closing using disks of 10 pixels at angles $0^\circ - 0^\circ$ D. Closing using lines of 10 pixels. The blue circles highlight the connections between the blood vessel parts, which unfortunately were not established despite the closing and the visible proximity of vessels parts. The yellow circles show where closing adds elements that are not really necessary. Indeed, at these points, the closing does not connect the vessels lengthwise, but creates a connection between two branches that form an angle, widening the branches widthwise, as shown in E. E. Blood vessel shown in green, left before closing and right with closing in red creating. It can be seen that closing create a connection between the two branches widthwise, which is undesirable.	54
5.9	Enlargement of the CALLLR tumor sub-volume as shown in Figures 5.7 and 5.8 A. Closing using lines of 10 pixels at angles $0^\circ- 0^\circ$ B. Closing using lines of 10 pixels at angles $90^\circ- 90^\circ$ was perfomed on A. C. Closing using disks of 10 pixels at angles $0^\circ- 0^\circ$ D. Closing using disks of 10 pixels at angles $90^\circ- 90^\circ$ was perfomed on C. The blue circles show the blood vessels that have been linked together by adding the second direction. The yellow circles surround the areas where surplus elements have been added due to the addition of closing in a second direction.	55

5.10	Visualization of CALLR tumor (Experience 114) to which a threshold was applied using <i>Interactive thresholding</i> . Next, a cleaning step was performed following the method explained in section 5.2.3. A. Closing with cube 10 pixels was applied to the tumor. B. Closing with ball 10 pixels was applied to the tumor. The images show from left to right : the whole tumor, then a first zoom on the central part of the tumor and then a second zoom on the blood vessels located on the left side of the central zoom (framed in green). The orange circle surrounds an area where many blood vessels are present and therefore more elements have been added.	56
5.11	Visualization of the fact that erosion in the cleaning process also leads to the loss of selected blood vessels at the edge of the tumor. A. CALLR tumor (Experiment 114) B. CALLR tumor (Experiment 114) but with a different viewpoint C. CALUR tumor (Experiment 114). The red represents the segmentation achieved only by applying a threshold using <i>Interactive thresholding</i> and the blue is the result after applying erosion (5% of the length of the smallest axis for CALLR and 2% for CALUR). The green circles surround vessels that were previously selected with the threshold but are no longer selected after erosion.	58
5.12	Illustration of the CALLR tumor (Experiment 113) with different segmentation techniques and corresponding blood vessels volumes. A. Application of <i>Method 3</i> using <i>Magic Wand</i> as defined in Section 5.2.3. B. Application of a threshold using <i>Interactive thresholding</i> C. Application of <i>Method 1</i> as defined in Section 5.2.3 meaning application of a threshold with <i>Interactive thresholding</i> followed by a cleaning process. D. Application of <i>Method 2</i> as defined in Section 5.2.3 meaning applying a closing to the segmentation performed in C. Here, the morphological element chosen is the ball with a size of 10 pixels. In blue is the segmentation as shown in C. and in red is what has been added by closing. . . .	60
5.13	Illustration of the CALUR tumor (Experiment 113) with different segmentation techniques and corresponding blood vessels volumes. A. Application of <i>Method 3</i> using <i>Magic Wand</i> as defined in Section 5.2.3. B. Application of a threshold using <i>Interactive thresholding</i> C. Application of <i>Method 1</i> as defined in Section 5.2.3 meaning application of a threshold with <i>Interactive thresholding</i> followed by a cleaning process. D. Application of <i>Method 2</i> as defined in Section 5.2.3 meaning applying a closing to the segmentation performed in C. Here, the morphological element chosen is the ball with a size of 10 pixels. In blue is the segmentation as shown in C. and in red is what has been added by closing. . . .	60

5.14	Identification of adipocytes on CALLL tumor from Experience 114. However, it can be seen that when applying segmentation with a very dark gray scale value in order to segment adipocytes, blood vessels that were not stained are also detected by the threshold. The two images show different views of the tumor. The segmentation performed with <i>Interactive thresholding</i> and a range from 5 to 101 is shown in blue. Blood vessels included in the adipocytes segmentation are circled in red.	62
5.15	Application of the ' <i>Label analysis</i> ' option of <i>Avizo</i> on a sub-volume of the C189 tumor - 2015. A. Selection of different independent labels without volume range selection applied B. Selection only of elements within the middle volumic range from $6.03312 \cdot 10^{-8}$ to $0.00447594 \mu m^3$ C. Selection only of elements within the small volumic range from $6.03312 \cdot 10^{-7}$ to $0.00447594 \mu m^3$	63
6.1	A. Illustration of the division of a tumor into 3 donuts-shaped parts representing: the outer, middle and inner parts. The first blue donut represents the outer part with an average of 50% of the tumor inside, then the green donut which is the middle part containing around 25% of the tumor and finally the orange inside which are the remaining 25%. B. Representation of the erosion of CBLLRLL tumor to divide it into 3 parts. The white area corresponds to the whole tumor. The red zone corresponds to the first erosion and the dark blue zone to the second erosion. C. Explication of correspondence between the various donuts-shaped parties and the erosions performed. The blue donut in A. corresponds to the subtraction of the red part (first erosion) from the white part (whole tumor) in B., the green donut in A. corresponds to the subtraction of the blue part (second erosion) from the red part (first erosion) in B. and the orange donut in A. corresponds to the blue part in B.	68
6.2	Visualisation of tumor CALLL (Experience 114) divided in half along the three directions X, Y and Z with its three axes drawn by colored lines and whose measurements are respectively equal to 8299, 8605 and 1102 μm . The smallest axe is highlighted in yellow and its measurement is the one used for the reduction of the whole tumor.	69
6.3	Sphere fitting method for measuring blood vessel thickness. It shows CALLL1 tumor cut in the XY plane. The different colors, in accordance with the legend, represent the spheres of different diameters that have been inserted into the various blood vessels. The maximum thickness for this tumor is 180 μm , but due to the absence of light blue or white on the image, vessels of this thickness are not present in this slice.	70

6.4	Graphical representation of the different tumor volumes calculated on the basis of the segmentation of CECT images. A non-significant difference was observed between control and pazopanib tumors. Furthermore, greater heterogeneity was observed between control tumors, which presented both very small and very large tumors, unlike pazopanib tumors, which were relatively grouped within the same range, except for one tumor that was larger than the others.	73
6.5	A., B., C., and D. are the CANML, CARUL, CALLL and CALUR control tumors respectively, while E., F. G. and H. are the following pazopanib-treated tumors: CBLRLLL, CBLULLR, CBLURUR and CBLURUL. These different images of blood vessels segmentation for the cited tumors illustrate the diversity of the vascular arborescence. However, some similarities can be seen between tumors in the same group. Indeed, a high vascular density can be seen at the edge in control tumors A., C. and D.. Control A. and D. tumors also showed a clear arborescent pattern of vascularization. The pazopanib tumors appear to have a more disorganized vascularization, with vessels that are sometimes very sinuous and sometimes very straight, as illustrated by tumors E. and H.	75
6.6	Graph comparing blood vessels density between control and pazopanib treated tumors based on CECT. Blood vessels density was calculated as the ratio VV/TV where VV is the blood vessels volume and TV is the tumor volume, both calculated on the basis of segmentation. There is a non-significant difference between the two groups. However, 3 control tumors were found to have relatively high vascular density compared to pazopanib tumors which remained in the same range. The majority of tumors (14 out of 16 tumors) had a vascular density of less than 0.5%.	76
6.7	A. Graph comparing blood vessels density between control and pazopanib treated tumors based on CECT (same as in Figure 6.6). There is a non-significant difference between the two groups. B. Graph comparing blood vessels density between control and pazopanib treated tumors based on the number of blood vessels counted in classical 2D histology. There is a significant difference between the two groups.	77
6.8	Illustration of blood vessels in CBLRLLL tumor. A. Slice number 1061 in XY plane. B. Slice number 1431 in XY plane. C. Enlargement of the green box in B. Differences in the quantity of blood vessels can be seen between the blue squares in A. and B., which represent the same area of the tumor but at different slices. Moreover, there is also a difference in blood vessels density between the blue and green boxes, which are on the same slice. Finally, image C. shows that a blood vessel can also intersect a slice at several points and therefore lead to being counted several times by classical 2D histology.	78

6.9	Graphs of blood vessels density as a function of the different donut-shape parts for control tumors on the left and pazopanib-treated tumors on the right. It can be seen that in both cases, blood vessels density is the highest for the outer donut and lowest for the inner donut. For control tumors, a small significant difference can be observed between the outer donut and the middle and inner donuts. For pazopanib tumors, moderately and highly significant differences could be observed between the outer and middle donuts and the inner donut respectively. In both groups, however, no significant differences were found between the middle and inner donuts.	80
6.10	Graph showing the density of blood vessels present in control and pazopanib tumors for each donuts-shaped part of the tumor. For the outer donuts, there is a non-significant difference between the control and pazopanib groups, although the control tumors had a higher density for this part. As for the middle donut, there is also no significant difference between control and pazopanib tumors. Finally, for the inner donut, there is a significant difference between the two groups, with higher values for pazopanib tumors.	81
6.11	Graph showing the percentage of blood vessels volume present in each donut relative to total blood vessels volume and according to each of the 3 donut-shaped portions observed. The graph on the left illustrates this percentage distribution for control tumors and that on the right for pazopanib tumors. In both cases, there is a highly significant difference between the outer donut and the middle and inner donut. However, there is no significant difference between the middle and inner donut.	82
6.12	Graph representing the percentage of blood vessels volume present in each donut compared with the total blood vessels volume for each 3 donut-shaped part observed and comparing these percentages between control and pazopanib tumors. There is a significant difference between the control and pazopanib groups at the level of the outer donut, with a higher percentage of blood vessels in the control tumors. There is no significant difference in the middle donut. There is a highly significant difference between the two groups for the inner donut.	83
6.13	3D visualization of blood vessels stained according to thickness. A., B. and C. are CALUR CARUR and CALLL control tumors respectively, while D. and E. are CBLURUR and CBLRLR pazopanib-treated tumors. F. represents a zoom on the CBLURUR tumor. Thicker blood vessels are red or yellow, medium vessels are green and smaller vessels are blue.	84
6.14	Comparison of the average thickness of blood vessels present in control and pazopanib tumors (expressed in μm). There was a non-significant difference between the two groups. The average thickness was less than 50 μm in both cases, but slightly higher in control tumors.	85

6.15	Thickness distribution graph representing the percentage of each mid-range value (μm) present for tumor control and pazopanib. The mid-range corresponds to the thickness of the blood vessels calculated using the "sphere-filling" method as explained in section 6.2.5	86
6.16	Correlation between blood vessel volume calculated with CECT (mm^3) and the number of blood vessels counted in classical 2D histology. This analysis led to an inconclusive correlation, with a very low correlation coefficient r of 0.005882.	87
6.17	Correlation between blood vessel density (VV/TV) calculated using CECT (expressed in %) and the number of blood vessels counted using classical 2D histology. The analysis led to a correlation factor r of 0.2180.	88
A.1	Magnetic resonance imaging (MRI) of brain metastasis. (A) with gadolinium-based contrast agent (Gd-BOPTA). (B) without contrast agent.[15]	98
A.2	Detection of pulmonary metastasis with ^{18}F -FDG PET/CT. From left to right, there is PET alone, CT alone and finally the combination PET/CT. (A) Transverse section and (B) coronal section. The white and yellow arrows indicate, respectively, the ^{18}F -FDG hypermetabolic zone on PET and a lesion with a density similar to that of water on the CT image. The red arrow on the PET/CT image confirms the location of the metastasis on the dorsal apex of the right lung. Physiologic tracer uptake is also visible in heart (h), kidneys (k), and bladder (b). [16]	103
C.1	Phase-contrast synchrotron-based X-ray CT system with two-crystal X-ray interferometer. The 3 different parts of the interferometer are visible: the splitter, the mirror, and the analyzer. Here, there are 2 mirrors, one for each wave resulting from the incident beam after splitter separation. Only one wave passes through the sample. Then, the two waves meet on the analyzer, allowing the diffraction difference between the two waves to be analyzed. [17]	105
C.2	Schema of the grating interferometer with the incident wave passing through the sample to view, then crosses the first grating (G1), the second grating (G2) and the detector. [18]	106
C.3	Propagation-base phase-contrast imaging system composed only of the source, the object, and the detector. [18]	106

List of Tables

4.1	Acquisition parameters for CECT of tumors	35
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List of abbreviation

ASL	Arterial Spin Labelling
BOLD	Blood Oxygenation Level Dependent
BV	Blood Vessels
CA	Contrast Agent
CDK4	Cyclin-Dependent Kinase 4
CECT	Contrast-Enhanced Computed Tomography
CESA	Contrast-enhancing staining agent
CT	Computed Tomography
DDLPS	Dedifferentiated Liposarcoma
FDG	Fluorodeoxyglucose
Hf-POM	Hafnium-based Wells-Dawson Polyoxometalat
H&E	Hemotoxylin and Eosin
IHC	Immunohistochemistry
I₂	Iodine
I₂E	Iodine in ethanol
LPS	Liposarcoma
MDM2	Mouse Double Minute 2
MRI	Magnetic Resonance Imaging
MVD	Microvascular Density

PCCT Phase-contrast computed tomography

PET Positron Emission Tomography

PTA Phosphotungstic acid

SPECT Single-Photon Emission Computed Tomography

SR μ CT Synchrotron micro-computed tomography

STS Soft Tissue Sarcoma

TGF- β Transforming Growth Factor- β

VEGF Vascular Endothelial Growth Factor

VEGFR Vascular Endothelial Growth Factor Receptors

WDLPS Well-Differentiated Liposarcoma

μ CT Microfocus X-ray Computed Tomography

Chapter 1

Introduction

As second most common global cause of death, cancer represents nearly one in six deaths, and a total of almost 10 million deaths worldwide in 2020 [19]. Caused by abnormal cells growing uncontrollably, this disease can affect most organs and tissues of the human body. There are therefore many different types of cancer but the most common types affect the lung, colon, breast and prostate [20].

Genetic mutations leading to cancer can be caused by the interaction of several agents or factors, such as genetic factors and external agents. External carcinogens can fall into 3 categories: physical, chemical, and biological. Physical carcinogens are, for example, ultraviolet rays and ionizing radiation; whereas chemical agents correspond to components of tobacco smoke, alcohol, some food or water contaminants. Biological factors are linked with infections caused by certain viruses, bacteria or parasites [1].

When suffering from cancer, patients can receive different types of treatment. These treatments are classified into three categories: surgery, radiotherapy and systemic treatment. Surgery consists in complete or partial tumor tissue removal which is the first-line treatment for localized tumors [21]. Radiotherapy uses high-energy X-rays to destroy cancer cells. These strategies can be considered alone or in combination with surgery or systemic treatment [22]. Finally, systemic treatments cover a wide range of treatment strategies, such as chemotherapy, which uses drugs to kill cancer cells [23]. This category includes also target therapies such as antiangiogenic drugs, which block the creation of new blood vessels essential to tumor development [24]. In addition, there are immunotherapies and hormonal therapies [23]. The use of systemic treatments can be curative on its own, adjuvant or neoadjuvant in combination with another strategy, or can be palliative [23, 25]. The choice of treatment depends on the tissues and organs affected, as well as the spread of the cancer, making diagnosis essential to selecting the most appropriate

treatment [26, 27].

The detection and staging of cancerous tumors are achieved through a number of different processes [22]. Firstly, examination of symptoms is essential for detecting cancer, and studies have shown that recurrent symptoms are present in different types of cancer [28]. Another way of diagnosing and characterizing a tumor is marker-based screening. This technique requires a tissue or liquid biopsy and is based on the analysis of various markers present, for example, in a sample of the tumor, or in a sample of blood or urine [29]. Finally, medical imaging is an essential diagnostic tool, with various *in-vivo* and *ex-vivo* modalities. When *in-vivo* imaging visualizes what is happening in a living being, *ex-vivo* imaging visualizes tissue extracted from a living being [30]. As far as *in-vivo* imaging is concerned, ultrasound, magnetic resonance imaging, positron emission tomography, computed tomography and single-photon emission computed tomography are the main techniques used for cancer diagnosis [30]. On the *ex-vivo* side, classical 2D histology is the standard examination for the study of tumor biopsies. However, 2D histology has its limitations, such as providing only a 2D visualization of the tumor, or being subjective. To overcome these limitations, new *ex-vivo* visualization techniques are emerging.[10]. The aim of this thesis will be to evaluate the complementarity and added value of contrast-enhanced microfocus X-ray computed tomography in visualizing tumors compared to 2D classical histology. To this end, contrast-enhanced microfocus computed tomography will be used for 3D visualization of tumor xenographs. The visualization of these tumors will be based on the assessment of their vascularization and necrosis in order to conclude on the response of the tumor to certain treatments.

Disclaimer: *This master thesis has been reviewed and corrected for spelling errors and sentence improvements using artificial intelligence, such as ChatGPT.*

Chapter 2

Literature review

2.1 Tumor ethiology

Cancer is a disease characterized by the uncontrolled growth of abnormal cells. The growth of an abnormal mass of tissue exceeds the growth of normal tissue, and it persists even after the stimulus that initially caused it has been removed. This phenomenon, also called neoplasia, is due to a change in the DNA of the cells [1, 22].

Many agents known as carcinogens can be risk factors for cancer. However, due to the complex process of cancer development, it is rarely possible to attribute a single cause. In numerous studies, however, radiation, certain chemicals and viruses have been shown to be carcinogens [1]. These radiations and chemicals known to be carcinogenic will mainly have the effect of damaging DNA and introducing mutations that lead to the onset of cancer. For example, a major cause of skin cancer is solar ultraviolet radiation [1]. Another well-known cause of most cancers is the carcinogenic chemicals contained in tobacco smoke [1, 22]. Indeed, a study showed that in 2018, one in five cancers in Europe was caused by smoking [31].

Following the ranking of risk factors for cancer, infections are just behind tobacco in developing countries. The three infectious most likely to promote cancer development are the bacterium *Helicobacter pylori*, human papilloma viruses and the hepatitis B and C viruses, followed by Epstein-Barr virus, human immunodeficiency virus (HIV) and the human herpes virus 8 [32, 33]. Another way in which carcinogens enhance cancer development is by promoting tumor cells proliferation through tumor promoters, such as certain hormones [1]. For example, sex steroid hormones such as oestrogens, androgens and progestogens influence the growth of certain cancers in women, notably breast, ovarian and endometrial cancers [22]. Other factors, such as lack of exercise, obesity or heavy alcohol consumption, can

contribute to the development of cancer and other diseases [22].

2.2 Tumor growth mechanism

One of the essential processes in the development of cancer is the abnormal proliferation of tumor cells following exponential replication of the same cell. This process is known as tumor clonality [1]. As explained previously, cancer begins with a single cell which undergoes a genetic mutation that leads to abnormal proliferation, as shown in Figure 2.1. This first step constitutes the tumor initiation. After that, tumor cells will replicate, giving rise to a clonally derived population of tumor cells. This process is called tumor progression. During this stage, certain cells will inherit additional mutations that confer a selective advantage in terms of growth speed, for example. This makes the new cells more dominant, and the process is called clonal selection [1].

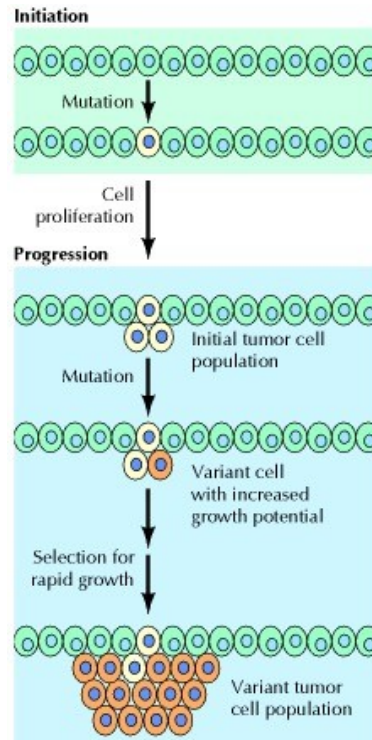


Figure 2.1: Tumor growth mechanism with tumor initiation as first step with the mutation of one cell. The mutated cell then proliferates. Finally, a variant cell appears due to progression process and natural selection causes the variant cell to invade everything because it reproduces faster. [1]

2.3 Tumor types

As cancer development can start from different cell types, there are many different types of cancer, whose behavior and response to treatment can vary significantly [1]. Depending on the type of cell affected, cancer can be classified into 2 categories: solid tumors and blood cell tumors. Firstly, among solid tumors, a distinction is made between carcinomas, which are cancers of epithelial tissue such as the breast, lung, colon, etc., and sarcomas, which are cancers of connective tissue such as muscle or bone. Next, hematological cancers, such as leukemia or lymphoma, are spread throughout the organism, occurring mainly in the bone marrow or blood [1, 34].

2.3.1 Soft tissue sarcomas

In this thesis, the focus will be on a group of rare tumors, representing only around 1% of all adult cancers, known as soft tissue sarcomas [35]. The origin of sarcomas is mesenchymal, specifically caused by genetic mutations in pluripotent mesenchymal stem cells which lead to connective tissue neoplasia. One of the main factors in the formation of sarcomas is ionizing radiation [36, 37]. Sarcomas can be divided into two categories: soft tissue sarcomas and bone sarcomas [36, 13].

As previously stated, this thesis focuses exclusively on soft tissue sarcomas (STS) which, as their name suggests, develop from soft tissue. STS are a very diverse group of entities, with over 80 histological subtypes, and can occur anywhere in the body, but the majority of tumors are located in the extremities [35, 36].

This thesis will mainly investigate a specific type of STS which is liposarcomas. This type represents the most common STS in adults and originate from adipose tissue. Liposarcomas (LPS) are classified into four subtypes: atypical lipomatous tumor or well-differentiated liposarcoma (WDLPS), dedifferentiated liposarcoma (DDLPS), myxoid liposarcoma and pleomorphic liposarcoma [38].

2.3.2 Metastasis

Metastases are produced by the complex process by which tumor cells escape from the original site of the tumor to other parts of the body. This can occur by way of the circulatory and lymphatic systems [1]. Metastasis is a multiscale phenomenon involving dissemination of cancer cells from the tumor source to distant organs, and is the principal cause of cancer lethality [21]. Indeed, most cancer patients die as a result of these metastases, rather than the original tumor. The development of metastases represents the most advanced stage of the disease [39].

The development of these metastases is governed by clonal selection, in other words, the ability of cancer cells to change their phenotypic state and cooperate with neighbouring immune and stromal cells. This enables them to grow and escape the immune system [39].

The metastatic cascade is made up of three phases, which may overlap in time: dissemination, dormancy, and colonization. During these phases, tumor cells penetrate deeper tissues, then invade peripheral blood or lymphatic vessels. Cells must survive transit to finally establish themselves in organs [39].

2.4 Tumor micro-structure

During its development, a tumor has the capacity to develop in three different tissular zones: the initial site, the mesenchyme of the initial site, which results in tumor invasion, and distant mesenchyme, meaning new tissue distant from the initial tumor site, which leads to tumor metastasis [40].

As can be seen in Figure 2.2, the micro-structure of a tumor is mainly composed of two parts: the tumor core containing tumor cells, and the stroma. In most cases, corresponding to solid tumors, the stroma is constituted by fibroblasts, connective tissue, blood vessels and immune cells. This transition zone between malignant cells and normal host tissue is necessary for nutritional support, tumor growth and waste elimination. In the case of leukemia or lymphoma, however, blood plasma serves as the stroma, since cancer cells are located directly in the blood [2, 40].

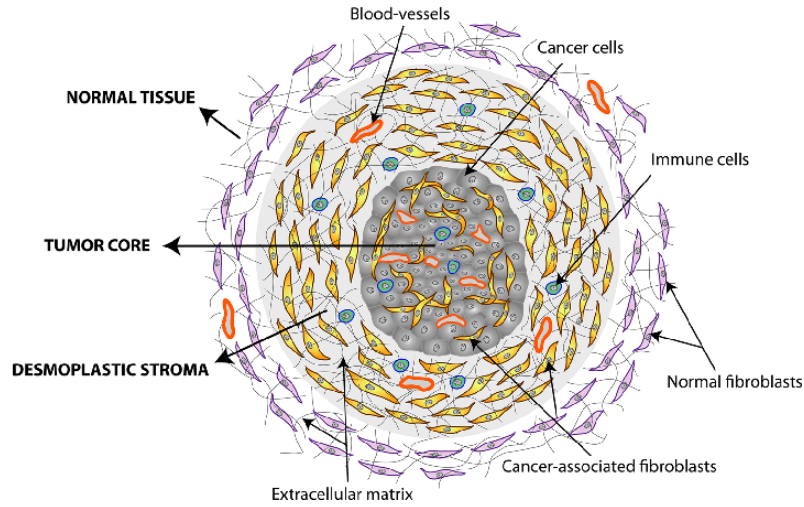


Figure 2.2: Details of the tumor structure containing the tumor core (dark grey) surrounded by the stroma (light grey). The tumor core is made up of tumor cells, while the stroma is the transition zone between tumor cells and normal tissue. There is the presence of cancer-associated fibroblast (yellow) as well as blood vessels (red) and immune cells (blue) in both regions : tumor core and stroma. The role of these different elements is nutritional support, growth and waste elimination for both tumor and healthy tissue. [2]

2.4.1 Angiogenesis

To expand, a tumor needs nutrients and oxygen supplied by the blood. The tumor will therefore send signals that stimulate the ingrowth of blood vessels and growth of new vessels, which will transport more blood in order to spread further [41]. The process by which new blood vessels form is called angiogenesis. In a healthy organism, this process occurs during wound healing, or the development of embryos [42]. However, studies have also shown that angiogenesis is a fundamental component in the development of tumors and metastases. Indeed, when tumors grow beyond 1 to 2 mm in diameter, passive diffusion is no longer efficient enough to ensure a sufficient supply of nutrients and oxygen for the vitality of tumor cells. Thus, the creation of new blood vessels becomes a necessity. The tumor will therefore trigger the "angiogenic switch". This creation of new blood vessels will enable the tumor to grow even more, and then to spread to other areas to create metastases as illustrated in Figure 2.3 [3, 42].

The main stimulus driving the tumor to trigger the angiogenic switch is oxygen deprivation, although other factors such as inflammation, oncogenic mutations and mechanical stress may also be involved [43]. This switch will then lead to the expression of proangiogenic factors such as growth factors or cytokines that will initiate a cascade of signalling. The most predominantly expressed paracrine chem-

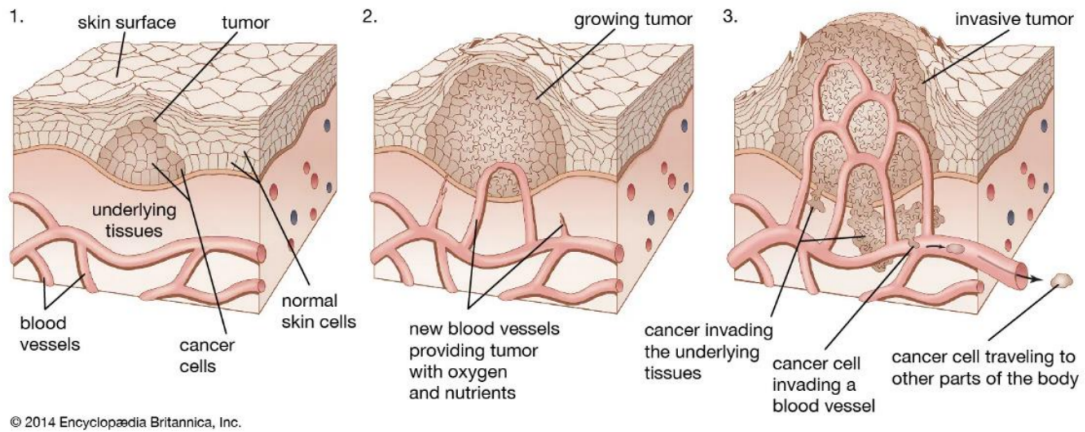


Figure 2.3: Angiogenesis in tumors showing early stage of tumor development (1) Tumor growth with a blood vessels (BV) network growing into the tumor region. (2) Tumor is growing and new BV network appears supplying the tumor with nutrients and oxygen (3) A massive tumor with fully penetrating BV network supplying the tumor but also leading to tumor cells traveling to the body with the blood upstream [3]

ical messenger is vascular endothelial growth factor (VEGF), but other factors may get involved such as angiogenin, fibroblast growth factor, platelet-derived growth factor, and transforming growth factor- β (TGF- β). These factors will stimulate the proliferation, migration, and invasion of endothelial cells, subsequently leading to the formation of neovascular structures derived from neighbouring blood vessels [44, 45].

As can be seen in Figure 2.4, VEGF will trigger the activation of endothelial cells, producing the secretion of proteolytic enzymes. That will lead to the degradation of the perivascular connective tissue and the creation of capillary sprouts into the extracellular matrix. The sprouts will then lengthen to form the lumen of the vessel. Then, they will be stabilized by pericytes and smooth muscle cells that will help for the maturation of the blood vessel. The formation of new capillaries will therefore arise from the branching of two or more sprouts before stabilization [4].

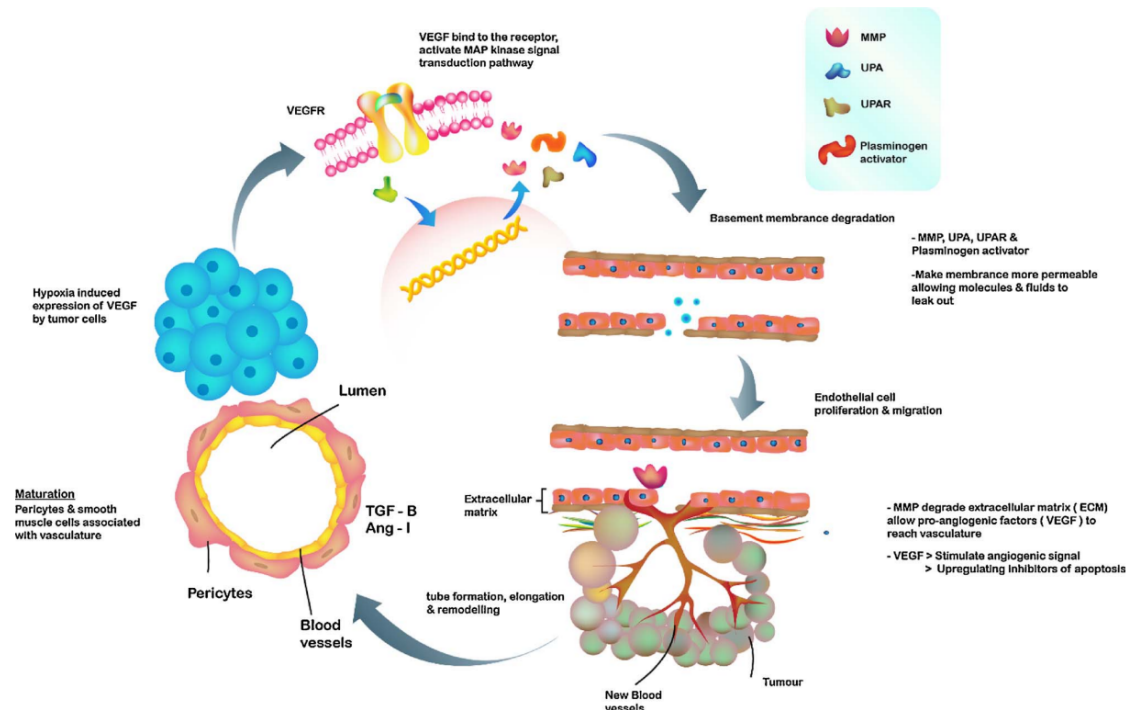


Figure 2.4: Mechanism of tumors angiogenesis. First, hypoxia will induce the expression of vascular endothelial growth factor (VEGF). Then, VEGF will bind to receptor and trigger cascade of signalling pathway. This pathway will result in the degradation of the basement membrane. After that, there is the endothelial cells proliferation and migration and finally, the maturation of the blood vessel. [4]

2.4.2 Necrosis

Necrosis is an abnormal and uncontrolled cell death caused by external and internal injuries. The etymology of the word necrosis comes from the Greek "nekrōsis" meaning "death" [5]. Unlike apoptosis, necrosis is uncontrolled, and the dying cell releases numerous chemicals that damage surrounding cells. Causes that can trigger necrosis are varied and include infection, hypoxia, trauma, extreme temperature or toxins, for example [5, 46].

Necrosis results from a process called "oncosis". Oncosis occurs when the mitochondria of a cell are irreparably damaged. As a result, mitochondria are no longer able to produce ATP (adenosine triphosphate), which causes the disfunction of cellular ion pumps and thus an imbalance in the ionic concentration of the cell. As a result, sodium and water penetrate the cell, which then swells until explosion occurs, releasing its entire contents, as illustrated in Figure 2.5. The released contents attract immune cells, which trigger inflammation [5]. Surrounding tissues

can be damaged, which in turn can result in organ disruption.

During necrosis, various changes in the nuclei can be observed, such as pyknosis, karyorrhexis and karyolysis. Depending on the type of cells affected and the type of damage that has occurred in the body, necrosis will present a specific morphological pattern [5, 46].

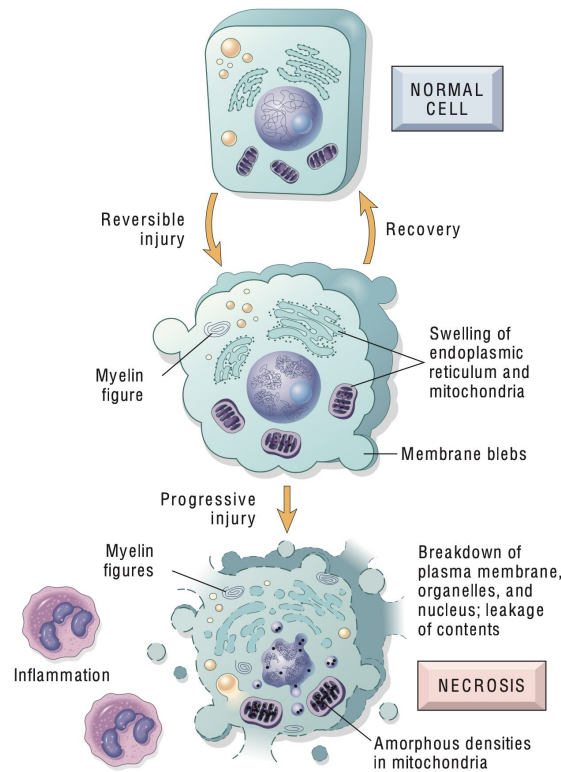


Figure 2.5: Development of necrosis. The first part represents a normal cell. Then, there is the swelling of the cells due to imbalance ionic concentrations. Finally, the explosion of these cells is shown and this explosion leads to the releasing its entire contents which trigger inflammation.[5]

2.5 Diagnosis

Diagnosis is an essential step, because the more accurate it is, the more effective the treatment will be [27]. Detecting cancer as early as possible is indispensable and reduces mortality. Cancer diagnosis includes several modalities which will be detailed below: symptom review, markers screening, biopsies, and finally imaging. Complete diagnosis of a patient requires one or more tests to be carried out successively [22]. When the diagnosis of cancer is confirmed, the next step is to carry out further tests to determine the extent of the disease. This step is called staging and is recorded in staging systems whose principle is common to all cancers, but some details may vary depending on the cancer [22]. The "TNM" system is one of the most common staging systems. Its initials correspond to what is being analyzed: T for the extent of the primary Tumor, N for the extent of spread to regional lymph Nodes, and M for the presence or absence of Metastases [22].

2.5.1 Symptoms review

As there is no single test to diagnose cancer, one of the first steps in the diagnostic process is to review personal and family medical history, to perform a physical examination and analyse the different symptoms [29]. The three symptoms that have been shown to be highly correlated with highly advanced cancer are neck lump, chest pain and back pain. It is therefore inevitable to pay attention to these symptoms, whether alone or in conjunction with other symptoms. In addition, 13 symptoms have been identified as linked to more than 50% of patients with lower stage of cancer. These symptoms include abnormal moles, breast lumps, fatigue, abdominal pain and many others [28]. Weight loss is also considered a frequent symptom [47].

DDLPS are often detected by the presence of large, painless masses that grow over time. The location of the primary tumor can sometimes give a clue, as DDLPS is infrequently in superficial soft tissues and most commonly found at the back of the abdominal cavity [48]. The fact that DDLPS are often located in the lower abdominal cavity is also linked to the fact that a frequent symptom is lower abdominal pain [47].

2.5.2 *In-vivo* imaging

Imaging plays an important role in early detection of cancer and is therefore an indispensable step for early and precise diagnosis [27]. The different imaging modalities can be grouped in a number of ways, depending on the energy source (X-rays, positrons, photons, etc.), spatial resolution (macro-, microscopic, etc.)

or the type of information obtained [49]. For example, ultrasound, X-rays, computed tomography (CT) and magnetic resonance imaging (MRI) are employed to visualize the anatomy of tumors. They deliver structural and morphological information about cancer. It allows a tumor to be visualized by its size and shape, hypertrophied lymph nodes or destructive lesions, for example [22, 50]. On the other side, nuclear imaging techniques such as positron emission tomography (PET) and single-photon emission computed tomography (SPECT) detect the metabolic activity of cells. They use functional and metabolic information to differentiate cancer cells on the basis of their activity levels [22]. These types of scanners use radionuclides that release low levels of radiation. These radiations will then be captured by special cameras that show where the tracer is accumulating [50].

The major advantage of *in-vivo* imaging is that it enables the tumor to be visualized directly in its environment, in the living organism. *In-vivo* medical imaging techniques mainly used for cancer diagnosis are X-ray, ultrasound, magnetic resonance imaging (MRI) and positron emission tomography (PET) [50, 51].

When choosing which modality to use for cancer detection or staging, it is important to consider the different spatial and temporal resolutions of each type of imaging. Indeed, choosing a modality that provides the best possible visualization of tumors and metastases is of paramount importance. For this reason, the type of imaging depends on the location and type of tumor [50]. Another important point when imaging a tumor *in-vivo* is the radiation dose the patient will receive. High-frequency electromagnetic radiations, such as gamma rays, X-rays, or ultraviolet light, are ionizing and can damage the human body or cause cancer. For this reason, dosage considerations are a key factor in the use of ionizing radiation-based imaging [52, 53]. More details of *in-vivo* imaging techniques are given in Appendix A, as the focus of this thesis is on biopsies and *ex-vivo* imaging.

2.5.3 Markers screening

Concerning markers screening, laboratory tests are used to quantify and analyse the level of specific substances present in blood, urine and other body fluids [29, 54]. If these specific substances are present in abnormally high or low quantities, this is an indicator of the occurrence of a disease or the response to a treatment. These substances are known as biomarkers [55].

2.5.4 Biopsies

Biopsies are an essential part of cancer diagnosis and can be divided into 2 categories: liquid and solid biopsies. Liquid biopsy can be used to analyse fluid samples

such as blood, urine and saliva, providing information about real-time progression as well as treatment response of the cancer [56]. One example of liquid biopsy is based on blood and is widely employed in the detection of cancers, but also of other diseases such as immune disorders [56]. Since blood supplies healthy organs as well as primary tumors and metastases, it contains the DNA of both normal and cancerous cells. Circulating blood is therefore a major source of information, containing traces of all tumor DNA variations [57]. Among the various materials contained in blood, the following can be cited as biomarkers of interest in cancer diagnosis: circulating free DNA; circulating free RNA, microRNAs, proteins and extracellular vesicles; as well as circulating tumor cells [54]. These biomarkers can therefore be easily analysed using a simple blood test, enabling the genomic profiling of cancer for real-time diagnosis [54]. Then, another part of liquid biopsies for cancer diagnosis is urine testing which combine various categories, such as genomic profiling, protein profiling and microfluidic techniques [54].

However, the main method of diagnosing cancer remains the solid biopsy of the presumed tumor, followed by its visualization using ex-vivo imaging [29, 6]. The solid biopsy may come from surgery performed during treatment to remove the tumor or part of it [58]. Another option is to perform the biopsy prior to surgery, using a variety of methods as shown in Figure 2.6. For example, a needle biopsy is used to take samples from the breast, prostate, and liver by inserting a special needle through the skin. Another example is endoscopic biopsy, in which a narrow, light-emitting tube called an endoscope is inserted in the body thanks to a natural orifice. This endoscope is used to extract all or part of the abnormal tissue. During a colonoscopy, for example, the endoscope goes through the anus and enables the colon and rectum to be examined for the diagnosis of colorectal cancer [29, 6]. Another type of biopsy is the skin biopsy used to diagnose skin disorders such as melanoma. Here, cells are taken directly from the surface of the skin. A final example is bone marrow biopsy, which is frequently performed to detect blood cancers such as leukemia, lymphoma and multiple myeloma [6].

However, biopsy tissue sampling has several disadvantages. Firstly, there is a risk of biasing the analysis when the operator takes the sample in a certain location. In addition, the tumor may be in a difficult-to-reach location, and in the case of metastatic cancer it is difficult to take repeated samples from each metastasis [54]. For example, in the case of lung cancer, the tumors are located close to the respiratory or blood systems, making biopsy a very complex operation. Moreover, extracting a small sample of tumor tissue is hardly representative of the entire tumor, due to its heterogeneity [59]. The various disadvantages of biopsy make it an invasive and costly examination, with results that may be ambiguous [54].

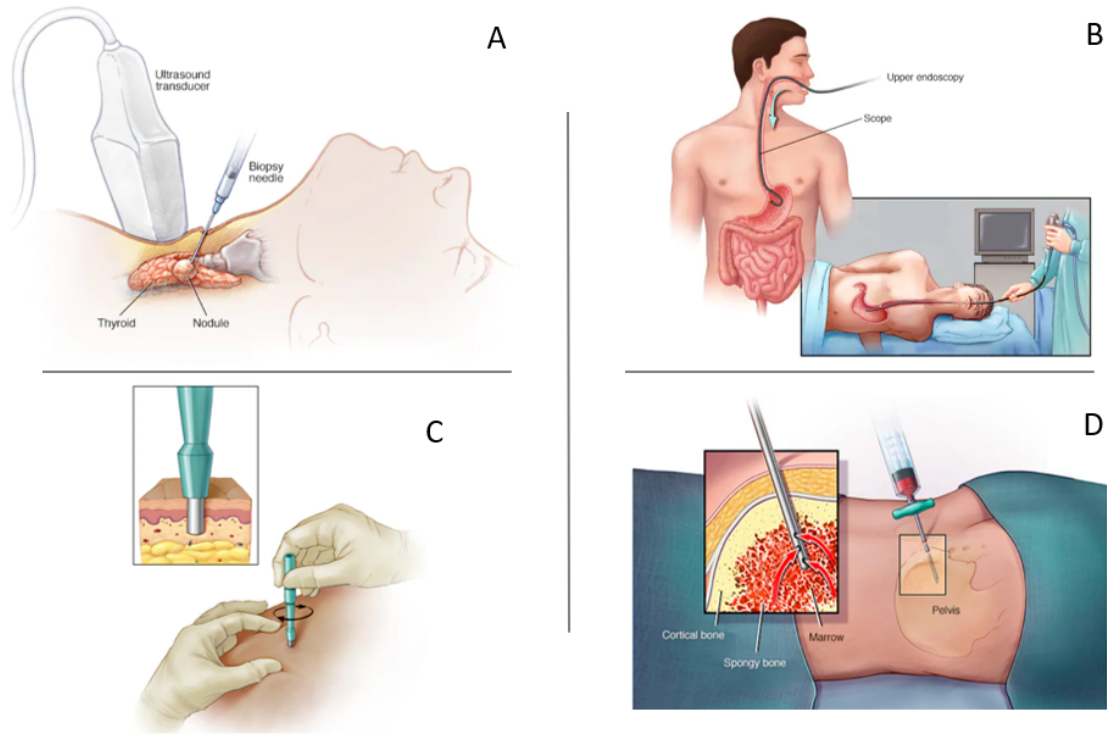


Figure 2.6: Different types of biopsy. (A) Needle biopsy, (B) Endoscopic biopsy precisely a bronchoscopy to get tissue from inside the lungs, (C) Skin biopsy and (D) Bone marrow biopsy [6]

2.5.5 *Ex-vivo* imaging

Ex-vivo imaging involves the extraction of cancer cells or of the entire tumor for analysis. As explained in previous section, this requires performing a biopsy to allow the visualisation of the tumor and its tissues. The main modality for diagnosing cancer remains imaging, and the most widely used technique is classical 2D histology [27, 60].

Classical 2D histology

Classical 2D histology consists of analysis under the microscope of the biopsy of the presumed tumor. Indeed, cancer cells are identified by their irregular size, shape, and borders, as well as larger and darker nuclei [61]. Histology is therefore a reference examination for tumor diagnosis [62, 63]. This technique involves anatomical study of tissues. To do this, cancerous tissue extracted by biopsy is prepared on histology slides and observed through a light microscope. Various steps for tissues preparation involve fixation in formalin, processing, embedding in paraffin, sectioning and, finally, staining of the tissues [57, 62, 63]. The last step is

very important, as the type of stain used will depend on the tissue to be visualized and analysed under microscope. Tissues can be stained with one or more dyes. The most commonly used stain is hemotoxylin and eosin (H&E), which visualizes the cytoplasm and stroma of cells in a pink tint and the nucleus in violet. Another widely used staining technique is immunohistochemistry (IHC) [57]. This technique is used to detect cancer type through the presence or absence of specific proteins using antigen-specific antibodies [57, 64].

In order to diagnose and differentiate the subtype of sarcoma present, there are 4 different parameters that are taken into account during histological analysis with H&E staining. Firstly, the predominant form of the cancer cells. In the case of sarcoma, 4 main categories can be distinguished, as shown in Figure 2.7: spindle, epithelioid, round (as high-grade myxoid liposarcoma) and pleomorphic (as pleomorphic liposarcoma or dedifferentiated liposarcoma). The next criterion is the pattern of growth. Indeed, among the different histological subtypes of sarcoma, some tend, for example, to arrange themselves in parallel to form fascicles of variable length, while others form roundish aggregates often surrounded by vascularized stroma. The third characteristic is the quality of the background, meaning the extracellular stroma in which cancerous cells are located. The final criterion is the architecture of the vascular network. In fact, the organization of blood vessels also helps to distinguish the sarcoma subtype. Blood vessels can be found in three architectures: plexiform, archiform and hemangiopericytoma-like architecture. In plexiform and archiform patterns, blood vessels are small and capillary-sized, but for plexiform they are forming a densely interconnected network whereas for archiform exhibit an arch-like shape. In hemangiopericytoma-like architecture, blood vessels branch widely, are dilated and have thin walls, creating a configuration resembling "deer antlers" [7].

On the other side, IHC plays a key role in tumor diagnosis, for example in soft tissue cancers [65]. Indeed, the characterization of well-differentiated liposarcomas (LPS) and dedifferentiated liposarcomas (DDLPS) can be made thanks to their specific molecular alterations such as the presence of extra ring chromosomes and giant markers. These structures contain amplified genetic material which contains several oncogenes, notably mouse double minute 2 (MDM2) and cyclin-dependent kinase 4 (CDK4), present in significant quantities [47, 65]. This overexpression of MDM2 and CDK4 makes their immunohistochemical staining extremely useful in the diagnosis of WDLPS and DDLPS, as shown in Figure 2.8 [47, 65, 8]. However, although nuclear reactivity for MDM2 and CDK4 is highly sensitive for these types of liposarcomas, it is not entirely specific as it can also be identified in other types of sarcomas. It is therefore preferable to use a combined positive stain for MDM2

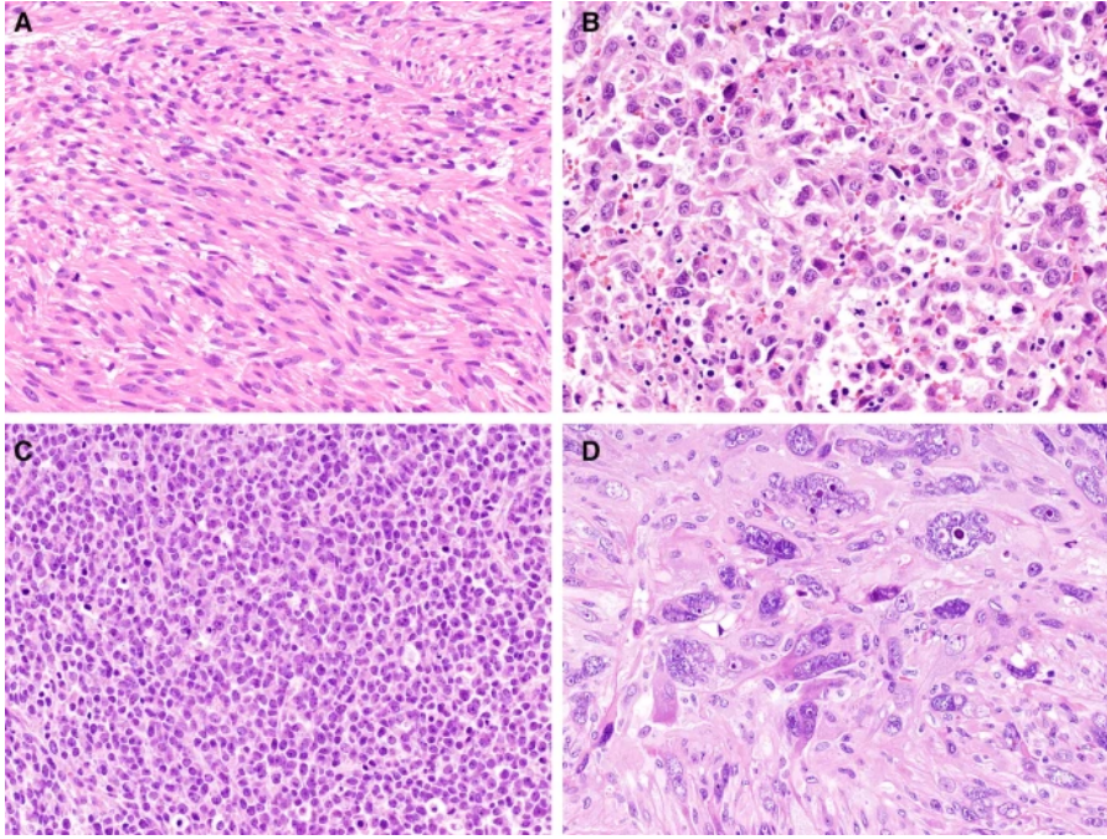


Figure 2.7: Different categories of cancerous cell shape on histological analysis with H&E staining. (A) Leiomyosarcoma representing characteristic spindle cell morphology with fascicle organization. (B) Epithelioid angiosarcoma forming abundant polygon-shaped cytoplasm. (C) Ewing sarcoma with round cell morphology (D) Undifferentiated pleomorphic sarcomas exhibit extreme nuclear pleomorphism. [7]

and CDK4 as a more specific diagnostic indicator [65].

However, histopathological assessment has some significant disadvantages. Firstly, it is highly invasive, costly, time-consuming. Indeed, after the tissue is sampled, it must go through various steps before analysis [62]. Secondly, histopathology requires specialists and pathologists to prepare and analyse histological sections [62, 60]. As diagnosis relies on the skills and experience of these pathologists, evaluation can be variable and subjective [60, 66]. A study of veterinary oncology analyses showed that when a second histological evaluation was carried out by another pathologist, in 20 to 34% of cases, a change in subtype or grade of the tumor, or another minor disagreement occurred. Furthermore, a complete disagreement, meaning a change between a benign and a malignant tumor, or two different

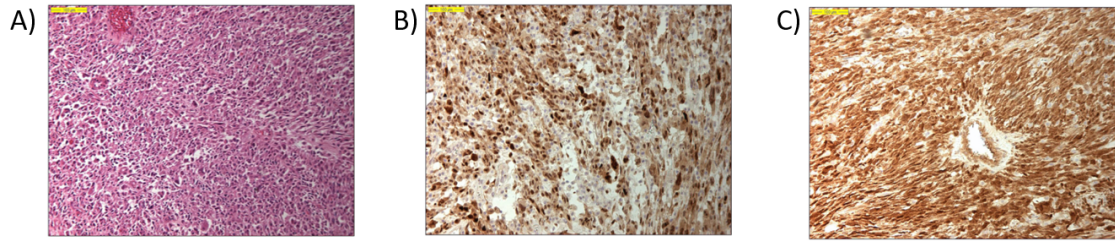


Figure 2.8: Dedifferentiated liposarcoma with different staining. (A) Hematoxylin and eosin, magnification 100x. (B) Mouse double minute 2 (MDM2) shows nuclear overexpression, magnification 200x. (C) Cyclin-dependent kinase 4 (CDK4) show nuclear overexpression, magnification 200x. [8])

diagnoses, occurred in 10 to 19% of cases [60]. This clearly demonstrates a variation in diagnostic sensitivity and specificity depending on the subjective assessment of the pathologist, but other factors such as the use of different antibodies or other fixation methods can also influence results [66, 67].

Regarding the analysis of tumor vasculature, a study demonstrated that immunohistochemistry significantly underestimates relative blood volume in contrast to the combination of *in-vivo* and *ex-vivo* CECT [68]. A commonly used method for quantifying angiogenesis is the histological estimation of microvascular density (MVD). This technique involves assessing the average number of micro vessels in a selected microscopic field [45]. For instance, one study used this technique to analyse tumor angiogenesis under different treatments. Four groups were investigated: a control group, a group with doxorubicin, a group with pazopanib, and a group with doxorubicin + pazopanib. To assess tumor vascularization, MVD was calculated as the average number of vessels positive for CD34 or VEGFR 2 immunostaining around the highest vascular density [47, 65]. Additionally, the total vascular area of vessels was calculated as the mean area of vessels positive for CD34. These data allowed for the evaluation of the antiangiogenic effect of pazopanib in liposarcomas. After a 2-week treatment, tumors in the control or doxorubicin treatment groups showed the highest vascular density, while two pazopanib-based treatments significantly reduced MVD by about two-fold and total vascular area of vessels by nearly three-fold [47, 65]. However, the use of MVD is invasive and cannot determine whether blood flow is occurring or if the vessels are hyperpermeable. Moreover, tumors are heterogeneous, and angiogenesis is often maximal at tumor periphery. As a result, MVD measurements will vary based on biopsy location, potentially leading to an underestimation or overestimation of the degree of angiogenesis [42].

Problem 1 : *Classical 2D histology requires the tumor to be cut and prepared before analysis. This preparation, as well as the subjectivity of the pathologist performing the analysis, can influence the results. In addition, quantification of the degree of angiogenesis can be overestimated or underestimated depending on the location of the biopsy, due to the heterogeneity of the tumors.*

3D histology

Classical histology and IHC are unfortunately limited to visualizing the tumor according to a single planar section of a sample. Furthermore, after the biopsy, tissues are embedded in paraffin, thus the orientation of the sectioning planes is set and is not easily adjustable [69]. To overcome the limitations of 2D visualization in classical histology, new techniques have emerged to perform serial sections to reconstruct the sample in 3 dimensions. An alternative is therefore to digitize glass slide images used for histology and reconstruct digital 3D images from serial glass slide images [63]. However, this approach requires considerable time and effort [63, 11]. In addition, scanning requires the entire paraffin block to be consumed in order to reconstruct the 3D image [63]. Another option is three-dimensional visualization of thicker biological samples using confocal or multiphoton microscopy techniques. Unfortunately, these imaging techniques have a very small penetration depth. Scanning and transmission electron microscopy were also considered, as both offer high-resolution 3D imaging. However, these techniques can only be applied to thin samples or samples near the cutting border [11].

Then, about tumor vascularization, serial histological sections or confocal microscopy have also been employed to reconstruct the vascularization of small regions. However, these techniques are severely limited in terms of reporting the complexity of trajectories and organization of vessels through tumor tissue [10]. However, even when performing 3D reconstruction of an entire tumor volume based on a series of histological sections, another drawback of histology remains. Indeed, cutting the tumor sample always induces tissue deformation, loss of certain parts of the tumor and creation of artifacts. All this leads to a fragmented representation of the tumor, which can interfere with the diagnosis [69, 12]. Furthermore, assembling the different sections into a single 3D image requires taking into account the artifacts caused by the cutting process [11].

Microfocus X-ray computed tomography

Microfocus X-ray computed tomography (μ CT) is based on the same principle as CT but has X-ray tubes with a reduced focal spot [70]. Indeed, micro-focus x-ray sources used in μ CT are composed of fixed tungsten anode and perform at lower

voltages and currents than CT [71]. This considerably improves the spatial resolution which reaches micrometres scale [70, 11]. μ CT has the advantage that analyzed tissues remain intact at the end of the examination and are not destroyed as in histology [63]. Another advantage is that μ CT is non-invasive [71]. Furthermore, this technique enables tumor samples to be visualized in three dimensions and cut at any point and in any direction, without destroying them [11, 72]. Moreover, the analysis of tumors by μ CT provided numerous structural parameters, such as tumor volume percentage, structural thickness, connectivity, porosity percentage and more [63, 63].

With regard to tumor vascularization, the 3D nature of μ CT enables visualization of vascular system, as well as quantification of angiogenesis by calculating, for example, vessel tortuosity [72]. However, although μ CT is very useful for *ex-vivo* tumor analysis, micro-CT technique can only be used to examine *ex-vivo* samples of human tumors. The reason for this is that the radiation dose used in micro-CT examination is much higher than in conventional tomography [63]. In addition, high-resolution scans can last several hours [71].

μ CT can be performed on formalin-fixed or non-fixed samples. However, one study demonstrated the presence of artifacts when using fresh lung samples. These artifacts are in fact caused by a too small contrast difference between air and tissue, or tissue shrinkage [63]. Another drawback is that, despite its advances, the resolution of μ CT remains inferior to that achieved with an optical microscope used for classical 2D histology. Consequently, it is still not practical to perform μ CT in place of classical histology for cancer diagnosis [63].

Synchrotron X-ray imaging

Synchrotron micro-computed tomography (SR μ CT) offers new perspectives in the 3D visualization of tissue samples with short acquisition times of the order of seconds or minutes [11]. SR μ CT achieves its high resolution by using a synchrotron to produce its X-rays. In general, synchrotron radiations are generated by moving charged particles at high speed along curved paths when applying magnetic fields. In the case of SR μ CT, X-rays are thus produced thanks to high-speed electrons circulating in a synchrotron, which significantly increase radiation intensity [73]. SR μ CT can be used for samples embedded in paraffin or agarose but also in combination with staining or phase-contrast protocols as seen in the next sections [11].

However, some disadvantages of SR μ CT are due to its high radiation dose of SR μ CT. The first problem is that SR μ CT radiations can interfere with the embedding material and damage it but fortunately it does not damage the tissue material itself. Moreover, these radiations create some requirements about the sample

preparation and staining. These drawbacks hinder SR μ CT to replace completely classical 2D histology. Nevertheless, this technique does seem to serve as a valuable complement [11].

Phase contrast imaging

Phase contrast imaging is of interest because of its greater sensitivity to density variations via phase changes. In fact, synchrotron-based X-ray phase contrast imaging offers high sensitivity and spatial resolution, enabling better tissue visualization [71, 17]. There are various methods for producing phase contrast imaging, and each technique has its advantages and disadvantages [18]. While some methods are based on integral calculations, most others play with the interference of x-ray waves passing through the object to be observed, as well as the diffraction and refraction phenomena of these waves. The principles behind techniques such as crystal interferometry, grating interferometry and propagation-based imaging are explained in Appendix C [17].

Synchrotron-based phase-contrast X-ray imaging has permitted the identification of various structures in tumors, such as necrotic tissue or blood clots, in any direction. These tissues were identified with a maximum spatial resolution of 26 μm which corresponds to the spatial resolution of low-power microscopic images. So, although the spatial resolution of synchrotron X-ray imaging based on phase contrast is still inferior to that of classical 2D histology, it has nevertheless enabled the identification of different tissue types inside a tumor. Furthermore, its ability to provide 3D information on whole organs offers a major advantage over classical 2D histology. Furthermore, this method also offers the advantage of being able to perform additional histological visualization after it [17].

Grating-based phase-contrast CT revealed small differences in tissue composition, whereas the differences in linear attenuation coefficients were too slight to be captured by a standard CT scan. Another major advantage is the visualization of vascular structures [9]. The results obtained with phase-contrast CT compared to other imaging techniques can be seen in Figure 2.9.

Propagation-based phase contrast imaging has improved image quality, even at lower radiation doses and is advantageous because it avoids damage to tissue samples or embedding material. In addition, propagation-based imaging improves soft tissue contrast, enabling virtual histology of unstained tissue samples [11]. Indeed, propagation-based CT enables 3D visualization and identification of tissue structures such as vessels, nerves and tumor stroma with good resolution [74].

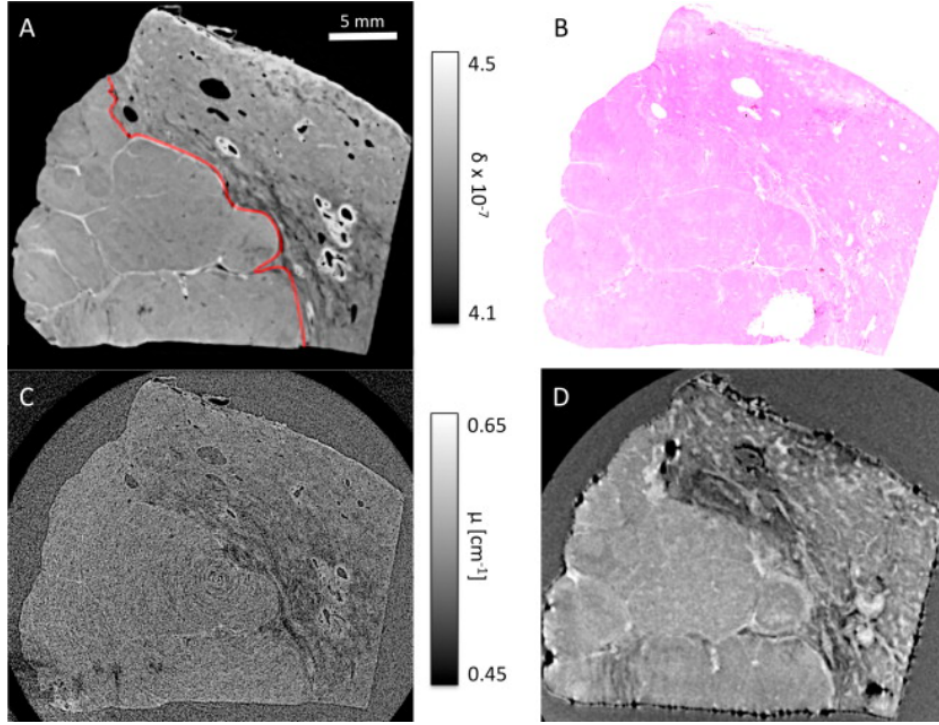


Figure 2.9: Imaging results of cancerous liver sample. (A) Phase-contrast CT (PCCT) with the red line delimiting the tumor present in the lower left corner from the healthy tissue above it. (B) Classical 2D histology (C) Absorption CT and (D) MRI. It can be seen that PCCT (A) and MRI (D) provide the best visualization of the morphological change associated with tumor pathology. In fact, the difference in color between tumor tissue and healthy tissue is more pronounced, enabling the edges of the tumor to be clearly seen. In addition, PCCT offers improved visualization of anatomical morphology such as soft-tissue and vascular structures. [9]

In summary, phase-contrast imaging techniques help solve the problem of low soft-tissue contrast found in standard X-ray imaging. In addition, these techniques offer high resolution identification of different tissues present within tumors, making them a promising tool for the diagnosis of different types of cancer [9, 75]. However, phase-contrast imaging setups like grating-based interferometers are specialized and less available than conventional X-ray systems. They require precise alignment and stability, making them sensitive to movement. Additionally, phase-contrast imaging currently has limited clinical experience and requires further technical improvements [76].

Contrast-enhanced micro-CT (CECT)

The introduction of contrast agents (CAs) into micro-CT will enhance the visualization of specific tissues by perfusing through or binding to them. The role of CAs is to increase the X-ray attenuation coefficient of the tissues through which they perfused or to which they have bound. There are therefore 2 types of CA: perfusion CAs (PCAs) and contrast-enhancing staining agent (CESA) [77, 78].

On one side, PCAs are widely employed for the visualization of blood vessels, as they infiltrate the vessels by perfusion. An example of a PCA for blood vessel analysis is Microfil [79, 78]. In fact, one study has used a polymerizing intravascular contrast agent (Microfil) and μ CT in combination with a computational method to visualize and quantify structural features of vessels *ex-vivo*. Microfil is a substance that solidifies inside blood vessels, enabling them to be visualized. Once μ CT data had been acquired, a direct three-dimensional maximum sphere analysis method was used to visualize and quantify three-dimensional structural features of blood vessels. This analysis enabled the identification of regions of hypoperfusion in tumors. These are areas in tumors where blood flow is insufficient, which could indicate necrosis. These newly generated images also helped to measure vessel thickness and intravascular distances, as seen in Figure 2.10 [10].

Another study also used the technique of Microfil perfusion-based vascular casting combined with *ex-vivo* CT imaging to visualize blood vessels and concluded that this allowed a highly detailed three-dimensional visualization of blood vessel distribution at a resolution of approximately 3.4 mm [68]. Furthermore, *ex-vivo* and *in-vivo* injection of Microfil were compared for visualisation of vasculature in lung and identification of the origin of vessels feeding the tumors using *ex-vivo* μ CECT. Both methods succeeded in filling pulmonary arteries to the level of alveolar capillaries and provided clear visualization of large vessels and microvessels in the lung. However, *in-vivo* injection of Microfil via the jugular vein filled small vessels more effectively than *ex-vivo* injection via the pulmonary artery. In addition, vessels filled *in-vivo* could be followed over longer distances and thus demonstrated better detection of vascular network and blood vessels origin. This difference between *in-vivo* and *ex-vivo* perfusion is due to differences in vascular pressures [72]. Indeed, perfusion pressure plays an important role in the use of PCAs. If excessive, it can damage tissues, whereas too low pressure will not completely fill blood vessels [79].

On the other side, CESAs stain tissue by passive diffusion when the tissue is immersed in the CA [79]. There are many different CESAs depending on the type of tissue to be targeted and each agent has its own advantages and disadvantages [11, 77]. For example, staining agents used with SR μ CT could be Phosphotungstic

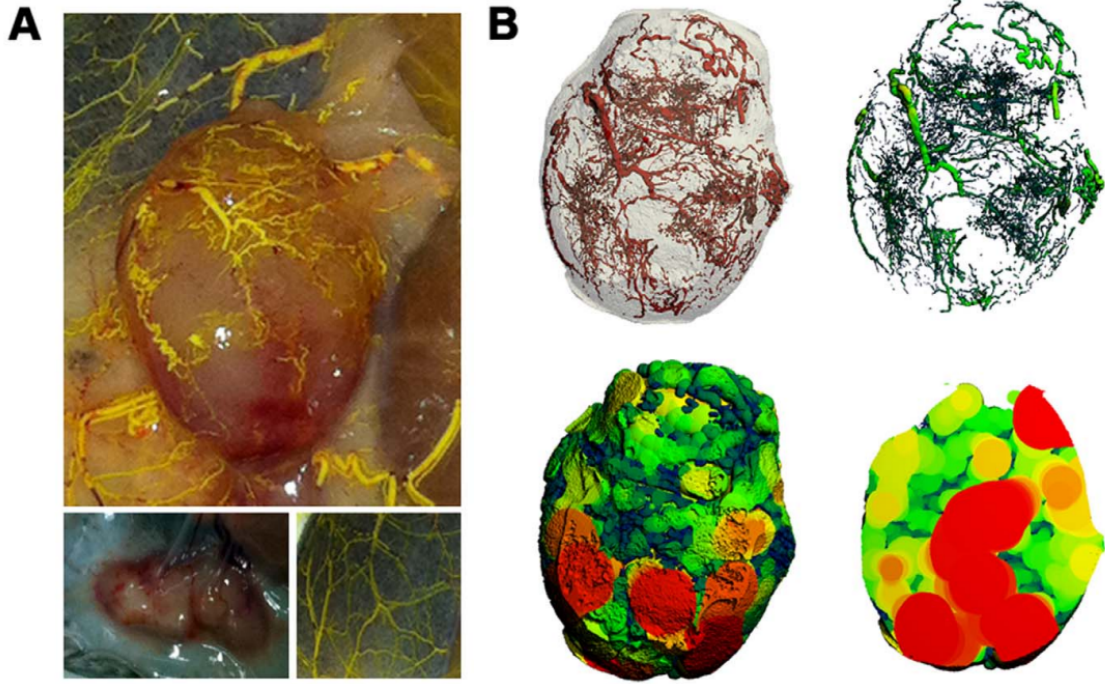


Figure 2.10: Microfil combined with μ CT (A) Microfil-perfused tumor with Microfil polymer in yellow. This polymer allows a better visualisation of blood vessels. (B) 3D computational reconstruction showing surface rendering of vasculature (top left), thickness of the vessels demonstrated by a heat map (top right) and vessel separation demonstrated with a sphere-filling model of the full tumor (bottom left) and of a cross-section through centre of tumor (bottom right). [10]

acid (PTA), Iodine (I_2) or chrome alum as shown in Figure 2.11. On the one hand, I_2 and chrome alum enable a short staining time but with a low contrast. On the other hand, PTA offers excellent contrast but requires a longer staining time. In addition, PTA and I_2 allows a good contrast for blood vessels visualisation [11].

Another study demonstrated the effectiveness of iodine in ethanol (I_2E) staining for μ CT to estimate tumor volume on an *ex-vivo* lung sample. As can be seen in Figure 2.12, staining with I_2E highlights tumors and unhealthy tissues significantly better than for unstained CT [12]. However, the size of estimated tumor regions diverges depending on whether it is calculated using I_2E staining μ CT or HE-stained histological sections. Indeed, μ CT image overestimates tumor volume, which can be explained by the fact that I_2E is not specific to tumor tissue, and therefore dead or inflammatory tissues can be assimilated to tumor. Furthermore, I_2E staining procedure can cause dehydration and shrinkage of the sample [12].

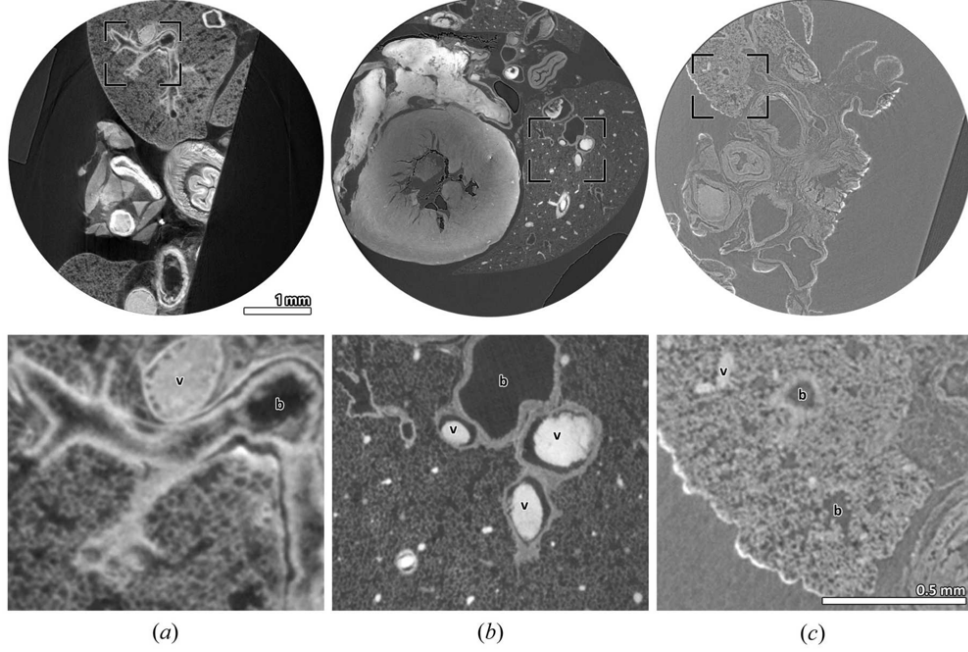


Figure 2.11: Comparison of 3 different staining agents for SR μ CT of mouse lungs. Mouse lungs was embedded in paraffin and scanned with SR μ CT scans with voxel size equal to 2.6 μ m. (a) Phosphotungstic acid (PTA) offers a very good contrast of the cartilage of the trachea as well as good visualization of the bronchi (*b*) and blood vessels (*v*). (b) Iodine (I_2) staining provided a strong contrast between blood vessels and bronchi. In fact, the clear staining of the red blood cells enabled the blood vessels to be distinguished from the respiratory system. (c) Chrome alum allows very little distinction of bronchi and lightly enhances contrast in blood vessels. [11]

Despite this, CECT has proved to be an interesting tool for tumor detection, as it provides high-resolution 3D information [12]. Concerning vasculature, CESAs like PTA or I_2 is beginning to emerge as a solution for better visualization of tumor vasculature using CECT [79]. Another example of a CESA used to visualize the vasculature is Hafnium-based Wells-Dawson Polyoxometalate (Hf-WD POM). This contrast agent is useful for analysing tumor vasculature as it binds to red blood cells and thus allows visualization of the vessel network [77].

Problem 2 : *The main concern when visualizing blood vessels by CECT is to check whether the contrast agent has been able to perfuse or diffuse correctly into the vessels. The main drawback of PCAs is that perfusion pressure impacts vessel visualization, which can be problematic in ex-vivo visualization where pressure is lower. The use of CESAs avoids this problem, although it is still necessary to ensure their proper diffusion in the vessels.*

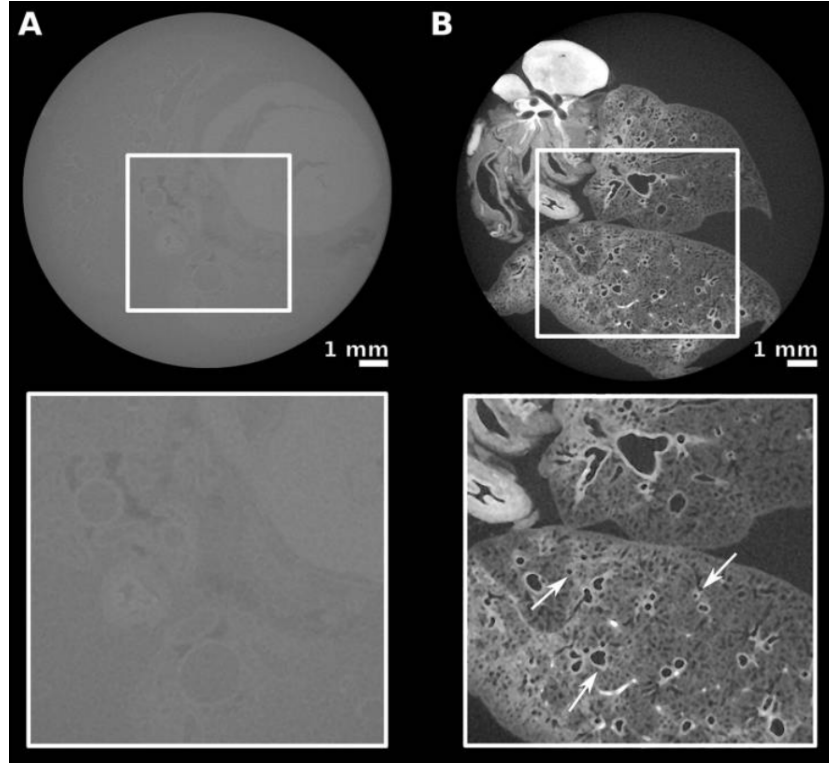


Figure 2.12: Comparison between unstained and stained CT of *ex-vivo* lungs sample. (A) Unstained CT and (B) iodine in ethanol (I_2E) stained pCT. The comparison between A and B demonstrates the usefulness of contrast agents for visualizing soft tissue in the lung. In image A, the gray values of the different lung tissues hardly change, which makes it difficult to visualize lung structure. However, in the image with the staining agent, high-contrast were achieved. I_2E enabled the identification of more detailed lung structures, such as connective tissue and vascular walls. The staining significantly improved the images contrast and the visualisation of the different tissues of the lungs such as lobe bronchi and small airways indicated by white arrows.[12]

2.6 Treatment

There is a wide range of strategies for treating tumors and there is no single treatment to cure cancer. The treatment chosen depends on the type of tumors and the stage of the cancer [80]. For sarcomas, like most cancers, conventional treatment strategies can be divided into three categories: surgery, radiotherapy and systemic therapies, including chemotherapy [36, 13]. These different treatments are illustrated in Figure 2.13.

In most cases, treatment involves a combination of different strategies. For example, local treatment like surgery or radiotherapy can be combined with systemic treatment, such as chemotherapy, to improve treatment efficacy. This combination

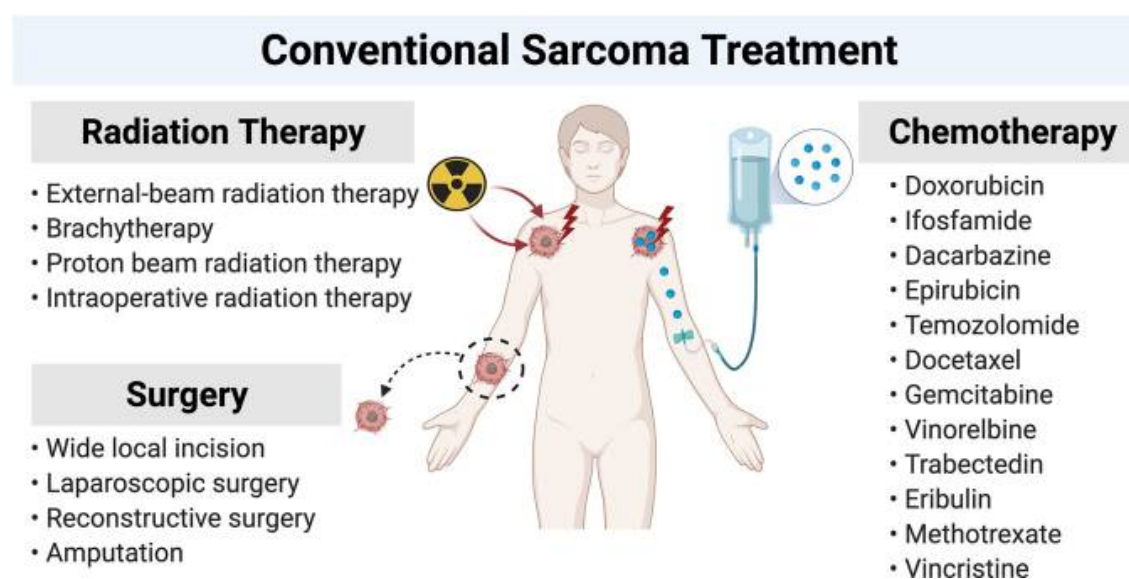


Figure 2.13: Three conventional categories of treatment for sarcoma : radiation therapy, surgery and chemotherapy with example for each type. In this image, systemic treatments are represented only by chemotherapy, but it is important to note that there are also targeted therapy, immunotherapy and hormonal therapy. [13]

can be neoadjuvant, meaning that chemotherapy is performed prior to surgery, or adjuvant, meaning that it is delivered postoperatively [36]. In the first case, neoadjuvant treatment helps reducing the volume of the tumor to facilitate surgery, or enhancing the effect of main treatment. In the second case, the aim of adjuvant treatment is to prevent cancer relapse due to the presence of micro-metastases [23, 81]. In addition, even for early, treatable stages, palliative care can be prescribed to manage and reduce symptoms and pain [22]. Indeed, once cancer reaches an advanced metastatic stage, patients are eligible for palliative therapy and the role of these kind of treatments is no longer curative. In fact, the aim of palliative treatments is to extend the survival of the patient by slowing the progression of the disease. In addition, these treatments are intended to improve the quality of life for the patient by attenuating the symptoms of the cancer [23, 25].

Surgery

Surgery is the main treatment for localized cancer, mainly corresponding to early stage disease, and is therefore the most commonly used treatment for localized liposarcomas [22, 45]. This treatment is widely used for early stage solid tumors, as it has been shown to reduce the risk of cancer-related death and increase long-term survival for different types of cancer [82]. In addition, surgical resection can be combined with other treatments, mainly radiotherapy and chemotherapy [82, 83].

Surgery involves removing the whole tumor, or the largest possible part of it. Whereas some tumors can be easily removed, others may be located in hard-to-reach or close to organs areas and prevent the total removal of the tumor. There exist several types of surgery, depending on the size and location of the tumor to be resected [83]. In most cases, the surgeon performs a total resection. This involves the entire removal of the tumor, with or without an additional amount of normal, healthy tissue around it, known as "surgical margin" [82, 84]. This margin enhances the chances of a successful operation and prevents the cancer from relapsing, making this procedure preferable. In other cases, the resection is partial and only a part of the tumor can be removed [82, 83].

For localized STS, the main treatment is surgical resection with negative microscopic margins, whatever the histological type. The aim of surgery with zero margins is to preserve the limb, particularly when the tumor is located in the extremities, in order to maintain limb function [84]. These negative margins make it possible to avoid amputations, and in the case of STS, around less than 5% of primary STS lead to amputation, compared with 9% to 14% for recurrent disease. These amputations are therefore avoided as much as possible, except when a resection with adequate margins is not feasible without compromising limb functionality [84]. However, although this is the first-line treatment for localized cancer, it is not inevitable that the cancer will relapse after surgery [41]. Indeed, it has been unfortunately shown that around 25% of STS cases will progress to metastatic disease, even after surgery of the primary tumor [84].

Radiotherapy

Developed in the middle of the 20th century, radiotherapy is used to treat cancer, either independently as single modality or in conjunction with surgical procedures or systemic treatment [22]. For example, combining radiotherapy and chemotherapy allows the effects of each modality to be linked for greater efficacy. Indeed, radiotherapy helps to treat large primary tumors, while chemotherapy will act on widespread small metastases. This also enables more powerful treatment without exceeding the dose-limiting toxicities of the two modalities [22].

Radiotherapy uses ionizing radiations such as X-rays, gamma rays, or radioactive particles as a source of treatment [22]. These radiations cause DNA breakage and consequently cell death. Radiation is delivered via a beam, which may be external or internal. The most common type is external radiotherapy, which uses radiation from a device positioned outside the body, such as proton therapy. Internal radiotherapy, conversely, is known as brachytherapy, and uses radioactive

material inserted inside the body adjacent to cancerous tissue. The two types of internal radiotherapy are permanent implants or temporary internal radiotherapy [85]. Further explanations of the principle of radiotherapy, the different types of treatment available for STS and the side-effects of this type of treatment can be found in Appendix D.

Systemic treatment

Metastatic cancers, conversely to localized cancers, affect several organs and therefore require more intensive treatment [39]. For this type of cancer, systemic therapies such as chemotherapy, targeted therapy, immunotherapy and hormonal therapy are preferred [23]. Other treatments, such as combination therapy or stem cell and extracellular vesicle therapy, can also be used [80]. However, metastases remain complex to treat, and most of them are even incurable due to their acquired resistance to existing therapies. Furthermore, the response to treatment varies radically between primary or metastatic disease in the same patient [39]. Systemic therapies can be used in different ways: curative, adjuvant, neoadjuvant or palliative [23, 81, 84].

The most common systemic treatment is chemotherapy which either interact with DNA, RNA, or target essential proteins required for cell division to disrupt the cell cycle and induce cell death by apoptosis [23, 81]. Unfortunately, chemotherapeutic agents can also be toxic to healthy cells, particularly bone marrow and mucous membranes. It is therefore necessary to plan cycles of chemotherapy to allow normal tissue to repair itself between each cycle. Unlike healthy tissue, tumors have less capacity for recovery, so repeated cycles of chemotherapy will reduce the tumor population over time [23].

The most commonly drug used for STS chemotherapy is doxorubicin, but epirubicin, ifosfamide or trabectedin may also be administered [84, 36]. Doxorubicin-based chemotherapy has shown the best results in terms of response in patients with locally advanced or metastatic STS [86]. Unfortunately, the side effects associated with chemotherapy are numerous. A major disadvantage is also toxicity, restricting the dose of doxorubicin that can be administered. Indeed, although doxorubicin is the most common drug for STS chemotherapy, it possesses a cumulative cardiotoxicity effect. This results in decreased left ventricular function and other adverse cardiac events [36]. Appendix E lists different uses of chemotherapy in STS and other possible side effects.

Following the many drawbacks of chemotherapy, other systemic treatments have been developed for STS such as targeted therapies. These targeted therapies

have been deployed thanks to a better comprehension of the molecular biology of tumor and the signalling pathways involved in their development [23]. For example, molecular targeted therapies have been developed to inhibit tumor growth by targeting cell signalling or angiogenesis, or by blocking protein degradation [81]. One example of targeted therapy on cell cycle progression is the use of cell cycle inhibitors, which can be used for the treatment of sarcomas [13]. Another solution is the use of immune checkpoint inhibitors such as PD1, PDL1 and CTLA 4 which are commonly used in the treatment of various forms of cancer [81]. Such inhibitors induce immune activation against cancer cells and play a key role in immunotherapy [24]. Moreover, hormonal treatments and stem cell-based therapeutic strategies are employed for cancer treatment as STS treatment [80, 84]. Nevertheless, the focus of this thesis is on targeted therapy using antiangiogenesis drugs.

As explained in Section 2.4.1, angiogenesis plays a major role in the development of tumors and metastases. This is why research has been trying for several years now to develop drugs that would prevent or slow down angiogenesis to stop the development of cancers. Therefore, antiangiogenic drugs are designed to inhibit the formation of new blood vessels [87]. These angiogenesis inhibitors have the advantage of being less toxic than conventional chemotherapy and have a lower risk of drug resistance. Currently, approximately more than 30 angiogenesis inhibitors are in clinical trials in the United States for the treatment of cancer, but also other diseases such as macular degeneration and diabetic retinopathy [43].

Antiangiogenic drugs can be classified into two main categories. On the one hand, there are analogues of natural angiogenesis inhibitors occurring naturally in the body, such as endostatin and angiostatin. On the other hand, there are inhibitors of proangiogenic factors, such as anti-vascular endothelial growth factor drugs [42]. Given that nearly 60% of malignancies have elevated VEGF concentrations, the focus of research on antiangiogenic drugs has shifted toward inhibition of the VEGF pathway. Approaches to inhibit this pathway involve the use of antibodies against VEGF or vascular endothelial growth factor receptors (VEGFR), soluble VEGFR/VEGFR hybrids, and tyrosine kinase inhibitors [43, 88].

In this thesis, the antiangiogenic drug used is pazopanib which is a tyrosine kinase inhibitor used and approved for most of the STS subtypes with the exception of gastrointestinal stromal tumor and adipocytic sarcoma [89, 36]. This tyrosine kinase inhibitor has multiple targets such as VEGFR 1, 2 and 3, platelet-derived growth factor receptors alpha and beta, as well as the tyrosine kinase inhibitor receptor [36]. Research has already supported the use of pazopanib treating patients with renal cell carcinoma and advanced non-adipocytic STS. *In-vivo* activity of

pazopanib in DDLPS was also studied by measuring the tumor volume using a calliper and calculating the relative tumor volume in percentage compared to day 0. This showed that the treatment with pazopanib significantly delayed tumor growth compared to controlled animals. After 14 days of treatment, tumor volumes in the control group increased approximately four to five times while with pazopanib administration, tumor volumes were one to two times higher than those on day 0 [45, 90].

Problem 3 : *Due to the numerous side effects of chemotherapy and especially the cumulative cardiotoxicity of doxorubicin, it is necessary to find new treatments. Antiangiogenic drugs show promise in reducing tumour size. However, their efficacy in reducing tumor vascularization remains to be demonstrated.*

Chapter 3

Problem statement and objectives

As seen in the literature review, there are several different techniques for visualizing tumors, both *in-vivo* and *ex-vivo*. However, each of visualisation techniques has its advantages and disadvantages. Indeed, when it comes to visualizing and quantifying tumor vascularization, current imaging techniques have certain limitations.

This thesis focuses exclusively on *ex-vivo* visualization of tumors, for which classical 2D histology remains the reference examination. However, this technique is time-consuming and does not allow very good visualization of blood vessels because of this 2D representation. Indeed, information provided by classical 2D histology depend on the subjectivity of the analysis by the pathologist and on the location selected since tumors are heterogeneous. Furthermore, the process of cutting tumor sample can induce tissue deformation, loss of certain parts of the tumor and creation of artifacts. All this leads to a fragmented representation of the tumor, which can interfere with the results obtained. This reflects the main disadvantage of classical 2D histology, which allows visualization of vasculature only through 2D slices, which can often lead to slice-dependent conclusions.

To counter these various limitations, the general aim of these thesis will be to assess the complementarity and added value of contrast-enhanced microfocus X-ray computed tomography (CECT) compared to classical 2D histology. Indeed, CECT is being developed to offer *ex-vivo* 3D visualization of tumor tissues, vasculature and necrosis by using contrast-enhancing staining agents (CESAs). This thesis will focus on the visualization with CECT of 2 tumor types called liposarcomas, which belong to the category of soft tissue sarcomas (STS).

The first objective will be to compare and evaluate the correspondence between tumor visualisation using classical 2D histology and with CECT. To do this, blood vessels, necrosis and adipocytes will be identified on the basis of the two imaging

modalities. This comparison will give a better idea of the appearance and composition of the tumors, as well as the visible features using each imaging technique. The second objective will be to improve the visualisation and analysis of vascularization in tumors during post-processing of CECT data. The main focus of this objective is to develop the segmentation of blood vessels in CECT images in order to demonstrate that CECT can identify blood vessels in a way that is comparable or even more complete than histology. The third objective is to investigate the efficacy of *ex-vivo* CECT visualization in quantifying the influence of antiangiogenic drugs on these tumors. The antiangiogenic drug used is pazopanib, a tyrosine kinase inhibitor. The visualization of tumors with or without antiangiogenic drugs will enable us to deduce whether the drug is indeed having the effect of inhibiting the growth of tumor blood vessels and thus reducing tumor development. To do this, it will be necessary to extract quantitative information from the vessels identified in the CECT scans and analyse it both visually and computationally. This step should show that CECT provides complementary information to classical 2D histology, such as analysis of the 3D structure of blood vessels by observing their organisation, volume fraction and thickness.

Chapter 4

Visual comparison of tumors imaged by CECT and classical 2D histology

4.1 Introduction

In this first chapter, images of tumors obtained by CECT and classical 2D histology will be analyzed and compared. The aim is firstly to carry out an initial analysis of the tumors, their morphologies and the identification of characterizing features such as blood vessels, necrosis and adipocytes. The identification of these various elements will enable tumors to be compared with each other. Nevertheless, the visualization of these various elements will above all help to establish a comparison between images visualized using CECT and those visualized using classical 2D histology. In fact, both imaging modalities will be evaluated on their ability to provide a clear and detailed visualization of tumors and their various components. In addition, this comparison will serve as a basis for justifying the segmentation that will be carried out in the following chapters.

4.2 Materials and methods

4.2.1 Preclinical models

To study the response of tumors to a new treatment, it is necessary to conduct preclinical trials during which cancer cells are exposed to the drug to be tested. Over the last few decades, the process of testing and harvesting these cancer cells has evolved. First of all, *in-vitro* cultured cells have been used, but this hardly represents the real environment in which tumors are located neither stroma nor

immune systems are present. However, this technique allows rapid cell growth [90].

A more realistic option is to use xenografts, meaning implanted cells or tissues from a species different from the one on which these cells or tissues are grafted. The first type of xenograft is created by subcutaneous injection of tumor cells into immunodeficient mice. These implanted murine models are referred to as cell line-derived xenografts. However, cell line-derived xenografts still have certain disadvantages which make them not fully representative of tumor growth environment in the patient. Indeed, there is only a modest diversity of molecular subtypes and limited intratumorally heterogeneity [90].

The second type of xenograft is the patient-derived xenograft. This type of xenograft is established by grafting human tumor tissue into immunodeficient mice. This patient-derived xenograft comes from a tumor removed during the surgical resection of patient and the patient-derived xenograft model has been shown to maintain the histological, molecular, and genetic characteristics of the donor tissue. As shown in Figure 4.1, patient-derived xenograft model is a valuable tool for preclinical testing of drugs and will be the model used in this thesis, as it offers a study environment closer to reality [90, 14].

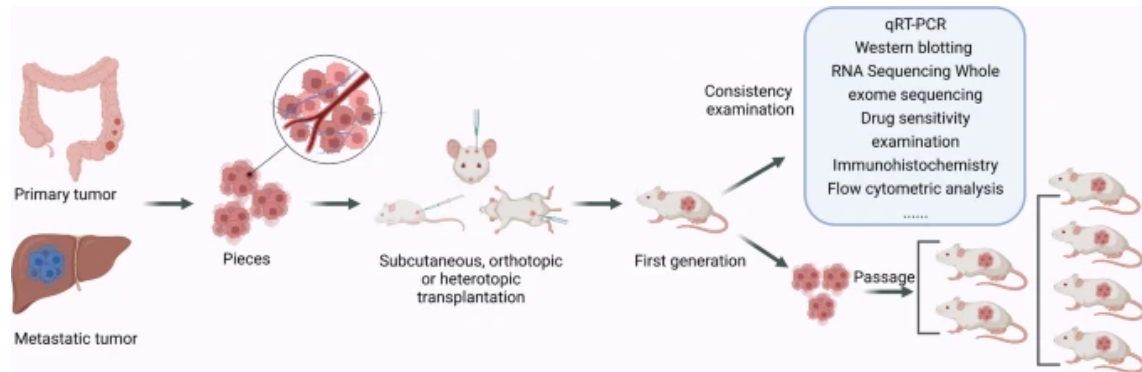


Figure 4.1: Use of patient-derived xenograft in cancer therapy. First, the tumors are achieved from surgical resection in human patients. Then, these tumors are cut into pieces and grafted into immunodeficient mice. These mice will undergo several tests and the tumors will then be collected to be transplanted again into other mice. [14]

4.2.2 Sample

The effects of an antiangiogenic drug called pazopanib were tested on immunodeficient mice, specifically NMRI nu/nu adult female mice. These mice were bilaterally subcutaneously transplanted with patient-derived STS on their backs. The STS

xenografts are human dedifferentiated liposarcoma, namely UZLX-STS3p.33 (for Experience n°113) and UZLX-STS5p.25 (for Experience n°114) and were surgically harvested in the Department of Surgical Oncology or Orthopedic Surgery, University Hospitals Leuven, Belgium. The mouse population was further expanded by mouse to mouse retransplantation of the tumor.

In the two experiments, mice were randomized into 2 groups: a control group and a group receiving pazopanib. Mice were orally treated twice daily with either water (control group) or 40mg/kg pazopanib. Treatment lasted for 2 weeks and tumor volume and body weight measurements were performed 3 times a week. At the end of treatment, mice were euthanized and tumors were collected. After harvesting, tumors were stained by passive diffusion with Hf-POM, a non-destructive contrast-enhancing staining agent (CESA). Then, *ex-vivo* CT scan and histopathological evaluation have been carried out. Histological evaluation was performed using 2 stains: H&E and CD31.

4.2.3 CECT image acquisition and reconstruction

After tumors were stained with Hf-POM and formaldehyde-fixed, CECT scans of them were acquired using a Phoenix Nanotom used in fast scan mode with parameters summarized in Table. 4.1.

Table 4.1: Acquisition parameters for CECT of tumors

Parameters	Values
Voxel size	6 μm
Number of images	2400
Exposure time	500 ms
Total time	20 minutes
Voltage	60 kV
Current	420 μA
Mode	0
Source-detector distance (ZD)	224.999375
Source-sample distance (ZS)	3.499250
Average	1
Skip	0

4.2.4 Classical 2D histological assessment

After CECT scanning, formaldehyde-fixed tumors were embedded in paraffin for histological analysis. The paraffin block was then cut into 4- μm sections and stained

with hematoxylin and eosin (H&E) and CD31 for immunohistochemical analysis. Histological sections were visualized and analyzed using *OlyVIA* software. Then, comparison between CECT scan and histological section of tumors was performed using *Avizo* software.

4.3 Results and discussion

As the aim of this master thesis is to demonstrate the matching of CECT with classical 2D histology, it goes without saying that one of the first steps is to compare what can be observed with these two modalities, and to highlight their similarities and differences. This comparison between tumors visualization with CECT and classical 2D histology also serves to justify that what has subsequently been segmented in CECT does indeed correspond to what is generally observed and quantified with histology.

The first aspect that stood out when visualizing the tumors in CECT was the presence of large dark areas on all the tumors of Experience 113. Indeed, very marked gray value differences were present in both control and pazopanib tumors. In contrast, the tumors of Experience 114 showed no such differences in gray values. Initially, these significant differences in gray value may suggest necrosis, but a comparison with classical 2D histology led to a reconsideration. As can be seen from Figure 4.2, the darker areas in CECT correspond to pinker areas in classical 2D histology. If one takes a closer look at these areas, as illustrated in Figure 4.3, there are no morphological differences, either in terms of cells or nuclei, between these pinkish areas and the surrounding areas, which are more bluish, purplish in color. There are therefore no characteristic features that could attest to the presence of necrosis. This difference in coloration on both CECT and histology is in fact due to a lack of CA. Indeed, Hf-POM, the CA used here, can have an influence on the staining of H&E and therefore alters its color without changing its specificity. The pinker-colored areas therefore retain their color from H&E staining in the absence of Hf-POM, while the neighboring areas, which have a more bluish/ purplish color, obtain this coloration from the mixing of Hf-POM with H&E. As far as CECT is concerned, the dark areas therefore also represent zones where CA has not diffused well. In addition, completely black zones attest to a total absence of CA and consequently it is not possible to visualize the differences in density at these points.

Detecting these areas where CA has not been diffused properly reveals a major problem for quantifying vascularization. Indeed, the absence of CA prevents the proper visualization of blood vessels and therefore may lead to an erroneous conclusion. It is therefore necessary to assess which tumors are affected by this

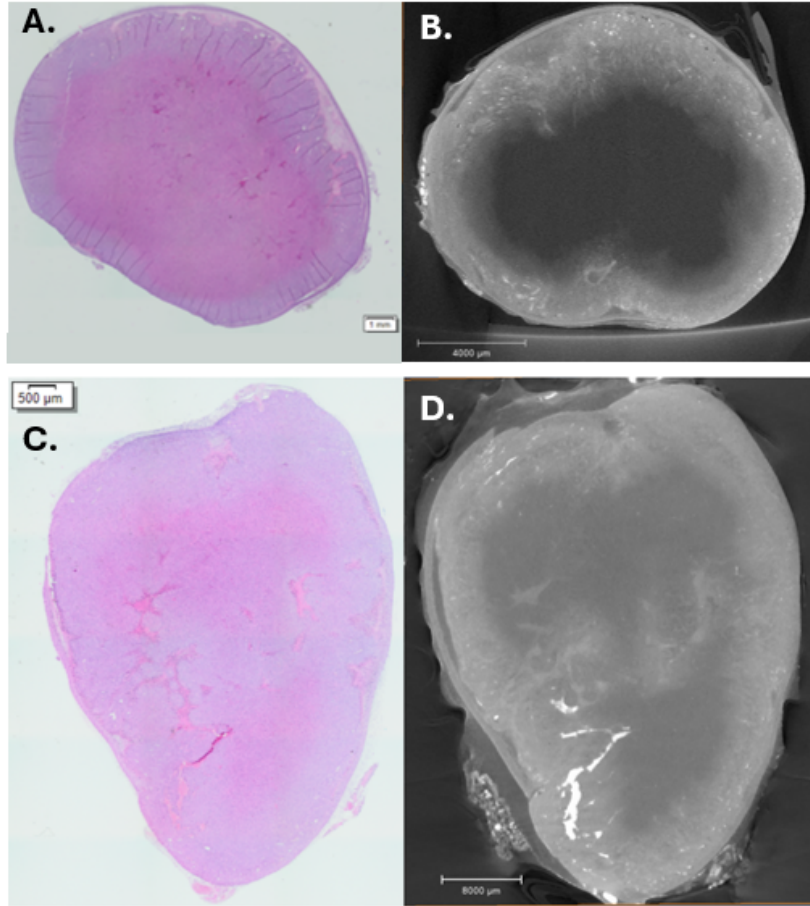


Figure 4.2: Visualization of control tumors of Experience 113 which present large dark areas in CECT that correspond to pinker areas on classical 2D histology. A. Classical 2D histology of CARLL tumor. It can be seen that the centre of the tumor is pinker in color than the edges of the tumor, which are bluish or purplish. B. CECT of CARLL tumor. The same central area as in A. is visualised as completely black on CECT with dark grey margins. The edges of the tumor, in contrast, are light grey with white dots which may be blood vessels. C. Classical 2D histology of CALLL1 tumor. D. CECT of CALLL1 tumor. There is also a correspondence between the pinker areas of the tumor on histology and the darker areas on CECT.

phenomenon, and measure the extent of the areas affected. These observations led to be able to attest to the reliability of each tumor. Indeed, reliability has been established on the basis of the appearance of tumors on CECT and on classical 2D histology, the diffusion of CA and the percentage of darker zones corresponding to areas where CA has not been diffused correctly. The various criteria and explanations are detailed in Appendix G. Following this analysis, it was concluded that only 4 tumors from Experience 113 could be considered reliable, whereas all 16 tumors from Experience 114 could.

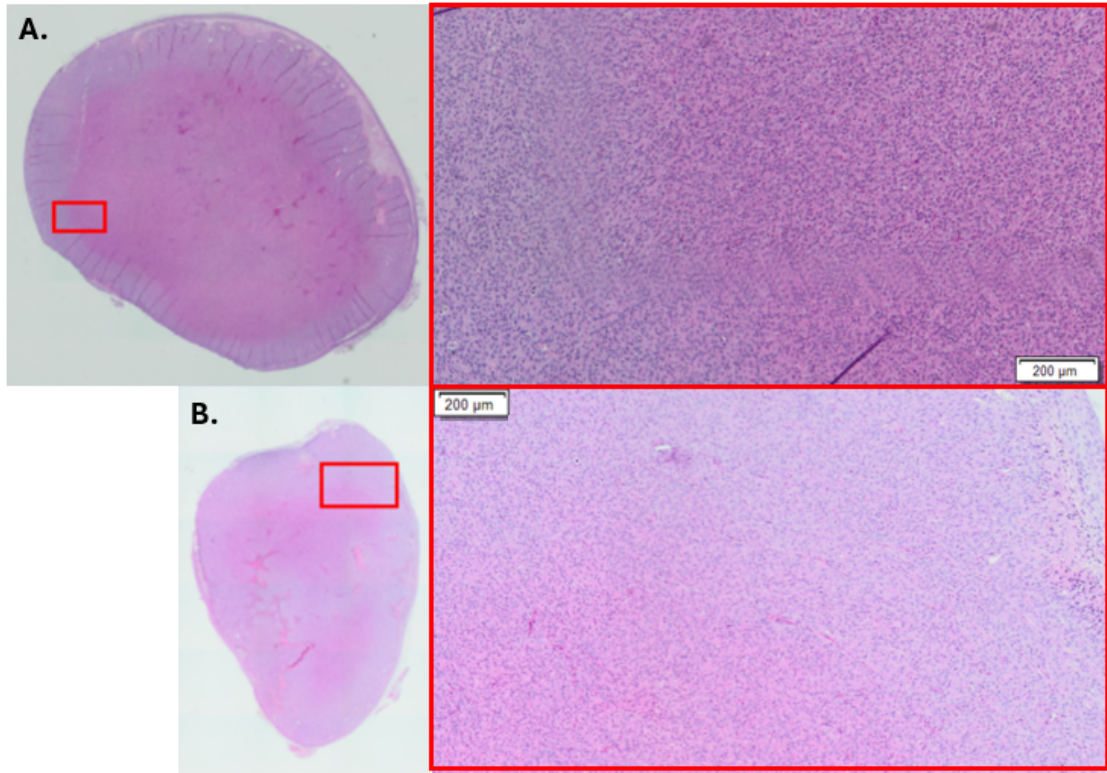


Figure 4.3: Classical 2D histology of CARLL tumors (A.) and CALLL1 (B.) from Experience 113. The right side shows an enlargement of the area in the red box located on the tumor on the left side. The areas observed correspond to the change from a pinkish to a bluish-purple color. It can be seen that apart from the difference in color, there is no morphological difference between the two areas. In fact, there is no change in terms of cell density or cell nucleus morphology that could, for example, indicate the presence of necrosis.

The importance of investigating the correct diffusion of CA is essential, as a difference in contrast diffusion can lead to altered visualization of blood vessels, and thus result in an erroneous conclusion due to an incomplete view of the vasculature. One point to note is that, even if tumors for which incomplete staining has been identified have been discarded, there is still a possibility that, among the tumors retained, some present deficient staining leading to missing blood vessels. This problem of incomplete CA penetration may be due to the fact that the tumors were small and, above all, very, very solid. The hardness of the tumors meant that staining could have taken a very long time, and even cause the CA to fail to reach the interior or densest parts of the tumor. During the experiment, these tumors were stained at different time intervals and the scans analyzed here are those from which no further improvement in staining was observed, but this does not confirm

that the CA had time to diffuse throughout the tumor. As can be seen from the figure 4.2, the lack of staining often appears in the center of the tumor, which is the most distant region and therefore the hardest to reach. However, some tumors present a particular phenomenon in which areas of the center have been stained while others have not, as shown in Figure 4.4.

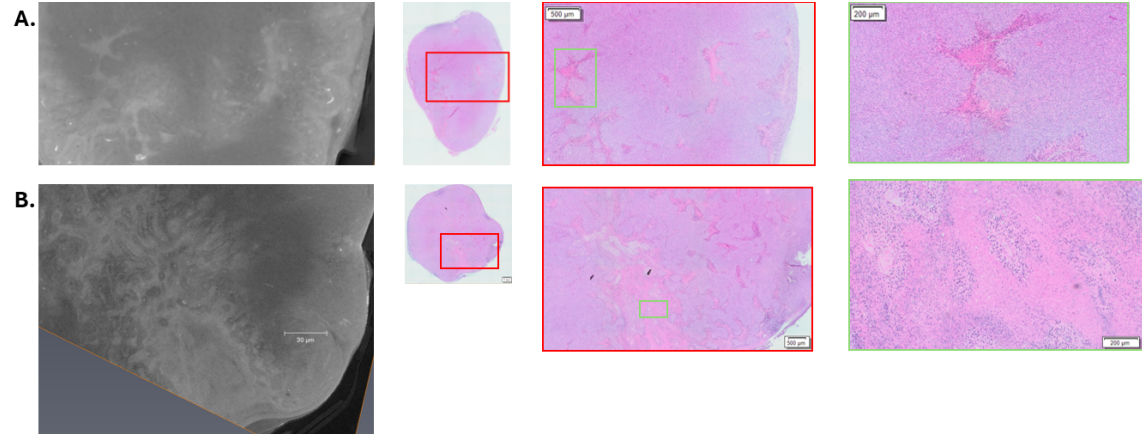


Figure 4.4: Tumors CALLL1 (A.) and CBLRL1 (B.) from Experience 113. From left to right: CECT of the tumors zoomed in on the lighter areas that appear in the centre of dark areas; then identification of these areas on classical 2D histology; enlargement of the area in the red box and finally enlargement of the area in the green box. It can be seen that the brightest areas on CECT correspond to the areas where cell density is lowest, or even absent on histology.

By observing these same areas with classical 2D histology, it can be seen that these areas correspond to zones where cell density is very low, and therefore to possible necrosis. As the areas are less dense, this can justify the fact that CA had an easier time diffusing into them than into denser healthy tissue. These areas of lower cell density therefore appear lighter on CECT than the surrounding healthy tissue, which will be darker or completely black, depending on the amount of CA that has managed to infiltrate. Incomplete diffusion of CA thus enables us to distinguish between more and less dense areas of the tumor, thanks to the difference in ease of penetration between the two types of tissue. However, this does not provide a sufficiently reliable means of identifying necrosis. In addition, the poor representation combined with the wide diversity of phenotypes led to an inconclusive comparison of necrosis identification between classical 2D histology and CECT. As far as histology is concerned, necrosis was mainly identified on Experience 113 tumors. However, as explained above, this experiment presents tumors for which CA diffusion was incomplete and are therefore considered unreliable on CECT. It goes without saying that no strategy for the identification of necrosis could be carried out on the basis of these tumors. On the other hand, with regard to

Experience 114, no necrosis could be identified with certainty on the histological sections, leading to an inconclusive comparison with CECT.

In terms of blood vessels identification, large and medium-sized vessels were easily matched between classical 2D histology and CECT, as shown in Figure 4.5. However, on classical 2D histology, a number of very small blood vessels were found, mainly in the center of tumors. On CECT, unfortunately, these blood vessels appear very faintly, relatively blurred in some cases, or even absent in others. The absence of these very small blood vessels on CECT can be explained by several reasons. Firstly, there is still uncertainty about perfect diffusion of CA within the tumors. As these small vessels are mainly located in the center of the tumor, they may not have received enough staining to be visible or simply distinguishable. Another reason for the absence of these very small vessels on CECT is that CA binds to blood cells such as red blood cells, so if there is no blood, there will be no staining. On the other hand, CD31 binds to a transmembrane glycoprotein expressed by endothelial cells and platelets [68]. It is therefore possible that CD31 could detect very small blood vessels even in the absence of blood, whereas CA cannot.

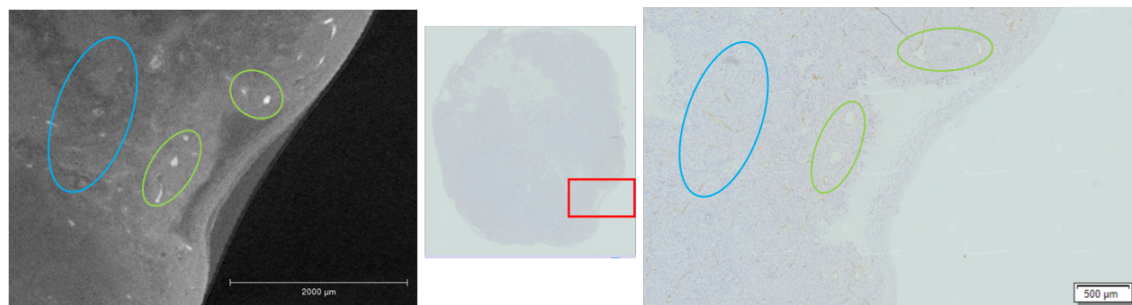


Figure 4.5: Tumor CARLR from Experience 113. Left: CECT of the tumor, showing blood vessels in white. Middle: location of the area visualised (framed in red). Right: Classical 2D histology of the tumor on which blood vessels appear orange/brown. The white dots on the CECT can easily be matched with the white dots surrounded by brown/orange on the histology as shown for the blood vessels circled in green. However, on the histology image, there are numerous orange/brown lines and dots on the inner side of the tumor which do not correspond distinctly to CECT features as can be seen in the area circled in blue.

However, a major shortcoming of classical 2D histology is its ability to assess the density of blood vessels distributed throughout the tumor. Indeed, 2D visualization of classical histology can lead to erroneous conclusions due to its limited vision and dependence on the slice analyzed. CECT, on the other hand, offers 3D visualization and identification of each vessel, as well as a representation of vascularization over the entire tumor. This information is invaluable for estimating

vascular density. To support this comparison, Chapter 6 will look at the quantification of blood vessels using these two modalities, and the correlation between them.

A characteristic feature of liposarcoma is the presence of adipocytes, fat storage cells. These adipocytes have been observed in a number of tumors and were visible on both classical 2D histology and CECT, as shown in Figure 4.6.

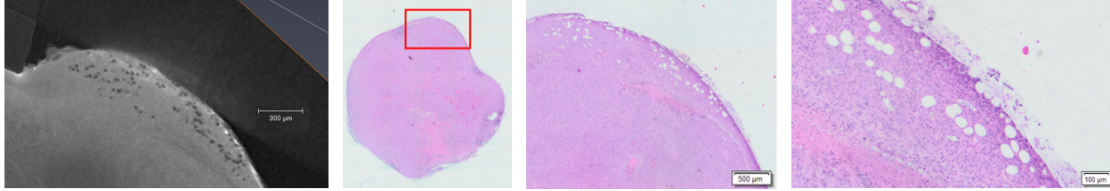


Figure 4.6: Tumor CBLRLRL from Experience 113. Left: CECT of the tumor, showing adipocytes as small black dots. Middle 1: location of the area visualised (framed in red). Middle 2: Classical 2D histology of the tumor on which adipocytes appear as small white dots. Right : Enlargement of the area of the classic 2D histology image where adipocytes are present. This shows that the very dark spots on the CECT correspond to the white spots on the histology, which are easily identifiable as adipocytes.

However, a non-negligible advantage of CECT is the 3D localization of these adipocytes throughout the whole volume of the tumor. Indeed, whereas classical 2D histology only allows visualization of a single slice in which adipocytes may not be present, CECT enables visualization of the entire tumor. Moreover, given the very dark color of the adipocytes on CECT, it is very easy to segment them and then get their location within the tumor, as well as their volume. The figure 4.7 shows the location of adipocytes within the tumor.

Finally, the visualization of tumors using both classical 2D histology and CECT revealed great diversity between tumors even within the same group. Indeed, differences in tumor size and morphological appearance can be noted. Furthermore, the very clear and visible presence of necrosis in Experience 113 tumors contrasts sharply with the more homogeneous appearance of Experience 114 tumors. It is clear, therefore, that certain differences are evident even within the control tumors. These differences may also have an impact on the results obtained, as it is clear that tumors of different sizes will not have the same requirements in terms of nutrients and oxygen, and will therefore also require different vascular organization. However, these differences within the same group also provide a better representation of clinical reality. Indeed, it is highly probable that different patients will not react the same way to a treatment. The use of patient-derived xenographs therefore offers the advantage of revealing the present heterogeneity when different individuals

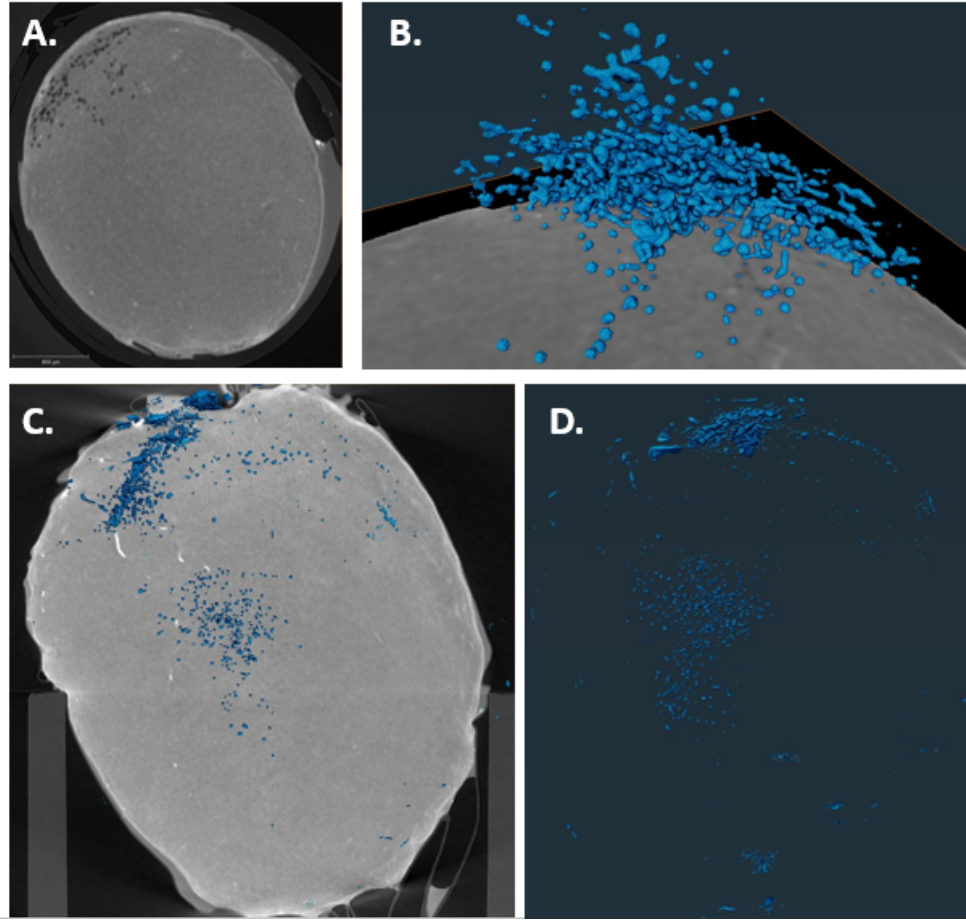


Figure 4.7: A. and B. Identification of adipocytes on CALLR tumor of Experience 114. A. CECT slice with adipocytes present in the upper left of the tumor. B. Application of a threshold to segment the adipocytes visible in blue. The image shows the same region as in A. C. and D. Identification of adipocytes in CBLULLR tumor of Experience 114. Segmentation of the adipocytes visible in blue allows their location to be visualised throughout the whole tumor.

suffer from a disease, and the different possible reactions of their organism to a treatment.

4.4 Conclusion

In conclusion, although CECT offers many advantages in terms of visualization, it unfortunately still has uncertainties concerning the homogeneous distribution of CA. Indeed, twelve tumors were found to have areas without CA, characterized by their black color on CECT and the absence of morphological changes on histology. Due to this problem of poor staining, these twelve tumors were considered unreliable

and excluded from the analysis. Moreover, although the least reliable tumors were discarded, it is not certain that the preserved tumors were perfectly stained. Indeed, unstained blood vessels were also detected in tumors that nevertheless showed correct CA diffusion. As far as blood vessels were concerned, it was also found that although large and medium-sized blood vessels could easily be matched between classical 2D histology and CECT, very small vessels remained mainly visible only on classical 2D histology and not on CECT. Moreover, no conclusive comparisons have been made for necrosis. Indeed, their identification on CECT was only possible thanks to the poor diffusion of CA, as they appear contrasted and therefore brighter than healthy tissue. However, no conclusions could be drawn from these observations, as the poor diffusion of CA in the tumors analyzed made them unreliable. Furthermore, it was not possible to determine how these necroses would have appeared in a normal situation in which CA would have diffused perfectly. It can therefore be concluded that the identification of necroses requires further investigation, especially as in this case they could not be identified without the aid of classical 2D histology.

Despite all these shortcomings, this chapter also highlighted the many advantages of CECT over classical 2D histology. Indeed, CECT offers the undeniable advantage of 3D visualization. This 3D visualization makes it considerably easier to identify tumor components such as blood vessels and adipocytes. Furthermore, CECT enables the identification and localization of blood vessels and adipocytes in the entire tumor, unlike classical 2D histology, which offers only a fixed, planar visualization, sometimes leading to missed elements. 3D CECT visualization enables blood vessel trajectories to be followed, as well as providing an overview of the distribution and organization of blood vessels and adipocytes. This makes it easy to identify and quantify the observed elements, by restoring their 3D architecture. Finally, observation of tumors on both classical 2D histology and CECT revealed the heterogeneity present between tumors, even when originating from the same patient or belonging to the same group (control or treated tumors).

Chapter 5

Segmentation of tumor vascularization

5.1 Introduction

The second chapter of this master thesis focuses on tumor segmentation to identify blood vessels as accurately as possible. Indeed, to best analyze tumors and their vascularization in Chapter 6, it is necessary to ensure that the segmentation provides the most accurate representation of reality possible. The aim of this chapter will therefore be to develop a good segmentation strategy by testing and comparing several methods to find the most appropriate strategy to visualize blood vessels as closely as possible to reality.

5.2 Materials and methods

5.2.1 Sample

In this chapter, as the aim is to focus on optimizing vascularization, tumors from both experiment 113 and experiment 114 were analyzed. Indeed, it is also of interest for the development of the segmentation strategy to work on tumors whose CA had less well diffused. Sample and image acquisition conditions are the same as those described in Chapter 4, in sections 4.2.1 to 4.2.3.

5.2.2 CECT image preprocessing

CECT images acquired were analyzed using *Avizo* software. First of all, preprocessing is carried out to make the image more easily readable and improve visualization of features. This necessary step consist in cropping the image to get as close as

possible to tumor edges, and applying a filter. The filter has the effect of smoothing the image, and the chosen one was *Median filter* (in 3D with Neighborhood = 26 - Iterations = 3). This filter provided convincing smoothing without blurring the blood vessels too much.

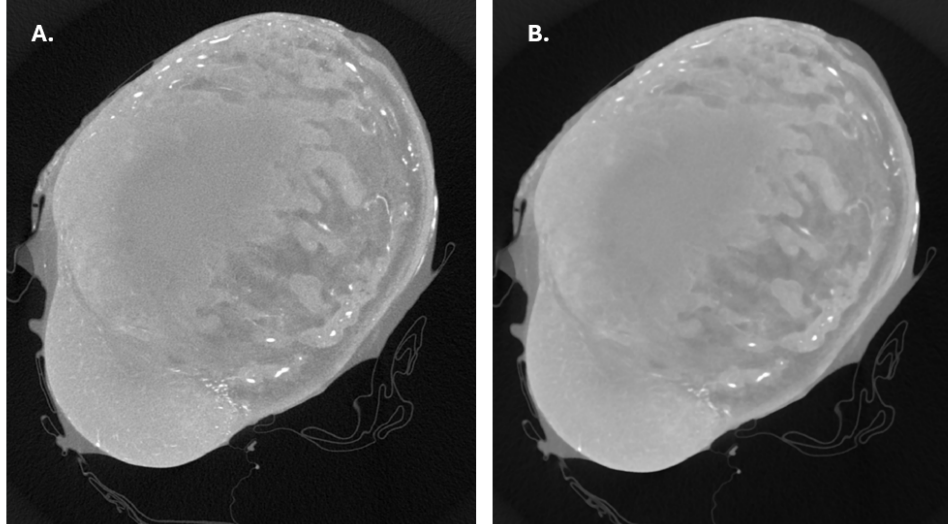


Figure 5.1: Images of tumor C189 – 2015. A. No filter applied, image is heavily pixelated. B. The Median filter in 3D with Neighborhood = 26 and Iterations = 3 has been applied, resulting in a smoother image.

5.2.3 CECT image segmentation

This important part of CECT scan processing involves segmentation of both tumor and blood vessels. The various thresholds and values used to segment these elements are listed in Appendix F.

Tools used

Two main semi-automatic segmentation tools on *Avizo* were used: *Interactive Thresholding* and *Magic Wand*. On one hand, *Interactive thresholding* consists of choosing a threshold and selecting all pixels whose intensity is greater than this threshold. On the other hand, *Magic wand* tool allows you to select directly or indirectly interconnected pixels within the same slice or across the entire image (“*All slices*” option selected), and which fall within the same user-defined color intensity range (“*Masking*”). In effect, the user chooses a grayscale range of interest and then clicks on a pixel and the software will then select all pixels belonging to the intensity range and directly or indirectly in contact with the selected pixel.

Tumor segmentation

First, tumors were segmented with an average *Masking* of 92-254. However, the range varied from tumor to tumor, with a minimum range of 65-254 and a maximum of 121-254. Two strategies were used here, depending on the tumor to be segmented. In the first case, the tumor was easily selected with *Masking* chosen and "All slices" option selected. In contrast, if too many external parts such as the holder were selected together with the tumor, it was necessary to adopt another strategy. For this, the "All slices" option was no longer used and the software only selected pixels in the current slice. All slices in the tumor are now scanned, and the tumor was selected every 30 slices. Next, "Interpolation" option was used between the slices where the tumors had been selected, to complete the selection and segment the entire tumor. Moreover, certain regions or small dots within the tumor were sometimes darker than the selected range, it was therefore necessary to fill the gaps with the "Fill interior" option to include these darker areas in tumor segmentation.

Blood vessels segmentation

In order to assess the best way of highlighting blood vessels, three strategies were mainly analysed in this chapter. The two first methods are closely related and use *Interactive Thresholding*. These strategies will thus select pixels without taking their connectivity into account and will then require cleaning to focus exclusively on blood vessels. However, what changes in *Method 2* compared to *Method 1* is the intention to recreate connectivity between the vessels by performing a closing after cleaning. The third proposed strategy is based on *Magic Wand* tool and takes advantage of the connectivity search offered by this tool.

In *Method 1*, blood vessels were segmented using *Interactive Thresholding* with a threshold adapted to each tumor, as listed in Appendix F. This choice of threshold was made in order to visualize as many vessels as possible, without selecting too many pixels that do not correspond to the vascularization. Once the threshold had been applied, it still remains a large number of selected elements that were not part of the vascularization. To remove them, erosion was applied to the binary tumor mask to reduce its size. This reduction depended on the tumors and how much had to be removed. However, to avoid removing too much of the tumor, a maximum limit of 5% of the length of the smallest axis of the tumor was set, but erosion was generally between 2 and 4% of this distance. Next, the reduced tumor mask was applied to blood vessel segmentation to retain only the portion of vessels within the tumor and remove unwanted elements. The results of this transformation are shown in Figure 5.2.

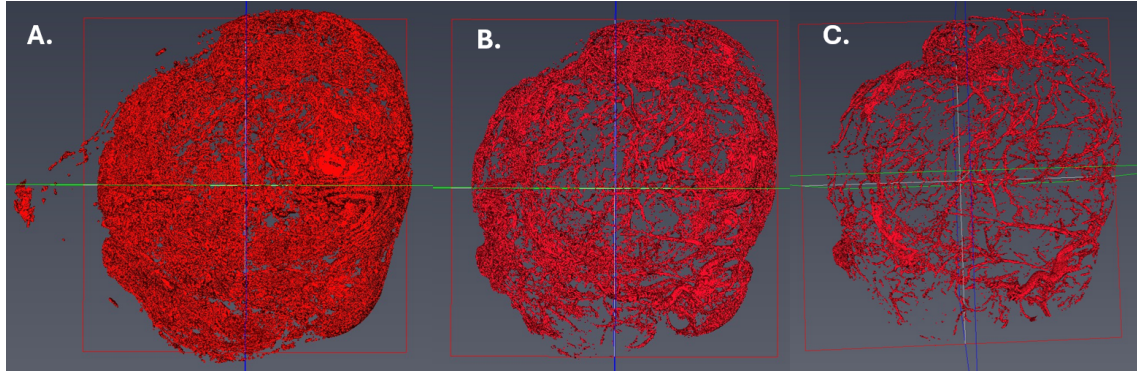


Figure 5.2: Erosion of tumor CBLULLL. A. No erosion has yet been applied to the tumor. Only *Interactive thresholding* has been applied with a threshold equal to 175. It can be seen that many features have been selected at the edges of the tumor. These features reflect both the interface of the tumor with the paraffin and the hemorrhagic zones. It is relatively difficult to distinguish the blood vessels at the edge, and those on the inside are barely visible. B. Erosion of 2% of the length of the smallest axis. Blood vessels are seen more clearly and distinctly. However, some plaques are still too present at the edge of the tumor. C. Erosion of 4% of the length of the smallest axis. The vascular tree is much more visible and blood vessels can be clearly distinguished. A few small plaques are still present, but these are located inside the tumor, so it is not possible to remove them with erosion without losing part of the tumor and therefore the blood vessels.

Once the large clusters have been removed, it is necessary to remove elements that are too small to be blood vessels. For this purpose, the *Remove islands* option in the *Segmentation* section of *Avizo* was used. This option allows you to remove voxel groups that are smaller than a specified number of voxels. In this case, the sets removed were those with fewer than 10 voxels, as illustrated in Figure 5.3. Finally, the result of the various steps can be seen in Figure 5.4.

In *Method 2*, the same steps as in Method 1 were first applied meaning blood vessels segmentation using *Interactive Thresholding* followed by the same cleaning process. However, afterwards a step is added to improve connectivity between blood vessels. Indeed, closing is performed. Closing is a method used to fill small holes and correct irregularities. This technique involves the application of dilation (addition of morphological element) followed by erosion (subtraction of morphological element). In this case, different morphological elements were tested to find the one that filled holes in the blood vessels in as many directions as possible, without adding too many surplus elements as discussed in section 5.3.1.

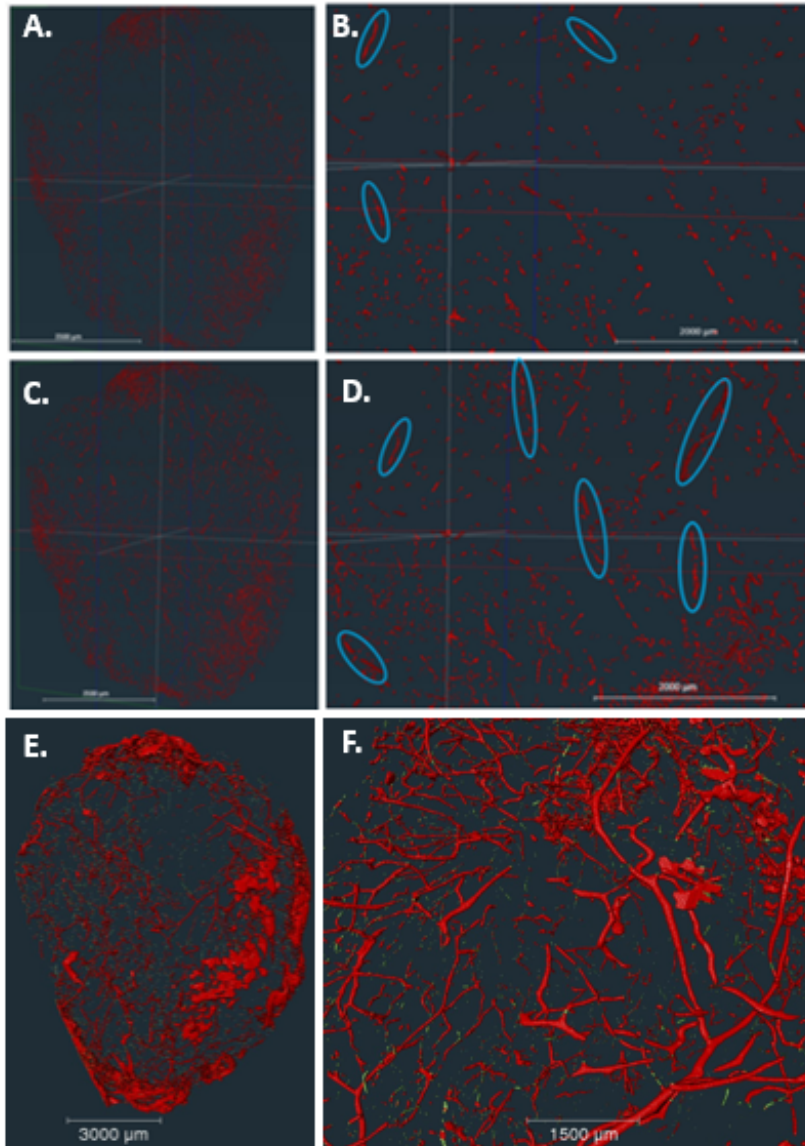


Figure 5.3: Application of the *Remove island* option from Avizo software on CALLL tumor (Experience 114). A. Elements smaller than 10 voxels are removed B. Zoom in on the central part of A. C. Elements smaller than 15 voxels are removed D. Zoom in on the central part of A. The blue circles frame the small blood vessels that are unfortunately removed when *Remove island* is applied. However, if the removal of 10 voxels is applied, the number of blood vessels lost is reduced. E. In red, blood vessel segmentation after *Remove island* has been applied to the tumor and what has been removed in green. F. Zoom in on the central part of E.

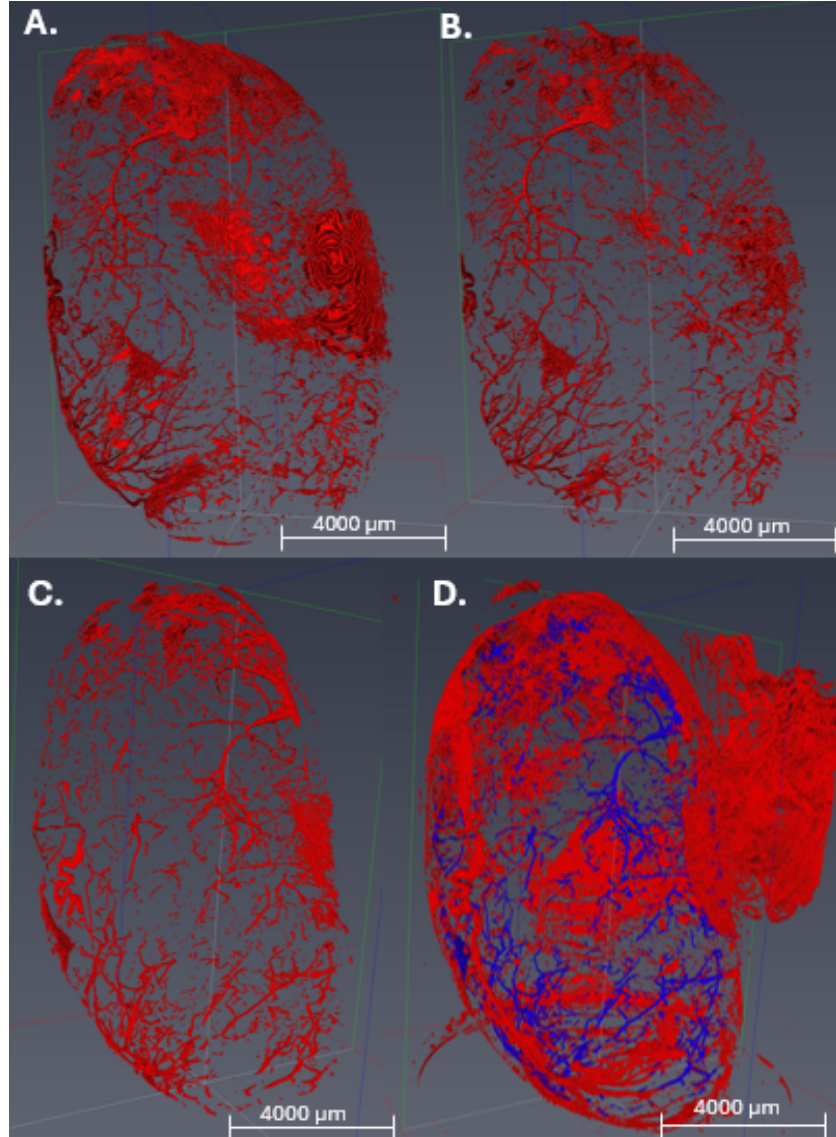


Figure 5.4: Various step of blood vessels segmentation shown on CALUR tumor (Experience 114). A. Erosion of 1% of the length of the smallest axis. Some plaques are still present at the edge of the tumor. B. Erosion of 2% of the length of the smallest axis. An improvement in plaque reduction is visible while preserving blood vessels. C. Final result with removal of selected volumes that were smaller than 10 pixels. D. Difference between the final segmentation result visible in blue and the initial segmentation obtained simply by applying a threshold value of 152 with '*Interactive thresholding*' option visible in red.

In *Method 3*, blood vessels segmentation was carried out using *Avizo's Magic Wand* tool and "All slices" option but in 3 stages. The first step involved a 50-by-50 pass through all the slides with a first *Masking* of 195-255. This range was chosen to enable good visualization of blood vessels while maintaining a certain selectivity for most of the tumors. The next step consisted in enlarging *Masking* to 180-254 in order to target more blood vessels in the center of the tumors, which generally have a less clear intensity, and to re-establish connectivity between certain blood vessels. Finally, the last step also consists of scanning all the 50-by-50 slices, but playing with 2 ranges. On one side, a smaller range (217-254 or 233-254) allows to separate large clusters of blood vessels at the edges of tumors and try to restore a tube shape to the blood vessels that better distinguishes their origin and trajectory. On the other side, a wider range (154-254 or 166-254) captures internal blood vessels of weaker intensity. The progression of the selection of blood vessels through the various stages is illustrated in Figure 5.5.

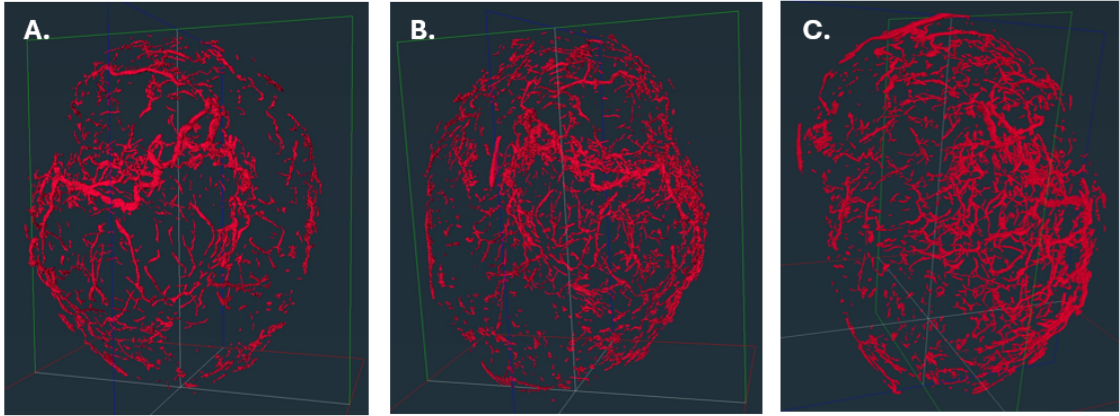


Figure 5.5: Illustration of the various stages of blood vessels selection when applying *Magic Wand* strategy (*Method 3*) on tumor CARLL (Experience 113). A. First step with the *Magic Wand* tool used with a *Masking* of 195-255. The largest blood vessels have been selected. B. Second stage: this time the *Masking* is 180-255. Most of the holes from the previous stage are filled in, and new connections have been created between blood vessels, mainly in the center of the tumor. C. Last and third step with the addition of small inner blood vessels (163-254) with bigger range and big outer blood vessels with smaller range (217-254).

5.3 Results and discussion

First, the simplest strategy for segmenting blood vessels is to apply a single threshold using the *Interactive Thesholding* tool. However, this strategy has a number of disadvantages. For example, once the threshold had been applied, it still remains a large number of selected elements that were not part of the vascularization. Indeed,

most of the tumors showed large hemorrhagic areas at their edges. These areas are due to blood clotting, and formed large clumps making it impossible to clearly distinguish blood vessels. In other cases, the simple interface between the tumor and the paraffin had formed a very clear contrast, which had therefore been selected by the threshold. In addition, elements outside the tumor, such as impurities in paraffin, were also selected by the threshold. The presence of these elements resulted in less accurate visualization of blood vessels delineation and trajectory. Furthermore, segment by applying only a threshold does not allow the selection of blood vessels only, so some sets of voxels are selected which have neither the shape nor the size required to be considered as blood vessels. As a result of all these observations, the use of *Interactive thresholding* alone is not an adequate solution for the segmentation of blood vessels, as it is not specific enough to them and leads to a segmentation that comes to overestimate them. To overcome these problems, certain modifications were made to the segmentation performed with *Interactive thresholding*, as explained in section 5.2.3.

After applying a cleaning process to remove disruptive elements which were not blood vessels and led to overestimating blood vessel volume, the result of *Method 1* was obtained. However, one problem can already be identified with this method. Indeed, some blood vessels are separated into small pieces that should normally be connected to each other, as easily observed in Figure 5.6. To overcome this problem, *Method 2* performs a closing to reconnect blood vessel fragments.

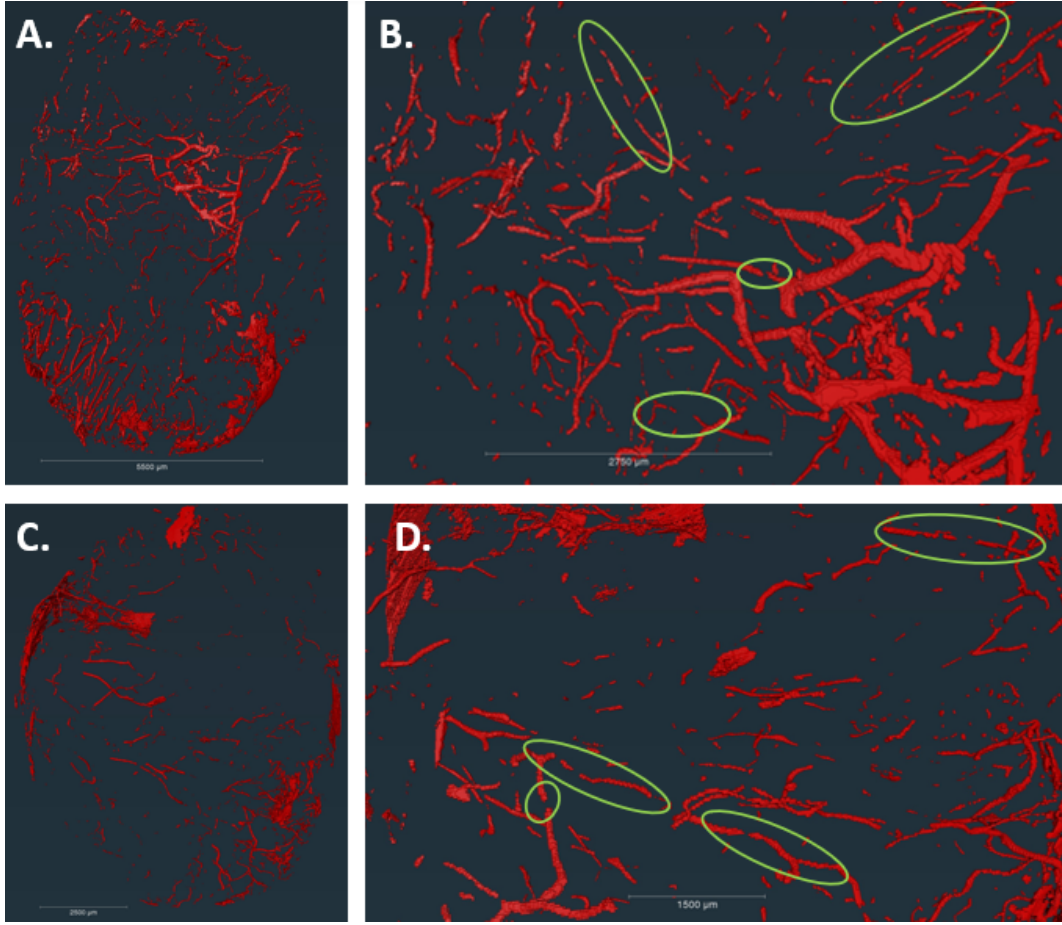


Figure 5.6: Illustration of the presence of cuts in blood vessel segmentation using Method 1 as defined in Section 5.2.3. A. Pazopanib CBRURLR tumor (Experience 114). B. CARUL control tumor (Experience 114). Visualisation of whole tumors on the left and zoom on the central part on the right. The green circles surround the different parts of a blood vessel that appear detached on segmentation when they should form a single vessel.

5.3.1 Closing

Avizo software provides different types of morphological elements when applying closing. Figure 5.7 illustrates the use of these different elements on a tumor sub-volume to better understand their effects and characteristics.

From this first visualization, it can be seen that closing with 10 pixels is more than sufficient, as closing with 20 pixels has the effect of closing too many holes and therefore adds too many elements. Secondly, elements with a more uniform structure in all three directions, such as cube and ball, are more likely to fill holes in the vessels in more directions, unlike flatter elements like lines and disks. This

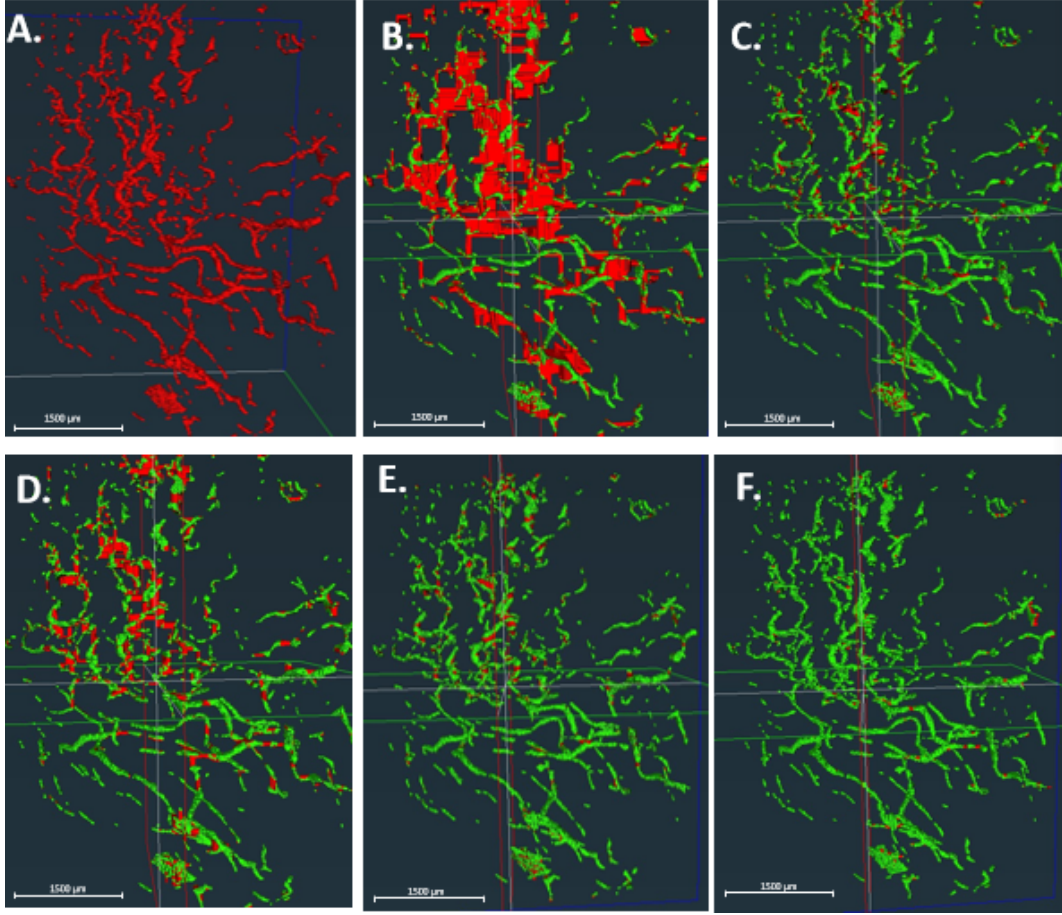


Figure 5.7: Illustration of the effects of different structural elements that can be used for closing. A. Sub-volume of the CALLR tumor (Experience 114) to which a threshold was applied using *Interactive thresholding*. Next, a cleaning step was performed. First, an erosion followed by the removal of all elements smaller than 10 voxels (segmentation method used in the chapter 6) B. A closing using ball of 20 pixels was performed on the sub-volume presented in A. C. A closing using balls of 10 pixels was performed on the sub-volume presented in A. D. A closing using cubes of 10 pixels was performed on the sub-volume presented in A. E. A closing using disk of 10 pixels at angles $0^\circ - 0^\circ$ was performed on the sub-volume presented in A. F. A closing using lines of 10 pixels at angles $0^\circ - 0^\circ$ was performed on the sub-volume presented in A.

initial analysis would therefore suggest that a morphological element with a cubic or round shape would be better suited to reconnecting blood vessels. However, it is interesting to take a closer look at where these morphological elements are added. In this sense, figure 5.8 shows an enlargement of the same sub-volume as figure 5.7.

The enlargement of the closing with different morphological elements shown in

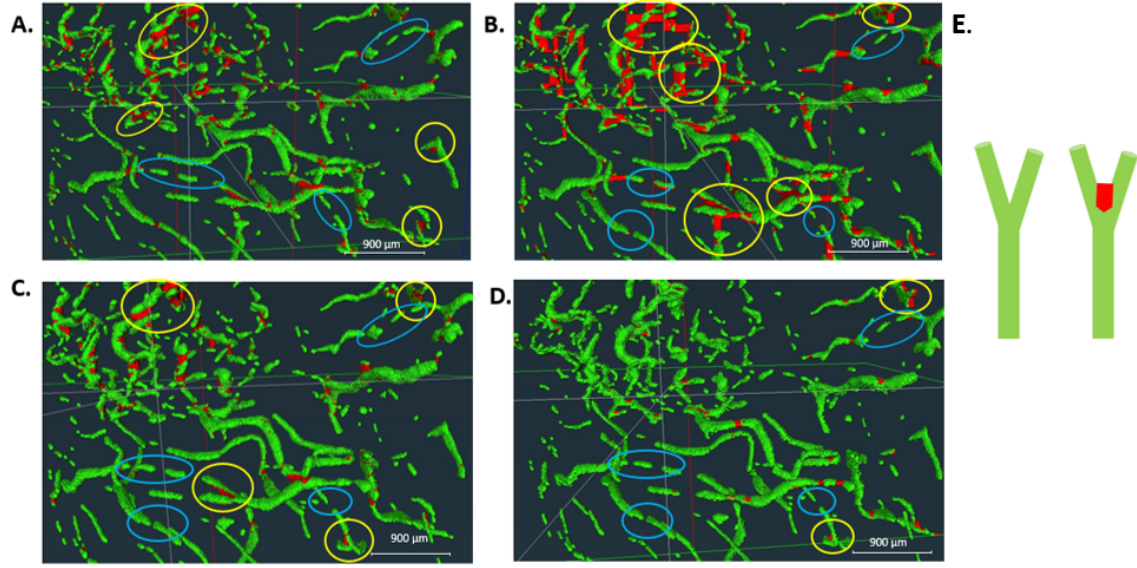


Figure 5.8: Enlargement of the CALLR tumor sub-volume to which closing with different morphological elements has been applied. A. Closing using balls of 10 pixels B. Closing using cubes of 10 pixels C. Closing using disks of 10 pixels at angles 0°- 0° D. Closing using lines of 10 pixels. The blue circles highlight the connections between the blood vessel parts, which unfortunately were not established despite the closing and the visible proximity of vessels parts. The yellow circles show where closing adds elements that are not really necessary. Indeed, at these points, the closing does not connect the vessels lengthwise, but creates a connection between two branches that form an angle, widening the branches widthwise, as shown in E. E. Blood vessel shown in green, left before closing and right with closing in red creating. It can be seen that closing create a connection between the two branches widthwise, which is undesirable.

figure 5.8 confirms that ball- and cube-shaped elements are the most suitable for blood vessel morphology. Indeed, it can be seen that they both enabled the creation of connections between certain vessels, whereas the other element types did not. However, the closer the vessels are to each other, the greater the number of elements added, and in the case of cubic elements, the blood vessels start to adopt a cubic architecture, which is not in line with reality. It is therefore preferable to use ball-type morphological elements to recreate links in the vascular architecture. In addition, this enlargement also revealed a number of defects in the closing process. When two vessels form an angle or are too close to each other in the width direction, the closing process can sometimes glue them together. This correction is unfortunately not correct, as the aim of closing is to connect vessels lengthwise, not to add elements that are not actually present. In addition, some connections are unfortunately still missing because the blood vessels are too far apart, and adding more elements would create too many surplus elements and defects in the areas of greatest vascular density. It is therefore important not to add too many

elements, as defects may become more pronounced and the blood vessels may stick together, losing their individuality. Another important point to note is that disks and lines add fewer elements because they are focused in a particular direction. However, they can be added in different orientations, as *Avizo* software allows the angles of their orientation to be varied. Indeed, as previously observed, these 2D morphological elements focus on creating connections along a specific direction. However, as blood vessels are oriented in a lot of various different directions, these morphological elements come to miss many connections. Figure 5.9 illustrates the difference between closing with lines and disks in one and two directions.

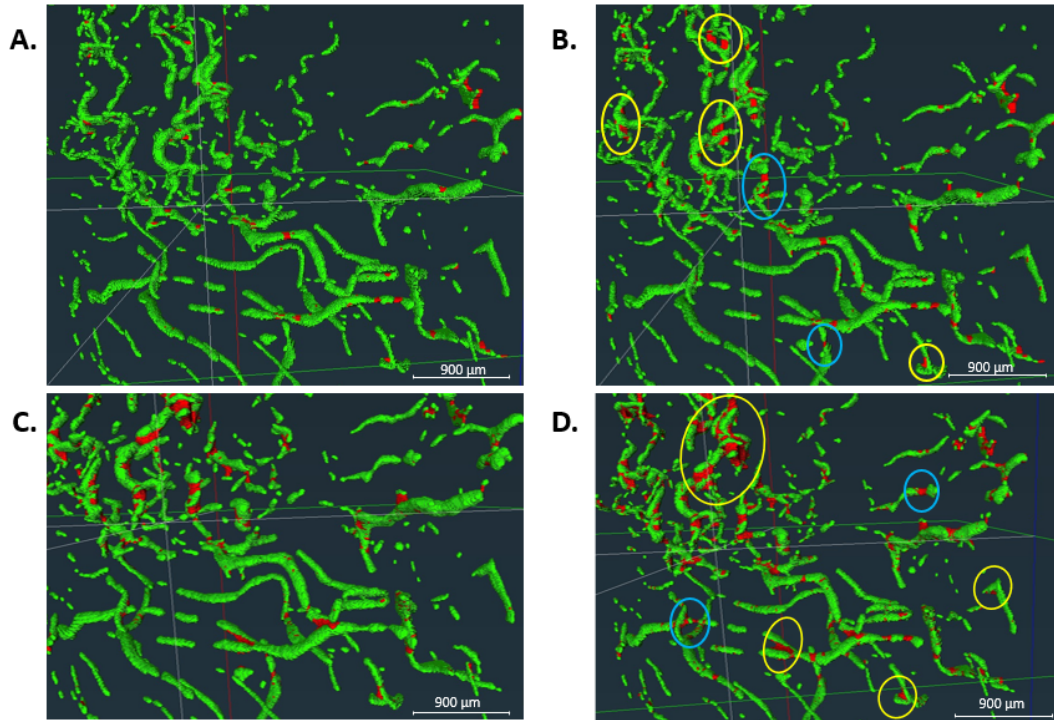


Figure 5.9: Enlargement of the CALLR tumor sub-volume as shown in Figures 5.7 and 5.8 A. Closing using lines of 10 pixels at angles 0° - 0° B. Closing using lines of 10 pixels at angles 90° - 90° was performed on A. C. Closing using disks of 10 pixels at angles 0° - 0° D. Closing using disks of 10 pixels at angles 90° - 90° was performed on C. The blue circles show the blood vessels that have been linked together by adding the second direction. The yellow circles surround the areas where surplus elements have been added due to the addition of closing in a second direction.

An improvement is seen when a second closing with a different direction is performed on the previous closing with 2D morphological elements. New blood vessels are connected, as their orientation is now taken into account. It can also be noted that the rounded shape of the disks blends in better with the architecture of the blood vessels. However, the second closing adds superfluous elements and

creates connections where none are supposed to exist. This situation raises similar problems to those encountered when closing with cubes or balls, meaning that the blood vessels are connected along the width rather than the length. Moreover, the addition of this second closing in another direction only allows us to consider two directions of connection, whereas blood vessels are oriented in many different directions. Unfortunately, it is impossible to consider all these directions. Indeed, even the addition of an extra closing would lead to an increase in defects, and in the end too many excess elements would be added, especially in areas where many blood vessels are present. Clearly, the best solution is to use balls as morphological elements because these elements offer better blood vessels fitting without adding too many defects. The morphological elements balls and cubes were used on a whole tumor and the result can be seen in Figure 5.10.

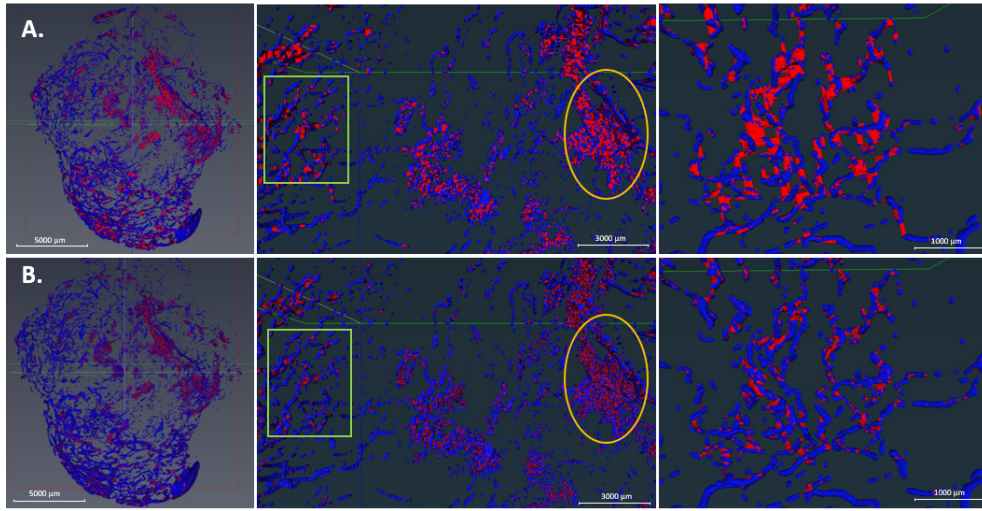


Figure 5.10: Visualization of CALLR tumor (Experience 114) to which a threshold was applied using *Interactive thresholding*. Next, a cleaning step was performed following the method explained in section 5.2.3. A. Closing with cube 10 pixels was applied to the tumor. B. Closing with ball 10 pixels was applied to the tumor. The images show from left to right : the whole tumor, then a first zoom on the central part of the tumor and then a second zoom on the blood vessels located on the left side of the central zoom (framed in green). The orange circle surrounds an area where many blood vessels are present and therefore more elements have been added.

Whether using the cube or the ball as morphological elements, it can be seen that the areas where many blood vessels are present have the most elements added after the closing. However, these elements do not always serve to link blood vessels together, but rather are often linked to the high blood vessels proximity that leads to the appearance of parasitic elements. In assessing the two types of morphological elements, however, cubes appear to add more elements in these areas than balls.

Furthermore, it can be seen that, in general, the cubic morphological element also leads to the addition of a greater number of pixel than balls, and this difference is also represented by the difference in volume of blood vessels. Indeed, whereas blood vessel volume was $3.16 \cdot 10^9 \mu m^3$ before closing, it rises to $5.00572 \cdot 10^9 \mu m^3$ after closing with cubes and $4.36207 \cdot 10^9 \mu m^3$ after closing with ball. Furthermore, the enlargement on the blood vessels clearly shows that closing with ball is more adapted to the blood vessels and offers the advantage of not adding too many surplus elements compared to closing with cube.

5.3.2 Comparison between the three proposed methods

Now that the morphological element with which the closing will be performed has been chosen, it is essential to focus on the comparison of the three methods explained in Section 5.2.3. To do this, the advantages and disadvantages of each method will first be discussed, followed by a comparison of the three methods using tumors.

First, the cleaning process used in *Method 1* and *Method 2* has been designed to bring the segmentation closer into line with real tumor vascularization. In fact, as already explained earlier, the use of a threshold alone is not possible, as it leads to the selection of undesirable elements. However, the cleaning process also has its drawbacks. Notably, it does not remove all the hemorrhagic plaques present, and these also lead to an overestimation of blood vessel volume. In addition, another disadvantage of the cleaning process is that the erosion also remove certain blood vessels present at the edges of the tumor or entangled in the hemorrhagic zones as shown in Figure 5.11.

Secondly, *Method 2* shares the advantages and disadvantages of *Method 1* listed above. However, the big change and advantage of *Method 2* compared to *Method 1* is the re-establishing connections between vessels. This re-establishment of connections between blood vessels is also of great interest, as this rupture can be caused by a variety of phenomena. Indeed, these broken connections may be due to incomplete diffusion of CA into blood vessels, or simply to an absence of blood in it. As tumors are viewed *ex-vivo*, blood may no longer be present in c vessels or at certain locations within them. This can represent a major obstacle to the visualization of vascularization. As a result, the absence of blood prevents the staining of vessels, since CA binds to blood cells such as red blood cells. The advantage of performing a closing is therefore to recreate this lost connectivity, whether due to a lack of CA, blood or simply the application of a too high threshold. However, this search for connectivity can also lead to inducing connectivity that does not exist in reality, or adding elements where none exists. That is why it is

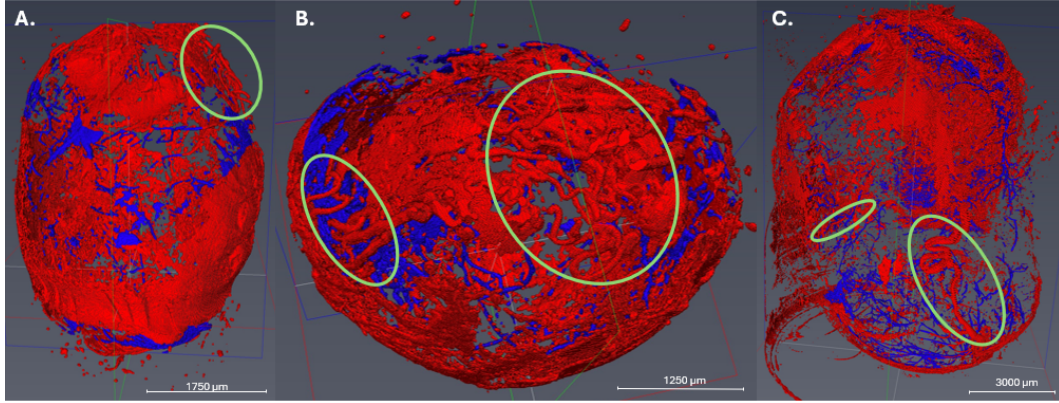


Figure 5.11: Visualization of the fact that erosion in the cleaning process also leads to the loss of selected blood vessels at the edge of the tumor. A. CALLR tumor (Experiment 114) B. CALLR tumor (Experiment 114) but with a different viewpoint C. CALUR tumor (Experiment 114). The red represents the segmentation achieved only by applying a threshold using *Interactive thresholding* and the blue is the result after applying erosion (5% of the length of the smallest axis for CALLR and 2% for CALUR). The green circles surround vessels that were previously selected with the threshold but are no longer selected after erosion.

essential to choose the closing that best suits the vascularization so that the closing best follows the architecture of the vascularization as discussed in Section 5.3.1. Furthermore, it is essential not to add too many elements, so that surplus ones do not have too much impact on blood vessel volume. In fact, it is preferable to add only a small number of voxels to induce connectivity without forcing it too much, rather than attempting to link all the blood vessels together at all costs and end up recreating vessel clusters that lead to the overestimation of blood vessels volume. Here, the closing value has been kept at 10 pixels, even if this does not enable the connection of a large number of blood vessel parts, but at least the range of surplus elements remains reasonable.

Thirdly, as for *Method 3*, it offers the advantage of playing on several ranges of intensity, and thus capturing more vessels when gray value intensities vary, such as between the center and edges of some tumors. Indeed, blood vessels located in the center of the tumor are sometimes more difficult to reach with the CA. This results in less marked staining and therefore a lower gray value for these central blood vessels. These blood vessels are often overlooked when using a single threshold. Unfortunately, when using a single threshold, it cannot be set too low for fear of losing selectivity. A single threshold will be generally set above the intensity of less contrasted central blood vessels and therefore not take them into account. The use of *Magic Wand* offers the advantage of greater selection of intensities. This selection results in greater accuracy in blood vessels detection, enabling more

detailed blood vessels visualization. The *Magic Wand* strategy offers the advantage of playing with several thresholds, which will also reduce the bias that can be caused by inhomogeneously distributed CA. Indeed, as explained in Chapter 4, if CA are not evenly distributed, some blood vessels may have a less pronounced intensity than others. The use of several intensity ranges ensures that intensity differences caused by poor diffusion are not affected. In addition, this tool induce connectivity between blood vessels right from the selection stage. However, this more progressive strategy is very time-consuming. In addition, although it aims to select interconnected pixels and thus induces connectivity during segmentation, it can also have the opposite effect. Indeed, selection criteria of *Magic Wand* can also lead to the creation of gaps. For example, if a blood vessel is fragmented into several segments, there is a good chance that some segments will not have been selected during segmentation. This can make it difficult to reconstruct the entire blood vessel because of the missing parts. Furthermore, one of the main concerns when using this strategy is the possibility of missing small blood vessels. Indeed, as the tumor slices are traversed at intervals of 50 slices, it is obvious that if a very small blood vessel lies within the interval, it will unfortunately be missed. This is very annoying, because in addition to the gaps already existing in the blood vessels due to lack of blood or CA, *Magic Wand* strategy creates new gaps. The fact that *Method 3* can miss blood vessels is unfortunately a major disadvantage that needs to be overcome.

In order to compare these three methods, 2 tumors from Experience 113 have been chosen (CALLR and CALUR). These tumors were chosen because, at first sight, they presented a relatively different distribution of vascularization, even though they were both control tumors. Moreover, as discussed in Chapter 4, they were among the 4 reliable tumors in Experiment 113. They therefore presented a good study case for tumors in which the CA had not diffused perfectly, but the blood vessels were still visible. The figures 5.12 and 5.13 show the different vascular tree structures and corresponding blood vessel volumes for the two tumors with the different methods.

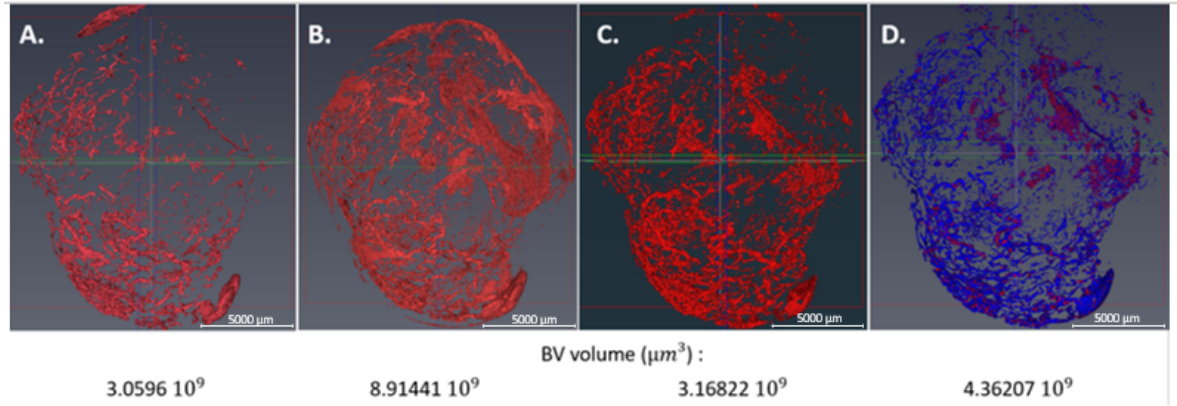


Figure 5.12: Illustration of the CALLR tumor (Experiment 113) with different segmentation techniques and corresponding blood vessels volumes. A. Application of *Method 3* using *Magic Wand* as defined in Section 5.2.3. B. Application of a threshold using *Interactive thresholding*. C. Application of *Method 1* as defined in Section 5.2.3 meaning application of a threshold with *Interactive thresholding* followed by a cleaning process. D. Application of *Method 2* as defined in Section 5.2.3 meaning applying a closing to the segmentation performed in C. Here, the morphological element chosen is the ball with a size of 10 pixels. In blue is the segmentation as shown in C. and in red is what has been added by closing.

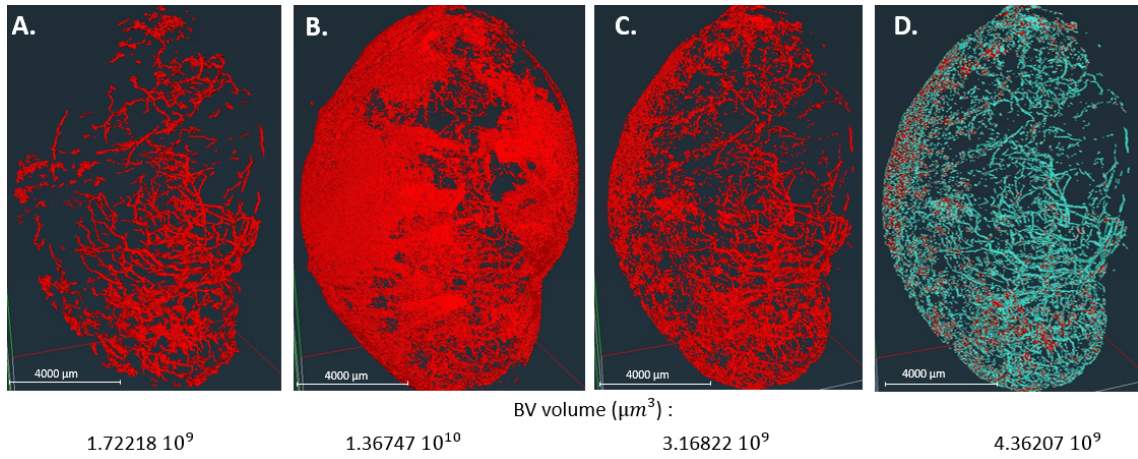


Figure 5.13: Illustration of the CALUR tumor (Experiment 113) with different segmentation techniques and corresponding blood vessels volumes. A. Application of *Method 3* using *Magic Wand* as defined in Section 5.2.3. B. Application of a threshold using *Interactive thresholding*. C. Application of *Method 1* as defined in Section 5.2.3 meaning application of a threshold with *Interactive thresholding* followed by a cleaning process. D. Application of *Method 2* as defined in Section 5.2.3 meaning applying a closing to the segmentation performed in C. Here, the morphological element chosen is the ball with a size of 10 pixels. In blue is the segmentation as shown in C. and in red is what has been added by closing.

As a first comparison between the three methods, it can be seen that *Method 3* presents a lower volume of blood vessels than the two others methods, and this finding is also visual, as more empty areas can be seen in the segmentation of *Method 3*. However, it can be pointed out that large hemorrhagic areas present in the center of the CALLR tumor and at the edge of the CALUR tumor are still segmented with *Method 1* and *Method 2*. In fact, it can be seen that *Method 3* avoids these areas, which tends to overestimate the volume of blood vessels. Indeed, although *Method 1* and *Method 2* have already made it possible to remove quite a few of these plaques compared to the single use of a threshold, there remain plaques that these methods cannot remove. Two cases are illustrated here. The first is when the plaque is located inside the tumor, as in the case of the CALLR tumor (Figure 5.12). In this case, erosion cannot be used to remove it, as this technique only removes plaques located at the edges of the tumor. The second case is when the hemorrhagic zones are too large and numerous at the edges, as illustrated by the CALUR tumor (Figure 5.12). In this situation, erosion cannot be carried out more extensively for fear of removing too large a part of the tumor. However, the presence of these hemorrhagic plaques is not the only cause of the fractional volume difference. Indeed, it can be observed that many small blood vessels are not taken into account in *Method 3*, even though the majority of blood vessels are present in all three methods.

In conclusion, each method has its advantages and disadvantages, and the reality of the vascular tree lies between Method 2 and Method 3. Indeed, Method 2 overestimates the volume of blood vessels, because despite its cleaning process, this method still takes into account elements that are not blood vessels. Method 3, on the other hand, underestimates the volume of blood vessels, as many small blood vessels are missing, even though most of the vascularization is present. Therefore, these methods need to be further improved, or even combined, in order to overcome their respective shortcomings.

5.3.3 Remaining segmentation problems

One of the major problems with CECT-based blood vessels segmentation is the poor diffusion of CA or even the absence of staining. A further illustration of this problem is when blood vessels appear totally uncolored and therefore dark. Indeed, when visualizing adipocytes, applying a threshold on very dark gray values to segment them could also results in segmenting unstained blood vessels. Although Figure 4.7 clearly shows adipocytes that are recognizable by their round shape and clustered in the same place, it is also possible that the small, very dark areas are also unstained blood vessels. Figure 5.14 shows that with the same high threshold used to segment adipocytes, blood vessels can also be found in this segmentation.

This raises another problem concerning the identification and correct visualization of blood vessels. Therefore, even in tumors where CA seems to have diffused over the entire tumor, blood vessels may not have been stained by CA. The problem of diffusion raised here is more complicated to identify. Indeed, tumors in which CA had not fully diffused were easily identifiable due to large differences in staining as shown in Chapter 4. However, the problem raised here requires a more detailed analysis of each tumor to be detected. Furthermore, the thresholds used to segment blood vessels will not take these blood vessels into account.

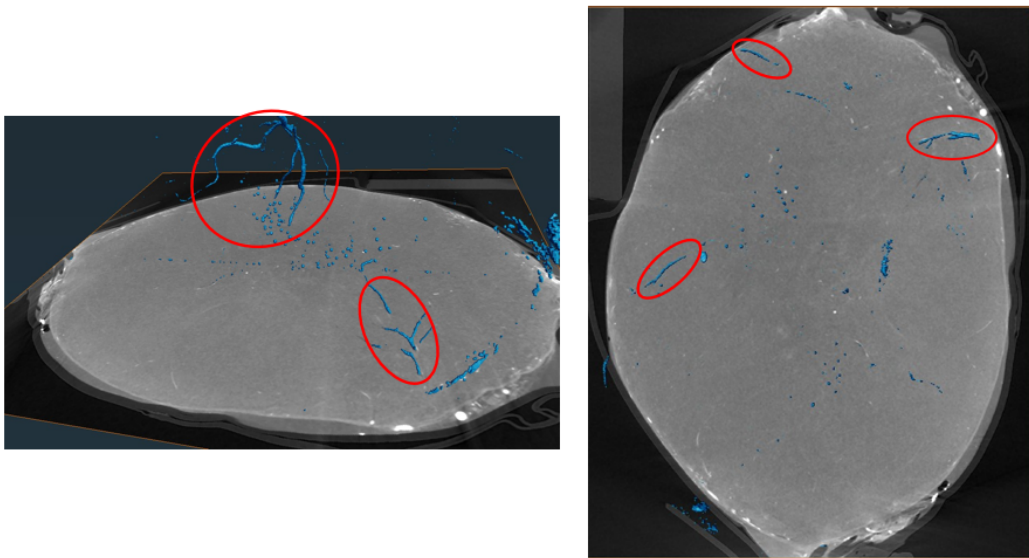


Figure 5.14: Identification of adipocytes on CALLL tumor from Experience 114. However, it can be seen that when applying segmentation with a very dark gray scale value in order to segment adipocytes, blood vessels that were not stained are also detected by the threshold. The two images show different views of the tumor. The segmentation performed with *Interactive thresholding* and a range from 5 to 101 is shown in blue. Blood vessels included in the adipocytes segmentation are circled in red.

5.3.4 Other methods

Other methods can also be used to overcome the segmentation shortcomings of the above methods. One possible solution for greater selectivity in the cleaning process would be to use a filtering tool for the volumes selected. This strategy would avoid the need for erosion, which would result in the deletion of blood vessels at the edge of the tumor, as well as greater precision in the deletion of elements too small to be vessels. This filtering strategy would work as follows. Once a threshold has been applied using *Interactive thresholding*, an option named “*Label analysis*” is used to

create an independent label for each element selected when the threshold is applied. This option then provides information on the various independent elements, such as their volume. It is then possible to filter these labeled elements according to a criterion, in our case : volume. As shown in Figure 5.15, it is then possible to keep only those elements present in a certain range of volumes. This filtering process removes elements that are too small to be blood vessels, as well as those that are too large. Furthermore, this strategy also offers the advantage of separating vessels by volume range, so that closing procedures more specific to their size can be applied. For example, for the smallest range in Figure 5.15, a closing with a larger number of pixels could be applied to connect the different parts of the large blood vessels contained in this volume range. This avoids applying this closing with a large number of pixels to all the vessels, as this would encapsulate and add too many surplus elements to the smaller vessels that do not require such a large closing. With this technique for separating selected elements, it would be possible to be more selective when eliminating elements that are too large or too small. Moreover, this technique would make it possible to apply different closings more suited to the size of blood vessels. However, this technique appears to be very time-consuming, since it requires analysis of the volume ranges of the segmented elements in order to choose the most suitable range for each tumor, and performing different closings for each of these volume ranges.

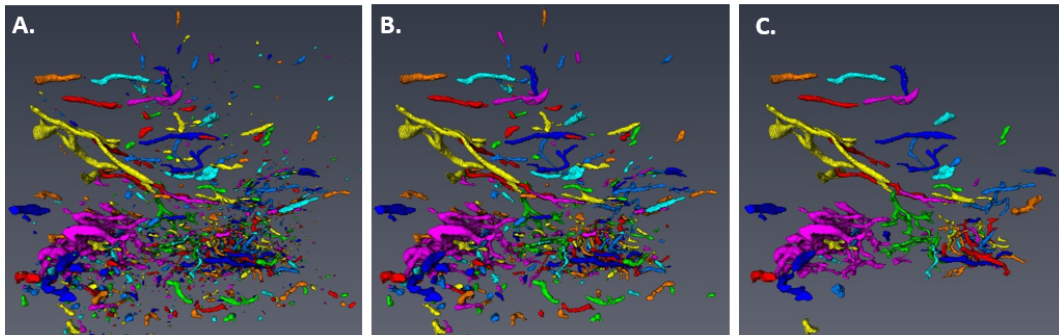


Figure 5.15: Application of the 'Label analysis' option of *Avizo* on a sub-volume of the C189 tumor - 2015. A. Selection of different independent labels without volume range selection applied B. Selection only of elements within the middle volumic range from $6.03312 \cdot 10^{-8}$ to $0.00447594 \mu\text{m}^3$ C. Selection only of elements within the small volumic range from $6.03312 \cdot 10^{-7}$ to $0.00447594 \mu\text{m}^3$.

Another solution that was not evaluated in this thesis but could have been considered is the use of automatic segmentation with methods such as *Otsu* or *Watershed*. However, given the irregularity of the blood vessels, it seems clear that manual or semi-automatic correction would have been necessary after the application of

these tools. In addition, the configuration of a process using artificial intelligence could also be envisaged, but given the small number of tumors present and the great variation and diversity of their vascular arborescence, it seemed a little too optimistic to be able to develop an algorithm in this respect.

5.4 Conclusion

Although various approaches were evaluated, none stood out as being the most suitable for segmenting the complexity of blood vessels. Indeed, although all three proposed methods offered numerous advantages and satisfactory results, defects and shortcomings still remain. Indeed, there are still a number of problems to be solved when it comes to segmenting blood vessels. The most complex and sensitive parts of segmentation are the search for inter-vessel connectivity, the elimination of hemorrhagic zones and the detection of small vessels mainly located in the center and generally less contrasted than those at the edge of the tumor. These three points have proved to be the biggest hurdles in improving blood vessel segmentation, and are still in need of improvement.

It was found that the cleaning process used in *Method 1* and *Method 2* led to the disappearance of blood vessels located at the edge of tumors, as well as those present in or near hemorrhagic areas. However, these strategies offer the advantage of selecting even very small blood vessels. A shortcoming of *Method 1* is that it had not been successful in creating connectivity between separate parts of the same blood vessel. To overcome this loss of connectivity, a closing was added in *Method 2* and the morphological element chosen are balls because best suited to blood vessels architecture. This closing has therefore enabled the linking of some blood vessel pieces without adding too many elements that do not exist in the real vascularization. However, some connections remained incomplete. Alternatively, *Method 3* focused on the creation of connectivity and offered the advantage of using different thresholds to compensate for differences in contrast between blood vessels due to inhomogeneous CA diffusion. This method also offers the advantage of avoiding hemorrhagic plaques and elements too small to be blood vessels. However, although based on connectivity, this method has the disadvantage of unintentionally creating gaps and missing small blood vessels. In addition, the application of this method is much more time-consuming than *Method 1* and *Method 2*.

Finally, differences in blood vessel volume were observed when using the different methods. Indeed, while *Method 3* shows an underestimation of blood vessels due to the non-selection of small vessels as well as the lack of other blood vessels or parts of blood vessels, *Method 1* and *Method 2* show an overestimation of blood

vessels volume because elements that are not blood vessels, such as hemorrhagic plaques, are still present. In order to select the segmentation method to be used in Chapter 6, a compromise was reached by choosing *Method 1*, which has a blood vessels volume between that of *Method 3* and *Method 2*. Indeed, although this method had not been successful in creating connectivity between separate parts of the same blood vessel, it offers a good compromise between *Method 2* and *Method 3*, which respectively over- and under-estimate blood vessels volume. However, it is necessary to underline that this method can still be improved to be more robust. Furthermore, there are many other strategies and methods that have not been tested or even imagined in this master thesis. This means that there are still a large number of possibilities other than the solutions evaluated here for improving blood vessel segmentation.

Chapter 6

Influence of antiangiogenic drugs on tumor vascularization

6.1 Introduction

The aim of this chapter is to compare control tumors with tumors treated with pazopanib, the antiangiogenic drug analyzed here. As Chapter 4 classified many tumors as unreliable in Experience 113, this chapter will only consider tumors from Experience 114, and therefore derived from the same patient. To observe the difference between control and treated tumors, the tumors and their blood vessels were segmented on the basis of CECT images. These segmentations were used to derive various data for comparing the two groups of tumors. Firstly, tumors will be compared by volume. Next, blood vessels vascularization will be analyzed both visually and quantitatively in terms of blood vessels volume, blood vessels density and blood vessels thickness. In addition, a comparison will be made between the results obtained with CECT and those obtained with classical 2D histology.

6.2 Materials and methods

6.2.1 Sample

As most reliable tumors were found in experiment 114, this chapter focuses only on tumors from this experiment and therefore from the same patient. Sample and image acquisition conditions are the same as those described in the previous chapters, in sections 4.2.1 to 4.2.3.

6.2.2 CECT image preprocessing

CECT images acquired were also analyzed using *Avizo* software and preprocessing is the same as in Section 5.2.2 meaning cropping of the image and applying *Median filter*.

6.2.3 CECT image segmentation

The tools used for segmentation are the same as those explained in Section 5.2.3. As for the preceding section, the different ranges used can also be found in Appendix F.

Tumor segmentation

The tumors were segmented using the same strategy as explained in Section 5.2.3.

Blood vessels segmentation

The method used to segment blood vessels corresponds to *Method 1* explained in Section 5.2.3 and involves the use of *Interactive thresholding* followed by a segmentation cleaning process.

6.2.4 Volume fraction calculation

Once the different parts of tumors had been segmented, volume fractions of tumor and blood vessels were calculated using "*Volume fraction*" tool from *Avizo* and based on the segmentation performed. Thanks to the volume fractions obtained, blood vessels density could be calculated for each tumor. Blood vessels density is the ratio (VV/TV) of blood vessels volume (VV) divided by total volume of tumor (TV).

Next, tumors were divided into three donuts-shaped parts as shown in Figure 6.1, in order to calculate the volume of blood vessels present in the outer, middle and inner parts of each tumor.

This division was achieved by approximating the shape of the tumors as an ellipsoid defined by three axes. Each tumor was divided in half along the three directions X, Y and Z in order to measure the length of these axes as illustrated in Figure 6.2. The smallest axis was taken as the reference because it is along this axis that tumor reduction will be the greatest. It turned out that reducing the tumor in each direction by 20% (first erosion) or 40% (second erosion) of the length of the smallest axis preserved an average 52% or 21% of the tumor volume respectively. In fact, the percentage of volume retained varied according to the proximity of tumor shape

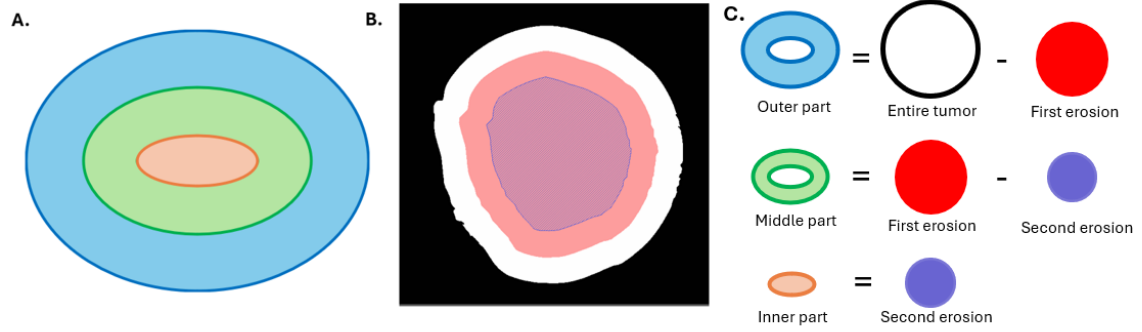


Figure 6.1: A. Illustration of the division of a tumor into 3 donuts-shaped parts representing: the outer, middle and inner parts. The first blue donut represents the outer part with an average of 50% of the tumor inside, then the green donut which is the middle part containing around 25% of the tumor and finally the orange inside which are the remaining 25%. B. Representation of the erosion of CBLRL tumor to divide it into 3 parts. The white area corresponds to the whole tumor. The red zone corresponds to the first erosion and the dark blue zone to the second erosion. C. Explication of correspondence between the various donuts-shaped parties and the erosions performed. The blue donut in A. corresponds to the subtraction of the red part (first erosion) from the white part (whole tumor) in B., the green donut in A. corresponds to the subtraction of the blue part (second erosion) from the red part (first erosion) in B. and the orange donut in A. corresponds to the blue part in B.

to a true ellipsoid and the regularity of tumor. To achieve this reduction, a binary image of tumor segmentation was used. The shape of the tumor was then reduced using "*Erosion*" tool in *Avizo*. The type of erosion chosen was *ball*, as it best suited the curving shape of tumors. As for the size of erosion, since it had to be given in pixels, the 20% or 40% of the smallest diagonal expressed in μm was divided by 6 to obtain pixels, since the size of a voxel is 6 μm in each of the three directions. The binary image of the reduced tumor was then used as a mask (thanks to "*Mask*" option in *Avizo*) on the image containing the segmentation of blood vessels. Once the segmented image has been reduced, volume of blood vessels was calculated using "*Volume fraction*" tool from *Avizo*. To obtain the volume of blood vessels corresponding respectively to the blue, green and orange donuts-shaped parts of tumors, subtractions were necessary as illustrated on the part C. of Figure 6.1.

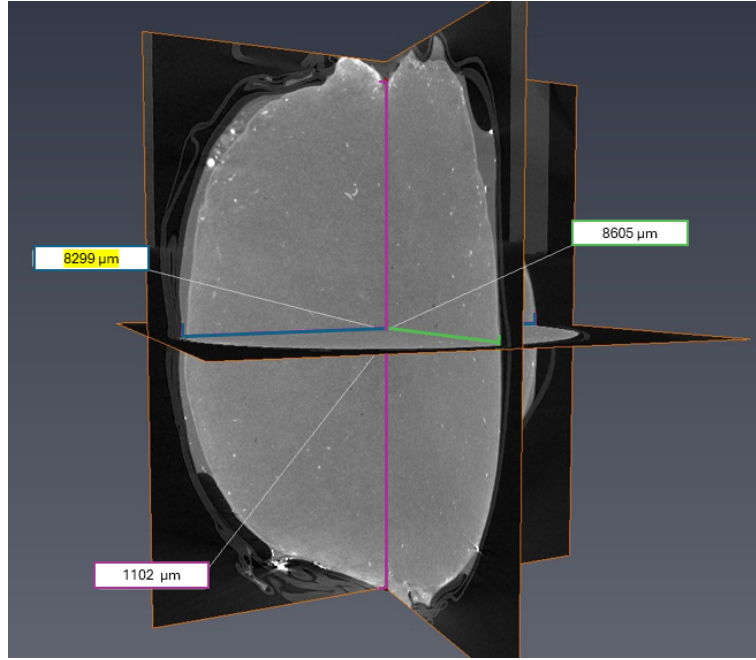


Figure 6.2: Visualisation of tumor CALLL (Experience 114) divided in half along the three directions X, Y and Z with its three axes drawn by colored lines and whose measurements are respectively equal to 8299, 8605 and 1102 μm . The smallest axe is highlighted in yellow and its measurement is the one used for the reduction of the whole tumor.

This division into donut-shaped parts enabled the analysis of two parameters calculated for each donut part in order to compare control and pazopanib tumors. The first analysis consists of comparing the volume of blood vessels present in each donut as a percentage of the total volume of the donut in question. This analysis gives an estimate of the density of blood vessels present in each donut. The second analysis consists of comparing, in percentage terms, the volume of blood vessels present in each donut in relation to the total volume of blood vessels in the tumor. This analysis quantifies the distribution of blood vessels within the tumor.

$$\text{Blood vessels density} = \frac{\text{Blood vessels volume in the donut}}{\text{Volume of the donut}}$$

$$\text{Blood vessels percentage} = \frac{\text{Blood vessels volume in the donut}}{\text{Blood vessels volume in the entire tumor}}$$

6.2.5 Characterization of blood vessel thickness

Based on blood vessels segmentation only, a binary image was generated containing highlighted blood vessels in white and the rest in black. This binary image was then analyzed by *CTAn* software to calculate average blood vessels thickness and thickness distribution. Thickness values are calculated using a “sphere-filling” method as illustrated in Figure 6.3. Indeed, the thickness of a blood vessel at a point corresponds to the diameter of the largest sphere to be fitted inside the blood vessel such that the following conditions are defined:

- "The sphere includes the point, without the point necessarily being the center of the sphere."
- "The sphere is completely inside the blood vessel in question." [91]

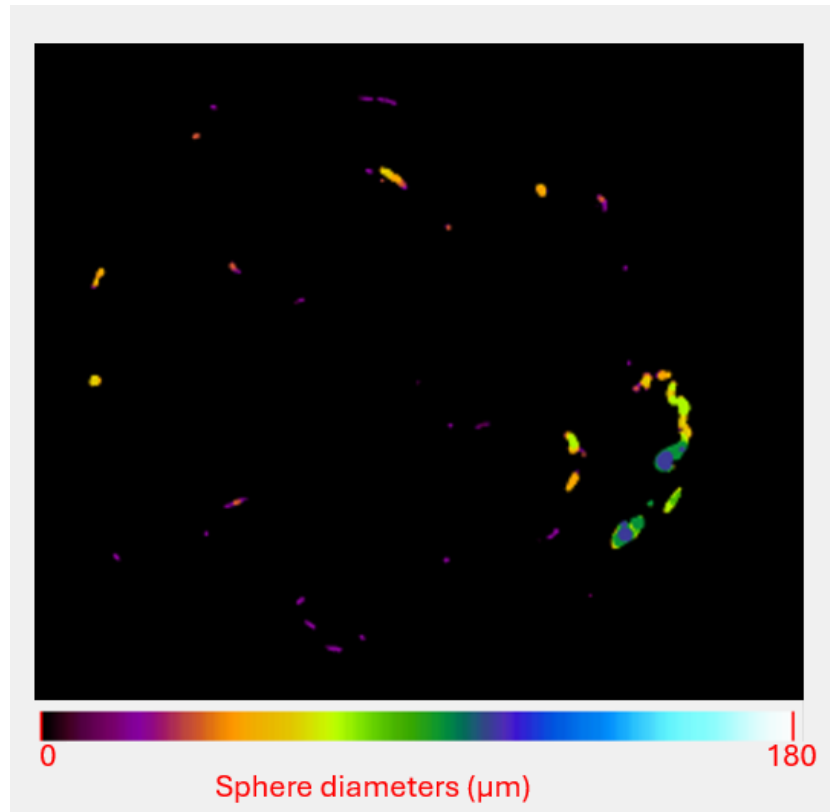


Figure 6.3: Sphere fitting method for measuring blood vessel thickness. It shows CALLL1 tumor cut in the XY plane. The different colors, in accordance with the legend, represent the spheres of different diameters that have been inserted into the various blood vessels. The maximum thickness for this tumor is 180 μm , but due to the absence of light blue or white on the image, vessels of this thickness are not present in this slice.

6.2.6 Classical 2D histology

As explained in the section 4.2.4, after CECT scanning, tumors were stained with H&E and CD31 for histological analysis. In addition, CD31-stained histological sections of all tumors were analyzed by a pathologist who established a blood vessel count on 5 high-power fields to quantify the density of blood vessels present in each tumor.

6.2.7 Statistical analysis

Finally, statistical analysis of the obtained data were calculated using *GraphPad Prism* software. For this, a similar process was followed to compare the obtained data for the control group with those obtained with the treated tumors. This process was used to compare differences in tumor volume, blood vessels density (VV/TV) and average thickness.

As regards the statistical analysis strategy, outliers were first identified and removed from the data. Next, the mean, the standard deviation, the minimum and maximum values were calculated. Then, a normality test of the residuals was performed to determine whether the distribution could be approximated as Gaussian. Finally, statistical significance between the control and pazopanib groups was assessed using the unpaired t-test method if the distribution can be approximated as Gaussian and Kruskal-Wallis test if not. A P significance level of less than 0.05 was considered to indicate statistical significance.

In addition, the previous strategy was also used to analyse blood vessels volume in each different donuts-shaped part of the tumor as illustrated in figure 6.1. For this part, the two parameters described in Section 6.2.4, meaning the density of blood vessels and the percentage of blood vessels, were compared within the same tumor group (control or pazopanib) according to the different parts of the tumor (outer, middle and inner donuts). To do this, outliers were first assessed within each group (control and pazopanib) and removed. Then, a normality test of the residuals was performed to determine whether the distribution could be approximated as Gaussian. Finally, to compare the obtained results for each donut within the same group, if the distribution could be approximated by a Gaussian, One-way ANOVA method was performed, if not Mann-Whitney test was performed instead. Multiple comparisons were used to evaluate any statistical significance (indicated by significance level P below 0.05) of the parameters between the different donuts within the same group.

Finally, *GraphPad Prism* software was also used to try to establish a correlation relationship between the data obtained using CECT and that obtained using

classical 2D histology. To do this, correlation lines were first drawn between the volume of blood vessels (CECT) and the number of blood vessels counted using classical 2D histology as explained in Section 6.2.6. Then, the second comparison was established between the blood vessels density (CECT) and the number of blood vessels counted using classical 2D histology.

6.3 Results and discussion

6.3.1 Tumor volume

A first and simple parameter to evaluate in order to compare control tumors with those treated with pazopanib is tumor volume. In this case, the use of CECT provides a precise measurement of tumor volume based on tumor segmentation. This simple analysis is, however, more accurate and closer to reality than conventional techniques, which involve weighing or measuring the tumor with a calliper to obtain its volume.

Although, as observed in Chapter 4, tumors present certain heterogeneities, even between tumors in the same group, the identification of outliers showed that tumor volume remained within a relatively coherent range as no outliers have been identified here. Comparison of tumor volume between the two groups can also provide evidence of tumor reduction after treatment. However, unfortunately this is not the case in this experiment. As can be seen in Figure 6.4, there was no significant difference between the volume of control tumors and the volume of treated tumors.

Therefore, the mean tumor volumes were relatively similar, with a value of $5.86 \cdot 10^{11} \mu m^3$ for the control tumors and $5.89 \cdot 10^{11} \mu m^3$ for the pazopanib tumors. However, there were differences between the distribution of volumes in the two groups. Indeed, the control tumors showed greater heterogeneity in terms of their value, with volumes ranging from $3.64 \cdot 10^{10} \mu m^3$ to $1.26 \cdot 10^{12} \mu m^3$. The volumes of the treated tumors were relatively similar, with the exception of one particularly large tumor. The range of volumes for the treated tumors thus goes from $3.36 \cdot 10^{11} \mu m^3$ to $1.06 \cdot 10^{12} \mu m^3$. This greater heterogeneity in the control tumors is also reflected in a greater standard deviation of $3.77878 \cdot 10^{11}$ for the control group compared with $2.2923 \cdot 10^{11}$ for the treated tumor groups.

There are several possible explanations for the lack of reduction in tumor volume after treatment. Firstly, one possible reason could be that the tumor did not respond to the drug. Indeed, although several tumors were analysed in the

Comparison of ex-vivo tumor volume between control and pazopanib tumors

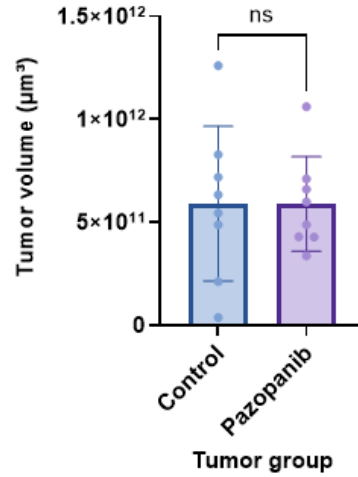


Figure 6.4: Graphical representation of the different tumor volumes calculated on the basis of the segmentation of CECT images. A non-significant difference was observed between control and pazopanib tumors. Furthermore, greater heterogeneity was observed between control tumors, which presented both very small and very large tumors, unlike pazopanib tumors, which were relatively grouped within the same range, except for one tumor that was larger than the others.

experiment, it is important to keep in mind that they all came from the same patient and therefore all have the same origin. It is therefore likely that this treatment does not work on this specific tumor, but works on a tumor derived from another patient. In order to confirm the absence of a reaction to the drug for this specific tumor, a likely approach would be to extend the experiment to xenographs from several different patients. Another possibility is that the observation time was too short. It is also possible that, after administration of the treatment, it may take some time before the drug has a real impact on reducing tumor vascularisation and tumor volume. It would therefore also be interesting to keep some of the mice alive after the end of the treatment in order to see whether the reduction in tumor does not appear later. A final explanation is simply that the treatment or dose was not adapted to the type of tumor treated and therefore had no effect on it. However, to confirm this last hypothesis, further analysis would also be required.

6.3.2 3D visualisation of blood vessels

One of the greatest advantages of CECT is that it enables 3D visualization of blood vessels over the entire tumor, unlike classical 2D histology, which offers only a planar, slice-dependent view. This 3D visualization of the vascular tree structure offered by CECT is invaluable for comparing control and pazopanib tumors. The result of the 3D visualization of blood vessels achieved with CECT can be seen in Figure 6.5. The objective of this 3D visualisation of segmented blood vessels is to analyse several characteristics of these vessels, such as their predominant location, the organisation of their arborescence and vascular density.

These images of the various tumors illustrate the heterogeneity of the vascular arborescence from one tumor to another. Although none of the tumors resemble each other, some similarities can be noted. First of all, it can be observed that control tumors generally have very intense vascularization at the edges of the tumors, with large vessels presenting numerous branches. This strong presence of vessels at the edges of tumors can be justified by the necessity for the tumor to be in contact with surrounding healthy tissue in order to access host oxygen and nutrients. As explained in section 2.4.1 and shown in Figure 2.3, tumors develop blood vessels at their extremities in order to take advantage of their host and invade it. This vascularization will then extend towards the center of the tumor, carrying nutrients and oxygen necessary for cell function. As a result, control tumors are characterized by a pronounced vascular arborescence. Indeed, a specific branching pattern can easily be identified in control tumors. The organization of these tumors is mainly tree-like, starting with a large vessel which then branches out into smaller and smaller vessels. As regards pazopanib tumors, they have fewer large blood vessels and, in general, their vessels tend to be finer than those observed in control tumors. There was also less vascularization at the edge of pazopanib tumors, in contrast to the control tumor. In the control group, blood vessels surrounding the tumors are more easily seen, whereas in the pazopanib tumors, blood vessels running from the edge inwards are the most visible. Vascular organization of pazopanib tumors is also less clear and distinct than that of control tumors. Indeed, some tumors have a tendency to have very sinuous and intertwined vessels, as in tumor E of Figure 6.5, whereas other tumors, on the contrary, have very straight vessels, as in tumor H of Figure 6.5. Furthermore, when comparing control tumors with pazopanib tumors, one would tend to say that control tumors have a greater vascular density, since at first sight it seems that blood vessels cover more surface area. However, this also depends on the tumors observed, tumor B in Figure 6.5 has very little vascularization, and is even less dense than the pazopanib tumor.

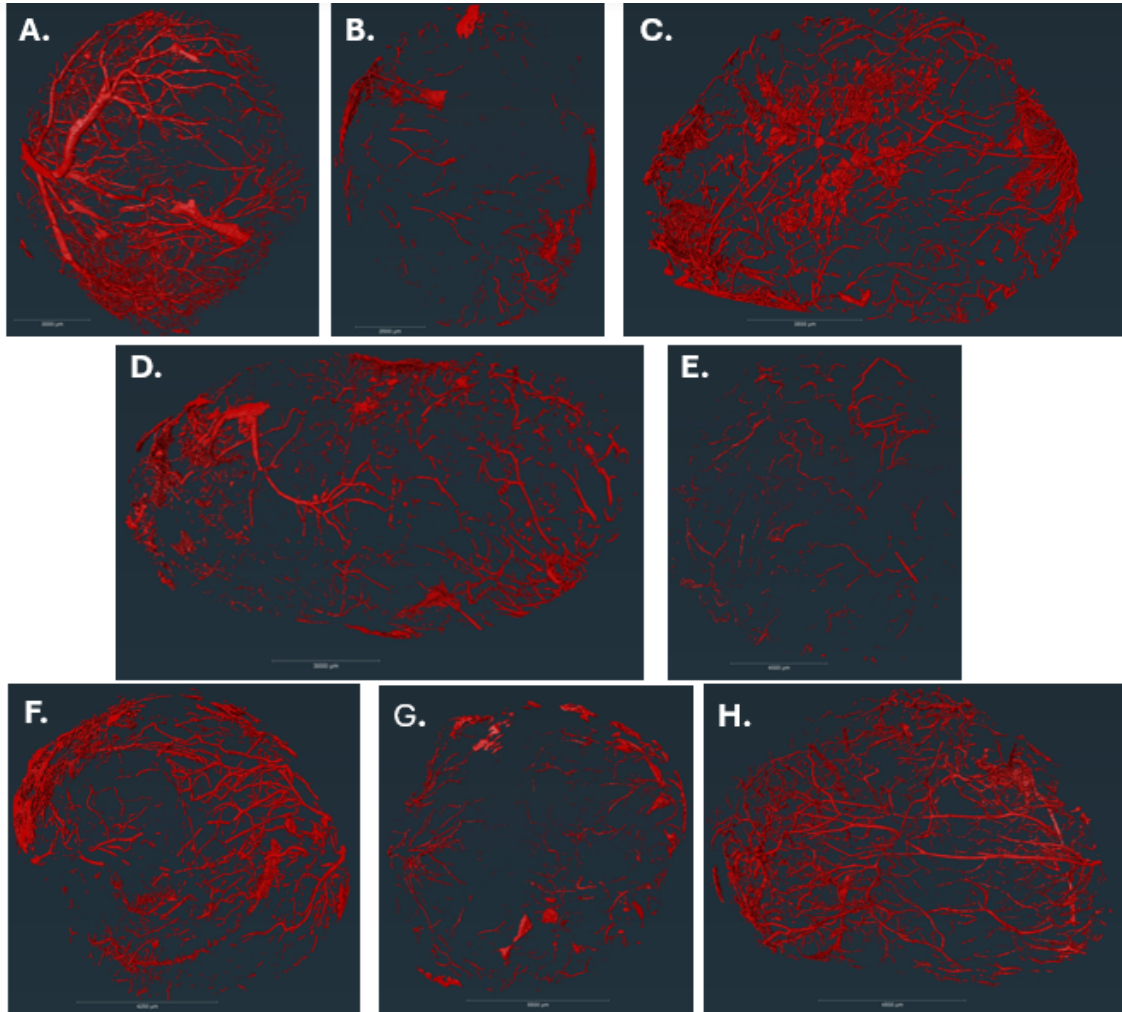


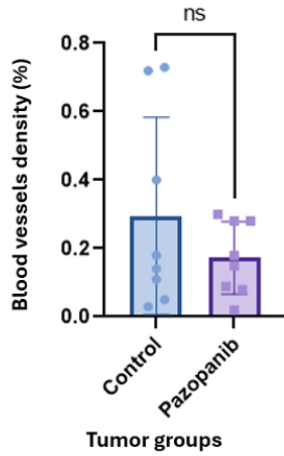
Figure 6.5: A., B., C., and D. are the CANML, CARUL, CALLL and CALUR control tumors respectively, while E., F. G. and H. are the following pazopanib-treated tumors: CBLRLRL, CBLULLR, CBLURUR and CBLURUL. These different images of blood vessels segmentation for the cited tumors illustrate the diversity of the vascular arborescence. However, some similarities can be seen between tumors in the same group. Indeed, a high vascular density can be seen at the edge in control tumors A., C. and D.. Control A. and D. tumors also showed a clear arborescent pattern of vascularization. The pazopanib tumors appear to have a more disorganized vascularization, with vessels that are sometimes very sinuous and sometimes very straight, as illustrated by tumors E. and H.

6.3.3 Blood vessels quantification

Once a visual analysis of blood vessels had been carried out, it is important to add more computational information. To do this, the percentage of the tumor covered by blood vessels was calculated on the basis of segmentation. This percentage

quantifies the density of blood vessels present in each tumor. Blood vessel density is also calculated using classical 2D histology. However, this analysis is performed by counting the number of blood vessels in random areas of the tumor, as explained in Section 6.2.6, and therefore remains approximative and highly dependent on the regions analyzed. CECT, on the other hand, provides a quantification of vascular density based on the entire tumor, thanks to its 3D visualization. The results obtained for the density of blood vessels computed on the basis of CECT is shown in Figure 6.6 and enable to compare the density of blood vessels in both group, control and pazopanib.

**Blood vessels density (VV/TV)
according tumor groups**



Blood vessels density (%)	Control tumors	Pazopanib tumors
Minimum	0,03	0,02
Maximum	0,73	0,3
Mean	0,295	0,1725
Standard. Deviation	0,2885	0,1059

Figure 6.6: Graph comparing blood vessels density between control and pazopanib treated tumors based on CECT. Blood vessels density was calculated as the ratio VV/TV where VV is the blood vessels volume and TV is the tumor volume, both calculated on the basis of segmentation. There is a non-significant difference between the two groups. However, 3 control tumors were found to have relatively high vascular density compared to pazopanib tumors which remained in the same range. The majority of tumors (14 out of 16 tumors) had a vascular density of less than 0.5%.

First of all, it is important to mention that blood vessels density did not exceed 1% and was even below 0.5% for 14 of the 16 tumors, including all treated tumors. Pazopanib-treated tumors tended to have a lower vascular density than control tumors. In addition, treated tumors had more similar blood vessels densities to each other than to control tumors. Indeed, the control tumors were more extensive in value, with 2 tumors in particular having a higher blood vessels density than all the others. The maximum blood vessel density for the control tumors was 0.73 %, whereas the maximum value for the treated tumors was only

0.3 %. However, there is a non-significant difference between the two groups of tumors with regard to their blood vessels density. This lack of significant difference undermines what had previously been observed visually. Indeed, in the section 6.5, a difference in blood vessels density between control and pazopanib tumors was noticed. However, the obtained data show that the difference is not large enough to attest to the impact of the antiangiogenic drug on vascularization. This lack of significant difference may therefore be justified by a lack of tumor response to the drug, as previously mentioned in the discussion of the lack of significant difference in tumor volume but requires further investigations to be verified.

Then, the obtained results for blood vessels density calculated on the basis of CECT are compared with those calculated with classical 2D histology, and both are shown in Figure 6.7.

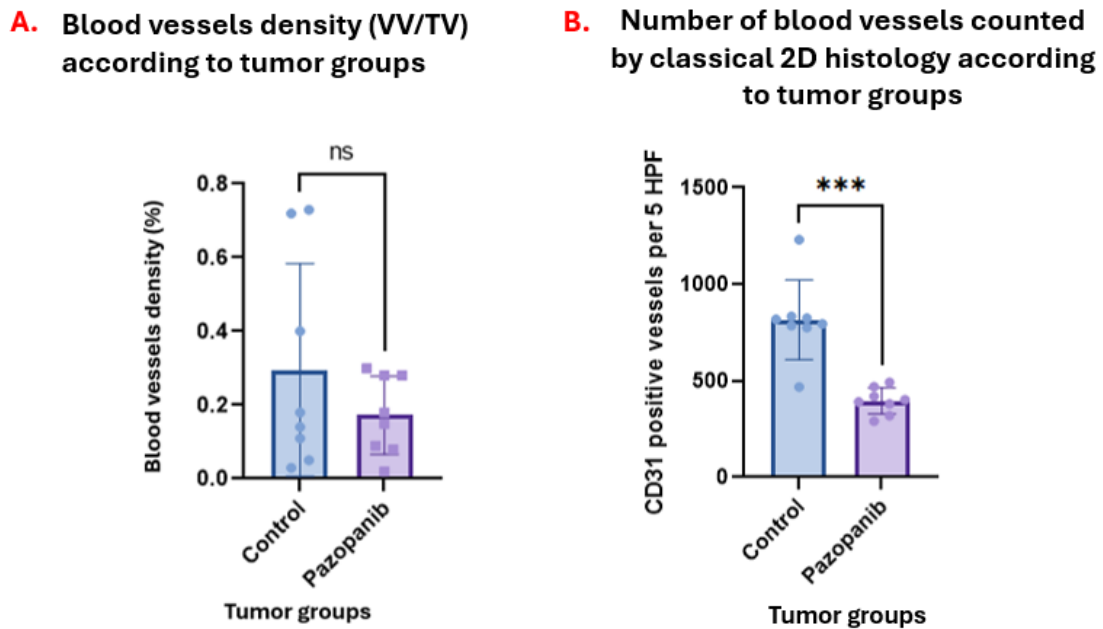


Figure 6.7: A. Graph comparing blood vessels density between control and pazopanib treated tumors based on CECT (same as in Figure 6.6). There is a non-significant difference between the two groups. B. Graph comparing blood vessels density between control and pazopanib treated tumors based on the number of blood vessels counted in classical 2D histology. There is a significant difference between the two groups.

A discordance between CECT and classical 2D histology can therefore be seen in the obtained results for the comparison of control and pazopanib tumors in terms of blood vessels density. While CECT showed a non-significant difference, classic

2D histology showed a significant difference between the two groups. This may be explained by the 2D and slice-dependent vision of classical histology, which may lead to shortcomings concerning its ability to assess the density of blood vessels distributed throughout the entire tumor. Indeed, as explained in section 6.2.6, in order to assess the density of blood vessels present in a tumor, a pathologist manually counts the vessels in randomly selected areas (5 areas in this case). This technique is therefore highly dependent on the observed areas, and can lead to conclusions that are not necessarily in line with reality, as they depend on only a few areas of the tumor and, above all, on a purely 2D visualization. In fact, the 2D visualization of histology can lead to the same vessel being counted several times, due to its intersection with the section at several points. Figure K illustrates errors that can occur when analyzing blood vessels using classical 2D histology and that could have been avoided by CECT. Indeed, CECT, on the other hand, offers 3D visualization and identification of each vessel, as well as a representation of vascularization over the entire tumor. This information is invaluable for estimating blood vessels density. It is therefore clear that the 3D information provided by CECT enables blood vessels density to be quantified more closely to reality. However, it is important to mention that blood vessels segmentation can also influence the obtained results, especially since, as mentioned in Chapter 5, there is still a need for improvement in the segmentation used in this case.

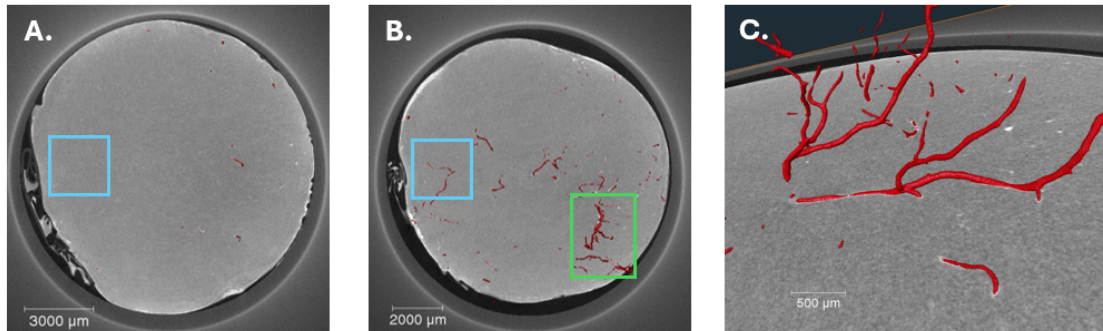


Figure 6.8: Illustration of blood vessels in CBLRL tumor. A. Slice number 1061 in XY plane. B. Slice number 1431 in XY plane. C. Enlargement of the green box in B. Differences in the quantity of blood vessels can be seen between the blue squares in A. and B., which represent the same area of the tumor but at different slices. Moreover, there is also a difference in blood vessels density between the blue and green boxes, which are on the same slice. Finally, image C. shows that a blood vessel can also intersect a slice at several points and therefore lead to being counted several times by classical 2D histology.

6.3.4 Blood vessels distribution

In this section, 3D spatial information provided on blood vessels by CECT is used to more closely analyse the distribution of blood vessels inside tumors. This study of the spatial distribution of blood vessels would not be possible with the purely 2D vision of classical histology.

Indeed, in order to better localize the distribution of blood vessels within the tumor, the latter was divided into three donuts-shaped parts as explained in Section 6.2.4. The aim of this division is to better quantify the proportion of blood vessels present in each part of the tumor. To achieve this, the analysis was separated into two phases. First, analysis of the density of blood vessels present in each part of the tumor and then, quantification in terms of percentage of the volume of blood vessels present in each part compared to the total volume of blood vessels in the entire tumor.

First, with regard to the blood vessels density present in each part of the tumor, it was found that the outer part had the greatest density, followed by the middle part and finally the central part. This lower density of blood vessels in the center of the tumor can be explained by morphological reasons. Indeed, as explained in Section 2.4.1, blood vessels develop in the tumor primarily to receive oxygen and nutrients from the host. It is obvious, then, that the vasculature will initially grow at the edge of the tumor to provide a link with healthy tissues. To connect with its surroundings, the tumor will have more and larger blood vessels at the edge. Another reason for the lower vascular density in the center is that blood vessels visualization is more complex. Indeed, as previously explained in Chapter 4, it is more difficult for the CA to penetrate inside the tumor. As a result, there is a greater likelihood that blood vessels have been more frequently missed inside the tumor than at its edges. Figure 6.9 illustrates the distribution of blood vessels density according to the different donut-shape parts for control and pazopanib-treated tumors.

It can be seen that for the control tumors, blood vessels density is around 0.5% for the outer part, with even 2 tumors having more than 1% density in this part, whereas the pazopanib tumors have a blood vessels density of no more than 0.6%. Moreover, for both groups, there was a significant difference between the density of the outer part and the density of the middle and internal parts. However, the differences were more marked in the case of pazopanib tumors, particularly between the middle and inner parts. In both groups, however, there were no significant differences between the middle and inner parts. The distribution of blood vessel density therefore appears to be fairly similar between the two groups, with the

Blood vessels density according to each donuts

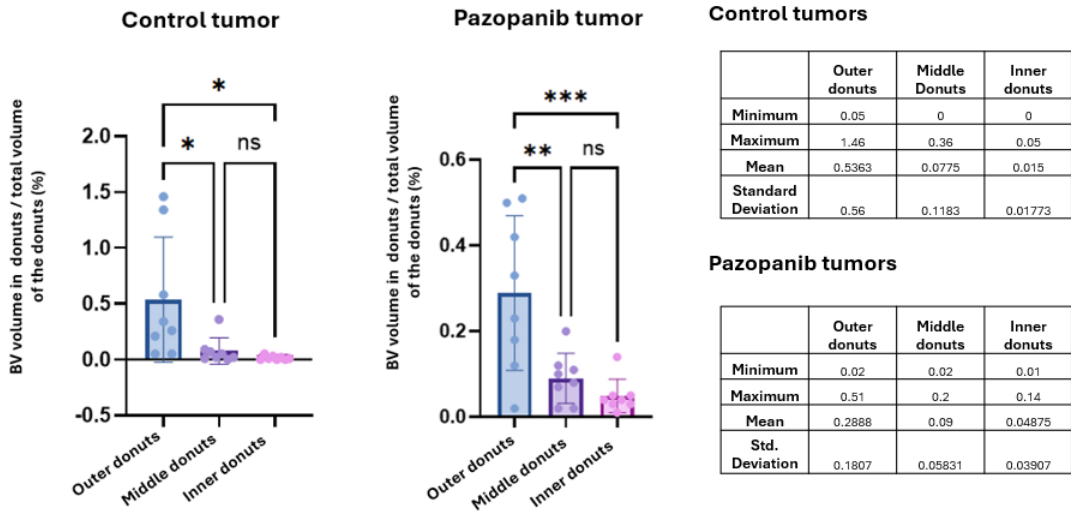


Figure 6.9: Graphs of blood vessels density as a function of the different donut-shape parts for control tumors on the left and pazopanib-treated tumors on the right. It can be seen that in both cases, blood vessels density is the highest for the outer donut and lowest for the inner donut. For control tumors, a small significant difference can be observed between the outer donut and the middle and inner donuts. For pazopanib tumors, moderately and highly significant differences could be observed between the outer and middle donuts and the inner donut respectively. In both groups, however, no significant differences were found between the middle and inner donuts.

exception that the density values are higher for control tumors. To confirm this impression of a similar distribution of the different donuts-shaped parts of the tumors, Figure 6.10 proposes a comparison between control and pazopanib tumors of the density of blood vessels within each part.

It can be seen that there is no significant difference between control and pazopanib tumors for outer and middle donuts. However, it can be noted that the density of blood vessels in the outer donut is higher for control tumors than for pazopanib tumors, which appear to be less extensive over a wide range of values. For its part, the average blood vessel density in the middle donut is very similar between the two groups. Finally, with regard to the inner donut, there was a significant difference between control and pazopanib tumors. Indeed, the pazopanib group has a higher mean value and therefore have a higher density of blood vessels in the center than control tumors.

Next, the second analysis performed on the division of tumors into donut-shape parts is the comparison of the percentage of blood vessels volume present in each

Blood vessels density in each donuts according to the tumor groups

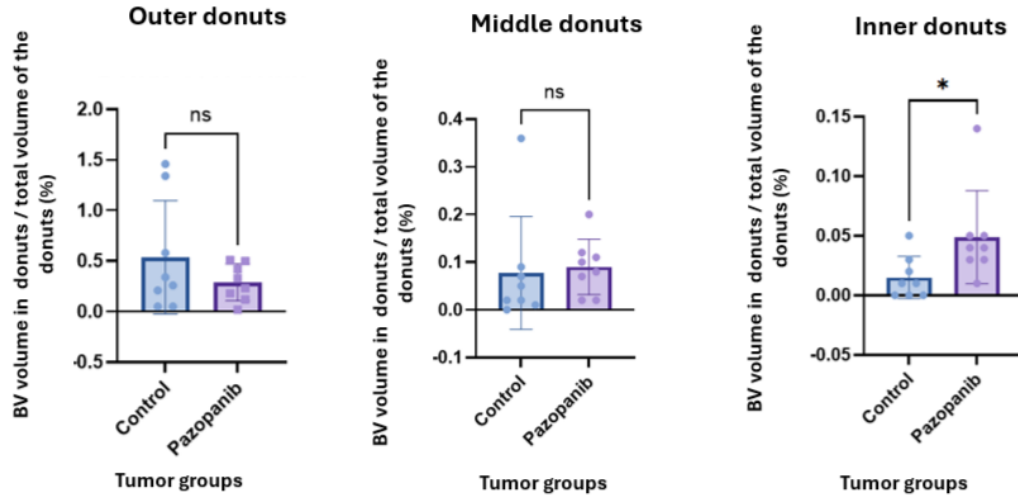


Figure 6.10: Graph showing the density of blood vessels present in control and pazopanib tumors for each donuts-shaped part of the tumor. For the outer donuts, there is a non-significant difference between the control and pazopanib groups, although the control tumors had a higher density for this part. As for the middle donut, there is also no significant difference between control and pazopanib tumors. Finally, for the inner donut, there is a significant difference between the two groups, with higher values for pazopanib tumors.

donut compared with the total blood vessels volume. Figure 6.11 illustrates the distribution of these percentages for control and pazopanib tumors.

The comparison of percentages leads to similar conclusions as the comparison of blood vessels densities. In fact, the outer donut seems to be very different from the other two, which show no significant difference between them. Moreover, for the control group, almost all the blood vessels were located in the inner part, with an average of 83.42% of vessels, compared with 74.85% for the pazopanib group. These high percentages of blood vessels on the outer parts of tumors can be justified, as explained above, by the need to interact with the tumor host. Next, Figure 6.12 illustrates the differences in blood vessels percentage between control and pazopanib tumors for each donut-shaped part.

The results observed are similar to those obtained for blood vessels density, with 2 exceptions. Firstly, there is a significant difference between the control and pazopanib groups in terms of the outer donut, with a higher percentage of blood

Percentage of the volume of blood vessels present in each donuts compared to the total volume of blood vessels

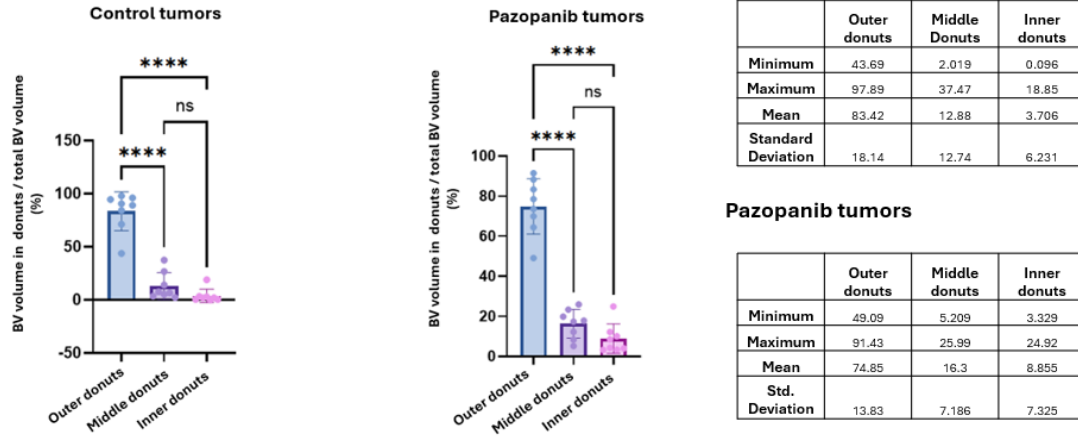


Figure 6.11: Graph showing the percentage of blood vessels volume present in each donut relative to total blood vessels volume and according to each of the 3 donut-shaped portions observed. The graph on the left illustrates this percentage distribution for control tumors and that on the right for pazopanib tumors. In both cases, there is a highly significant difference between the outer donut and the middle and inner donut. However, there is no significant difference between the middle and inner donut.

vessels in the control tumors. This was not the case in terms of density, which was considered insignificant. Secondly, the difference between control and pazopanib tumors at the inner donut level is considered highly significant in terms of percentage. In fact, the percentages obtained for pazopanib tumors are much higher than those for control tumors.

In conclusion, a very inhomogeneous distribution of blood vessels is observed for control tumors, with a high percentage of blood vessels at the edges of the tumors and low percentages of blood vessels in the middle and center. In the case of pazopanib tumors, the distribution of blood vessels percentages is also heterogeneous, but less uneven. The problem with this finding is that it is not in line with what might have been expected. Indeed, a study demonstrated that tumors with low levels of VEGF had vessels located mostly in the tumor periphery and the most angiogenic tumors displayed a vascularization more homogeneously [68]. Unfortunately, this would mean that the less homogeneous control tumors would be less angiogenic and therefore possess less VEGF than the pazopanib tumors, whereas the aim of pazopanib is to diminish angiogenicity and therefore reduce the presence of VEGF. It is therefore necessary to find an explanation

**Percentage of the volume of blood vessels present in the concerned donuts
compared to the total volume of blood vessels in the tumor
according to each tumor groups**

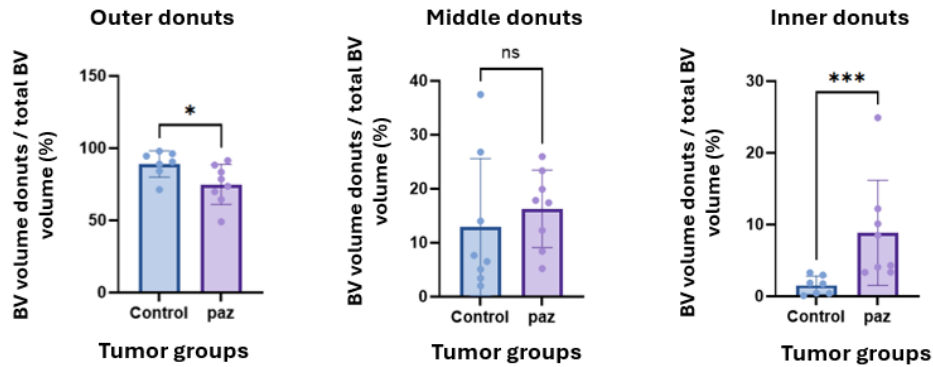


Figure 6.12: Graph representing the percentage of blood vessels volume present in each donut compared with the total blood vessels volume for each 3 donut-shaped part observed and comparing these percentages between control and pazopanib tumors. There is a significant difference between the control and pazopanib groups at the level of the outer donut, with a higher percentage of blood vessels in the control tumors. There is no significant difference in the middle donut. There is a highly significant difference between the two groups for the inner donut.

for this observation, which contradicts what could be expected. A first possible explanation is, as mentioned previously, the possibility that the drug did not have the desired effect on the tumors, and consequently vascularization is not reduced in pazopanib tumors. Secondly, it is still probable that incomplete diffusion of the CA in certain tumors prevented good visualization of the blood vessels and therefore led to altered results. Finally, it is important to highlight that both groups of tumors show a non-homogeneous distribution of blood vessels. It is therefore possible that the greater heterogeneity of control tumors compared with treated tumors is simply due to the small number of tumors compared. Indeed, it would be preferable to extend the comparison to a larger number of tumors to see whether the differences are still significant or not.

6.3.5 Blood vessels characterization

Another interesting parameter to analyze in relation to vascularization is blood vessel thickness. Indeed, although the aim of antiangiogenic drugs is mainly to reduce vascularization in itself, these kind of drugs can also have an impact on blood

vessel dimensions. CECT, with its 3D view of blood vessels, offers the significant advantage of quantifying blood vessels thickness throughout the entire tumor. This overcomes the limitation of classical 2D histology, which can only assess a single slice at a time and therefore has a limited representation of blood vessels thickness. Figure 6.13 shows a 3D visualization of colored vascular tree according to blood vessels thickness. In the control tumors (A., B. and C. on Figure 6.13), a greater

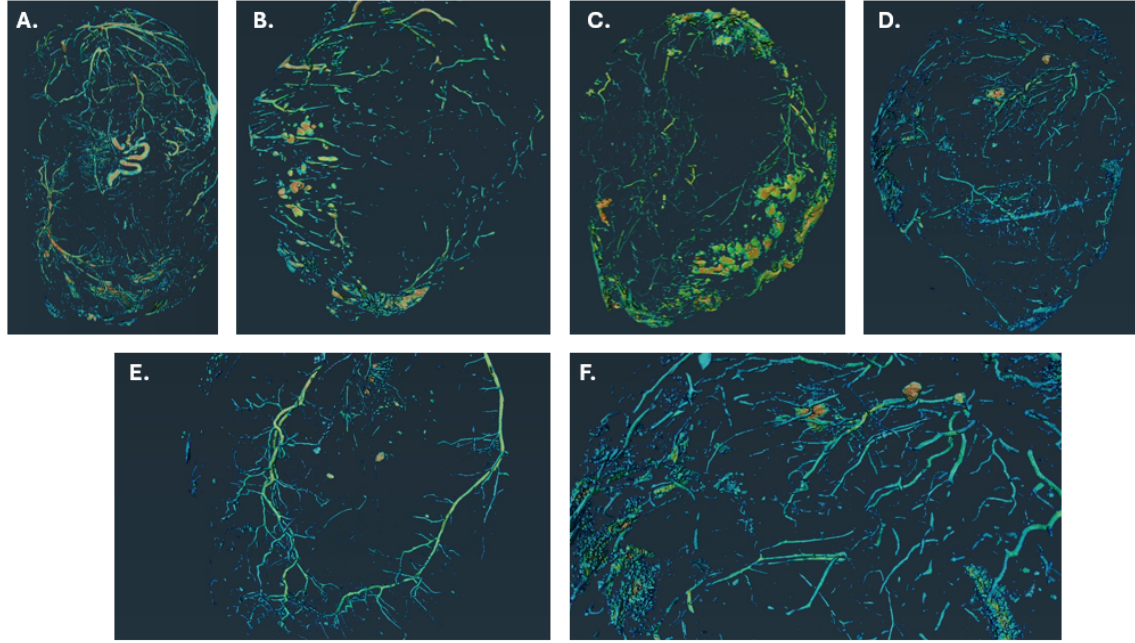


Figure 6.13: 3D visualization of blood vessels stained according to thickness. A., B. and C. are CALUR, CARUR and CALLL control tumors respectively, while D. and E. are CBLURUR and CBLRLR pazopanib-treated tumors. F. represents a zoom on the CBLURUR tumor. Thicker blood vessels are red or yellow, medium vessels are green and smaller vessels are blue.

number of thick blood vessels were observed, whereas the tumors treated with pazopanib showed more medium and thin blood vessels. It can also be seen that the thicker vessels are mainly located at the edges of the tumors. Furthermore, on the CBLURUR tumor enlargement shown in F. on Figure 6.13, it can be seen that there are many very fine vessels inside this pazopanib tumors. Finally, it is very important to mention that some of the thickest parts may also be remains of hemorrhagic plaques that could not be removed during the segmentation cleaning process, as shown in tumor C. in Figure 6.13.

After a visual analysis, a more computational analysis is also necessary. To this end, the thickness averages of control and pazopanib tumors were compared, as shown in Figure 6.14. There was a non-significant difference between control and pazopanib

Average thickness comparison between control and pazopanib tumors

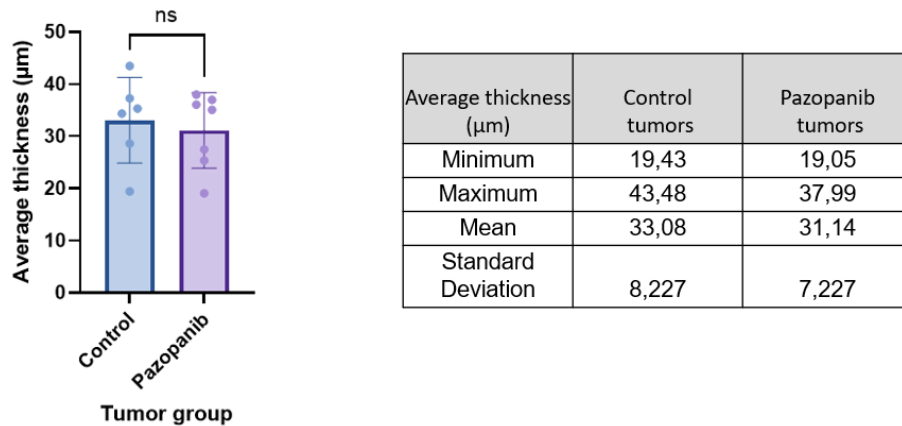


Figure 6.14: Comparison of the average thickness of blood vessels present in control and pazopanib tumors (expressed in μm). There was a non-significant difference between the two groups. The average thickness was less than 50 μm in both cases, but slightly higher in control tumors.

tumors with regard to the average thickness of their blood vessels. Indeed, both groups had average thicknesses of less than 50 μm with a slightly higher average thickness in control tumors. The non-significant difference between both groups means that blood vessel thicknesses are similar in control and pazopanib tumors. Therefore, the antiangiogenic drug would have no influence on blood vessel thickness. To confirm this hypothesis, thickness distributions were assessed for control and pazopanib tumors, and are shown in Figure 6.15. It can be seen that control and pazopanib tumors have similar blood vessel thickness profiles. In fact, both groups have the highest percentage of blood vessels with a thickness of 24 μm . However, it can be seen that pazopanib tumors generally have a higher percentage of blood vessels with smaller thicknesses than control tumors. This can also be justified by the fact that, conversely, control tumors have higher percentages of thicker blood vessels. In addition, although they represent on average only 0.23% of control tumors, it is important to note the absence of blood vessels with a thickness equal to 228 μm in pazopanib tumors, for which the greatest thickness detected was 216 μm . Finally, it is also important to mention that while the pazopanib tumors show a constant decay after their peak at a thickness of 24 μm , the control tumors show two small peaks following the main peak, also at a thickness of 24 μm . The next two peaks are of very small amplitude, appearing at thicknesses of 120 and 180 μm . This illustrates the tendency of control tumors to possess a greater number of blood vessels

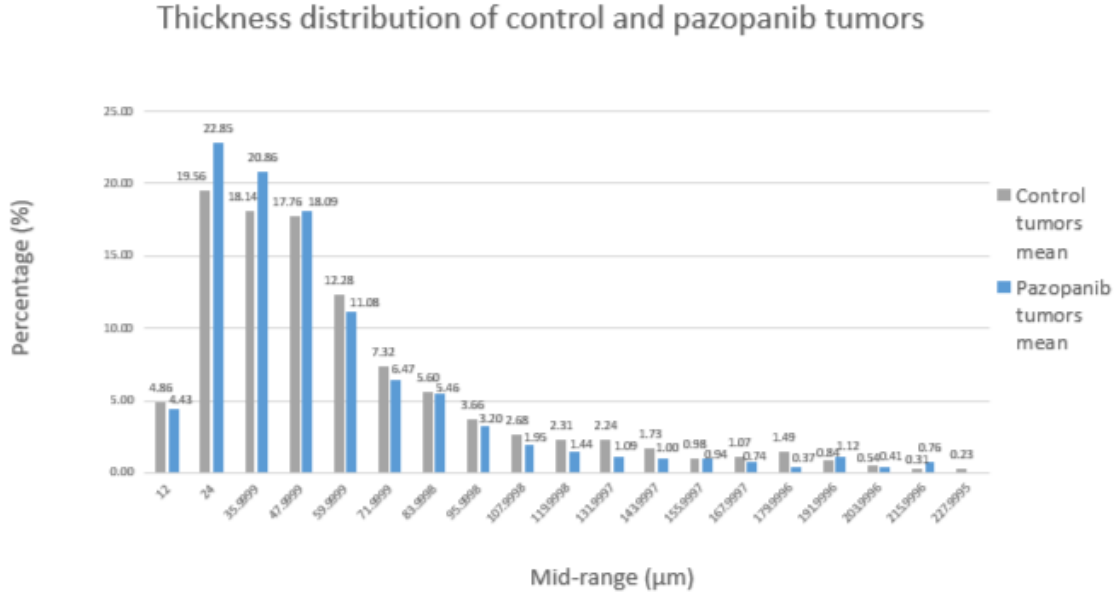


Figure 6.15: Thickness distribution graph representing the percentage of each mid-range value (μm) present for tumor control and pazopanib. The mid-range corresponds to the thickness of the blood vessels calculated using the "sphere-filling" method as explained in section 6.2.5

of greater thickness compared with pazopanib tumors as also outlined in Figure 6.13

However, it is important to mention that, as shown in Figure 6.13, some tumors still have hemorrhagic plaques in their blood vessel segmentation. Due to their larger volume than that of the blood vessels, these plaques can lead to an overestimation of average thickness, as well as influencing thickness distribution. It is therefore important to bear in mind that these segmentation defects could lead to distorted results. To confirm the obtained results, it would therefore be preferable to carry out the same analyses with other segmentation methods, to see if the results change or not.

6.3.6 Correlation between CECT and histology blood vessel quantification

The aim of this section is to assess whether the results obtained with CECT are in line with what could have been obtained with classical 2D histology. To this end, a correlation was attempted between what is analyzed with CECT, such as blood vessels volume or blood vessels density, and what is analyzed with classical 2D histology, meaning the number of blood vessels counted in 5 randomly selected areas. The first established correlation was between the quantification of blood vessels

made with CECT on the basis of volume and the number of blood vessels counted with classical 2D histology. This first analysis led to an inconclusive correlation, with a very low correlation coefficient r of 0.005882. Figure 6.16 illustrates the relationship between blood vessels volume calculated using CECT and the number of blood vessels counted using classical 2D histology. There was a poor correlation

Correlation between the number of blood vessels counted in histology and the blood vessels volume computed on CECT data

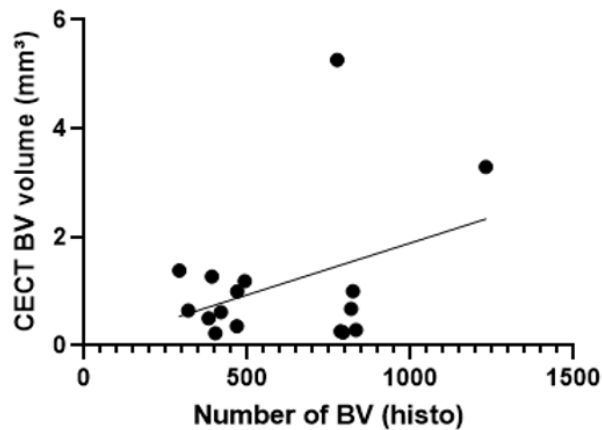


Figure 6.16: Correlation between blood vessel volume calculated with CECT (mm^3) and the number of blood vessels counted in classical 2D histology. This analysis led to an inconclusive correlation, with a very low correlation coefficient r of 0.005882.

between the two parameters evaluated. Indeed, a good correlation would have been represented by an arrangement of the data around an oblique line whose slope would be close to 1 and therefore an r value close to 1 as well. However, this is not the case here. Instead there are two clusters of data for which similar numbers of blood vessels counted by histology correspond to different values of blood vessels volume calculated by CECT. In addition to these two clusters of data, there are also two tumors that stand out for having higher blood vessels volume values than the others. One of these tumors also has the highest histology value. The fact that this tumor has a high value for all two imaging techniques shows that it is indeed more vascular than the others, regardless of the technique used to assess its vascularity. The second tumor, on the other hand, has a similar number of blood vessels to other tumors, but the highest volume of blood vessels. This may point out a problem with the histology. Indeed, it is highly probable that by selecting 5 random regions in the tumor to assess the vascularization of the whole tumor, the number of blood vessels will be underestimated or overestimated, depending on the areas analyzed. Indeed, as highlighted in previous sections, tumor vascularization is

heterogeneous, and classical histology has the disadvantage of leading to conclusions that are highly dependent on the regions analyzed, and therefore may vary from one analysis to another. This lack of correlation may therefore be linked to the fact that CECT takes into account the entire tumor to obtain blood vessels volume, whereas histology is strongly influenced by the regions analyzed.

Secondly, the number of blood vessels counted in the histological sections was compared with blood vessels density. However, the correlation was again inconclusive too, although it showed a higher value for the correlation factor r of 0.2180.

Correlation between the number of blood vessels counted in histology and blood vessels density computed on CECT data

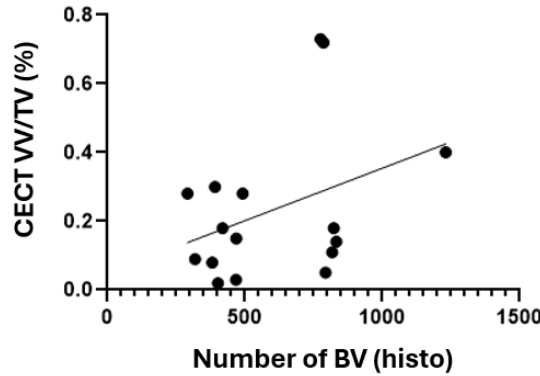


Figure 6.17: Correlation between blood vessel density (VV/TV) calculated using CECT (expressed in %) and the number of blood vessels counted using classical 2D histology. The analysis led to a correlation factor r of 0.2180.

This lack of correlation between blood vessel quantification performed with CECT and those obtained with classical 2D histology is in line with what was also obtained in Section 6.3.3. These observations confirm that classical 2D histology, with its slice-dependent view and analysis limited to specific regions, leads to a blood vessel count that under- or over-estimates the real quantity of blood vessels. Therefore, 3D information provided by CECT is an essential improvement in overcoming the limitations of classical 2D histology. Finally, a very important point to underline is that the blood vessels volume and therefore density calculated at CECT can also be influenced by the segmentation performed. Therefore the correlation between CECT and classical 2D histology can also vary with different segmentation methods. It is therefore essential to improve blood vessels segmentation in order to obtain

results that are closer to the real vascularization of tumors.

6.4 Conclusion

After evaluating various parameters and comparing them for control and pazopanib tumors, it turned out that many of them showed no significant difference between the two groups. Indeed, tumor volumes were compared and showed no reduction in the case of treated tumors. Moreover, no differences between the two groups were detected in terms of blood vessel density or average thickness. However, differences were detected according to other factors. Firstly, by observing the vascularization of the tumors, it was found that the control tumors had more massive vessels at the edges of the tumors, whereas the pazopanib tumors had predominantly medium and small vessels, and these were mainly located towards the interior of the tumors. Furthermore, the analysis of the vascular structure of the tumors revealed a prominent tree-like organization in the control tumors, whereas this characteristic was less pronounced in the pazopanib-treated tumors. In addition, the pazopanib tumors showed particular characteristics: their vessels were either very sinuous or very rectilinear. Significant differences were also detected in the distribution of blood vessels within the tumors. Indeed, differences were observed in terms of density and percentage of blood vessels present in the inner part of the tumors, as the pazopanib tumors had significantly more vessels in this area than tumor controls. Additionally,, it was found that control tumors also had a greater proportion of their blood vessels present in the outer part of their tumors, as their percentage of blood vessels was significantly higher than that of pazopanib tumors. Finally, although the average thickness was similar for control and pazopanib tumors, the thickness distribution showed some divergences between the two groups. Indeed, there was a greater tendency for control tumors to have thicker blood vessels than pazopanib tumors. Conversely, pazopanib tumors had higher percentages of thinner blood vessels.

Unfortunately, given these mixed results, no conclusions could be drawn regarding the influence of pazopanib on the tumors analyzed. Indeed, although visual and analytical differences could be observed, the results obtained did not provide sufficiently significant and quantitative information to be able to confirm or not the hypothesis of blood vessel reduction in pazopanib tumors. In addition, it is important to note that this analysis was only carried out on tumors originating from the same patient, and therefore does not represent the wide clinical diversity that exists. Indeed, it is highly possible that this specific tumor is simply not reactive to pazopanib. It would therefore be necessary to carry out a more quantitative analysis involving not only a larger number of tumors, but also tumors derived

from different patients.

Finally, divergent results were observed concerning blood vessel quantification with CECT and classical 2D histology. Indeed, the 2D view, limited to specific regions, of classical histology can lead to a quantification of blood vessels that does not reflect reality. Conversely, the 3D visualization offered by CECT provides a more realistic representation. However, it must be highlighted that the results obtained by CECT can also be influenced by the segmentation method. Indeed, it has been shown in Chapter 5 that blood vessels segmentation still possesses certain imperfections. It is therefore necessary to improve this segmentation as much as possible to obtain a visualization of the vascular arborescence that matches reality as closely as possible, and which will therefore enable us to assert with greater confidence the influence of a drug on tumor vascularization.

Chapter 7

Conclusion and future perspectives

In this master thesis, the advantages of using contrast-enhanced microfocus X-ray computed tomography (μ CECT) were highlighted for various aspects of *ex-vivo* tumor visualization and vascularization. Indeed, although classical 2D histology is the gold standard for detailed visualization of tumor and its various structural elements such as blood vessels, necrotic areas, and adipocytes, this technique is limited by its 2D perspective. As a result, CECT represents a highly promising method for resolving the limitations of classical 2D histology in the visualization and study of tumor vasculature.

First, CECT has proven to be a technique that offers the undeniable advantage of 3D visualization and quantification of blood vessels over the entire tumor. This 3D visualization enables blood vessel trajectories to be followed, as well as provides an overview of the distribution and organization of vessels. The spatial distribution of blood vessels visualized using CECT is therefore invaluable in representing all the heterogeneity of vascularization that exists between different tumors. Indeed, the complexity and variability of the vascular arborescence was observed both between tumors belonging to different groups (control or pazopanib tumors) and within the same group. These findings would have been difficult to achieve with classical 2D histology due to its limited visualization of tumors according to a single planar section of a sample. The use of CECT allows tumor samples to be visualized and cut at any point and in any direction, without destroying the sample. In addition to blood vessels, CECT enabled the identification and localization of adipocytes in the entire tumor. This 3D visualization of adipocytes using CECT also showed that the slice-dependent vision of classical 2D histology can lead to missed elements. By restoring 3D architecture of tumors, CECT therefore makes it easy to identify and quantify the elements such as blood vessels and adipocytes.

Secondly, this thesis investigated the ability of blood vessel segmentation to best represent the reality of the vascularization occurring in tumors. For this purpose, it became clear that the use of a threshold alone was not sufficient, and several aspects had to be taken into account when segmenting blood vessels. Indeed, good cleaning is essential to eliminate the selection of undesirable elements that are not actually present in the vasculature. For example, most tumors showed large hemorrhagic areas at their edges due to blood clotting, making it impossible to distinguish blood vessels clearly. In other cases, the interface between the tumor and paraffin created a contrast that was mistakenly identified as blood vessels by the threshold used. Additionally, small elements like impurities had gray values similar to blood vessels and were falsely selected. It was therefore necessary to establish a segmentation method that avoided these elements. In addition, another challenge highlighted in this research is the appearance of a single blood vessel fragmented into several distinct parts. Indeed, one of the main objectives of segmentation is to recreate inter-vessel connectivity and reattach the different parts of the same vessel. This thesis has shown the advantages of closing to enable the reconstruction of fragmented vessels. However, further improvements are still required to achieve the most accurate results possible. In this thesis, three different strategies are developed and analysed. The study of these different segmentation methods has demonstrated the importance and impact of segmentation on the accurate representation of blood vessels.

Thirdly, untreated control tumors were compared to tumors treated with an antiangiogenic drug called pazopanib, in order to assess the influence of this drug on tumors. The main aim of this part of the thesis is to quantify the efficacy of this drug in reducing tumor development by inhibiting tumor vascularization. To this end, patient-derived xenographs of liposarcoma were transplanted into immunodeficient mice to illustrate the heterogeneity present between different individuals. The use of ex-vivo CECT allowed detailed assessment of tumor vasculature and the identification of changes in response to antiangiogenic treatment through various parameters. The first finding was that there was no significant difference between control and pazopanib tumors in terms of tumor volume. This meant that no significant reduction occurred after treatment with pazopanib. Secondly, with regard to blood vessel density using CECT, no significant difference was also observed. This finding contradicts the significant difference detected by comparing the number of blood vessels per 5 HPF calculated with classical 2D histology. This discrepancy highlights that the assessment of blood vessel density based on classical 2D histology could lead to under- or over-estimation. Indeed, classical 2D histology quantifies blood vessels by counting them in a predefined number of

randomly selected zones. The results obtained by histology will therefore depend on the slice and zones analysed, and may lead to erroneous conclusions. CECT, on the other hand, enables blood vessel quantification by calculating the volume or density of blood vessels over the entire tumor, which is an undeniable advantage of 3D visualization of blood vessels. Thirdly, 3D CECT visualization revealed differences between the vascular tree structure of control and pazopanib-treated tumors. Control tumors generally show intense vascularization at the borders, with large, highly branched vessels following a tree-like pattern. In contrast, pazopanib tumors have less large vessels, which are mainly oriented from the periphery to the interior of the tumors. The vascular organization of pazopanib tumors is also less clear, with sinuous, intertwined vessels in some tumors, and very straight vessels in others. Next, differences in vascular distribution between control and pazopanib-treated tumors were analyzed in detail by quantifying blood vessels in outer, middle and inner areas of the tumors. This spatial analysis of blood vessels would not have been possible with classical 2D histology due to its planar nature. By examining the distribution of blood vessels in the different zones, it was found that blood vessels were generally predominantly located at the edges of tumors, illustrating the heterogeneity of their distribution. In addition, control tumors showed a significantly greater percentage of their blood vessels in the outer part compared with pazopanib tumors. The pazopanib tumors, on the other hand, had a significantly higher density and percentage of blood vessels in their inner part. Finally, the last parameter analyzed to compare control and pazopanib tumors was blood vessel thickness. This showed a non-significant difference between control and pazopanib tumors with regard to the average thickness of their blood vessels. However, thickness distribution analysis revealed that even though the average thickness could be considered similar between the two groups, control tumors tended to be composed of blood vessels of greater thickness than pazopanib tumors, which were composed of blood vessels of smaller thickness.

Although some changes in tumor vascularization were observed with the use of pazopanib, they were not sufficiently conclusive to demonstrate a real impact of the drug. Indeed, as no significant differences were observed in terms of blood vessel density or tumor volume, this could suggest that pazopanib does not have the desired effect of reducing tumor vascularization and thus tumor growth. However, it is preferable to carry out further analyses, as the non-significant results obtained could also be due to the fact that all tumors came from the same patient xenograph. It is also possible that the drug simply has no effect on this particular tumor. In addition, short observation times, a too low dose of pazopanib or an insufficient number of tumors analyzed may also be factors influencing the results.

Despite all the advantages and advances made possible by the use of CECT, this technique has yet to overcome certain limitations. First of all, dark areas where CA has not been properly diffused have been detected on many tumors, and have represented a major problem for quantifying vascularization. Indeed, the difficulty of obtaining homogeneous staining and confirming good diffusion of CA in the blood vessels has been highlighted. It is therefore necessary to find a way of improving CA diffusion to obtain better staining, as the absence of staining is impairing blood vessel visualization. Furthermore, it has been found that even with good diffusion of staining, some blood vessels are not fully visible, leading to fragmented visualization of the same blood vessel. Indeed, another problem with staining is that it requires the presence of blood in the blood vessels, and its absence can therefore also compromise blood vessel visualization. This also relates to the limitation of visualizing small blood vessels located mainly in the center of tumors. Indeed, it has been observed that classical 2D histology shows very small blood vessels that are not necessarily visible on CECT. One possible reason for this may be that Hf-POM binds to blood cells like red blood cells, so if there is no blood there will be no staining. CD31, used in histology, binds a transmembrane glycoprotein expressed by endothelial cells and platelets. It is therefore possible that CD31 can detect blood vessels even when there is no blood in them, whereas CECT cannot. Another limitation of CECT is the visualization of necrosis. Indeed, their identification on CECT could only be performed on tumors for which the CA had not completely diffused, and consequently tumors that had been classified as unreliable. Moreover, these necroses could never have been identified on CECT without the help of classical 2D histology. Therefore, no conclusions can be drawn regarding the identification of necrosis on CECT, and further investigations are required. As far as segmentation is concerned, although various methods have been considered and have proved effective, there are still points for improvement to be mentioned. Indeed, the complexity of the vascularization requires solving various problems such as eliminating hemorrhagic zones, recreating connectivity between blood vessels and detecting small vessels located in the center of tumors. It is therefore necessary to continue investing in other methods, or in ways of improving these methods to obtain the most robust segmentation possible, and to homogenize and consolidate the results obtained.

The limitations cited here suggest a number of possible improvements and future perspectives concerning the use of μ CECT for ex-vivo tumor visualization. Indeed, one of the major challenges is to find a way to improve CA diffusion. To achieve this, various parameters can be investigated, such as the concentration of Hf-POM used or the sample temperature, as well as the use of other CESAs.

Secondly, to assess the influence of pazopanib on tumors and their vascularization, it is essential to carry out additional tests to obtain more detailed information. One option, for example, would be to apply an automated tool from *Avizo* called *Autoskeletization* to the binary image of segmented blood vessels. Indeed, *Autoskeletization* tool recreates the tree-like organization of the blood vessels, by constructing graphs made up of segments and nodes linking these segments. The aim of this tool is then to collect various parameters such as the number of segments making up a blood vessels tree, the average length of blood vessels, the number of branches, the tortuosity of blood vessels... These characteristics would therefore provide new investigative elements for comparing control tumors and tumors treated with pazopanib. In addition, it would be necessary to carry out further experiments with a larger number of samples to gain a clearer picture of tumor reactivity, as well as to adjust various parameters such as treatment dosage, treatment duration, and the length of tumor observation. It would also be interesting to extend the experiment to xenographs of a larger number of different patients, in order to obtain a better representation of clinical reality. Finally, it is essential to evaluate and investigate other blood vessels segmentation methods to establish the most robust segmentation method possible. For example, it may be possible to filter blood vessels by volume to remove selected elements that are too large or too small to be blood vessels. In addition, filtering by volume would also make it possible to apply different closings more specific to the range of blood vessel volumes and thus improve the connectivity of blood vessels that have been separated into several parts. Furthermore, automatic segmentation with methods such as *Otsu* or *Watershed* as well as artificial intelligence and machine learning can also offer solutions for blood vessels segmentation.

It should be mentioned that the data analyzed in this master thesis dates from 2016, and the CECT scans were performed at that time. Since then, imaging techniques have already evolved. For example, the process of normalizing gray values had not been used and therefore did not allow homogeneity of gray values between tumors for features of the same density. It is therefore interesting to point out that CECT is an imaging technique that continues to improve, and each improvement enables clearer and more detailed visualization. Future advances in CECT technology, as well as in segmentation methods and improvements in CA diffusion, could further enhance the accuracy and benefit of this technique in tumor visualization. It is therefore inevitable that future improvements in CECT technology will enable 3D visualization of blood vessels as accurate as, or even more detailed than, the 2D visualization of classical histology.

Appendix A

In-vivo imaging

A.1 Ultrasonography

Ultrasonography is based on the quantity of echoes received after ultrasound waves have been sent through different tissues, which have reflected or scattered them [2].

Unlike most other imaging techniques, ultrasonography does not use ionizing radiation, which could damage tissues [50]. The fact that no ionizing radiations are emitted is one of the main advantages of ultrasonography, enabling it to be used in cases where radiations must be avoided, for example, with pregnant women [52, 53].

In addition, ultrasonography offers the advantage of real-time images to visualize organs structure and movements, as well as blood flow in blood vessels [2]. In the case of blood vessels, special ultrasonography equipment can also be used to show the speed and direction of blood flow in the vessels. These devices are called Doppler flow machines and are very useful for quantifying the difference in blood flow between tumor tissue and normal tissue [92].

Ultrasonography is a good first-line examination because of its ease of use, good spatial resolution and capability to distinguish between solid and cystic lesions. In addition, this technique can be beneficial to identify certain STS [93]. Moreover, this technique has also the advantage of been less expensive than the other imaging modalities [2]. However, ultrasonography images are not as accurate as those from other imaging techniques such as CT or MRI [92]. Another major drawback is that this imaging technique is limited in depth of visualization, and can therefore only visualize tumors to a depth of a few centimetres [49].

A.2 Magnetic resonance imaging (MRI)

Magnetic resonance imaging (MRI) is one of the main imaging techniques for the detection and diagnosis of cancer, particularly brain cancer [94]. This technique is based on the magnetic moment of nucleus of atoms, and in particular the hydrogen nuclei of water molecules present in the body. Therefore, MRI scanner produces an external magnetic field that forces the nuclear magnetic moment to align with this external magnetic field. What is measured during MRI is the speed at which nuclei disturbed by electromagnetic radiation return to their initial state [15]. This speed is quantified by tissue relaxation times and can be expressed in various ways, but the main ones for MRI are T1 and T2. T1 stands for spin-lattice or longitudinal relaxation time, which is the time for the longitudinal component of the tissue magnetization to restore to its balanced value. In T1-weighted MRI, tissue with a short T1 will appear bright because it will recover its equilibrium value faster than tissue with long T1. T2, on the other hand, quantifies transverse relaxation time. In T2-weighted MR images, a tissue with short T2 relaxation time appear dark compared to those with long T2. This is a result of the faster decay of transverse magnetization of short T2 tissue [95].

Moreover, the speed of tissue reversion to their equilibrium state depends on the environment in which their nuclei are located. Contrast agents, for example, can cause the observed nuclei to return more rapidly to their initial state. This increased relaxation will lead to an increase in signal at the location of the agent [15]. In tumor imaging, agents such as Gadolinium (Gd(III)) can facilitate and enhance the quality of tumor detection, as illustrated in Figure A.1 [96, 15].

MRI is a reference imaging technique for the diagnosis of soft tissue tumors, as most STS are highly intensified on T2-weighted MRI scans, as are myxoid tumors, for example. In addition, certain tumor types are easily recognized by their specific MRI features [93].

However, MRI has the disadvantage of being costly and time-consuming, requiring the expertise of an imaging professional. Moreover, MRI has limited resolution and encounters several obstacles such as patients suffering claustrophobia, side-effects from contrast agents, or the presence of pacemakers or metal implants [51, 97]. The presence of non-removable metal prostheses requires a MRI scan with a magnetic field intensity of 1.5 Tesla (T) instead of the usual 3T. This lower magnetic field limits susceptibility artifacts and image distortions caused by metal implants [98].

Another use of MRI for cancer diagnosis is whole-body MRI (WB-MRI), which can detect tumors in several organs at the same time. Indeed, this technique can offer widespread body mapping, at least from head to pelvis, and without exposing

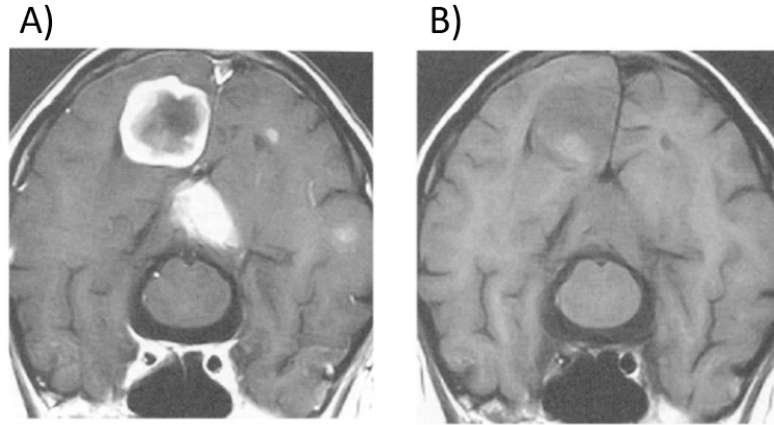


Figure A.1: Magnetic resonance imaging (MRI) of brain metastasis. (A) with gadolinium-based contrast agent (Gd-BOPTA). (B) without contrast agent.[15]

subjects to ionizing radiation as with a CT scan, for example [98]. In addition, the detection of primary tumors and distant metastases with BM-MRI has been demonstrated to be as effective as with PET/CT [99].

Concerning tumor vascularization, MRI offers relatively good spatial resolution and specificity, without the need for ionizing radiation, and can be used in 2 different ways to visualize blood vessels. The first is Arterial Spin Labelling (ASL), and the second is Blood Oxygenation Level Dependent (BOLD) signal. These 2 techniques provide indirect measures of angiogenesis, such as blood flow and blood volume, without the need for external contrast agents [42].

To measure tissue perfusion, arterial spin labelling uses radiofrequency pulses to invert the nuclear spin of protons present in arterial blood. As the protons in arterial blood pass through the region of interest, the radiofrequency pulses invert their nuclear spin. This creates a kind of magnetic marking of the blood. Once in the region of interest, the tagged blood exchanges magnetically with the water protons present in the surrounding tissue [42]. This exchange modifies the magnetization of the tissue. The effect is low-intensity and cannot be measured directly, but it is highly quantitative and does not require the use of contrast agents. Arterial spin labelling is mainly used as a non-invasive means of indirectly monitoring angiogenesis in brain tumors by measuring blood flow [42, 100].

On the other hand, Blood Oxygenation Level Dependent (BOLD) signal was originally developed as a functional MRI technique and quantifies changes in the

deoxy/oxyhemoglobin ratio [42]. One study showed that using BOLD could be useful for detecting hypoxia, which is a problem in angiogenesis leading to insufficient oxygen supply to tissues. Hypoxia is considered a good indicator of a radioresistant tumor and therefore provides important information on the treatment to be administered to the patient [55]. While BOLD imaging is highly effective in brain activation studies, it has proved more difficult to apply this methods to tumors, as the relationship between signal and angiogenesis is not always clear. In addition, the technique is limited by susceptibility artifacts, poor resolution and lack of precision [42].

However, other research is attempting to use MR contrast agents to detect angiogenesis, for example with dynamic contrast-enhanced-MRI [42, 49].

A.3 Positron emission tomography (PET)

Positron emission tomography (PET) belongs to the category of functional imaging, which use radionuclides that release low levels of radiation for detecting metabolic activity. These radiations will then be captured by special cameras that show where the tracer is accumulating. The main functional imaging techniques used are bone scintigraphy and PET, but also thyroid scintigraphy, multi-managed acquisition (MUGA) scintigraphy and gallium scintigraphy [20].

PET is based on the use of molecules labelled with radioactive isotopes that produce a positron. This positron will travel a short distance before encountering an electron and being annihilated. This annihilation process generates two photons, each with an energy of 511 keV, ejected in opposite directions. These photons interact with the ring detector, resulting in a visible line indicating the location of the annihilation event. The detection of the photon at the same time is called a coincidence, and the accumulation of several coincidence occurrences constitutes the final PET image [101, 102].

Radionuclides are important elements in PET imaging because choosing the right one is essential to obtain the most accurate information. These radionuclides are produced in cyclotrons, and their energy determines the distance that can be crossed through the tissue to reach the final target location. Furthermore, the half-life of the isotope must be taken into account, and must be consistent with the imaging process [102]. The most widely used radionuclide is [18F]fluorodeoxyglucose (FDG), with a half-life of 109.7 minutes. Other frequent radioisotopes are carbon-11, oxygen-15, and nitrogen-13 [51, 101]. However, new tracers are still being developed to target new metabolic processes providing new sources of information for cancer

diagnosis, monitoring and prognosis [102].

Regarding cancer diagnosis, PET imaging is used to acquire a wealth of information about the cancer and its progression in a non-invasive manner [103]. PET is a particularly effective imaging method for detecting cancer, identifying the level of malignancy of tumors thanks to their ability to absorb a tracer and localizing small primary tumors or undiagnosed metastatic cancers [102].

A recurrent technique is [18F]FDG PET to image glucose metabolism in the brain, prostate, pancreas, bladder, and other cancers. This technique quantifies glucose overconsumption, which is mainly present in tumors [102, 103]. However, PET scans with [18F]FDG have certain drawbacks because it has some difficulties when differentiating tumor from infection or acute inflammation [102]. Other disadvantages of PET imaging are its high cost, due to the need to produce radiotracers. Moreover, there is a risk of radiation exposure via the administration of radioactive tracer and the spatial resolution of PET is limited compared to the other modalities [51, 102].

About vascularization imaging, although PET can measure picomolar concentrations of tracer molecules, there are few commercial radiotracers available to correctly identify vasculature, and those available offer poor spatial resolution. One option would be the use of O-water (oxygen-15 labelled water) in PET to estimate tumor perfusion non-invasively. However, studies are difficult to perform due to short half-life of the radiotracer and poor imaging features of dynamic PET images, which result in low-resolution images [20].

A.4 X-ray and computed tomography (CT)

X-ray imaging is based on the acceleration of electrons under high voltage, which then collide with an anode made of a high-atomic-number material, usually tungsten. Then the interactions between the electrons and the anode produce X-rays. These X-rays are afterwards sent through the object to be observed, and the absorption of these rays by the object enables visualization [70].

X-rays are an essential imaging technique for diagnosing cancer using a fixed x-ray tube [104]. Indeed, a very common example of the use of X-ray imaging for tumor detection is the mammography to detect breast cancer [20, 96]. X-rays offer the advantages of low cost and simplicity, making them a highly suitable imaging technique for primary screening [105]. Indeed, X-rays provide a good initial assessment of the cancer, particularly when it is located at the extremities

[93].

A better alternative is employing computed tomography (CT) which uses X-rays to provide a three-dimensional view of the patient. This technique uses a beam of X-rays that rotates around the patient, producing signals that are collected by detectors and transmitted to a computer. The computer then processes the signals and generates images in different slices from different angles, which are collected and digitally stacked to form a 3D image [104, 51]. In addition to precise spatial resolution, CT also provides high temporal resolution of tumors [51, 59] and the possibility of "*Fast Scan*", which helps to decrease motion artifacts [51].

CT scans visualize basic structures in the patient as well as possible abnormalities and is therefore a diagnostic technique for hemorrhage, blood clots, or cancer [104].

Unfortunately, the main disadvantage of CT is that the use of X-rays produces ionizing radiations, and these radiations can have biological effects on living tissue, such as the further development of neoplasia. It is therefore necessary to find solutions to reduce the radiation dose received, and this has been one of the major concerns of CT since its introduction in the 1970s [104, 106]. Another disadvantage is the presence of artifacts created by metallic objects, such as implants and prostheses. These artifacts can lead to the dissimulation of anatomical structures, thus hindering diagnosis [104].

Then, another weakness of CT is its low visibility of soft tissues. Indeed, soft tissues have a low capacity to stop X-rays, so they require the use of contrast agents to enhance visualization [104]. The main contrast agents (CAs) are based on iodine or barium, due to their high X-ray attenuation [104, 71]. The use of contrast-enhanced computed tomography (CECT) is an essential diagnostic tool for retroperitoneal sarcomas for example. CECT allows visualization of the site of origin of the cancer, providing information on tissue composition which is fundamental to define the type of retroperitoneal sarcomas. In addition, different types of retroperitoneal sarcomas can be differentiated on the basis of their appearance on CT imaging, thanks to visualization of the amount of fat, heterogeneity or calcification of the sarcoma [107]. However, the use of CAs can be problematic, as iodine as well as other agents can cause allergic reactions [104, 51] or, in extreme cases, temporary renal failure [104].

Regarding tumor vasculature with CT, the main limitation of this imaging technique is also the absence of soft tissue differentiation. Therefore, to overcome this limitation, CAs are indispensable to visualize vascularization. In general, used CAs

can be classified into two categories: blood pooling and targeting. Blood pooling CAs move through vascular structures, highlighting the blood vessels of each organ they pass through before being eliminated from the body. In comparison, targeting CAs bind to specific extracellular matrix proteins or cell receptors and accumulate in the organ concerned to increase its visibility [108].

One study showed that *in-vivo* dual-energy CT with an iodine-containing contrast agent allowed a good visualization of the relative blood volume. However, one of the main disadvantages of this technique was the low resolution of the *in-vivo* CT which was about 35 mm per voxel whereas the majority of newly formed blood vessels in tumors are smaller than that. In addition, *ex-vivo* CT was necessary in order to have a 3D visualization of the vasculature [68].

A.5 PET/CT

The combination of structural and functional imaging is becoming increasingly common, as it enables better visualization and localization of tumors. One example is the development of hybrid imaging systems such as PET/CT, which combine metabolic sensitivity of PET with temporal and spatial resolution of CT [52, 51]. Indeed, PET has the major disadvantage of lacking anatomical information and must therefore be combined with CT. In this case, high specificity of PET tracers for their molecular targets provides few anatomic landmarks but it is difficult to track the initial tumor and metastasis. The combination with CT allows to localize the origin of the signal and thus to improve this weakness of PET by providing a better anatomical representation. This multimodality imaging method allowing visualization of metabolic information within an adequate anatomic framework. In addition, research has made it possible to adapt whole-body imaging techniques to small animals so that tumor and metastasis can be visualized and quantified in a single scan as can be seen in Figure A.2 [16].

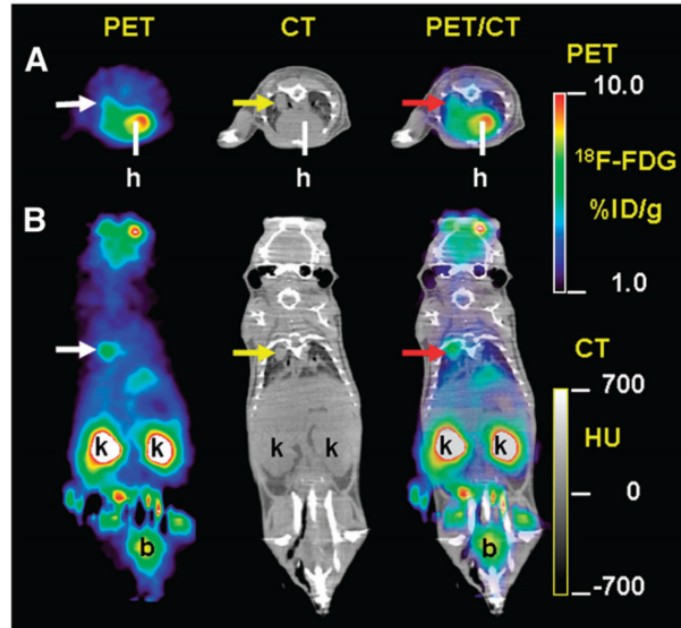


Figure A.2: Detection of pulmonary metastasis with ^{18}F -FDG PET/CT. From left to right, there is PET alone, CT alone and finally the combination PET/CT. (A) Transverse section and (B) coronal section. The white and yellow arrows indicate, respectively, the ^{18}F -FDG hypermetabolic zone on PET and a lesion with a density similar to that of water on the CT image. The red arrow on the PET/CT image confirms the location of the metastasis on the dorsal apex of the right lung. Physiologic tracer uptake is also visible in heart (h), kidneys (k), and bladder (b). [16]

Appendix B

More information on immunohistochemistry (IHC) of sarcomas

The various biological markers used in immunohistochemistry (IHC) for the visualisation of sarcomas can be divided into three categories: lineage-restricted transcription factors, protein correlates of molecular alterations and gene expression profiling [65].

First, regarding lineage-restricted transcription factors, this category involves the use of antibodies that recognize nuclear transcription factors. The most widely used transcription factor for soft tissue tumors is myogenin (MYF4). Myogenin is essential for the diagnosis of rhabdomyosarcoma, but is also helpful in identifying the presence of differentiating rhabdomyoblasts in other tumor types, such as dedifferentiated liposarcoma and malignant peripheral nerve sheath tumors [65]. Another marker of lineage-restricted transcription factors consist in the special AT-rich sequence-binding protein 2 (SATB2), a nuclear matrix protein. SATB2 enables osteoblastic differentiation in bone and soft tissue tumors, as this protein is essential for osteoblastic lineage recruitment [65].

Secondly, protein correlates of molecular alterations is the category discussed in Section 2.5.5.

The third category in IHC corresponds to diagnostic markers identified by gene expression profiling. These markers can be used to distinguish between histologically similar classes of tumors. One example is transducin-like enhancer of split 1 (TLE1), which identifies synovial sarcoma from other types of sarcoma [65].

Appendix C

Phase contrast imaging techniques

While some methods are based on integral calculations, crystal interferometry imaging directly detects phase shift through wave superposition [17]. A phase-contrast imaging system with an interferometer is shown in Figure C.1 and consists of three parts: a splitter, a mirror, and an analyzer. The role of the splitter is to divide the incident beam into two waves. Between these two waves, only one will pass through the object to be observed, not the other. After the splitter, the mirror part allows the waves to converge towards the analyzer. The two waves formed a diffraction pattern on the analyzer, which showed the difference in the beam paths caused by refraction from the object on only one of the two waves [17, 18].

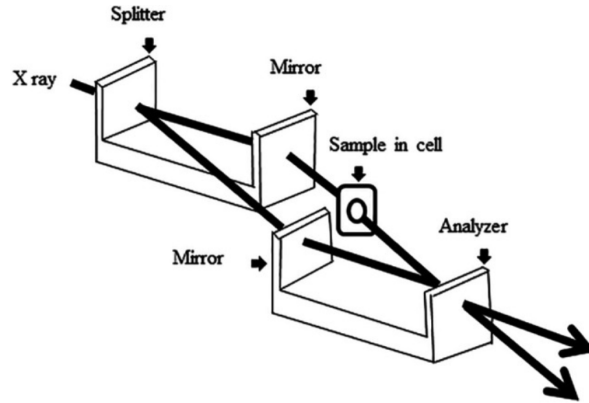


Figure C.1: Phase-contrast synchrotron-based X-ray CT system with two-crystal X-ray interferometer. The 3 different parts of the interferometer are visible: the splitter, the mirror, and the analyzer. Here, there are 2 mirrors, one for each wave resulting from the incident beam after splitter separation. Only one wave passes through the sample. Then, the two waves meet on the analyzer, allowing the diffraction difference between the two waves to be analyzed. [17]

Another technique for phase-contrast imaging involved using a grating interferometer, as shown in Figure C.2. For this, a first grating (G1) is placed after the object to be observed, and its role is to split the incident beam into two first-diffraction orders. These two beams then interact with each other, resulting in linear periodic fringe patterns. A second grating (G2) with the same period as the first is then located at a specific distance from G1. This second grating enables the diffraction fringes to be restored on its detector [18].

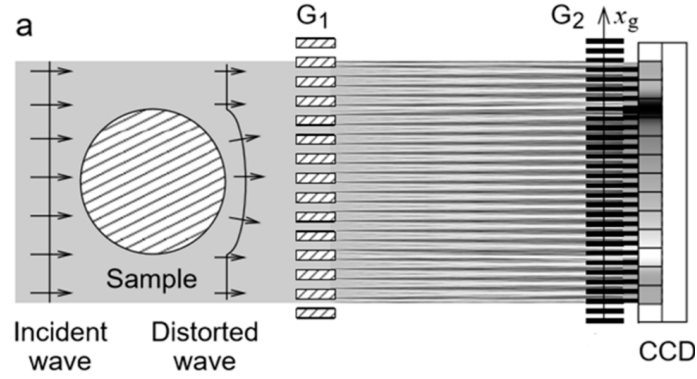


Figure C.2: Schema of the grating interferometer with the incident wave passing through the sample to view, then crosses the first grating (G1), the second grating (G2) and the detector. [18]

Another technique used for phase-contrast CT is propagation-based phase contrast imaging. This method requires only the source, object and detector, and no other components, as shown in Figure C.3. The phase contrast information is therefore obtained from the intensity measured in the detector [18].

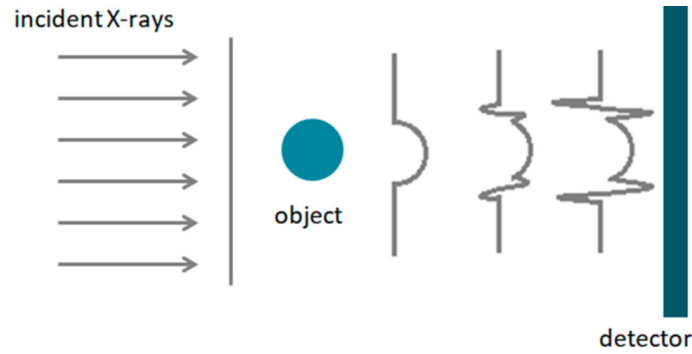


Figure C.3: Propagation-base phase-contrast imaging system composed only of the source, the object, and the detector. [18]

Appendix D

Radiotherapy : fractionating process, types of treatments and side effects

Radiotherapy is based on a process called fractionating. Indeed, fractionating consists of administering the total dose of radiation over several days or weeks. The total dose is thus delivered by a beam in several sessions, called a fraction [22]. This fractionating process is used because the time between different radiations allows normal tissues to repair much of the damage caused by radiation, while tumor cells, which are less capable of repair, do not recover and are therefore eliminated [22, 85]. However, some tumor types are more sensitive to radiotherapy than others [109, 110].

Radiotherapy can be used to avoid surgical removal of an organ when it is possible to achieve comparable tumor control. When the tumor is larger, radiotherapy is used in conjunction with surgery to reduce the size of the tumor, or to reduce the risk of tumor recurrence [22]. Moreover, radiotherapy can be used as an adjuvant treatment when surgical margins are insufficient, or after highly challenging surgical procedures [36]. This use of adjuvant radiotherapy has demonstrated positive effects on the local control of STS, improving the 5-year local control rates to 82% for patients who received adjuvant brachytherapy, compared to 69% for those who did not [84]. A recurrent example where radiotherapy is employed is for the treatment of DDLs of the extremities when they are deep and with a diameter exceeding 5 cm, or in the case of resection with positive margins where enhancement is not possible without causing serious complications [84]. Considering the advantages of combining radiotherapy with surgery in terms of reduced tumor recurrence and improved local control, the National Comprehensive Cancer Network advises the use of radiotherapy for stage II, IIIA and IIIB extremity STS, either preoperatively

or postoperatively. For less advanced tumors (stage IA or IB), surgery only can be performed if resected with large margins [84].

The doses used for palliative radiotherapy are then lower than those used for curative purposes. In this case, radiotherapy aims to reduce or eliminate the need for analgesics, used to reduce pain [22]. In the case of metastatic STS for which surgery is not an option, palliative treatment typically also includes radiotherapy [25]. Radiotherapy is used for palliative purposes to treat a variety of problems, including pain associated with bone and brain metastases, or patients pain with terminal lung or prostate cancer [22].

The two types of side effects that can be observed with radiotherapy are early effects and late effects. Early radiation toxicity occurs within a few weeks of treatment and generally affects skin and mucous membrane cells. For example, radiation therapy to an abdominal tumor can damage the lining of the small intestine, causing malabsorption and diarrhea. However, these side effects disappear after a few weeks once the cells have been replaced. Late side effects are much less frequent, occurring months or years after the end of treatment. They can result from tissue damage that cannot compensate for cell death. As with most treatments, these side effects depend on and vary according to the body area treated, the volume of normal tissue irradiated, the total dose and the accumulation rate of the dose [22, 85].

Appendix E

Systemic treatment : types of treatments and side effects

The most commonly drug used for STS chemotherapy is doxorubicin, but epirubicin, ifosfamide or trabectedin may also be administered [84, 36]. However, the use of systemic chemotherapy for STS is mainly considered for high-risk lesions, such as those over 5 cm in diameter or at acute recurrence potential. Indeed, the rarity and diversity of the biological behavior of STS subtypes hinders any conclusion regarding the efficacy of neoadjuvant or adjuvant treatment in STS [84].

Furthermore, chemotherapy can also be used for palliative purposes for different types of cancer, including advanced STS with metastases [25, 111]. Indeed, the main role of chemotherapy is palliative, with the aim of controlling and slowing the development of advanced or inoperable sarcomas and their symptoms [36]. When a patient is diagnosed with inoperable or metastatic STS, standard palliative treatment includes a single drug regimen of doxorubicin, dacarbazine, epirubicin or ifosfamide, or a combination of drugs. Doxorubicin-based chemotherapy has shown the best results in terms of response in patients with locally advanced or metastatic STS. However, sensitivity to these compounds depends on histological features [84, 36]. In this case of liposarcomas, doxorubicin is the most commonly used first-line palliative treatment for advanced liposarcomas, but it has limited effectiveness [45].

Unfortunately, the side effects associated with chemotherapy are numerous. Common side effects include nausea and vomiting, and hair loss. Other effects are linked to the cytotoxic effects on normal dividing cells in the digestive tract, bone marrow or blood. These include thrombocytopenia, anemia, mucositis, and alopecia. Other toxic effects are less widespread, as they are more specific to each drug or drug class [22, 23]. A major disadvantage is also toxicity, restricting the dose of doxorubicin

that can be administered. Indeed, although doxorubicin is the most common drug for STS chemotherapy, it possesses a cumulative cardiotoxicity effect. This results in decreased left ventricular function and other adverse cardiac events [36]. In addition, some metastatic STS respond poorly to chemotherapy [13].

Appendix F

Ranges and thresholds used for segmentation

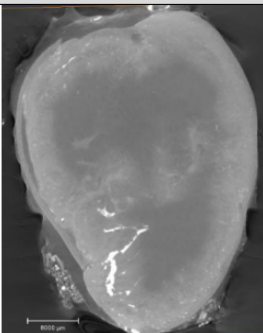
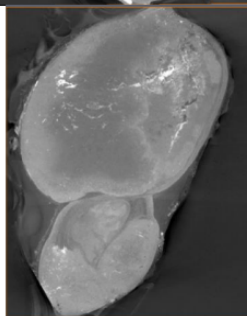
This appendix shows the different thresholds used to segment tumors, blood vessels and dark areas. Where the upper value is not specified, this means that it was set to the maximum (= 254). The “erosion” column specifies the percentage of the length of the smallest axis used for erosion when segmenting blood vessels, as explained in Section 5.2.3.

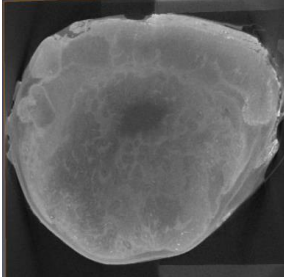
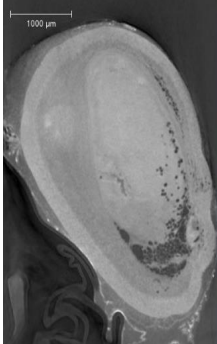
Experience 114			
Tumor names	Tumor seg.	Blood vessels seg.	Erosion
CALLL	65	162	3%
CALLR	45	131	4%
CALUL	86	160	2%
CALUR	116	152	2%
CANML	112	180	3%
CANMR	95	157	4%
CARUL	96	160	5%
CARUR	84	160	4%
CBLLRLL	105	196	3%
CBLLRLR	65	144	4%
CBLULLL	92	175	4%
CBLULLR	121	185	3%
CBLURUL	108	155	3%
CBLURUR	107	150	3%
CBRURLL	105	172	3%
CBRURLR	96	155	4%
Experience 113			
Tumor names	Tumor seg.	Blood vessels seg.	Erosion
CALLR	79	185	2%
CALUR	116	196	3%

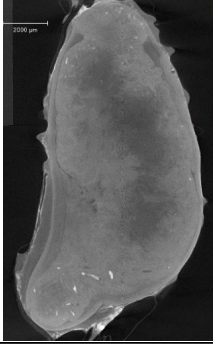
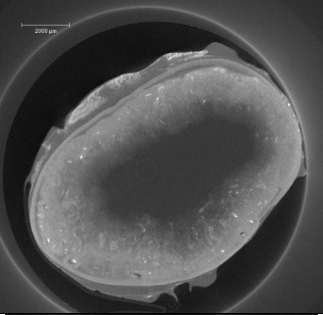
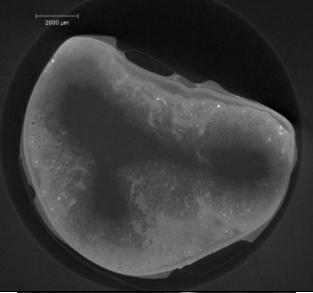
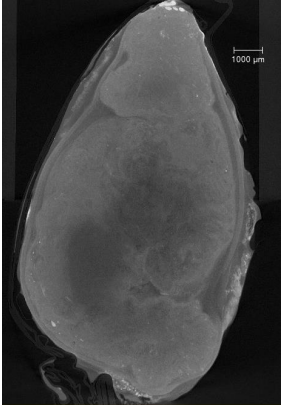
Appendix G

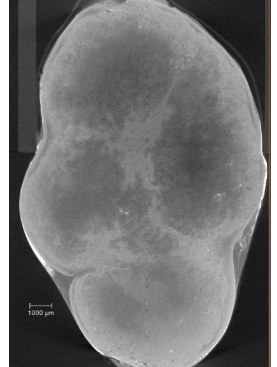
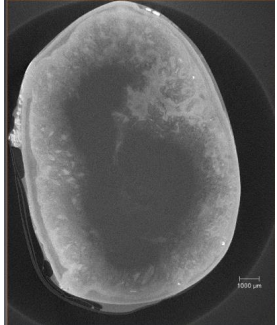
Reliability evaluation of tumors

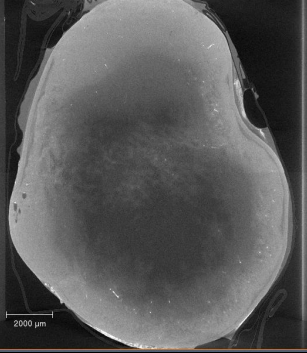
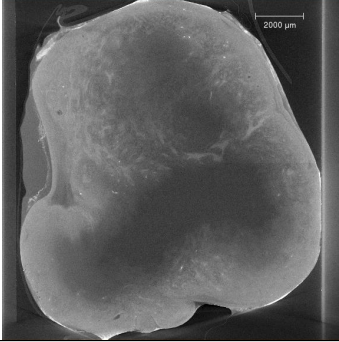
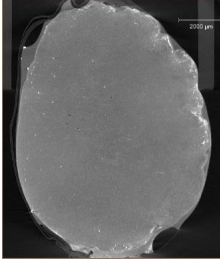
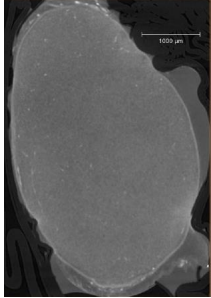
The following pages illustrate and discuss tumor reliability. For some of them, a percentage of dark area is indicated, obtained through segmentation performed using *Magic wand* tool with "All slices" option selected. *Masking* varied between 31 and 128 with a mean *Masking* of 61-102.

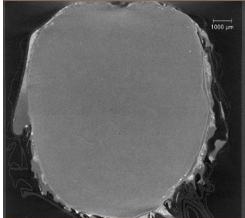
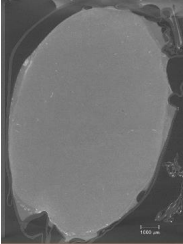
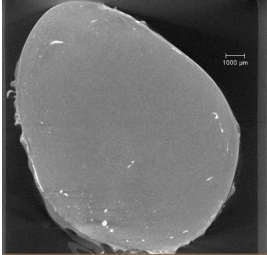
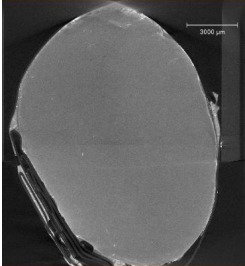
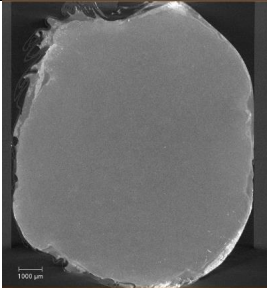
Tumor name	Observations	Image of the tumor		Reliability
RUN 1				
CALLL1	Large portion of tumor center into which CA does not appear to have penetrated (dark area representing around 16% of total tumor volume). + Large white spot that could be mistaken for a BV but is not (no CD31 coloration)			Not reliable
CALLL2	Many very bright areas that do not correspond to BV, the interior of the tumor is relatively dark due to incomplete CA penetration (dark area representing around 11% of total tumor volume), but above all the tumor is composed of 2 distinct mini-tumors, which may affect the results.			Not reliable

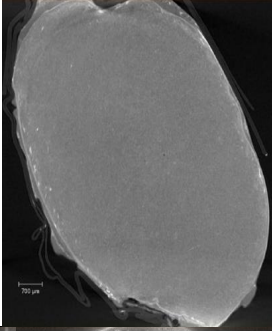
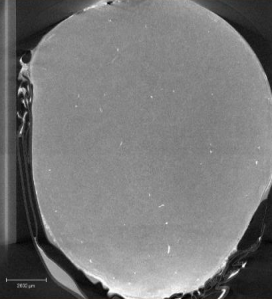
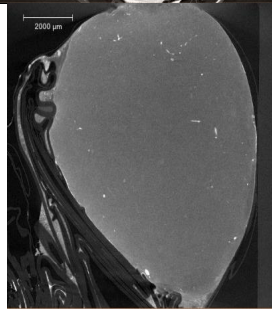
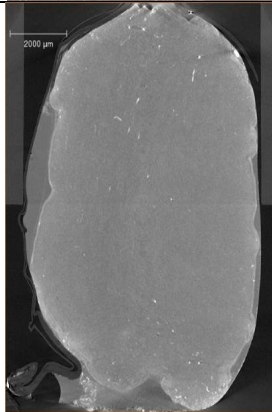
CALLR	The CA appears to have penetrated fairly deep into the tumor, with just a very small very dark zone (dark area representing around 8% of total tumor volume).				Reliable
CALUL	Large medium-dark area, but the real very dark part without AC is quite small (dark area representing around 20% of total tumor volume).				Not reliable
CALUR	A small part of the tumor shows absent staining, but the inside of the tumor has been stained, so it's more a case of incomplete repetition of CA than a real lack of staining. (dark area representing around 10% of total tumor volume).				Reliable
CANML	CA diffused everywhere as the tumor is fully stained, but BV does not seem to have been distinctly marked. In addition, many adiposites were present within the tumor.				Not reliable

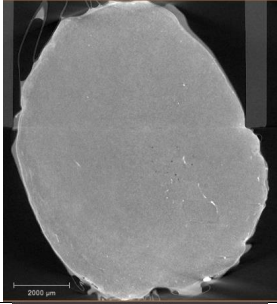
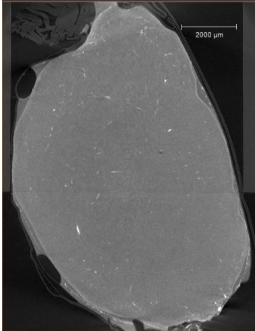
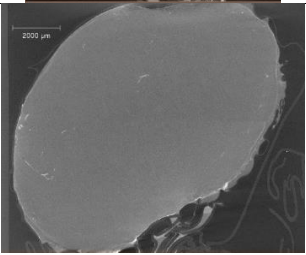
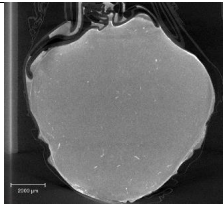
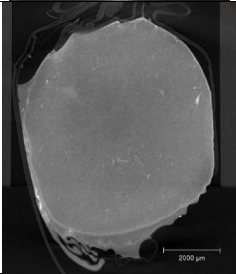
CANMR	Part of the tumor has less diffusion of CA but the difference is small in gray values. (dark area representing around 10% of total tumor volume).		Reliable
CARLL	No diffusion inside the tumor Large dark area (dark area representing around 25% of total tumor volume).		Not reliable
CARLR	CA has not reached the inside of the tumor, as the center is completely black (dark area representing around 16% of total tumor volume).		Not reliable
CARUR	CA seems to have diffused a little less well in the center, but nothing to worry about as there is still a slight change in contrast. (dark area representing around 9% of total tumor volume).		Reliable

CBLRLL	CA less well distributed in the center but BV still distinct (dark area representing around 15% of total tumor volume).				Not reliable
CBLULL	Some parts where the diffusion is less complete but the CA has reached the center (dark area representing around 17% of total tumor volume).				Not reliable
CBLULLR	CA did not spread inside the tumor (dark area representing around 26% of total tumor volume).				Not reliable
CBLURUR	CA did not spread inside the tumor (dark area representing around 34% of total tumor volume).				Not reliable

CBRURLL	CA didn't go deep in the tumor (dark area representing around 17% of total tumor volume).				Not reliable
CBRURLR	The CA did not penetrate deep into the tumor (dark area representing around 17% of total tumor volume).				Not reliable
RUN 2					
CALLL	Not a single darker area due to a lack of staining - CA diffused throughout the whole tumor				Reliable
CALLR	Not a single darker area due to a lack of staining - CA diffused throughout the whole tumor. However, blood vessels appear to be weakly marked				Reliable

CALUL	Not a single darker area due to a lack of staining - CA diffused throughout the whole tumor. However, blood vessels appear to be weakly marked		Reliable
CALUR	Not a single darker area due to a lack of staining - CA diffused throughout the whole tumor. However, blood vessels appear to be weakly marked.		Reliable
CANML	Not a single darker area due to a lack of staining - CA diffused throughout the whole tumor. In addition, blood vessels are clearly highlighted.		Reliable
CANMR	More variability in shades of gray within the tumor but no major differences that might indicate poor diffusion of CA. However, blood vessels appear to be weakly marked.		Reliable
CARUL	Not a single darker area due to a lack of staining - CA diffused throughout the whole tumor. However, blood vessels appear to be weakly marked		Reliable

CARUR	Not a single darker area due to a lack of staining - CA diffused throughout the whole tumor. However, blood vessels appear to be weakly marked		Reliable
CBLLRL	Not a single darker area due to a lack of staining - CA diffused throughout the whole tumor. In addition, blood vessels are clearly highlighted.		Reliable
CBLLRLR	Not a single darker area due to a lack of staining - CA diffused throughout the whole tumor. In addition, blood vessels are clearly highlighted.		Reliable
CBLULL	Not a single darker area due to a lack of staining - CA diffused throughout the whole tumor. In addition, blood vessels are clearly highlighted.		Reliable

CBLULLR	Not a single darker area due to a lack of staining - CA diffused throughout the whole tumor. In addition, blood vessels are clearly highlighted.		Reliable
CBLURUL	Not a single darker area due to a lack of staining - CA diffused throughout the whole tumor. In addition, blood vessels are clearly highlighted.		Reliable
CBLURUR	More variability in shades of gray within the tumor but no major differences that might indicate poor diffusion of CA. Moreover, some BV are clearly visible, while others show very little contrast.		Reliable
CBRURLL	Not a single darker area due to a lack of staining - CA diffused throughout the whole tumor. In addition, blood vessels are clearly highlighted.		Reliable
CBRURLR	More variability in shades of gray within the tumor but no major differences that might indicate poor diffusion of CA		Reliable

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