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Insight box

In many tumour cases there is a need for an accurate delineation of tumour growth and infiltration pattern in a tumour-specific manner. The objective of this study was to build a mathematical model able to describe the growth and real invasion pattern of an avascular microtumour under different microenvironmental conditions. We found experimentally that multicellular spheroids from an epithelial mammary tumour cell line immersed in a collagen I gel presented two very different invasion patterns depending on the presence or absence of an adipocyte-conditioned media in the matrix. Both infiltration patterns observed (collective and individual) were modelled changing the way the diffusion coefficient is made spatially variable, making use of a shape function and a fractal matrix generated by the diffusion-limited-agregation (DLA) method, respectively. We believe this kind of experimental/numerical interaction has many potential applications both at basic research and clinical level.

Microenvironmental influence on microtumour infiltration patterns: 3D-mathematical modelling supported by *in vitro* studies

Emmanuel Luján,^{a,b} Daniela Soto,^c María S. Rosito,^d Alejandro Soba,^e Liliana N. Guerra,^{c,f} Juan C. Calvo,^c Guillermo Marshall,^a and Cecilia Suárez.*^a

Mathematical modelling approaches have become increasingly abundant in cancer research. Tumour infiltration extent and its spatial organization depend both on the tumour type and stage as well as on the bio-physicochemical characteristics of the microenvironment. This sets a complex scenario that often requires a multidisciplinary and individually-adjusted approach. The ultimate goal of this work is to present an experimental/numerical combined method to develop a three-dimensional mathematical model able to reproduce the growth and infiltration pattern of a given avascular microtumour in response to different microenvironmental conditions. The model consists on a diffusion-convection-reaction equation that considers logistic proliferation, volumetric growth, a rim of proliferative cells at the tumour surface, and invasion with diffusive and convective components. Parameter values of the model were fitted to experimental results while radial velocity and diffusion coefficients were made spatially variable in a case-specific way through the introduction of a shape function and a diffusion-limited-aggregation (DLA)-derived fractal matrix, respectively, according to the infiltration pattern observed. The *in vitro* model consists of multicellular tumour spheroids (MTS) of an epithelial mammary tumour cell line (LM3) immersed in a collagen I gel matrix with standard culture medium ("naive" matrix) or conditioned medium from adipocytes or preadipocytes ("conditioned" matrix). It was experimentally determined that both adipocyte and preadipocyte conditioned media are able to change the MTS infiltration pattern from a collective and laminar to an individual and atomized one. Numerical simulations were able to adequately reproduce qualitatively and quantitatively both kinds of infiltration patterns, which was determined by area quantification, analysis of fractal dimensions and lacunarity, and a Bland-Altman analysis. These results suggest that the combined approach presented here could be established as a new framework with interesting potential applications both at the basic and clinical levels of the oncology area.

Introduction

Cancer invasion is one of the hallmarks of cancer and a prerequisite for cancer metastasis. However, the invasion process is very complex and depends on multiple and correlated intrinsic and environmental factors. This makes it difficult to be studied in a fully controlled way. Since *in vitro* models represent an oversimplification of the *in vivo* system, in these years it has been

attempted to increase the level of complexity of *in vitro* assays to create models that could better mimic the behaviour of cells *in vivo*. This levels of complexity include, among others, the dimension of the system, moving from two-dimensional to three-dimensional models¹.

Multicellular tumour spheroids (MTS) has been largely proposed as a predictive tool in cancer research, as they are physiologically useful *in vitro* models of a microtumour in the avascular stage^{2,3}. When MTS are immersed in a three-dimensional biological matrix, the system is suited not only for therapeutic screening, but also for studying the growth and infiltration of the microtumour in response to the physico-chemical and biological characteristics of the microenvironment^{4,5}. The re-creation of the tumour microenvironment including a three-dimensional structure with tumour-stroma interactions, cell-cell adhesion and cellular signalling is essential for a deeper understanding of the

^a Instituto de Física del Plasma, CONICET-UBA, Buenos Aires, Argentina.

^b Centro de Simulación Computacional para Aplicaciones Tecnológicas, CONICET, Buenos Aires, Argentina

^c Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, UBA, Buenos Aires, Argentina.

^d Instituto de Astronomía y Física del Espacio, CONICET-UBA, Buenos Aires, Argentina

^e Comisión Nacional de Energía Atómica, CONICET, Buenos Aires, Argentina

^f Departamento de Ciencias Básicas, Universidad Nacional de Luján, Luján, Argentina

* Corresponding author. E-mail: csuarez@dc.uba.ar

tumour behaviour in general and the invasion process in particular⁶.

In the context of a breast tumour, mammary fat may affect human breast cancer development. Recently it has been recognized the pro-tumourigenic ability of matured adipocytes promoting proliferation and invasion of tumour cells *in vitro* and *in vivo*⁷. Conditioned medium from adipocytes significantly increases proliferation and survival of human mammary tumour cells *in vitro*⁸. Preadipocytes are also able to stimulate the growth of mammary tumour cells in culture⁹.

In mathematical oncology, numerical modelling emerged in the past decades with the intention of predicting the growth and infiltration of a tumour and its possible response to a given treatment^{6,10}. So-called *in silico* trials aimed to predict patient-specific responses to various dose schedules or treatment combinations are becoming an invaluable tool to optimize patient care¹¹⁻¹³. Agent-based tumour growth models are commonly used to describe the behaviour and interaction of individual cells in different environments^{14,15}. Among continuous models, the use of ordinary differential equations (ODE) is an useful tool to simulate the evolution of total tumour cell number over time, though it lacks of spatial considerations¹⁶. Nevertheless in cancer, where the infiltration of tumour cells into normal tissue and the establishment of new distant metastasis are such crucial spatial aspects of the disease, the use of partial differential equations (PDE) are often more convenient. Relatively simple models based on reaction-diffusion equations of this kind that describe tumour proliferation and invasion into peripheral host tissues have proved to be of clinical relevance^{17,18}.

Computational simulation approaches focused on MTS can advance our understanding of cancer invasion and treatment¹⁹⁻²¹. Nevertheless, taking aside some specific cases²²⁻²⁴, continuum models related to microtumour invasion have in general centered in the description of spherical tumour invasion areas. It is necessary at present to develop new strategies to better determine non-spherical tumour infiltration borders as well as to predict as much as possible tumour spread characteristics to optimize treatments such as surgery or radiotherapy. In a recent work, we introduced a two-dimensional mathematical model of microtumour growth and invasion in a collagen matrix, considering non-spherical invasion shapes²⁵.

Here we extended this work to a three-dimensional model able to reproduce, in a case-dependent manner, two very different infiltration patterns derived from different environmental conditions. In a first step, we tested experimentally the growth and infiltration of MTS from mammary epithelial origin with or without the presence of a conditioned medium from adipocytes or preadipocytes (fibroblasts) in the microenvironment. We then simulated the two types of invasion patterns observed *in vitro*. The approach presented here is applied in this case to a breast tumour type but it may be used with other tumour types, especially those where infiltration is a most important factor, like glioblastomas. This makes this experimental/numerical approach a potentially useful complementary clinical tool to help in tumour prognosis and treatment definition in a patient-specific way.

Methods

1- *In vitro* model

Adipocyte differentiation and conditioned medium extraction

In this method, described in²⁶, 50.000 cells of the 3T3-L1 cell line (fibroblasts derived from murine embryonic tissue) were seeded in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich) with 10% fetal bovine serum (FBS, Natocor). This line behaves as preadipocytes as cells can be differentiated to mature adipocytes in culture. For this, differentiating agents (Sigma-Aldrich) such as insulin 2 μM , 3-isobutyl-1-methylxanthine (IBMX) 0.5 μM and dexametasone 0.1 μM were added to the medium when cells are at 60-80% of confluence. Two and four days after, the medium was changed and insulin was renewed. At the sixth day, approximately 90-100% of the cells were differentiated, changing from fibroblastic to amoeboid shapes and evidencing lipidic intracellular vesicles. Conditioned media were obtained from not-differentiated and differentiated (preadipocytes and adipocytes, respectively) cell cultures after three days in DMEM/FBS and stored at -20°C until their utilization.

MTS formation

Multicellular tumour spheroids of the LM3 cell line (mouse epithelial and metastatic mammary tumour cells²⁷), were generated by the hanging-drop method²⁸. This technique has the advantage of producing homogeneous spheroids and consists on seeding drops of 20 μl with 1.500 cells each, in the inner surface of a Petri dish cap. Once seeded, phosphate buffer solution (PBS) was placed in the base of the dish to maintain humidity and the cap returns to their natural position. Drops hang from the cap surface by superficial tension. After four days in culture at 37°C and 5% CO_2 , one spheroid is formed at the bottom of each drop.

MTS seeding

Once formed, spheroids were recovered from the drops and immersed in a collagen I gel²⁹. At this point two different experimental conditions were tested: spheroids immersed in collagen/DMEM ("naive" collagen) and spheroids immersed in collagen/conditioned medium from mature or immature adipocytes ("conditioned" collagen). For this, a rat tail collagen I (Gibco) solution 2 mg/ml in DMEM with 10% FBS (control group) or conditioned medium (treated groups) was prepared and placed on a six multi-well plate (0.5 ml/well). Ten spheroids were then placed on the surface of each well. After half an hour of incubation at 37°C , collagen solution becomes a gel and spheroids get immersed in it. Spheroids begin to invade the surrounding gel a day after seeding. Photographs were taken daily with an inverted optical microscope (Olympus) for seven days. Spheroid core and invasion areas were measured from photographs through the ImageJ software (<http://imagej.nih.gov/ij>). Experiments were repeated three independent times.

2- *In silico* model

The three-dimensional mathematical model represents an invading microtumour as a composition of two tumour cell populations with their own phenotype and behaviour: proliferative core cells

and invasive peripheral cells. This defines two domains under different mathematical modelling with a moving boundary determined by the core radius (r_{core}) between them: an inner domain corresponding to the growing core (core domain) and an outer domain corresponding to the infiltration area (infiltration domain). The model initiates with a single tumour cell located at the core subdomain and considers two stages: an initial benign stage and a later malignant stage. The benign stage is estimated in 10 days. During this time there is only core growth and the spatial domain only consists on the core subdomain. This lasts until the simulated microtumour reaches the radius corresponding to an specific experimental spheroid when seeded in collagen (invasion radius or r_{inv}). After that moment the spheroid goes on growing but invasion in the gel matrix is also incorporated, then both spatial subdomains are present. The invasion process has two components: an spherical (non-directional) and a radial/planar (directional) one.

Core subdomain

Core subdomain is an expanding area that describes the growth of the spheroid core. The inner boundary of this subdomain is defined by the minimum core radius (r_{min}). The outer moving boundary is defined by the core radius (r_{core}) calculated in spherical coordinates by:

$$r_{core}(t) = r_{min} + V_{core} t \quad (1)$$

being V_{core} the core growth velocity and t the time. Tumour cell concentration C inside this domain is equal to the tumour cell carrying capacity of the system C_{max} . Initial conditions at $t = 0$ are $r = r_{min}$ and $C(r_{min}, \phi, \theta) = C_{max}$. This corresponds to the placement of a single cell with a radius of $5 \mu m$ in this subdomain. For both subdomains, ϕ is the azimuthal angle ($0 \leq \phi < 2\pi$) and θ the polar angle ($0 \leq \theta \leq \pi$).

Infiltration subdomain

In this domain, infiltrative tumour cell concentration follows a mass balance:

$$\frac{\partial C}{\partial t} = s - \nabla \cdot j \quad (2)$$

where s is the reaction term and j is the mass flux. The reaction term is composed by two components:

$$s = P C \left(1 - \frac{C}{C_{max}}\right) + S \delta(r - r_{core}) \quad (3)$$

The first corresponds to the basal proliferation of the tumour cell population (logistic law), being P the net proliferation rate. The second component represents a cell source (S) located at the spheroid surface (r_{core}) by the Dirac's delta function δ , being r the radius from the spheroid center. This corresponds to an external rim of specially proliferative cells present in this kind of microtumours.

On the other hand, the mass flux correspondent to tumour cell invasion is composed of a diffusive and a convective term:

$$j = -D \nabla C + V C \quad (4)$$

with D being the diffusion coefficient and V the invasion velocity. The diffusive term is based on the Fick's law and represents the spherical non-directional component of the tumour invasion while the convective term represents the radial or planar (directional) component. Then the model can be expressed by a three-dimensional diffusion-convection-reaction equation²⁵:

$$\frac{\partial C}{\partial t} = \nabla \cdot (D \nabla C) - \nabla \cdot (V C) + P C \left(1 - \frac{C}{C_{max}}\right) + S \delta(r - r_{core}) \quad (5)$$

In spherical coordinates:

$$\begin{aligned} \frac{\partial C}{\partial t} = & \frac{1}{r^2} \frac{\partial}{\partial r} \left(r^2 D \frac{\partial C}{\partial r} \right) + \frac{1}{r^2 \sin(\theta)} \frac{\partial}{\partial \theta} \left(\sin(\theta) D \frac{\partial C}{\partial \theta} \right) \\ & + \frac{1}{r^2 \sin^2(\theta)} \frac{\partial}{\partial \phi} \left(D \frac{\partial C}{\partial \phi} \right) - \frac{1}{r^2} \frac{\partial}{\partial r} \left(r^2 C V_r \right) \\ & - \frac{1}{r \sin(\theta)} \frac{\partial}{\partial \theta} \left(\sin(\theta) C V_\theta \right) - \frac{1}{r \sin(\theta)} \frac{\partial}{\partial \phi} \left(C V_\phi \right) \\ & + P C \left(1 - \frac{C}{C_{max}}\right) + S \delta(r - r_{core}) \quad (6) \end{aligned}$$

It is worth noticing that, in the case of simulations of individual infiltration, the convective term is null as planar invasion is achieved by the spatial variation of the diffusion coefficient D .

The inner boundary of the infiltration subdomain is $r_{core}(t)$ and the outer boundary is r_{max} . Due to the symmetry of the problem, in laminar simulations θ varies between 0 and $\pi/2$ instead of 0 and π . V_r , V_θ and V_ϕ are the components of the invasion velocity but, as the biology of the problem only conserves a radial component, V_ϕ and V_θ are null. The boundary condition at $r = r_{max}$ is $\frac{\partial C}{\partial r}(r_{max}, \phi, \theta) = 0$ (Neumann's conditions). Initial conditions at $t = 0$ are $r = 0$ and $C(r, \phi, \theta) = 0$.

Implementation

Main parameter values are presented in table 1. Case-specific values were obtained from each individual experimental image. In order to achieve a more realistic description of the invasion pattern, we made V_r or D spatially variable for the laminar and individual infiltration cases, respectively. V_r was made variable by the introduction of a shape function and D , by the incorporation of a DLA-derived matrix. Otherwise, all infiltration areas would be spherical. In this way we make case-specific simulations in relation to core growth rate, infiltration pattern (laminar or individual), infiltration velocity and infiltration shape. The model was solved by finite differences with standard relaxation techniques and centered discretization of order $O(h^2)$. It was implemented in C++ and parallelized through shared memory technology (OpenMP). Simulations were performed in an Intel(R) Core(TM) i7 processor, 2.2 GHzx8, 5.8 Gb, under Ubuntu Linux 16.04 LTS. In all cases, Paraview was used for image visualization (<http://www.paraview.org/>).

Table 1 *In silico* model parameters

Parameter	Value	Parameter	Value
C_{max}	0.0007 cells/ μm^3	r_{min}	5 μm
r_{inv}	case-specific	r_{max}	450 μm
V_{core}	case-specific	P	0.03 cells/h
S (laminar)	$4e^{-7}$ cells/ $\mu\text{m}^3\text{h}$	S (individual)	$9e^{-5}$ cells/ $\mu\text{m}^3\text{h}$
D (laminar)	$1.7 \mu\text{m}^2\text{h}^{-1}$	D (individual)	$70 \mu\text{m}^2\text{h}^{-1}$
V_r (laminar)	case-specific		

Laminar infiltration pattern simulations

In these simulations, a case-specific basal V_r was made spatially variable along the azimuthal angle ϕ depending on a shape function. The shape function is extracted by image processing (through a C++ code written for this purpose) from each experimental image, and describes the invasion area contour of this tumour case, as it is explained in²⁵. This function assigns weight along ϕ in the 360° domain based on the distance from the spheroid center to the external limit of the infiltration. Then the invasion contour curve is normalized and the correspondent invasion distance is calculated. Finally, the shape function was used to feed the main model to reproduce each experimental infiltration shape in a case-specific manner. The whole process is shown in figure 1.

Individual infiltration pattern simulations

This kind of simulation makes use of fractal structures. This was achieved by making basal D spatially variable by the generation of a fractal 3D matrix derived from a modification of the DLA (diffusion limited aggregation) method^{30,31}. Briefly, this method simulates the location of an original particle in the center of a three-dimensional box. Then other particles are released successively from the periphery of the box, eventually move inside it and settle nearby another established particle. This finally achieves aggregated structures with fractal characteristics. Then the binary 3D fractal matrix determines the points where D will have a zero or a non-zero value. 1000 different DLA matrices were generated by this method. This initial pool was used to select the best ones to fit with each experimental case of individual infiltration in base on shape similarities. Each DLA-matrix chosen was then used to feed the model and run a case-specific simulation.

Fractal characteristics (fractal dimension and lacunarity^{32,33}) of all images were determined by the box-counting³⁴ and the gliding-box³⁵ method, respectively. Then, these characteristics were compared between experimental and simulated images by the Bland-Altman analysis^{36,37} to determine the accuracy of the model. This analysis quantifies the level of similarity between two groups of study through the comparison of their main parametric statistics. This is achieved by constructing a linear regression curve that tends to be non-significant as groups are more similar between each other.

Results and Discussion

Figure 2 shows two very different infiltration patterns observed experimentally. In both cases, a clear central spheroid area can be well differentiated from the peripheral invasion. MTS immersed in "naive" collagen present a collective and laminar invasion pat-

tern, with polyhedral epithelial cells attached between each other (figure 2, left). On the contrary, the same spheroids immersed in collagen with adipocyte-conditioned medium ("conditioned" collagen) yield an individual and atomized invasion pattern, with rounded amoeboid cells separated from each other (figure 2, right). This was observed both with adipocyte- and preadipocyte-conditioned medium. It remains to be experimentally determined which specific soluble cell secretions are responsible for the observed effects.

Two main patterns of cancer cell invasion have been described so far in bibliography: collective and individual. Among them, each specific pattern depends on the tumour cell type but also on tumour microenvironment and stroma organization^{38,39}. Human cancer pathology usually shows tumour cells invading collectively as strands, cords or clusters. *In vitro* studies display from single isolated cells with round or elongated phenotypes (amoeboid and fibroblast-like shapes, respectively) to loosely streams of cells or collective migration of cell strands or sheets.

Experimental MTS cultures have revealed that collective epithelial cell invasion shows a leader-follower behaviour that is accompanied by an extensive extracellular matrix reorganization involving proteolysis and matrix alignment⁴⁰. On the other hand, the passage from a laminar pattern to one with isolated cells suggests an epithelial-mesenchymal transition (EMT) of the infiltrating cells. This phenomenon is very common in the initial phases of cancer invasion and implies a phenotypic change marked by the loss of epithelial characteristics and the acquisition of invasive mesenchymal properties⁴¹. *In vivo*, both adipocytes and fibroblasts, main cellular components of the breast microenvironment, are known to secrete factors able to induce the EMT in epithelial mammary tumour cells (leptin and interleukin-6 for instance).

Figure 3 presents some examples of numerical simulations performed by the 3D model correspondent to the laminar infiltration pattern (MTS immersed in "naive" collagen), after five days of invasion. All parameter values were kept constant among the different simulations, except those relative to case-specificity, as V_{core} , r_{inv} , V_r and the shape function. There is good qualitative agreement between each experimental case and its correspondent numerical simulation concerning both core growth and tumour infiltration. A whole simulation video about the first five days of infiltration is included as additional material (*Laminar – infiltration.avi*).

Table 2 depicts the quantification of the experimental and simulated image areas both for the MTS core and invasion zone, at the fifth day of invasion; the difference between them (calculated in relation to total invasion area); and the average difference. Averaged differences between experimental and simulated images are 12.57 % and 10.37 % for core and infiltrative areas, respectively. Thus, simulations reproduce both qualitatively and quantitatively well the experimental cases.

Figure 4 shows some examples of numerical simulations performed by the 3D model correspondent to the individual infiltration pattern (MTS immersed in "conditioned" collagen), after five days of invasion. It can be observed good qualitative correspondence between each experimental case and its fractal simulation. A whole simulation video about the first five days of infiltration is

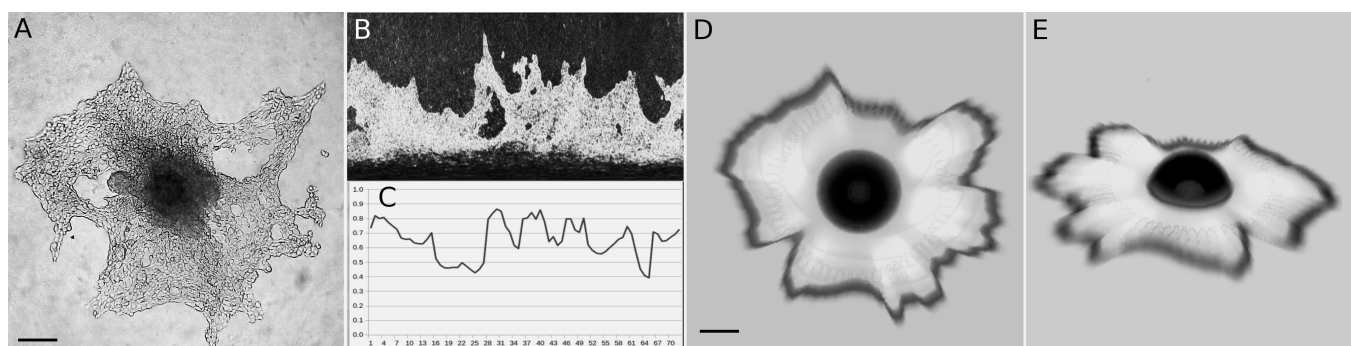


Fig. 1 Image processing – A) Initial experimental image of an MTS invading a collagen matrix (100x). The central tumour core can be well differentiated from the peripheral invasion area. B) Image binarization and transformation from polar coordinates to Cartesian coordinates. C) Generation of the shape function (invasion distance vs. azimuthal angle ϕ). D) and E) Different views of the final simulation that incorporates data from the shape function.

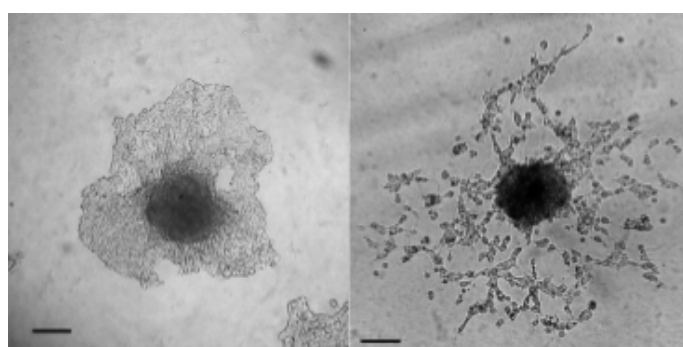


Fig. 2 Experimental infiltration patterns – Micrographs of epithelial mammary MTS immersed in "naive" collagen (left) or in collagen with adipocyte-conditioned medium (right), both at the fifth day of invasion (scale bar: 100 μm).

Table 2 Experimental and simulated core and invasion areas ($1 \times 10^4 \mu\text{m}^2$) with their correspondent differences and final average, for the MTS cases presented in figure 3.

Case	Exp core	Sim core	Diff (%)	Exp inv	Sim inv	Diff (%)
1	1.329	1.273	4.22	11.461	10.527	8.15
2	1.765	1.737	1.58	5.096	6.127	16.83
3	0.804	0.819	5.26	10.989	11.905	7.69
4	1.858	1.502	19.16	18.745	21.822	14.1
5	1.766	1.474	16.53	5.498	6.316	12.95
6	2.013	1.547	23.13	13.221	14.658	9.81
7	5.404	4.935	8.68	20.972	22.397	6.36
8	3.427	2.674	21.98	28.668	30.687	7.04
	Average		12.57	Average		10.37
	St. dev.		8.05	St. dev.		3.57

included as additional material (*Individual – infiltration.avi*).

In order to achieve a more quantitative analysis of these similarities, fractal properties (fractal dimension and lacunarity) of both types of images were compared. These two fractal properties are complementary, as fractal dimension quantifies how much of the total space is filled out by the object, while lacunarity indicates how this space is filled out, giving an idea about image symmetry and inner lacunar spaces. Experimental images about the laminar infiltration pattern were also included in the comparison, as it is expected that DLA-derived simulations were more similar to the individual than to the laminar experimental cases.

Average fractal dimensions are significantly different among the three study groups (1.57 \pm 0.04, 1.49 \pm 0.03 and 1.68 \pm 0.07 for simulations of the individual infiltration pattern, experimental individual infiltration pattern and experimental laminar infiltration pattern, respectively). Although local fractal dimensions (figure 5), determined by the box-count method, show a characteristic and distinctive pattern for each study group, the one presented by simulations of individual infiltration cases seems to be more similar to the individual than to the laminar experimental cases, as expected.

Figure 6 depicts some characteristic lacunarity curves, derived by the gliding-box method, correspondent to the three types of images. Each subfigure presents the curve of a given individual infiltration case along with its correspondent simulation. The average lacunarity curve for laminar experimental cases was also included in all subfigures for comparison. It can be observed great qualitative similarity between lacunarity of simulated curves and their correspondent experimental cases. On the other hand, lacunarity curves from laminar infiltration cases are very different.

In order to achieve some quantitative idea about the level of accuracy between individual infiltration experimental cases and their corresponding simulations, a Bland-Altman analysis about lacunarity curve correspondences was performed. A resultant non-significant linear regression intercept derived from this analysis is a sign of maximum similarity, while a non-significant regression slope indicates an independence between the similarity level and the magnitude of the statistic considered.

Figure 7 shows a comparison, by this method, of three lacunarity curve statistics (rank, standard deviation and average) derived from simulations of individual infiltration patterns with their correspondent experimental cases, on the one hand, and with experimental cases of laminar infiltration, on the other. It can be observed that, when comparing simulations with their correspondent experimental cases (first row), both linear regression intercepts and slopes are non-significant for the three statistics considered (significance level at 5%). On the contrary, when comparing statistics from these simulations with the ones derived from laminar experimental cases (second row), the observed differences, evidenced by the intercepts, are significant. Nevertheless, as linear regression slopes are not significant, these differences seem

not to be related to the magnitude of the statistic considered.

These results mean that lacunarity curve statistics (and consequently lacunarity curves) derived from simulations of individual infiltration are significantly like the ones derived from their correspondent experimental cases and not like the laminar experimental ones. This indicates that the DLA-derived fractal approach is adequate to reproduce the individual infiltration pattern observed experimentally.

Since the onset of the concept of fractal geometry present in biological structures (stated by Mandelbrot in 1977⁴²), science has tried to approach to the cancer study through fractals, issue nowadays aboarded by the systems biology⁴³. Recent work has been attempted to use fractal tools in tumour differential diagnosis to achieve a kind of cookbook ready for the pathologist⁴⁴. Fractal characteristics have been related to K1725 mouse melanoma clones and spheroid invasion *in vitro*⁴⁵, to surface growth patterns in endometrioid endometrial adenocarcinomas⁴⁶ and to glioma detection based on magnetic resonance imaging⁴⁷. Even, a fractal anomalous diffusion model has been developed to study leiomyoma spatiotemporal dynamics and to propose methods of cancer control by manipulating the microenvironment plasticity⁴⁸.

Although present personalized-medicine therapeutics tend to make increasing usage of *in vitro* three-dimensional models (in particular, tumour-derived spheroids) to test chemotherapy options in a patient-specific way^{49–51}, the *in vitro* model presented here does not intend to reproduce the *in vivo* histological characteristics of the tumour invasion, as it is well known that the invasion pattern of a given tumour may differ from *in vivo* to *in vitro* conditions. Nevertheless, tumour cells do conserve *in vitro* much of their physiopathological characteristics and may give relevant information about tumour subtype and aggressiveness. The approach proposed in this work aims to reveal these characteristics (specifically, spheroid growth rate, invasion growth rate and invasion pattern) that could feed a patient-specific predictive (or at least useful for prognosis estimation) mathematical model.

Conclusions

Infiltration issues are specially critical in many tumour types, as their correct determination is often essential to achieve a good tumour prognosis and/or surgery/radiotherapy optimization. Infiltration extent and its spatial organization depend both on the tumour type and stage as well as on the bio-physicochemical characteristics of the stroma it encounters when spreading. This sets a complex scenario that often requires a multidisciplinary and individually-adjusted approach.

In a previous work we have proposed a combined method to study the invasion pattern of MTS immersed in a collagen matrix. Here, we proved that this approach is viable and useful applied in the context of an epithelial mammary microtumour that presents two very different infiltration patterns depending on the microenvironmental conditions. We showed experimentally that adipocyte and preadipocyte secretions are able to alter this pattern from a laminar and collective to an individual and atomized one.

We also presented a case-specific, 3D numerical model based

on a system of partial differential equations able to describe the microtumour growth as well as the two different infiltration patterns evidenced experimentally, in a more realistic way than previous models. This is achieved by making the radial velocity or the diffusion coefficient spatially variable. Numerical simulations were able to adequately reproduce these empirical data qualitatively and quantitatively for both infiltration patterns, as it was revealed by area quantification, fractal analysis (fractal dimensions and lacunarity), and a Bland-Altman analysis.

The *in vitro* model of multicellular tumour spheroids, combined with its correspondent mathematical modelling, has many experimental as well as clinical interesting potential applications in oncology. Results presented here suggest that our experimental/numerical approach could be established as a combined method to study possible invasion patterns that may result from different tumour types and microenvironmental conditions with potential usefulness for estimating clinical prognosis and designing tumour-treatment strategies in a context of a personalized medicine.

Conflicts of interest

There are no conflicts of interest to declare.

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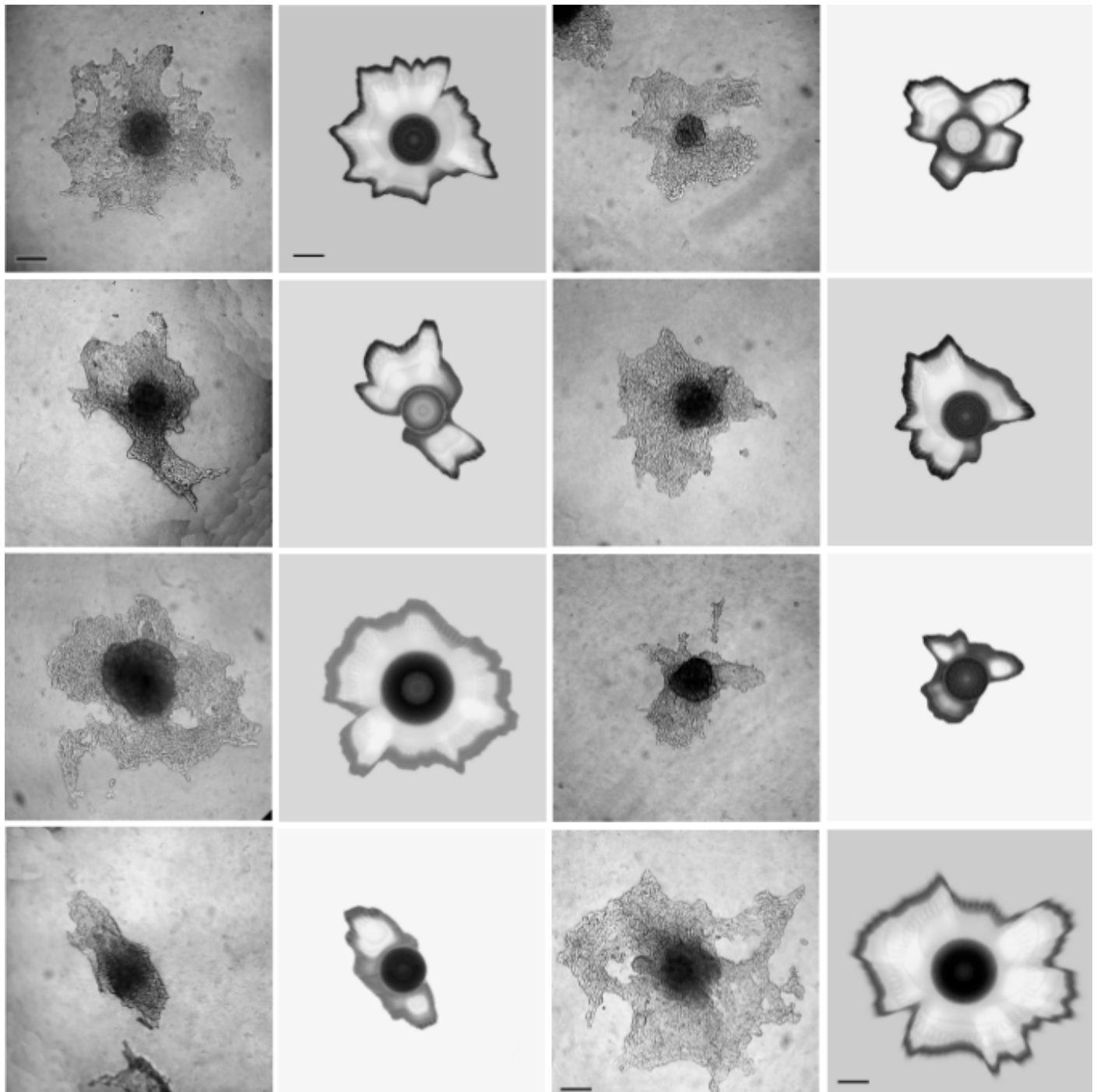


Fig. 3 Laminar infiltration simulations – First and third columns: experimental images of epithelial mammary MTS at the 5th day of laminar infiltration into a "naive" collagen matrix (100x). Second and fourth columns: Numerical simulations corresponding to each experimental case, also at the 5th day of invasion (scale bar: 100 μm).

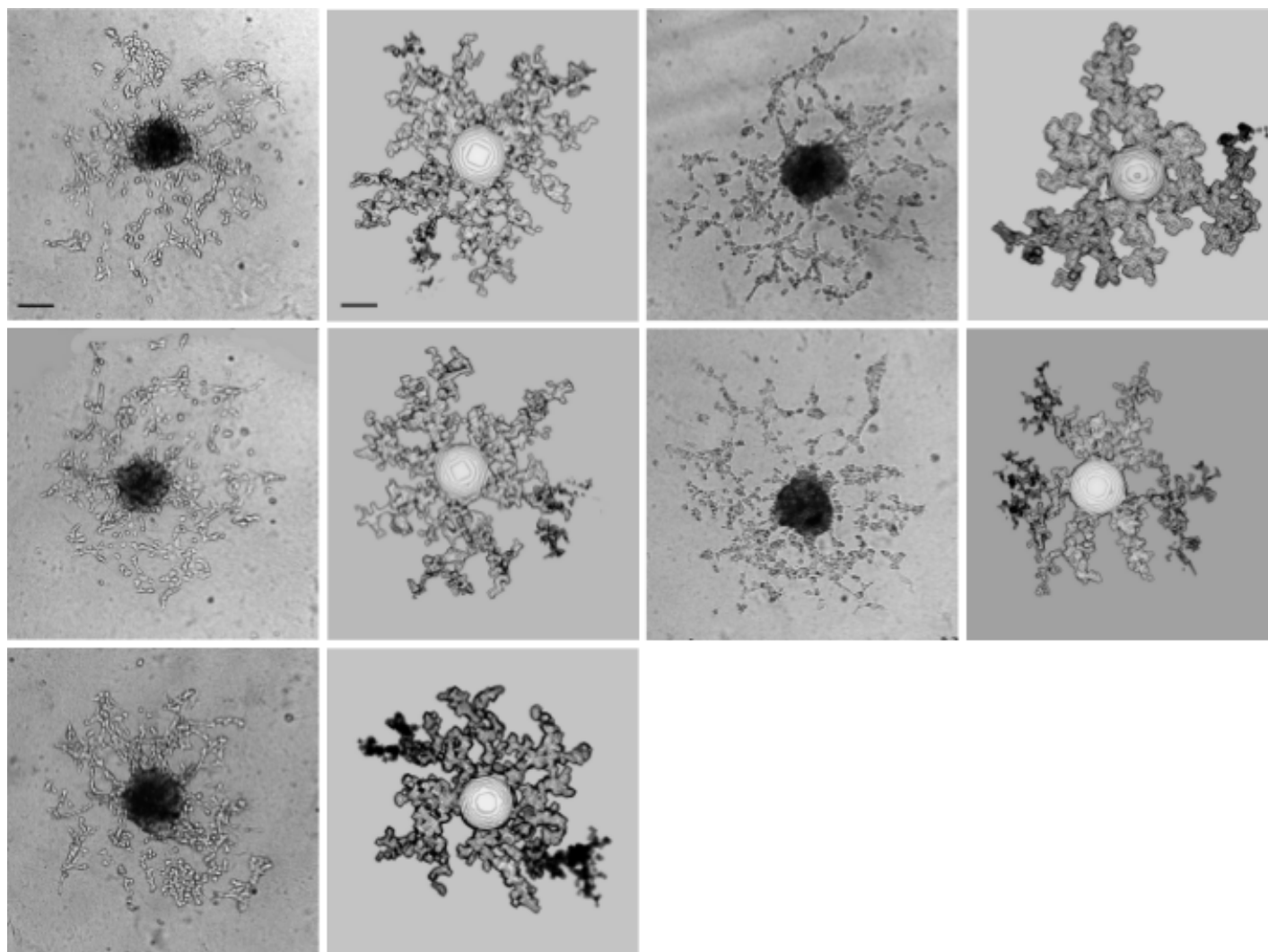


Fig. 4 Individual infiltration simulations – First and third columns: experimental images of MTS at the 5th day of individual infiltration into a "conditioned" collagen matrix (100x). Second and fourth columns: Fractal simulations corresponding to each experimental case, also at the 5th day of invasion (scale bar: 100 μm).

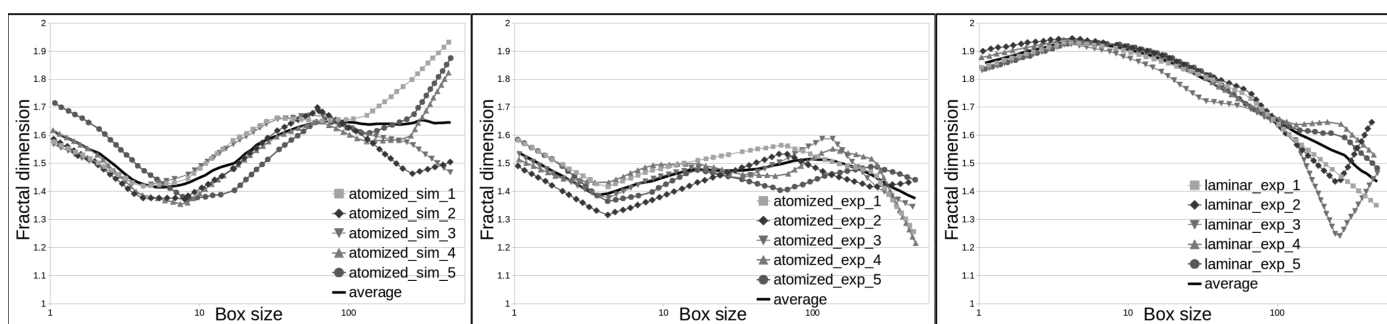


Fig. 5 Local fractal dimensions – Fractal dimension vs. box size for five example cases of each type of image, with their correspondent average curve. Left: Curves derived from simulations of individual (atomized) infiltration cases. Center: Curves derived from experimental atomized infiltration cases. Right: Curves derived from experimental laminar infiltration cases. All curves were obtained by the boxcount method.

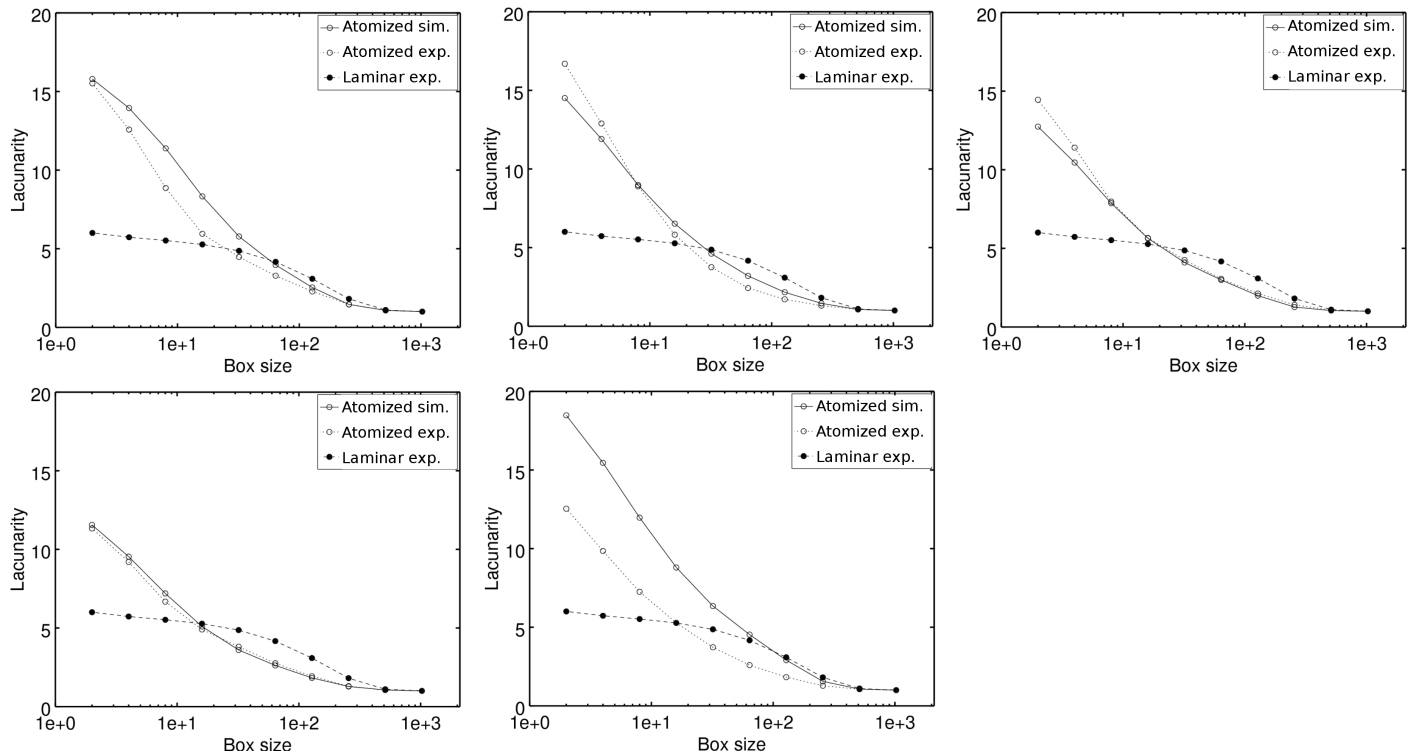


Fig. 6 Lacunarity analysis – Lacunarity vs. box size for five examples of the individual (atomized) infiltration pattern. Each subfigure compares one experimental image (atomized exp.) with its correspondent simulation (atomized sim.). The average lacunarity curve resultant from laminar experimental cases was also included for comparison (laminar exp.). All curves were obtained by the gliding-box method.

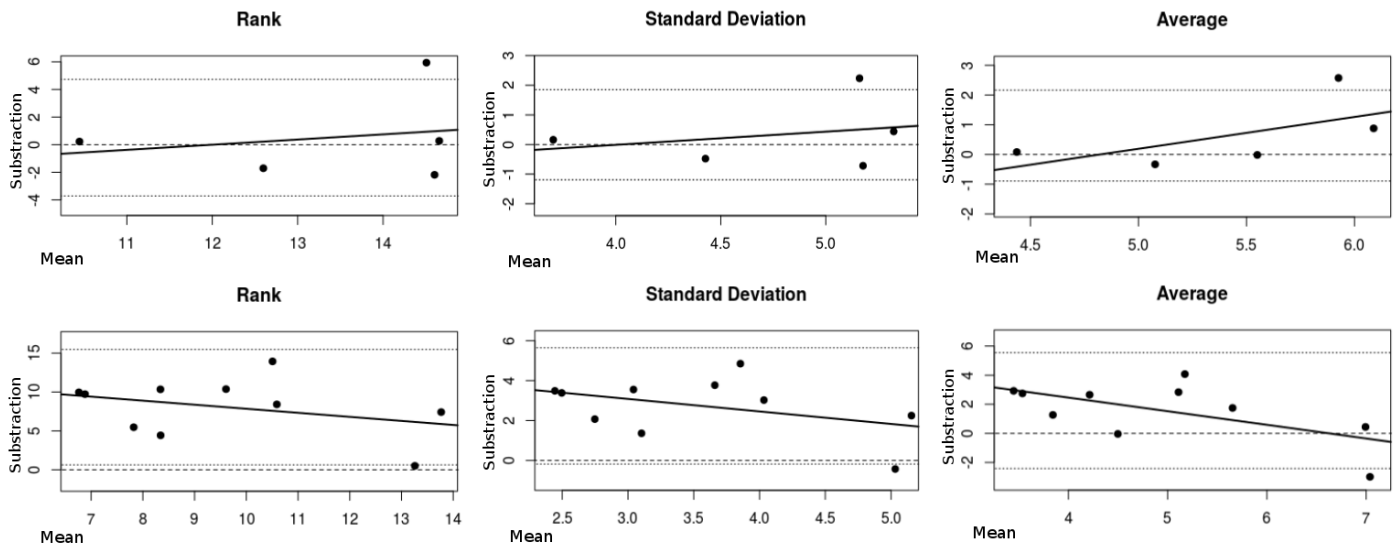


Fig. 7 Bland-Altman analysis of lacunarity curves – Subtraction of the compared statistic vs. its mean, for three statistics (rank, standard deviation and average) derived from the lacunarity curves considered. First row: comparison between curves derived from simulations of individual infiltration cases and their correspondent experimental ones. Rank intercept: -4.49 ($p=0.758$), slope: 0.374 ($p=0.73$). Standard deviation intercept: -1.79 ($p=0.72$), slope: 0.445 ($p=0.67$). Average intercept: -5.17 ($p=0.318$), slope: 1.07 ($p=0.269$). Second row: comparison between curves derived from the same simulations and those from the laminar experimental pattern. Rank intercept: 13.04 ($p=0.0328$), slope: -0.52 ($p=0.34$). Standard deviation intercept: 4.96 ($p=0.0251$), slope: -0.626 ($p=0.238$). Average intercept: 6.22 ($p=0.0241$), slope: -0.939 ($p=0.0647$).

Microenvironmental influence on microtumour infiltration patterns: 3D-mathematical modelling supported by *in vitro* studies

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Table of contents entry

We present a new mathematical model able to describe the growth and infiltration pattern of multicellular tumour spheroids immersed in different microenvironmental conditions.

