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Cancer, an Ecological Manifesto of Metazoan Life: The Multi-scale Ecology of Complex Cancer Ecosystems

by
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‘Make rhizomes, not roots, never plant! (…)
The tree imposes the verb ‘to be’, but the fabric of the rhizome is the conjunction, ‘and ... and ... and ...’ − G. Deleuze and F. Guattari

‘It is by logic that we prove,

but by intuition that we discover.’− H. Poincare

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Resumen General

Mas allá de ser una enfermedad exclusiva de los genes, el cáncer exhibe una estrecha relación con el ambiente biofísico, lo que lo constituye como un fenómeno ecológico. Esta tesis se enfoca en el estudio del cáncer bajo aproximaciones teóricas, analíticas, y experimentales bajo una mirada ecológica; aplicada desde la emergencia del cáncer (oncogénesis) hasta la migración de propágulos metastáticos entre órganos. En el primer capítulo, trabajamos en una conceptualización de la oncogénesis como un cambio en el fenotipo celular en respuesta al envejecimiento del organismo. Proponemos que en respuesta a los cambios en el ambiente celular que ocurren durante el envejecimiento no se mantiene el contexto bajo el cual se mantiene la multicelularidad traduciéndose en un cambio fenotípico celular que se aproxima a condiciones atávicas 'unicelulares' de disgregación estructural y funcional. Discutimos acerca de las nociones de individualidad, co-determinación individuo-ambiente y la enacción. El envejecimiento participa en la determinación de los rasgos de historia de vida celulares; y por lo tanto, las interacciones entre distintos tipos celulares. En el segundo capítulo abordamos la complejidad de interacciones competitivas entre estrategias celulares, cancerosas y no-cancerosas, y el ambiente físico. Desarrollamos un modelo analítico a partir de observaciones de competencia en un modelo *in vitro* (línea HEY-GFP de cáncer de ovario y línea MET5A de mesotelio de ovario) en condiciones de cultivo asociadas al envejecimiento del tejido ovárico (Matrigel con concentración variable de colágeno I). Nuestros modelos proponen que mecanismos competitivos jerárquicos influenciados por el envejecimiento modularían las ventajas competitivas de las células no-cancerosas; permitiendo que en tales ambientes la estrategia cancerosa logre invadir el ecosistema residente. En el tercer capítulo, abordamos la dimensión espacial el fenómeno a partir del estudio de la estructura espacial de los ecosistemas de cáncer mamario (ER^{+}DCIS) bajo terapia endocrina neoadyuvante anti-proliferativa (ensayo clínico fase III POETIC, UK).

Utilizando algoritmos supervisados de *machine learning* identificamos de forma automatizada células individuales en muestras de tumor con tinción inmunohistoquímica Ki67 (marcador de proliferación). Identificamos zonas de mayor concentración celular (*hotspots*) y proyectamos las células en tales hotspots como nodos de una red espacialmente explícita. La detección y cuantificación de la comunidad espacialmente estructurada y fragmentada de células cancerosas y del sistema inmune junto con la expresión de Ki67, permite predecir con mayor poder la respuesta patológica a terapia anti-proliferativa. Además de operar como articulador de patrones espaciales, la diversidad de historias de vida celular, en interacción con las condiciones locales del ambiente celular, modulan la probabilidad de que células tumorales puedan invadir otros órganos. En el último capítulo, basados en registros recopilados de literatura, cuantificamos la invasibilidad y la variabilidad en invasividad de canceres metastásicos y analizamos el patrón emergente de tal variabilidad. A través del análisis de la metástasis como una red entre órganos fuente y receptores, un gradiente de invasividad e invasibilidad subyace el patrón macroscópico que resulta ser modular, anidado y libre de escala. Discutimos algunos mecanismos relacionados al gradiente de invasibilidad y la invasividad dando cuenta de la estructura global de la red. El trabajo desarrollado en esta tesis evidencia que el pensamiento ecológico y su aplicabilidad está más allá del paradigma ecológico tradicional, y que, por lo tanto, una visión multiescala del cáncer inspirado por relaciones entrelazadas en la naturaleza, podría dar luces de los mecanismos que subyacen la evolución del cáncer en el contexto del programa de organización multicelular, con las implicancias ecológicas, evolutivas y clínicas que ello contiene.

General Abstract

Beyond genes, at different scales, cancer exhibits a narrow dialectic interaction with other cells and with its physical environment, undoubtedly an ecological phenomenon. This thesis focuses on theoretical, analytical, and experimental approaches at different scales; from cancer emergence (oncogenesis) to metastasis. In the first chapter, we conceptualise oncogenesis as the cellular phenotype-shift of individual cells in response to ageing. We propose that under ageing conditions, the multicellularity cannot be sustained. Hence, rewiring internal cellular processes enacting a particular phenotype in the cell characterised by structural and functional disgregation. We discuss the notions of individuality, cell-environment co-determination, and the enaction of the external environment of cells. Besides to impact on life-history cellular traits, ageing also shapes the ecological interactions between cells. In the second chapter, we attempt to integrate the complexity of competitive interactions between cellular strategies at different environmental contexts. We developed an analytical model which allows us to study the invasion fitness of a cancer strategy invading a population of noncancer cell inspired by observations of *in vitro* competition (HEY-GFP ovarian cancer cell line and MET5A mesothelial ovarian cell line) in an ecosystem model associated with the ageing of the ovarian tissue (modifying collagen I concentration). Our model suggests that hierarchical competitive mechanisms may underlie cancer occurrence during late-life stages, where ageing lessen the competitive advantages of non-cancer cells allowing a cancer strategy to invade. In the third chapter, we tackle the spatial dimension of the phenomenon by studying the spatial structure and composition of ER+ breast cancer ecosystems under antiproliferative endocrine therapy. Using supervised machine learning algorithms, we identified individual cells in biopsies immunolabelled for Ki67 (proliferation marker). In each sample, we identified spatial hotspots of cells (zones of higher density), and we projected the cells in these hotspots as nodes of a spatially explicit

network. The detection and quantification of spatially fragmented and structured communities of cancer cells and lymphocytes together with Ki67 expression allow us to predict the patients' pathological response to antiproliferative treatment stronger than traditional non-spatial metrics, such as Ki67 expression alone. Besides operating as a driver of spatial patterns, the diversity of life-history attributes interacting with organ's local conditions also determines the likelihood to metastasise. In the last chapter, based on literature records, we analyse the metastatic pattern for different primary metastatic tumours. Through the analysis of metastasis as a bipartite network between source-acceptor organs, we show that a gradient of invasiveness and invasibility underlies the modular, nested, and free-of-scale macroscopic network pattern. We discuss several mechanisms behind the network structure based on the gradient of invasiveness and invasibility. The work developed in this thesis shows that ecological thinking and its applicability is beyond the traditional ecological paradigm. And that a multiscale view of cancer may expound some mechanisms underlying cancer evolution in the context of the multicellular organisation with the ensuing environmental, evolutionary, and clinical implications.

General Introduction

'There are many kinds of individuals out there and some of them are still waiting to be discovered'

B. Santelices

The evolutionary transition from the single-cell ancestors to multicellular organisms opens the question about how the multicellular organisation emerges after single-cell individuals reach a spatial structure and intertwined functional divergence (Michod 2007; Nedelcu 2012; Szathmary 2015). The evolution by natural selection based on competition provides a conceptually complete paradigm for the divergence of new strategies, but it lacks explanatory power for life transitions such as the emergence of multicellularity from single-cell ancestors (Kikvidze and Callaway 2009; Rainey and De Monte 2014; Szathmary 2015). The development to multicellularity can be explained through the integration of different mechanisms associated to fitness, for instance, multi-level selection, division of labours, and the decoupling of fitness at the level of the group from the scale of the individual cell (Michod and Nedelcu 2003; Hanschen et al. 2015). Also, the evolution requires spatial convergence and cell-cell communication between the single-cell pre-metazoan individuals (Romeralo et al. 2013; Du et al. 2015), emphasising the role of space and local conditions as the quintessential characteristic of the multicellular program.

An ecological manifesto of metazoan life

The origin of multicellularity in animals and cell differentiation have parallels in terms of the underlying spatial convergence and functional divergence or division of labours (Arendt 2008; Brunet and King 2017). The division of labours entails a social contract where the individuals cooperate in favour of the collective over individual success (Herron and Michod 2008). Here, the analysis of the intricate emergence of a cancer strategy within the

multicellular ecosystem resembles the evolutionary transition from pre-metazoan single-cell individuals to metazoans, wherein a set of rules has evolved to solve the conflicts of coexistence leading to cooperative metabolic cycles within the multicellular schema (Michod and Nedelcu 2003; Michod 2007; Rainey and De Monte 2014; Szathmáry 2015); but in the case of the onset of cancer, the opposite evolution seems to occur, i.e., an override of the cell's multicellular algorithm back to a unicellular-like strategy (Davies and Lineweaver 2011; Bussey et al. 2017; Cisneros et al. 2017; Axelrod and Pienta 2018; Trigos et al. 2018). The emergence of a cheater strategy is a likely scenario of the evolution within cooperative cycles (Szathmáry and Maynard Smith 1997; Herron and Michod 2008), and in the multicellular organisation, it challenges the mechanisms supporting the homeostatic multicellular life. Within this ecosystem of complex interactions, the role of other cellular lineages has been considered recently, highlighting that a tangled set of interactions orchestrate the evolution of cancer within the metazoan ecosystem, from oncogenesis to metastasis (Condeelis and Pollard 2006*a*; Merlo et al. 2006; Xing et al. 2010; Korolev et al. 2014; Aktipis et al. 2015; Nawaz et al. 2015; Ganesh et al. 2020).

Cancer, a multi-scale complex adaptive ecosystem

The multicellular organisation fits in the criteria of complex adaptive systems (Gell-Mann 1994), wherein different individual elements interact and produce emergent behaviour that evolves adaptively in their environment; in this case, the emergent property is the multicellular organisation. Complex adaptive systems (CAS) reproduce emergent patterns at different ecological spatiotemporal scales (Levin 1992), representing a challenging phenomenon for the study of their ability to cope with external disturbances, i.e., their robustness and stability (MacArthur 1955; May 1977, 2001). In this view, ecological systems can be studied as CASs, whose patterns result from the interactions between entities behind the flux of matter, energy, and information (Gell-Mann 1994; May et al. 2008).

Cancer is a phenomenon emerging at the cellular scale where the traditional wisdom defines it as a 'disease of genes'. Such a view promises that through the understanding of the most reduced biological constituents of cells, we will be able to develop better detection systems and, eventually, treat the disease. However, it is clear that cancer corresponds to the overproliferation of a cellular strategy, i.e., a population phenomenon; then, it is not evident how cancer can be fully understood only through genes without considering the environment where the cell or a collective of cells, exist. The past development of the biomedical research in cancer has focused its attention on the intracellular mechanisms and processes; however, the emerging paradigm is that either the external environment of the cell (Merlo et al. 2006) as the external environment of the organism (Hochberg and Noble 2017), shape cancer's ecology and evolution.

Cancer has been considered, at least theoretically, as a complex adaptive system at a broader sense (Schwab and Pienta 1996; Kitano 2004). A novel view from ecology and evolution on cancer uphold that ecological and evolutionary principles can help us to spell out some of the complexities underlying cancer and contributing to its detection and treatment (Merlo et al. 2006; Chen and Pienta 2011*a*; Greaves and Maley 2012*a*; Noorbakhsh et al. 2020). However, the difficulties of studying cancer ecosystems are that complexity is produced at every scale of the phenomenon where the cancer phenotype interacts recursively with its environment, i.e., genes/intracellular environment, genes/extracellular environment, cell/extracellular environment, organs/physiology, and organism/organismal environment. Therefore, the consideration of cancer as a multiscale phenomenon is needed, and actually, it can fuel our understanding of its evolution (Deisboeck et al. 2011).

In this thesis, we analyse how an ecological paradigm of cancer contributes to the understanding of this disease as a shift in the organisation of diversity within-organisms' ecology at different scales. This thesis has four chapters. The first two chapters allow us to

integrate how ageing as a shift in the cellular environment may shape oncogenesis and facilitate the cancer cell's invasion in a competitive context with non-cancer cells. In the third chapter, we study the spatial dimension of cellular interactions in breast tumour biopsies seeking to discover how spatial ecological measures can contribute to predicting tumour's response to antiproliferative adjuvant therapy. Finally, in the fourth chapter, we analyse the metastatic phenomenon by using network theory applied to the medical records of metastases obtained from the literature seeking to understand the diversity of invasiveness and invasibility in metastatic cancers and how such diversity entails emergent macroscopic structures. Through these four chapters, we aim to highlight the matching between cells' complexity and their environment required to give rise and maintain a cellular strategy and resulting in particular patterns at each scale, from the individual cell to the metastatic spread between organs.

Chapter I Oncogenesis, Cell-Environment Codetermination, and the Erosion of the Multicellular Algorithm with Ageing

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Abstract

Cancer represents the emergence of a singular cellular state that destabilises the normal organismal evolutionary steady state, reached under the multicellular algorithm, and maintained by homeostatic mechanisms. We present the idea that the onset of the cellular disobedience to the metazoan algorithm, known as the cancer phenotype, is triggered by changes in the cell's external environment occurring with ageing, making cells no longer able to sustain the social contract of multicellular life characteristic of Metazoans. We propose that in an aged context, the environmental information leading to a multicellular organisation is eroded, rewiring internal processes of the cell, and resulting in an internal shift enacting the ancestral unicellular condition, and expressing the cancer phenotype.

The multicellular algorithm and the cellular social contract

Metazoan cells' internal environment delimited by the cellular membrane, has evolved in agreement to its local external environment (Ratcliff et al. 2012) but keeping encrypted in the space of possible strategies the atavistic behaviour from its unicellular ancestors (Bussey et al. 2017; Cisneros et al. 2017) or cells embedded in a noncondensed and nondifferentiated collection of cells. The transition from unicellular to multicellularity is vital to comprehend the nature of the emergence of the cancerous phenotype as a knockdown of the metazoan algorithm (Chen et al. 2015). To do so, it is necessary to distinguish the most likely state of a multicellular as one ruled by a contemporary division of labours in a condensed state whereby homeostasis is achieved through the cooperative metabolic replication of different cell lineages and conflict mediation among them by a set of rules that underly their continuous co-existence (Szathmary and Smith 1995; Michod and Roze 2001; Michod and Nedelcu 2003; Axelrod and Pienta 2018) in a spatially and functionally structured landscape, i.e., the 'multicellular algorithm' (Figure I.1).

Oncogenesis involves overriding some rules of the multicellular algorithm; in particular, the local rupture of the social contract that maintains the multicellular architecture (Aktipis et al. 2015; Axelrod and Pienta 2018). The metaphor of the social contract is explicit in the functional divergence emerging from a cooperative metabolic network among coexisting cell lineages which favour the collective over the individual scale, as a necessary condition for multicellularity (Szathmary and Smith 1995; Michod and Roze 2001; Arendt 2008; Brunet and King 2017; Doolittle and Booth 2017). In this case, oncogenic cells find a way to hack the norm and progressively erode the homeostatic social contract. In physiological terms, the cancer phenotype is a cellular strategy characterised by a myriad of hallmarks and innovations (Hanahan and Weinberg 2011) often viewed as a mosaic of characters chiefly determined by mutational steps and clonal expansion, as envisioned under the somatic

mutation theory (Soto and Sonnenschein 2011; Montévil et al. 2017), which emphasises the onset of cancer as an endogenously mutation-driven process. The somatic mutations model states that each cancer cell has a history of accumulation of mutations and epi-mutations in somatic cells. In other words, with each stochastic mutational event, a cell might display a new strategy, navigating through its fitness landscape (Simpson 1944; Wright 1988; Huang et al. 2009). Recently, however, empirical data show that despite the presence of mutations in common cancer-associated genes (e.g., TP53, NOTCH), tissues do not show signs of cancer growth (Martincorena et al. 2018; Yizhak et al. 2019); therefore the emergence of the cancer phenotype cannot be reduced to genetic modification only, challenging the paradigm of genetic mutations as exclusive determinants of the cancer phenotype. Alternatively, an emerging paradigm highlights the role of the external environment studied at the scale of the organism (Hochberg and Noble 2017) but also at the cellular scale as a driving force of cancer dynamics (Bissell et al. 1982; Greaves and Maley 2012*a*) In particular, the cell's external environment shapes the internal environment and this interaction can modulate the emergence (Chaudhuri et al. 2016) and progression (Ingber 2002; Nelson and Bissell 2006) of the cancer phenotype. Henceforth, we consider the external environment as the environment where the cell is embedded (extracellular matrix).

Besides the somatic mutation model, another evolutionary hypothesis was proposed to explain oncogenesis considering cancer behaviour as a cellular atavism (both reviewed in (Thomas et al. 2017)). We propose that these two hypotheses are complementary in the light of ecological computability of the external environment as we will explain. Here, we use the term computation to refer to the capability of the system (a cell or a collective of cells) of interpreting, creating, and modifying its external environment (Trautteur 2007). It raises the question of whether the external cellular environment contains the elements that cells transduce and interpret as symbols (physical patterns) (Bissell et al. 1982) and whether such

representation of the outside lead to a cellular behaviour of a particular kind. In this framework, the notion of computability links with the concept of virtuality borrowed from computational systems (Trautteur 2007) that includes the capability of focal systems of interpreting external signals and adjust their internal environment (Ashby's homeostat (Ashby 1968)). Hence, these concepts consider the notion that biological machines can modify- and be modified by- their external environment, integrated through the dialectic co-emergence of the 'self' as the cellular individuality and the enacted extracellular environment. This dialectic co-emergence already has biological mechanisms such as evolution through natural selection (Darwin 1859) and niche construction (Laland et al. 2016). The ability of cancer cells to modifying their immediate external environment and change as a consequence of these changes (niche construction) are essential characteristics which contribute to explain their success in invading an organ either in primary tumours or in distant metastases (Yang et al. 2014; Qian and Akcay 2018), but this begs the question of what determines the appearance of such innovations in cancer cells that allow them to proliferate despite the prevailing social contract of cells wherein they are embedded. In this essay, under the idea of cellular computation, we propose that the cancer strategy co-emerges with its external environment (at the cellular scale) as a consequence of dynamic cell/environment interactions underlying the rupture of the spatial and functional structure characteristic of the multicellular organisation (Figure I.1). The emergence of the collective organisation, underpinned by the division of labours, enable an efficient process of the flux of matter, energy, and information (Michod and Nedelcu 2003). Moreover, it is worth to make a point about the efficiency of the computation of the external variables, which might be facilitated by collective computation and the flux of information between individual systems (Flack 2017); thus, cells beyond being single-isolated entities, modify their

behaviour and environment as a collective system, and the emergence of cancer strategies and their evolution is mediated by the collective, heterogeneous, and enacted environment.

Oncogenesis and mutual codetermination cell/environment

Before addressing the evolutionary dynamics leading to the neoplastic phenotype shift, it is necessary to introduce some simple notation. Let us first define a cell as the focal system, wherein we can discern observables, such as phenotypic strategies $\omega_i(t) \in \Omega$, where Ω recapitulates and stores the contemporary and ancestral space of possible strategies or cellular phenotypes (Fig. 1). A phenotype is the realisation of a set of continuous life-history traits at a given spacetime; in other terms, the expression of the internal environment as a representation of the fluxes with the external environment. The internal environment is a multi-layered network from genes' co-expression to metabolic reactions which enables the cell's maintenance over time. Now, let us define a process $P: \omega_i \rightarrow \omega_o$, as the shift from a given cellular strategy to another one, in the case of cancer, the process P is called oncogenesis. This transition between cellular strategies might be related to a rewiring of the internal environment represented by a shift in the gene co-expression network (Anglani et al. 2014; Trigos et al. 2017) which can be mediated by the environment (Bissell et al. 1982). From an evolutionary point of view, this shift and the resulting appearance of an ecological novelty within the population of non-cancer cells represents the invasion of a mutant phenotype to a 'stable' structured community of cellular strategies, where the fate of this novel phenotype depends on its interaction with the biophysical environment (Gatenby 1991; Orlando et al. 2013; Keymer and Marquet 2014). Notice that we denote the cancer strategy as ω_o, to emphasize that it does imply a reversion to an ancestral strategy that existed before multicellularity, resembling a strategy with a lower degree of spatial and functional structure (Figure I.1).

The somatic mutation theory has driven the understanding of oncogenesis, effectively treating it as an endogenous process, and in so doing closing a system that is intrinsically open and complex (Bissell et al. 1982; Schwab and Pienta 1996). Current data that show the conspicuity of mutations in cells without the expression of cancerous phenotype challenges that view (Martincorena et al. 2018; Yizhak et al. 2019), calling for the emergence of a new theoretical framework where cells are envisioned as open autonomous systems that co-emerge with their external -biophysical- environment (enactivism *sensu* Francisco Varela (Varela 1991; Thompson 2010)). The enactive principle implies a dialectic co-construction individual-environment; hence, any deformation or perturbation in the external environment is sensed and integrated (e.g., through a point mutation, DNA methylation) by the cell into its internal environment, which in turn affects the external environment and how the cell enacts it. In this context, cells emerge as autonomous 'selves' thanks to their organisational closure (Varela 1979), whereby it becomes an autonomous entity at a given context with which it is in a continuous co-transformation (Varela 1991; Thompson 2010). This mutual specification or co-determination between a living entity and its environment is the result of a history of coupling that involves the exchange of matter, energy, and information between the cell's internal environment and its immediate neighbourhood carried out by a multilayered network of interactions involving genes and their products (Varela 1991; Oyama 2000). This flux determines the state of the focal system (i.e., the cell's strategy), the associated population-level effects (Suderman et al. 2017), and the transformation of the cell's immediate environment (Varela 1991; Thompson 2010; Yang et al. 2014; Laland et al. 2016). This dialectic view of cell-environment co-transformation, we argue, is essential to understanding the phenotypic transition of cells from a healthy to a cancerous phenotype (oncogenesis) and its evolutionary trajectory. Here, codetermination arises because the

environment and the entity specify each other through selection, drift, epi-mutation, and niche construction (Varela 1991; Yang et al. 2014; Laland et al. 2016; Larson et al. 2020).

The erosion of the spatial and functional multicellular ecosystem

Revisiting the evolutionary hypotheses in the light of cellular environmental effects, we propose that in the context of the human body as an ecosystem, the external environmental conditions that enable the transition to the atavistic phenotype are the changes in the extracellular matrix due to ageing (Benz and Yau 2008; Sprenger et al. 2010). Ageing erodes the external information computed by a cell from which it reproduces a singular strategy in a multicellular context, equalising the transition between the condensed and functionally-coupled collective (metazoan) and the noncondensed cellular collectives (pre-metazoan), hence increasing the likelihood of oncogenesis, the enaction of the pre-metazoan strategy, by selection on the cellular strategy with higher proliferative rate (Greaves 2002). Now, it can be linked with the hypothesis of atavism, i.e., the idea of cancer as a reversion to an ancient cellular state (Davies and Lineweaver 2011; Bussey et al. 2017; Cisneros et al. 2017). As an extension, the relationship between environmental conditions due to ageing, the transduction of those environmental signals into cells internal environment (Bissell et al. 1982), the rewiring of the internal cellular architecture and the restructuring of the external network of interactions are reflected in the epidemiological patterns of cancer incidence increasing as a function of age (Rozhok and DeGregori 2019).

The construction of a robust theory of oncogenesis demands its applicability in a biomedical context. This framework stresses attention on the role of the external environment, other cellular strategies and the physical conditions of the extracellular matrix involved in oncogenesis. For instance, epigenetic reprogramming could be an alternative implying that the phenotype switch: $P: \omega_i \rightarrow \omega_o$, is reversible through manipulation of the external environment (Suvà et al. 2013), but also age-related changes at tissue level might serve as

early warnings of potential oncogenesis (García-Nieto et al. 2019). In another venue, targeting the tumour biophysical environment, such as through cytokines or growth factors, also is gaining attention within the potential application to therapeutics (Martin et al. 2016), with the benefits of controlling short and long-distance spread of cancer propagules. Also, at the level of intratumoural genetic diversity, the role of the environment is gaining importance (Swanton 2012), with the corresponding study of the clinical implications of such level of biodiversity (Junttila and de Sauvage 2013; McGranahan and Swanton 2015).

The current understanding of cancer is shifting from the traditional reductionist and closed system view towards a view where the configurations of the biophysical, cellular environment have a fundamental role shaping cancer's fitness landscape and hence, its evolution. Here, we discussed the idea of cancer as the result of a dialectic cell-environment interaction. For the sake of simplicity, we restricted ourselves to analyse the impact of environmental changes of the cellular environment associated with ageing; however, it is clear that the cellular environment also changes in response to the environment that is experienced and enacted by the organism as a whole, which is known to affect the risk of cancer development (Hochberg and Noble 2017). It requires further research to test the interaction between the organismal and the cellular environment in affecting the cellular behaviour and maintaining or destabilising the multicellular structure.

Cancer emergence can be understood in an integrative way adding ecological and evolutionary concepts and tools, since as we discussed here, cancer cells are not isolated entities, and the multicellular ecosystem is not merely a collection of cells. To the contrary, cell-environment and cell-cell interaction in the context of a biofilm or a functional multicellular organism are paramount; no living entity exists in isolation. Ecology and evolution are quintessential to tackle the complexity of the human ecosystem, understanding

the emergence of the cancer phenotype within it, and in using this knowledge to reach a robust and multidisciplinary understanding of cancer onset and evolution.

Figures

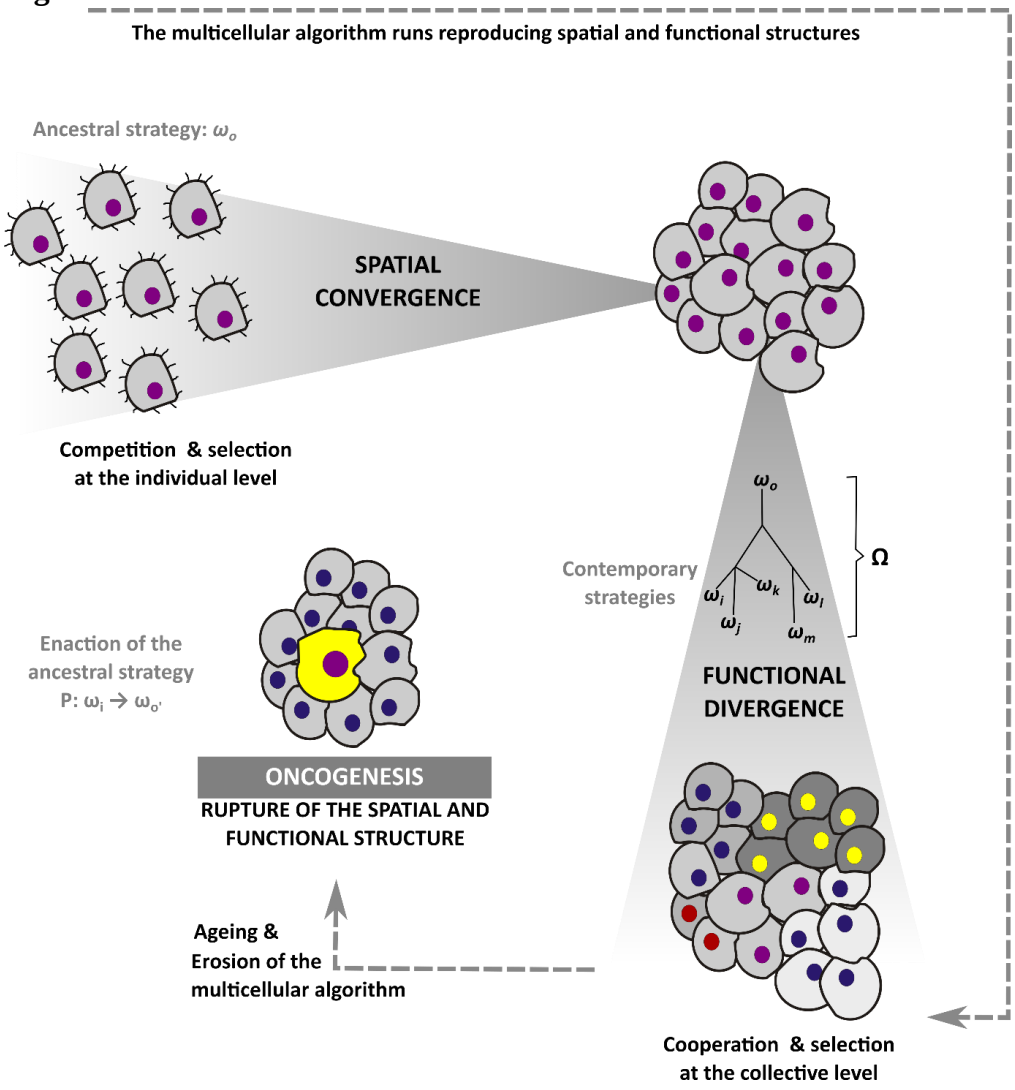

Figure I.1The emergence of the cancer phenotype in response to the erosion of the spatial and functional structure reached under the multicellular algorithm. The external environment of cells promotes the spatial convergence of the individualised cells into aggregates. Mechanisms related to the selection of varieties and the resolution of conflicts lead to functional divergence, cooperative metabolic cycles, and multicellularity. These steps append possible cellular strategies to the evolutionary history of the cell stored in Ω. With ageing, the erosion of the multicellular environment is transduced and interpreted by cells, enacting the ancestral strategy and breaking the spatial and functional structure.

Chapter II Invasion Fitness in Ageing Landscapes

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Abstract

Ageing entails the intertwined evolution of both the internal and external environment of cells along with the organism life; hence, shaping the ecosystem at the cellular scale. Here, we study the ecology of the beginning of cancer cells' invasion by integrating hierarchical competitive interactions between cancer and noncancer strategies and external environmental contexts shaped by ageing. Inspired by our observations of *in vitro* ecosystems of co-cultured high-grade serous ovarian cancer cells and mesothelial ovarian cells coexisting in collagen I environments; we proposed a numerical ecosystem based on evolutionary game theory and adaptive dynamics, analysing how the environmental parameter and the noncancer's competitive resistance to invasion drive the initial invasion fitness of the cancer strategy. Our results show how the environmental properties, which we might associate with ageing, shape fitness dynamics of both cancer and non-cancer cells, increasing the cancer cells' invasion fitness through lessening the competitive resistance of non-cancer cells.

Understanding the natural laws governing the entangled bank of species packing in nature is a fundamental issue in population and community ecology, where some permanent questions are related to the relationship between species traits, population dynamics, community structure, spatial and temporal environmental dynamics, and invasibility (MacArthur and Levins 1967; Sutherland et al. 2013). Cancer ecosystems are very close to these questions; they occur in multicellular organisms (Aktipis et al. 2015) where the emergence of the cancer strategy, its traits, and its population dynamic modify the community structure of cells menacing the organismal homeostatic state evolutionary and ontogenetically achieved by different cell types (Pienta et al. 2008; Chen and Pienta 2011*b*; Hanahan and Weinberg 2011; Axelrod and Pienta 2018; Noorbakhsh et al. 2020). From this starting point, it is clear that ecological interactions between cancer and non-cancer cells play a key role within cancer ecosystems determining the internal structure, its evolutionary trajectory, and the ensuing clinical implications (Rak et al. 1996; Li and Neaves 2006; Giannoni et al. 2010; Pietras and Ostman 2010; Bertolaso and Dieli 2017; Heindl et al. 2017; Amend et al. 2018). For instance, different players within the stroma play underly the faith and spread of cancer cells. In breast cancer, macrophages are associated with tumour migration, intravasation, and invasion (Condeelis and Pollard 2006*a*). Also, metastasis may be influenced by cancer-associated fibroblasts (CAFs) (Zhang et al. 2013), mesothelial cells promoting cancer cells migration to other tissues (Karnoub et al. 2007), or as was showed recently, some cells (L1CAM+ cells) in human colorectal cancer have metastasis-initiating capacity (Ganesh et al. 2020). Therefore, the study of how a cancer cell population evolves at a given context needs to scale up from the individual scale to the interactions between different cellular strategies and the external physical environment.

Competition within tumour ecosystems

Competition is one of the possible interactions between strategies. It has had a prolific development in ecology, pioneered by Charles Darwin's observations (Darwin 1859), Charles Elton's (Elton 1946), and Robert Macarthur's work (MacArthur and Levins 1967). Despite a substantial evolution of theoretical, mathematical, and experimental treatment in ecology, their applicability to within-organism ecologies is rare. In fact, with the exception of Robert Gatenby's work (Gatenby 1991, 1995), ecological interactions in general, and competition in particular, within tumour ecosystems, has been sparsely latent only during the last decade (Moreno 2008; Baillon and Basler 2014; Nelson and Masel 2017; Amend et al. 2018; Qian and Akcay 2018). The idea of competitive interactions rests on the fact that, at a given time, individuals can perform a particular strategy defined by a set of traits (e.g., birth and death rates), and that different strategies compete for the same finite resource. Inspired by the competition models used in ecology (MacArthur and Levins 1967; May 1974; Orlando et al. 2013) and the hypothesis of competitive hierarchies (Kinzig et al. 1999), the baseline argument here is that cancer cells, as a strategy initially scarce in density, can proliferate at a community of noncancer cells if and only if their traits are sufficiently different from resident cell's traits (Keymer and Marquet 2014).

Before moving forward, let us succinctly talk about the hypothesis and hierarchical competition-colonisation trade-off (Kinzig et al. 1999; Orlando et al. 2013). A strategy, in this case, is defined by the life-history traits that an individual display in a given context. These traits draw a competitive shadow, limiting the similarity in traits, in favour of the strategy and restricting the space of traits that an invasive strategy can display to achieve a positive fitness. The hypothesis imposes a constraint in the performance of life-history traits, such that individuals are limited in their capabilities to be a strong competitor or a good coloniser; determining the state of the focal system, its stability and evolution (Levin and

Paine 1974; Nee and May 1992). An essential element of this hypothesis is the establishment of a competitive hierarchy, wherein in a two strategies interaction, there is a superior competitor which population is not affected by the inferior competitor's dynamic; while the latter's fitness is affected by all the strategies (superior and inferior competitors). Previously it was discussed this idea of asymmetric competition within cancer ecosystems (Keymer and Marquet 2014), suggesting that cancer cells (the inferior competitor) must perform a higher reproductive rate and/or lower death rate to survive and sustain an invasive population at a suitable habitat governed by noncancer cells playing as superior competitors.

The interaction between different strategies has recursive implications on the populations' fitness. In this work, we use a simple analytical framework governed by the motion equations for biomass x_i for a given strategy *i* as a function of a fitness parameter f_i, with *i:{C, NC}*:

$$\dot{x}_i = f_i x_i \qquad \text{Eq. II.1}$$

The competitive hierarchy is introduced later on the fitness parameter f_i, which in the case of the superior competitor's fitness (f_{NC}) is depending only on its dynamics; whereas for the subordinated competitor, the initially scarce cancer strategy, its fitness parameter f_C depends on its dynamic as well as its competitor's. In this particular case, a missing link which has not received enough attention in the literature is related to the question of how this ecological interaction works throughout the human lifetime. It arises from the fact that the external environment under which cells display their life history is dynamic (Keymer et al. 2012), following the inexorable phenomena of ageing which might help to explain cancer's success in late stages of human life (Rozhok and DeGregori 2019).

Ageing a permissive context for invasion

A known determinant involved in the success of invasive species is the invasibility of a recipient community; it includes the biophysical properties of the native habitat (Richardson and Pyšek 2006). In the case of within-organism ecosystems, such as the human body, the

changes in the flux of biochemical materials influence the community structure of cells, where some of these changes could be related to local or microenvironmental conditions at the tissue level (Croucher et al. 2016) or organism-level processes such as ageing (Benz and Yau 2008).

Ageing has two dimensions. The first dimension represents the change in the physical environment that cells inhabit, i.e., the extracellular matrix (ECM). These changes determine cells' behaviour and proliferation (Sprenger et al. 2010; Kurtz and Oh 2012; Spadaccio et al. 2015); where besides being an entangled network of structural support, it also initiates crucial biochemical cues required for cell growth, proliferation, differentiation, and homeostasis (Frantz et al. 2010). One of the most notorious changes in ECM composition is related to structural proteins concentration (e.g., collagen I and IV) which modifies rheological properties of the ECM, which in turn modify the invasiveness of cancer cells (Baker et al. 2010; Swaminathan et al. 2011; Xu et al. 2012; Heindl et al. 2018). The second dimension, even though not independent of the first one, is related to the noncancer cell's internal ageing exhibiting a reduction in their proliferative capacity (Fehrer and Lepperdinger 2005; Bonab et al. 2006; Collado et al. 2007), representing a shift in one of the cellular life-history trait, and under our view, reducing their competitive ability to face invasive cells.

In summary, the main biological principles of our hypothesis are three: (1) a resident noncancer strategy projects a competitive shadow along a niche axis, determining a space of traits where an invasive cancer strategy may hardly invade the resident habitat; (2) noncancer cell's life-history traits (birth and death rates) experiment changes along ageing; and (3) along with ageing, the cellular microenvironment is eroded, impacting the noncancer cells/environment codetermination, hence in its competitiveness against cancer cells (Figure II.1). In this work, initial experimental observations on *in vitro* coexistence of ovarian cancer

(HEYGFP) and noncancer (MET5A) cell lines, inspired us to develop an analytical model to evaluate how the interaction between vital histories of two cellular strategies (cancer and noncancer) and the external environment shapes the invasion of the cancer strategy in an ageing context.

Methods

In vitro ecosystems

Cell lines used in this study are a high-grade ovarian cancer cell line (HEY-GFP, cancer strategy) and a mesothelial cell line (MET5A, noncancer strategy) cultured under two different environmental conditions given by matrigel (Corning Inc., USA) with different collagen I concentration. Considering that in the ovarian environment collagen I is one of the components that shows a more notorious change with ageing; default manufactured conditions of matrigel was considered as a case of the high-quality environment, representing a non-aged environment, whereas matrigel with an increase of three times the concentration of collagen I relative to the high-quality environment, represents the low-quality environment. We added purified collagen I from rat tail. We incubated cells using DMEM medium. Cancer cells express GFP (green fluorescent protein), and MET5A cells were pre-treated with a red cell tracker (Corning Inc., USA).

We set a transwell migration setup described as follows. First, we cover the outer face of the bottom of the insert (8μm pore size) with matrigel (one of the two types). Once matrigel solidified, we incubated MET5A cells (30000 cells) during 24h, allowing their attachment on the matrigel and growth. Then, we added an estimated 30000 cancer cells through the inner face of the insert. Since the interest of this study is not only on the invasion, also in the temporal dynamics of the system, the experimental setup was left a maximum time of five days from the culture of MET5A; which imply the growth and proliferation of both strategies, a competitive scenario with demographic results. The first temporal cohort (day

0) corresponds to 17h after the addition of HEYGFP cells representing the invasion of cancer cells to the non-cancer population, a second temporal cohort (day 1) was retired from incubation 24h after cohort 1, and finally, the third cohort (day 2) was retired 24h after cohort 2. An important point to mention is that 17h after the addition of HEYGFP cells, i.e., cohort 1 - day 0, we removed the remaining cells that did not migrate through the membrane using a cotton swab; therefore the observed population at the cohorts 2 and 3 (day 1 and 2, respectively) approaches chiefly to proliferation of the cancer cells which trespass through the membrane. After we removed each cohort from incubation, cells were rinsed with PBS 4%, fixed with PFA 4%, and the membrane of the insert was manually removed and mounted over a microscope slide to photograph cells using confocal fluorescence microscopy. Due to the high spatial heterogeneity of the Matrigel on the membrane of each insert, noncancer cells did not form a homogeneous monolayer over the substrate; hence, we took photographs only where we could visualise both strategies.

Experimental design - A two factors design with three fixed factors is considered in this experiment: cells in culture, culture conditions, and day. The 'culture conditions' factor has two levels according to the composition of matrigel: matrigel, and matrigel with an increase of three times of collagen I concentration. The factor called 'day' has three levels according to the time each assay lasts. The replication level consists of the number of experimental units where each combination of levels of factors was applied, for our case, it is incomplete due to logistic constraints, with two replicate for some combinations and three in others, summing a total of 16 observational units (inserts).

Numerical ecosystem: underlying assumptions, concepts, and notation

Our model deals with the process after a cancer strategy appears. We studied the invasion of a mutant cellular strategy (cancer, C) in a monomorphic resident population of cells (noncancer, NC) where both populations are characterised by particular and fixed

reproductive (β) and death (δ) rates defining each strategy and its intrinsic population dynamics. Our formulation assumes that all individual cells maintiaind their identity (*C* or *NC*) and that within each population type are equivalent, there is no stage or metabolic differences. We define a game, based on evolutionary game theory, as pairwise interactions between individuals performing particular strategy (*C* or *NC*), where by-product of an interaction individuals get specific pairwise payoff (p) times the counterpart's relative abundance, being the overall payoff for a strategy, i.e., its fitness parameter (here denoted by f_i) the sum of its pairwise payoffs. The last concept to define is the environmental parameter (ω), it intends to represent the quality of the physical conditions where cells display their strategies, i.e., growth, die, and interact. For the sake of simplicity, the environmental parameter is an abstract and dimensionless value representing the quality of the extracellular matrix for cell growth, lower the parameter lower is the quality of the ECM for cell proliferation and tissue renewal. For this model, the parameter associated with the environment (ω) and the life-history traits remain fixed over the time of each trajectory since our interest is on to evaluate how it shapes the interaction between strategies, and how this shapes the invasion fitness of the cancer strategy.

Let us start with a well-known model in ecology representing denso-dependence within each population; it generalises the growth of a population through a fitness parameter (f_i) and denso-dependency, which apply for cells responding to contact inhibition by proliferation. Thus, we can track the population growth over time ($\dot{x}_i$) of a given strategy i: $\{C, NC\}$, as a function of its fitness parameter f_i and population size x_i.

Let us start with the dynamic of the resident, noncancer strategy *NC*

$$\dot{x}_{NC} = f_{NC} x_{NC} \qquad \text{Eq. II.2}$$

In this case, we define the fitness parameter by the interactions within the *NC* population, hence

$$f_{NC} = p_{NC,NC} x_{NC}$$ Eq. II.3

The pairwise payoff between noncancer cells ($p_{NC,NC}$) can be approximated by the dot product of the strategy's life-history traits ($\vec{s}$) and the environmental parameter ($\vec{\omega}$) representing the habitat where the interaction occurs (Keymer and Marquet 2014). The first vector, $\vec{s}$, represents two key events underlying a cell's vital history: reproduction (β) and death (δ), hence, a cellular strategy *i:{NC, C}* is defined by a particular pair of traits $\vec{s}_i = (\beta_i, \delta_i)$. The second vector, $\vec{\omega}$, includes the environmental quality within the model; this vector $\vec{\omega} = (\omega_+, \omega_-)$ with $\{\omega_+, \omega_-\} : [0,1]$ acting as a control of the local external environment on population growth (sensu (Keymer and Marquet 2014)). Assuming that the environmental parameter increases the proliferation and diminishes the death of a singular cellular strategy, we consider $\omega_+ = \omega$ and $\omega_- = -1/\omega$, with ω: (0,1]. Considering this notation, for the resident noncancer strategy we have that its payoff can be approached by

$$p_{NC,NC} \equiv \vec{s}_{NC,NC} \cdot \vec{\omega} = (\beta_{NC}, \delta_{NC}) \cdot (\omega_+, \omega_-)$$ Eq. II.4

We incorporate a denso-dependent population dynamics operating over the proliferation; hence the population dynamic can be expressed as

$$\dot{x}_{NC} = (\beta_{NC}\omega(1 - x_{NC}) - \delta_{NC}/\omega)x_{NC}$$ Eq. II.5

It seems realistic to assume that the noncancer population is at its demographic attractor, i.e., $\dot{x}_{NC} = 0$, which represents the homeostatic organismal state; then, the population at the equilibrium (x^*_{NC}) follows

$$x^*_{NC} = 1 - \frac{1}{\omega^2 R^*_{NC}}$$ Eq. II.6

Where $R^*_{NC} = \frac{\beta_{NC}}{\delta_{NC}}$.

As an alternative case, we can consider that the noncancer strategy is not affected by the environment occurring in the extracellular matrix; hence, its population state is reduced to

$$x^*_{NC} = 1 - \frac{1}{R^*_{NC}}$$ Eq. II.7

Henceforth, we consider the scenario showed by the Eq. II.5 and II.6 assuming that noncancer's proliferation and death are mediated by the environmental parameter ω. Now, the resident population, at its equilibrium ($x_{NC}(t) = x_{NC}^*$) , face the appearance of the cancer strategy (*C*). Considering that *C* is initially scarce, and it runs with disadvantages on a multicellular context, we will consider it as an inferior competitor, while the noncancer strategy holds the superior competitor category. This hierarchical structure implies that the noncancer dynamic holds its intra-strategy and environmental dependency, imposing a resident competitive resistance against the invasive strategy, the cancer strategy. The latter, in turn, experience a growth given by its life-history traits but subjected to denso-dependency imposed by the *NC* population and a competitive pressure given by the proliferation of the *NC* strategy; hence its initial population dynamic can be defined by

$$\dot{x}_C = \beta_C \omega x_C (1 - x_{NC}^*) - \delta_C x_C - \beta_{NC} \omega x_{NC}^* \qquad \text{Eq. II.8}$$

Which led us to define the invasion fitness (*S*) of the cancer strategy as

$$S = \frac{\dot{x}_C}{x_C} = \beta_C \omega - \delta_C - \omega x_{NC}^* \left(\beta_C + \frac{\beta_{NC}}{x_C}\right); \; x_{NC}^* = 1 - \frac{1}{\omega R_{NC}^*} \qquad \text{Eq. II.9}$$

We study the deterministic behaviour of the noncancer population and the invasion fitness of the cancer strategy seeking to identify the conditions which allow the cancer strategy to invade an ecosystem dominated by noncancer cells. The main point of our model is that an initially scarce cancer strategy can, at least initially, invade an ecosystem, i.e., $S>0$, by the interaction between the environmental properties defined by the parameter and the difference in the life history traits (R^*) with the resident strategy.

Results

The results from the co-culture of cancer cells (HEYGFP) and noncancer cells (MET5A) under two culturing conditions open new views about the coexistence of these strategies and how the biophysical environment shape cancer patterns. We show that the initial invasion of

cancer cells to the population of noncancer cells seems higher in the aged environment (with higher collagen I concentration) in comparison with the non-aged environment. However, such initial advantage seems to be reverted with time (Figure II.2). These results feed the development of the numerical ecosystem that shows that the resident's resistance to invasion changes with the environmental quality (Figure II.2) and so it does the invasion fitness (Figure II.3 and Figure II.4).

Further, the numerical ecosystem allows us to distinguish scenarios where invasion is a possible outcome. The condition for a ppositive invasion fitness (S>0) entails that the life history traits of the cancer strategy recapitulated by R_C^* must to b higher than the traits of the noncancer strategy R_{NC}^*. It implies that there is a difference in the proliferation rate ($\beta_C > \beta_{NC}$) and/or in the death rate ($\delta_C > \delta_{NC}$). The invasion fitness also is mediated by the environment, which boosts the proliferation and reduce the growth suppression.

If the consider that the environmental parameter ω is a representation of the content of collagen in the ovarian environment, then we could consider that an aged fibrotic environment increases the proliferation of cancer cells and the evasion of growth suppressors. While, in this aged context, the noncancer strategy, resident of the ovarian environment, has a reduced competitive ability, either by a reduction in its proliferation and/or an increase in the apoptotic events.

Discussion

The role of ageing shaping cancer prevalence is complex involving several biophysical changes within-organism ecologies, involving deregulation and impairing of the immune response (Derhovanessian et al. 2008; Shaw et al. 2010), cellular senescence (Collado et al. 2007; Campisi 2013), and changes in the physical environment of cells (Sprenger et al. 2010; Kurtz and Oh 2012; Spadaccio et al. 2015). Our consideration of ageing includes either intrinsic changes related to the proliferating capacity of resident cells (Nelson and Masel

2017) and extrinsic changes related to the transformation suffered by the extracellular matrix along with ageing (Briley et al. 2016). In our model, even though through a very simplistic approach, both effects shape the competitive shadow of the resident strategy to face the invasive cancer strategy. Similar terms are used in population ecology referring to these effects as vertical and lateral perturbation effects, those which modify reproductive parameters and those which modify the environmental capacity of supporting a population, respectively (Royama 2012). However, in our case, both are variations which shape the strategy's life-history traits through modifications of the reproductive parameters and on the other hand, perturbations which modify the biophysical environment but that also shape the interaction between life-histories. That is to say that either vertical and lateral age-related perturbations coalesce shaping the competitive shadow of noncancer strategies, likely resulting in the non-linear occurrence pattern of cancer along with human ageing (Rozhok and DeGregori 2019). The results presented here show that two aspects are the key for an increase of cancer fitness: a decreasing of competition pressure mediated by environmental deterioration due to ageing, and a reduction of the proliferative capacity of noncancer cells. As we have said, the eroded environment represents an unsuitable habitat for noncancer cells proliferation, this case can be further explored in chronic inflammatory conditions related to ageing (Bonafè et al. 2012).

Despite our simple model, the formalisation of the effect of the environment on the competition between cell strategies is the first step into the formal consideration of ecological forces shaping tumour ecosystems(Gatenby 1991; Korolev et al. 2014). The determination of parameters associated with environmental quality is crucial to achieve a better understanding of cancer evolution and improve its diagnostic and prognosis. For this work, we considered fixed values of environmental quality, notwithstanding, in the future, it could be more informative a competitive game where the players are subjected to a

particular form of environmental fluctuation which represents the natural trajectory of the cellular environment. Although our approach does not deal with the emergence of cancer strategy, a call has been done towards the consideration of micro-environment as a driver of oncogenesis besides genetic instability (DeGregori 2017). In terms of environmental fluctuations, studies oriented to the physical properties of the extracellular matrix as well as cell's intrinsic modifications with ageing have shed light into the understanding of how vertical and lateral perturbations affect cell behaviour from a physical point of view (Baker et al. 2010; Soto and Sonnenschein 2011; Wirtz et al. 2011; Mierke 2015).

The approach through competitive hierarchies in ageing landscapes points to explain how and why cancer ecosystems evolve in a certain way. Its relevance aims to the idea of cells are interacting individuals at a confined and limited space compete between them, and this competition is underpinned by the codetermination of the strategies with the local environment along with the ageing process. Notwithstanding, is necessary to highlight something that we have not incorporated here, that this dialectic interaction individual/environment is recursive, in sense that the environment could shape community structure and the community can modify the local environmental conditions, hence modifying its selective pressures (Laland et al. 2016; Ibrahim-Hashim et al. 2017; Qian and Akcay 2018). Our hypothesis is bounded in the sense that it only tries to explain what happens under a competition framework at the initial step of the invasion, no more and no less; however, other interactions could be incorporated, such as cooperation (Krtolica and Campisi 2002; Xing et al. 2010; Wei et al. 2017; Ganesh et al. 2020), increasing the number of strategies in the ecosystem. This work sought to study particular biological phenomena integrating different approaches, more complex biophysical mechanisms could be incorporated to model habitat quality and spatial structure; for instance, it can be analysed how strategies coexist in stochastic environments or the invasion fitness of a new strategy

invading a dynamic environment (Ripa and Dieckmann 2013) or how pro-inflammatory environmental conditions could trigger a neoplasm's cascade since it similarities with ageing (Franceschi et al. 2006; Bonafè et al. 2012), there are many ways to add ecological complexity to one formulation, whereas this should be added only when it is necessary and first principles of the ecology and evolution of the system are clear. In this way, ecology can help to elucidate different population mechanisms underlying cancer manifesto, way beyond the metonymic sense.

Figures

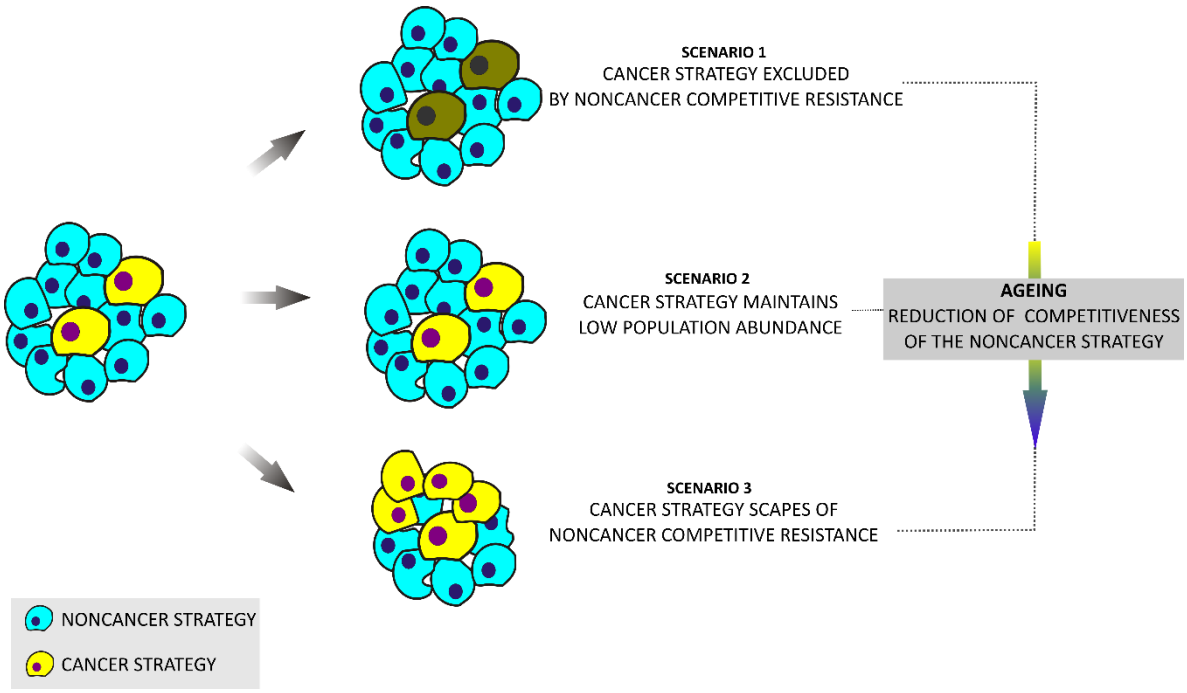

Figure II.1 Invasion of the cancer strategy driven by ageing, which implies a decrease in the competitiveness of the noncancer, resident, strategy. Hypothesised scenarios of local competitive interactions and population outputs for cancer and noncancer cellular strategies.

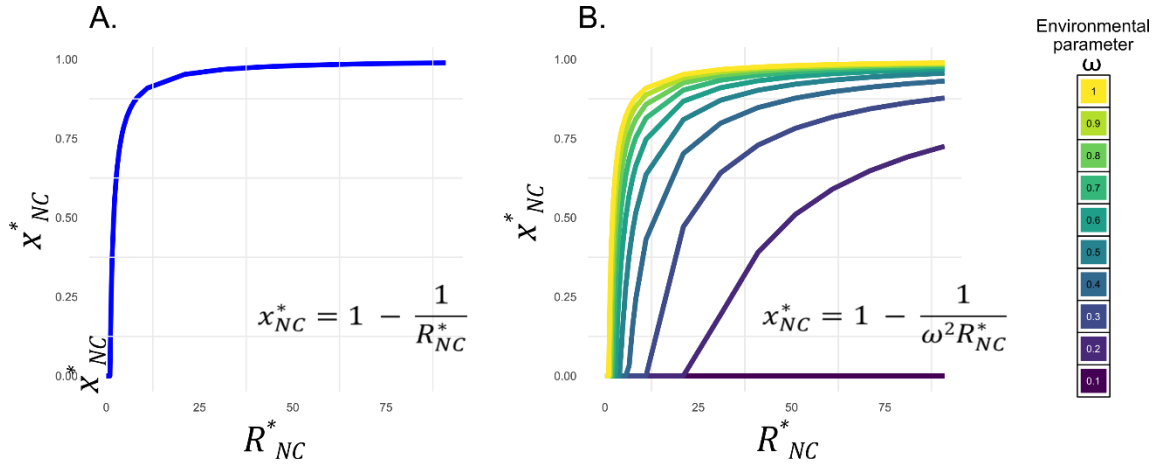

Figure II.2 Life-history traits and the environment shape the noncancer strategy's competitiveness and its population homeostatic steady state. (A) The canonical model where the environment does not influence proliferation and death; (B) environment-dependent proliferation model, where the proliferation increases with the environmental parameter; and (C) environment-dependent dynamic model where both proliferation and death depend on the environmental parameter. Note that $R^*_{NC} = \beta_{NC}/\delta_{NC}$.

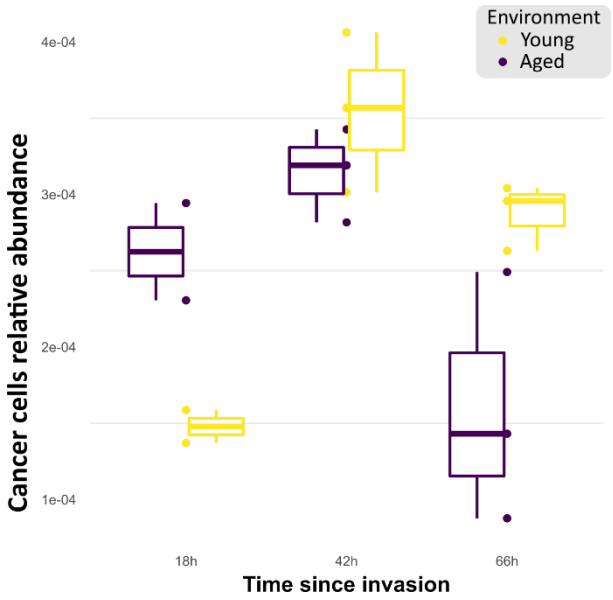

Figure II.3 Environmental impact on cancer cells invasion and proliferation in fabricated in vitro ecosystems. Each dot corresponds to one replicate where both cell lines were cultured, and the relative abundance was calculated as the density of each strategy over the total density of cells.

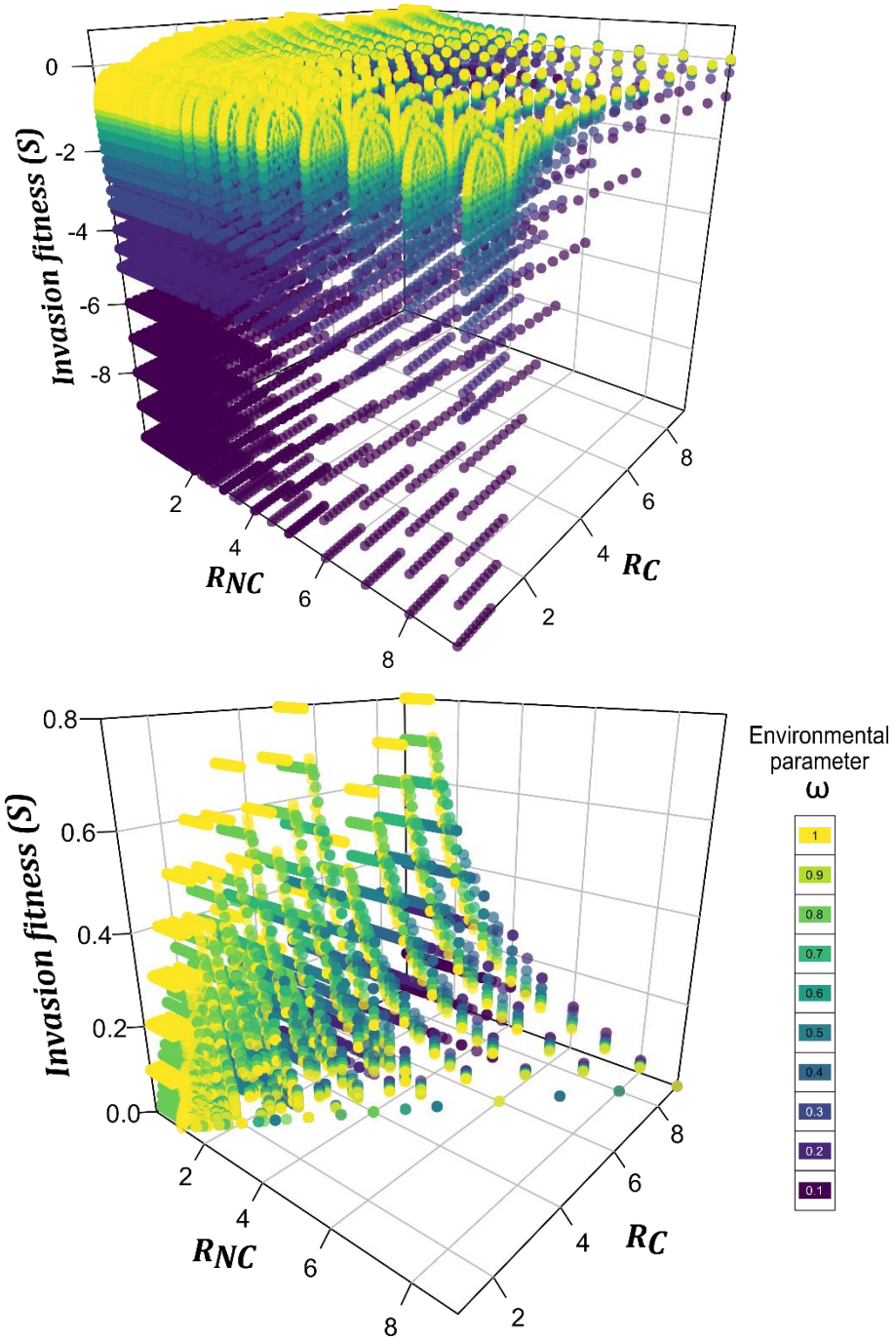

Figure II.4 Codetermination of the invasion fitness. The cancer life history traits (R_C^*) interact with he noncancer life history traits (R_C^*) and the environmental condition (ω) in shaping the initial success of the invasion. The bottom figure shows the condition under which the invasion fitness is positive ($S > 0$). The colour key applies for both figures.

Chapter III Spatially Fragmented Coexistence of Cancer and Immune Cells Predicts the Tumoural Response to Endocrine Therapy in Breast Cancer

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Abstract

How the spatial structure of the tumour ecosystem determines the response to therapy remains unclear. Here, we study the spatial ecology of breast tumours of 387 patients using samples before and after endocrine therapy (clinical trial POETIC). We draw on the geospatial information in pathological specimens, exploring the spatially fragmented coexistence between cancer and immune cells by using deep learning and community composition analysis. We found that patients with poor antiproliferative response exhibit a lower level of pre-treatment proliferation, spatial coexistence, and fragmentation of the interaction between cancer and immune cells, compared with patients with a good antiproliferative response. Community structure and fragmentation inferred from pre-treatment samples predict antiproliferative response stronger than existing metrics. Our findings provide new insights into how cancer evolves therapeutic resistance, which can help direct precision medicine and identify novel cancer vulnerabilities that will lead to the development of new treatments.

Space is a quintessential dimension to study patterns in heterogeneous systems of interacting adaptive components. To consider the role of space is essential to understand pattern formation, patterns of diversity and coexistence, and the overall functioning of ecosystems (Durrett and Levin 1994*a*, 1994*b*; Tilman and Kareiva 1997). A spatial approach has emerged during the last decade in cancer research (González-García et al. 2002; Yerushalmi et al. 2010; Nawaz et al. 2015; Yuan 2016; Corredor et al. 2019). This work has highlighted the importance of accounting for spatially-explicit interactions between different cellular lineages, and the need to consider how different cell strategies coexist in space to develop more informative and robust spatial biomarkers for linking the state and evolution of these particular ecosystems to a clinical outcome.

In histopathological samples, zones of higher concentration of cancer and immune cells, or hotspots, are associated with good disease-specific survival; improving understanding the short and long-term responses of tumours (Nawaz et al. 2015; Nawaz and Yuan 2016; Yuan 2016). However, cancer ecosystems do not always respond as expected; some may develop resistance to endocrine therapy. In oestrogen receptor-positive breast cancer (ER^{+}BC), some patients do not show a reduction in cancer cell proliferation after neoadjuvant treatment with peri-operative aromatase inhibitors (POAI), and even some of them exhibit recurrent malignant cell growth after surgical resection (Dowsett et al. 2007; Gao et al. 2014). Understanding the causes of this tumour resilience to treatment is, therefore, a clinical imperative.

The spatial distribution of entities generates a macroscopic pattern which may contribute to the understanding of the system's response to external changes (Levin 2005; Gao et al. 2016). A spatial ecological system evolves by changes in the diversity of its components (Ives and Carpenter 2007; May et al. 2008) (e.g., number of species and their abundances for ecological systems), their interactions, driven by the environment in which the system is

embedded (Gao et al. 2016), or by the spatial continuity of the system (Durrett and Levin 1994*a*; Bascompte and Sole 1996; Hanski and Ovaskainen 2000; Keymer et al. 2000*b*; Levin 2005). The continuity or fragmentation of population is vital for its persistence; it reflects the integration between life-history traits (survival, reproduction, and migration)(Keymer et al. 2000*b*; Ovaskainen and Hanski 2001) and local ecological interactions (e.g., competition)(Tilman et al. 1997). How this generalise to cancer, hence explaining tumoural persistence to antiproliferative therapy, remains unclear; presenting new challenges for an integrative understanding of tumours. Here we seek to apply the understanding of the spatial ecology to understand the success of cancer cells proliferating in the breast tissue and overcoming therapy, and associating how the spatial dimension through a measure of fragmented coexistence between proliferating cancer cells and immune cells can explain and predict the tumoural vulnerability to the percolation of therapy through the cancer ecosystem, therefore its effectiveness. To address this challenge we sum up the information revealed by gene expression profiling (Veer et al. 2002; Calabrò et al. 2009) and molecular markers of proliferation such as Ki67 expression (Urruticoechea et al. 2005; Yerushalmi et al. 2010; Penault-Llorca and Radosevic-Robin 2017; Gao et al. 2019) to test empirically how well spatial (e.g., hotspot and patch distribution and composition) and non-spatial (e.g., Ki67 expression) parameters measured in patients before therapy can help us to decipher and predict a potential response (Figure III.1). Consequently, we could hypothesise that local coexistence between cancer cells and tumour infiltrating lymphocytes (TILs) integrated with topological analysis of hotspots within ER^{+}BC ecosystems, can help to explain a tumour's treatment response.

Methods

Patients, samples and global scale ecology

From the POETIC trial, 387 postmenopausal women with ductal carcinoma in situ ER^+BC were randomly selected, corresponding to 774 formalin-fixed paraffin-embedded (FFPE) tissue samples. Two samples from each patient, one biopsy before treatment with endocrine therapy aromatase inhibitors (hereafter, pre-ET) and another sample after the two-weeks treatment (hereafter, post-ET). Each sample was immuno-stained for Ki67 before our handling and was digitalised at 20x. The expression of Ki67 changes throughout the different phases of the cell cycle being expressed in G1, S and G2, but not during the resting phase G0, making it a useful biomarker for cell proliferation (Yerushalmi et al. 2010). For cell detection and classification, we used a previously developed (Narayanan et al. 2018) supervised convolutional neural network (CNN) algorithm, which allows us to detect different cell classes according to nuclear morphology and response to immunostaining for Ki67 antigen. There are four possible cell classes identified by the CNN algorithm: $Ki67^+$ (proliferating cancer cells), $Ki67^-$ (non-proliferating cancer cells), tumour infiltrating lymphocytes (TILs) and stromal cells (non-immune cells). This approach enables the quantification of the number of cells corresponding to each class divided by the tissue area obtaining global densities of each cell class for each sample (whole-slide image -WSI- densities) and Ki67 expression (number of $Ki67^+$ cancer cells over the sum of $Ki67^+$, $Ki67^-$, immune cells and stromal cells).

Local-scale ecology: hotspots, network, and patches

After cell detection, Getis-Ord hotspot analysis was carried out between $Ki67^+$/TILs, allowing us to focus the next steps of this work on biologically-relevant zones of higher occurrence cancer cells and lymphocytes within these ecosystems (Nawaz et al. 2015). This

analysis allowed us to calculate cell densities that are part of hotspots for each sample (HS Ki67$^+$, and HS ITLs, where HS stands for hotspot). For the next step, we distinguished two scales of spatial ecological complexity based on the spatial resolution: global (WSI) and local (HS) scales. At the global scale, for each patient, we obtained pre-ET and post-ET cells class densities and Ki67 expression. The local scale refers to the spatial co-existence of Ki67$^+$/ITLs within hotspots. At this scale, we built the community network of Ki67$^+$ and ITLs. Each network reconstruction was made following a Delaunay triangulation method using as input the spatial coordinates of each cell in each sample and obtaining as output a list of distances between a pair of neighbour cells. Each network was analysed as a symmetric-weighted matrix. In this approach, each cell is a node of the network *P*, and the weight of the link between two cells is inverse to the distance separating a pair of cells normalised by the maximum distance between two nodes observed in each sample *P*. In this way, we calculated a weighted link between two cells $\{a,b\}$ as

$$w(a,b)_P = 1 - \frac{dist(a,b)_P}{max\,(dist_P)} \qquad \text{Eq. III.1}$$

For each network, we computed sub-group detection within them (henceforth 'patches', spatially discrete associations of cells) using the Girvan-Newman algorithm for large networks based on the nodes' betweenness (Newman and Girvan 2004) implemented by the R-package igraph. This analysis adds the quantification of patch-level coexistence between Ki67$^+$ cells with ITLs and the level of fragmentation of the community before endocrine therapy. Given that the algorithm might consider isolated cells as patches, for the computation of the coexistence index we only considered patches with more than one cell, reducing the sample size to 336 patients. We consider the local coexistence with immune cells because their action is the main control on tumour development, and also previously it has been shown to have prognostic value (Nawaz et al. 2015). For each sample *P*, we calculated a pre-treatment coexistence index which corresponds to a Pearson's correlation

coefficient between patch-level abundances of $Ki67^+$ cancer cells and lymphocytes. The coexistence index takes values between -1 and 1, where values close to -1 indicate that $Ki67^+$ cells and ITLs do not coexist within patches, i.e., when in one patch the density of cancer cells is high the density of ITLs is low, and vice-versa. While, coexistence values close to 1 indicate when within-patches cancer cell's density is high, ITLs is high too.

A priori we categorised patients according to their difference in Ki67 expression after antiproliferative treatment in two groups: good antiproliferative response (GAR, with $\Delta Ki67<0$ and poor antiproliferative response (PAR, with $\Delta Ki67>0$). Since Ki67 expression is used as a metric for response in ER^+BC and considering the propagule pressure hypothesis; we started testing the explanatory power of the pre-ET Ki67 expression on the post-ET Ki67 expression for each patient category. We built linear models of the form *post-ET Ki67 ~ pre-ET Ki67*AP category,* and in the same way for the tumour response to therapy (measured as the change in Ki67 expression), we built linear models of the form $\Delta Ki67$ *~ pre-ET Ki67*AP category*; these models allow us to test the interaction between pre-ET Ki67 expression and the antiproliferative category of patients as explanatory variables of the response variables. The R^2 value was taken as a measure of the explanatory power of the predictor variable (pre-ET Ki67 expression) and a statistical evaluation of the estimated slope (null hypothesis slope = 0). Furthermore, we studied the relationship between the coexistence index, the pre-ET WSI $Ki67^+$ expression, and the degree of fragmentation (number of patches). Using a t-test, we compared these variables between the antiproliferative response categories (GAR vs PAR), here we only consider patients a statistically significant value of the coexistence index (152 patients).

Prediction of the antiproliferative response

With the subset of 152 patients with a statistically significant coexistence index, we built three binomial logistic regressions (link function logit) where the response variable is the

antiproliferative response (i.e., GAR or PAR) and the independent variables were: model 1: pre-ET Ki67 expression; model 2: pre-ET coexistence index and Ki67 expression; and model 3: pre-ET number of clusters and Ki67 expression. Models 2 and 3 include the interaction term. Each resulting model was used to predict the antiproliferative response of another subset of 184 patients that do not have a statistically significant coexistence index. For this analysis, we considered a good antiproliferative response as the positive value; therefore, the sensitivity of the prediction refers to correct detection of GAR patients after endocrine therapy while the specificity refers to the correct prediction of PAR patients. We considered a probability threshold of 0.5 on the probability result for each binomial logistic regression to assign a predicted GAR category.

For all the statistical analyses, we used a type I error rate of 0.05; after testing homogeneity of variances, we used t-test or Wald test if it corresponds when comparing two samples. Spearman correlation was used in the analyses including the number of patches as a predictor. All analyses were performed in R, except the convolutional neural network for tissue segmentation and cell classification and the Getis-Ord hotspot analyses done in Python.

Results

After two weeks of antiproliferative treatment with aromatase inhibitors, 304 (78.6%) patients showed a good antiproliferative response (GAR) while 83 (21.4%) showed a poor antiproliferative response (PAR), considering the expression of $Ki67^{+}$ as a surrogate of cancer proliferation. According to our results, the pre-treatment Ki67 expression, $Ki67^{+}$/ITLs' within-patch spatial coexistence, and the degree of spatial fragmentation of the community quantitively improve the understanding and prediction of the pathological response measured by the change in Ki67 expression (a surrogate of cancer cell proliferation) after antiproliferative therapy.

Positive population feedback in cancer ecosystems

Our results indicate a positive statistical dependence of the post-therapy Ki67 expression on the pre-treatment Ki67 expression (*Figure III.2*A), giving us some insights into how these variables are connected. That positive population feedback in the cancer population may apply. The effect of pre-ET Ki67 expression explaining post-ET Ki67 expression varies between antiproliferative response categories (table III.S1; *pre-ET Ki67*AP category*: $F_{[1,383]=}267.92$, p-value=4.93×10^{-46}). Overall, pre-ET Ki67 expression explained 82.04% of the variance in post-ET Ki67 expression in the case of the PAR patients (estimated slope=1.99, p-value=2.58×10^{-32}; R^2=0.82). For GAR patients, the pre-ET Ki67 expression shows a lower, but high and significant explanatory power of post-ET cancer cell proliferation (estimated slope=0.36, p-value=2.05×10^{-74}; R^2=0.67). The pre-ET Ki67 expression also emerged as a good predictor of the change in cancer cell proliferation (*ΔKi67*) after two weeks of treatment either for GAR and PAR patients, explaining the 86.6% and 53.03%, respectively, of their change (Figure III.2B); but equally in terms of its effect between antiproliferative categories (table III.S2; pre-ET Ki67*AP category: $F_{[1,383]=}1.34$, p-value=0.25). In the case of PAR patients, we find support for propagule pressure to explain the success in terms of cancer cell's invasion since a higher pre-ET $Ki67^+$ cancer cell density relative to the other cell classes was positively associated with the antiproliferative response.

Fragmentation and coexistence in the tumour ecosystem

The pre-ET $Ki67^+$ expression allows us to estimate the magnitude of response, but we cannot a priori, identify patients that could not show the expected clinical result. To test how spatial patterns might predict response to therapy in addition to existing metrics such as Ki67 expression, we quantified how local-scale patterns of fragmentation and coexistence in the hotspot landscape might influence the sign of the patient's response to therapy in addition to

traditional metrics such as the Ki67 expression. The detection of spatially-structured subcommunities within each sample allowed to obtain a measure of the community fragmentation as the number of patches detected (Figure III.3A); which is negatively associated with the antiproliferative response (Spearman's correlation ρ=-0.49, p-value=$2.15x10^{-10}$), it means that higher fragmentation of the community, higher is the decrease in Ki67 expression, hence, better response to antiproliferative therapy (Figure III.3B). In fact, after the fragmentation corresponding to five patches, the confidence interval (CI) of the mean antiproliferative does not include 0 ($CI_5^{\Delta Ki67}$= 0.001--0.003; $CI_6^{\Delta Ki67}$=-0.002−-0.007), suggesting that there could be a critical fragmentation size between PAR and GAR patients. Also, the degree of patchiness is positively associated with the coexistence between immune and cancer cells within hotspots (Figure III.3C; Spearman's correlation ρ=0.77, p-value=$3.41x10^{-31}$), implying an interaction between ecological interaction and spatial patterns of community fragmentation. We detected a significantly lower value of pre-ET Ki67 expression (Welch t-test $t_{[69.98]}$=-6.86, p-value=$2.26x10^{-09}$), pre-ET within-patch coexistence (t-test $t_{[150]}$=-2.93, p-value=0.004), and pre-ET fragmentation was found in PAR in comparison to GAR patients (t-test $t_{[150]}$=-3.3, p-value=0.001) within the patients' subset who had a statistically significant coexistence index (p-value < 0.05, 152 out of 336 patients)(Figure III.4).

Predicting the antiproliferative response based on coexistence in fragmented landscapes

Finally, the binomial logistic regressions built to predict the likelihood of patient response to antiproliferative therapy of 184 patients show that adding the pre-treatment ecological metrics of coexistence and fragmentation to the pre-ET Ki67 expression, independently, increases the specificity, hence the power, of the models (i.e., the detection of PAR patients), without showing a critical reduction in the sensitivity (detection of GAR patients), hence in the increase of false negatives (type II error) of the predictions (Figure III.5). We show that

the model that only considers pre-ET Ki67$^+$ expression is very general, predicting the 100% of GAR patients and only classifying the 2% of PAR patients correctly (Figure III.5A). The consideration of the interaction between Ki67$^+$ expression and coexistence can predict the response of 99% of GAR patients and increasing to 11% the correct prediction of PAR patients (Figure III.5B). Hence, the consideration of spatial coexistence between ITLs and Ki67$^+$ in spatially structured communities increases 5.5 times the detection of patients while keeping the prediction of GAR patients high (99%). Moreover, the model that considers the interaction between Ki67$^+$ expression and the pre-ET patchiness (Figure III.5C) maintains high sensitivity (0.94) and low rate of type II error equals to 0.06 (1-sensitivity = 1-0.94), predicting the 96% of GAR patients correctly and increasing the detection of PAR patients to 40%, i.e., an increase of 20 times the detection of patients showing a poor antiproliferative response compared to the model that only considers the pre-ET Ki67 expression (Figure III.5A).

Discussion

Emergent properties in a complex adaptive system (e.g., an ecosystem) are the result of local non-linear interactions between its agents (e.g., species) (Gell-Mann 1994; Levin 2005; May et al. 2008). The main system evolves by changes in the diversity of its components (Ives and Carpenter 2007; May et al. 2008) (e.g., number of species and their abundances for ecological systems) or their interactions, driven by the environment in which the system is embedded (Gao et al. 2016). Cancer, as a complex adaptive system (Schwab and Pienta 1996) reflects these properties wherein its composition and complexity of interactions, determine its evolutionary trajectory and response to external perturbations, such as therapy.

Treatment resistance is the major clinical challenge in the treatment of cancers. As we show here, with an integrated approach, we are closer to predict the antiproliferative response to

therapy in ER^+ breast tumours. Based on the current evidence, Ki67 expression quantifying cancer cell proliferation has been strongly suggested to be included in the standard pathological assessment of early breast cancers (Urruticoechea et al. 2005; Dowsett et al. 2007; Yerushalmi et al. 2010; Penault-Llorca and Radosevic-Robin 2017; Gao et al. 2019). Moreover, the proliferative status measured with the Ki67 expression is strongly associated with proliferative cancer cells' density after therapy (Figure III.2), where the effect of POAI, which has been previously documented (Pohl et al. 2003; Urruticoechea et al. 2005; Dowsett et al. 2007; Gao et al. 2014; Penault-Llorca and Radosevic-Robin 2017), is shown on the different responses of patients. In this work, we contribute evidence to support the need of including Ki67 immunostaining within the protocol from breast cancer; that would allow a quantification of the proliferative status of the tumour and also the ER^+BC tumour´s community structure, abundance and distribution of cells.

Nevertheless, just a visual inspection is enough to state that pre-treatment Ki67 expression alone cannot predict the sign of change, i.e., whether a patient will have a decrease or increase in proliferating cancer cells after treatment (FiguresFigure III.2, Figure III.5C). The second challenge was to estimate whether a patient will show a reduction in cancer cell proliferation in response to therapy, based only on pre-treatment tumour metrics obtained from the heterogeneous landscapes of cells in 2D images. In our cohort, we show that ecological spatial patterns generalise to cancer ecosystem in terms of the viability of population and resistance to perturbations shaped by fragmentation and coexistence patterns. The observed positive feedback in the population of cancer cells under a critical value of fragmentation (Allee 1931; Liebhold and Bascompte 2003) separating PAR and GAR patients show the vulnerability of the cancer ecosystem to the percolation of the control performed by the antiproliferative therapy and immune cells (Mocellin et al. 2001; Mao et al. 2016; Burugu et al. 2017). Considering that these are primary tumours, the most

straightforward explanation for the observed non-uniform –patchy– patterns and the implication it has to cancer population fitness emerges from the interaction between the two life-history traits: local dispersal ability and proliferation rate (Merlo et al. 2006). The dispersal ability of individuals within any population, from cells to human societies, is a crucial trait for exploration and colonisation of the environment. The limitation to local dispersal modulated by the physical properties of the extracellular matrix properties, i.e. niche construction is driven by cancer cells' metabolism (Carmona-Fontaine et al. 2013) and/or interactions with other cell types such as immune cells (Bellone and Calcinotto 2013; Nawaz et al. 2015; Yuan 2016) may underlie the observed clumped pattern, the fitness of cancer cells, the positive population feedback, and the critical fragmented coexistence separating the good and poor responses to therapy.

Our findings come to contribute to the local coexistence patterns between lymphocytes and cancer cells as has been discussed for breast cancer (Manjili et al. 2012; Nawaz et al. 2015; Savas et al. 2016; Burugu et al. 2017), summing evidence to that Ki67 expression, local coexistence, and the degree of fragmentation matter shaping tumour's response (magnitude and direction) (Pohl et al. 2003; Penault-Llorca and Radosevic-Robin 2017), emerging from the local spatial interaction between individual cells. Despite the effect, positive or negative, that ITLs may exert on cancer cells (Mao et al. 2016), we have found that PAR patients are not evenly distributed in the coexistence nor patchiness axis (they fall towards the lower values of coexistence and patchiness), meaning that cancer cells reach higher fitness as they spatially coexist less with lymphocytes within tumours, opening the question about the effect of ITLs on local migration/proliferation patterns of ER^{+}BC cells, and if ITLs can promote local dispersal or inhibit local proliferation of cancer cells (Man et al. 2013). Notwithstanding, here, the coexistence in strongly associated with fragmentation, linking a

potential control driven by immune cells contributing to the fragmentation of the community, hence to the spatial diffusion of antiproliferative therapy.

The development of predictive oncology is gaining evidence from the new approaches (Brindle 2008; Yuan 2016). We show that through an integrated approach considering traditional and novel spatial metrics is possible to predict patients' response antiproliferative therapy. We claim that through the consideration of tumours as complex adaptive systems, the field can gain predictive power to anticipate emergent tumour ecosystem responses to therapy where the local interactions between the components of the ecosystem shape its responsiveness (May 2001; Thébault and Fontaine 2010; Gao et al. 2016), which is imperative in the case of the patient's prognosis.

In conclusion, despite each patient being a particular ecosystem responding in a different way to treatment, we think that there are general patterns across patients that can be discovered and interpreted using the knowledge of different fields, in our case from ecology and neural network analyses. We suggest that these results need to be taken carefully because they are based on single tumour sections before, and after therapy. The POETIC clinical trial database does not have multi-region sampling, making it impossible for us to test if the patterns found here are consistent across space and time within a 3D landscape such as a tumour, or how sampling variability might affect our results. In our analyses, such sampling variability is integrated into the error variance through the study of different patients. Nevertheless, there are other studies which may help to expand the results presented here; for instance, an analysis carried out from 245 ER-BC patients from the METABRIC consortium (Nawaz et al. 2015) averaging three sections within a tumour showed that the amount of co-localised cancer and immune hotspots correlates with a better prognosis. There are limitations associated with our analyses since we reduced the complexity of a 3D tumour to single region 2D landscapes, and we have a limited resolution of lymphocyte diversity.

These limitations certainly can not be ignored; nevertheless, we expect our findings to ignite further research into the spatial ecology within tumours and the tools that the interaction between clinical, ecological and machine views can serve to disentangle the complexities behind tumours ecosystems.

Figures

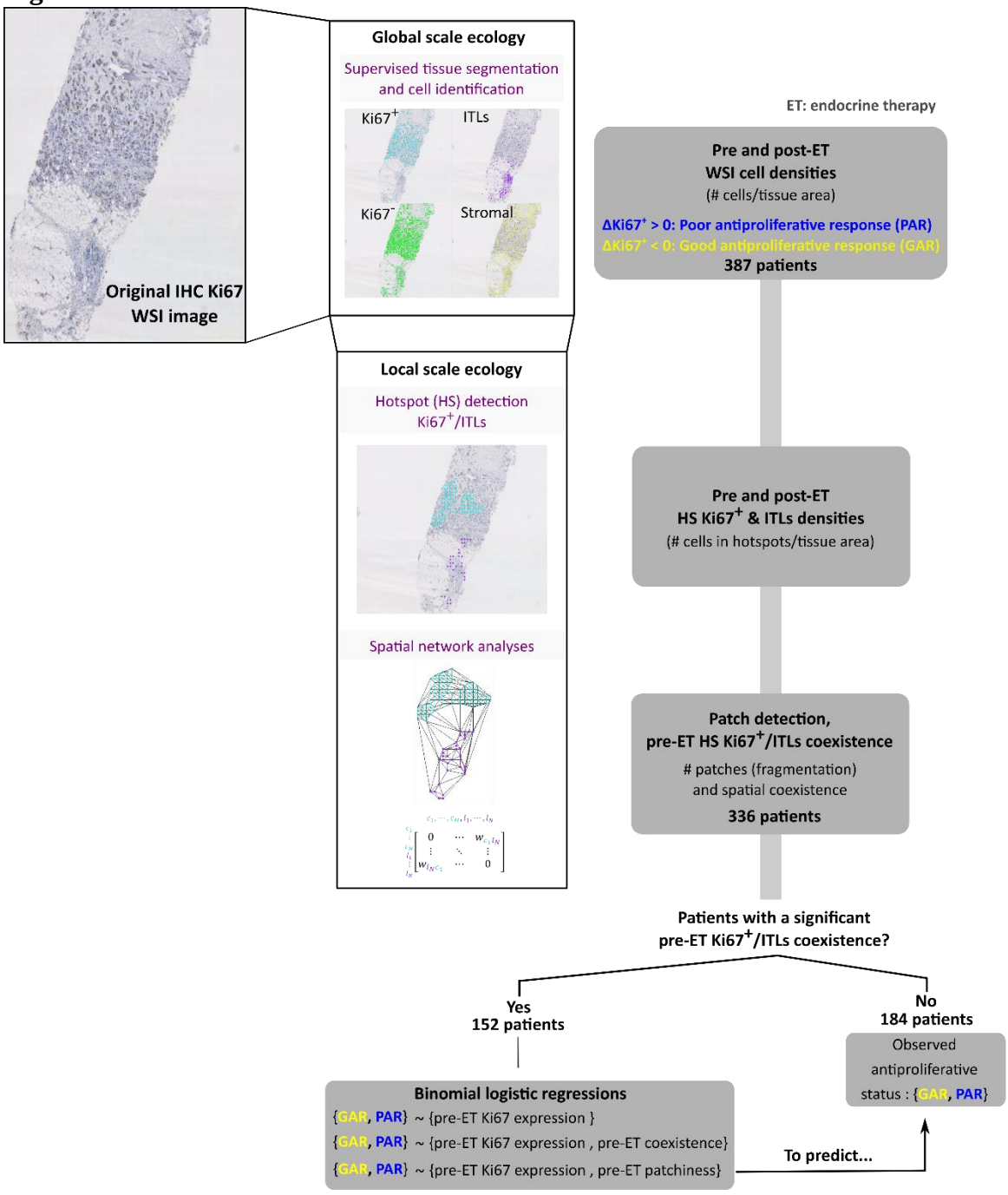

Figure III.1 Dissecting ER+ breast tumour's ecology under endocrine therapy. The pipeline followed seeking to extract global-scale metrics acquired from WSI after cell detection and local-scale metrics from hotspot detection and spatial network analyses (based on matrices analyses). Finally, a set of binomial logistic regressions allows predicting patients' response to therapy.

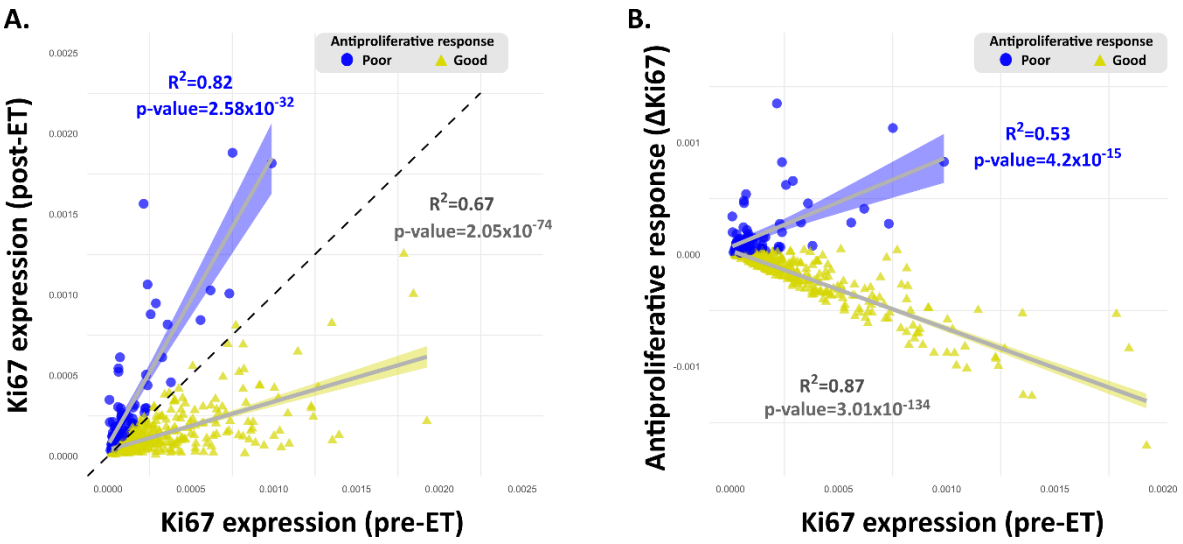

Figure III.2 Propagule pressure and the explanatory power of the pre-treatment Ki67 expression. (A) post-treatment Ki67 expression and (B) the change in the Ki67 expression (post-ET – pre-ET). Each dot corresponds to one patient in the categories of good antiproliferative response (yellow triangles) and poor antiproliferative response (blue circles). For each category, a linear model is shown (confidence interval 95%) with the corresponding statistics. For (A) and (B) we tested the interaction between pre-ET Ki67 expression and the antiproliferative response.

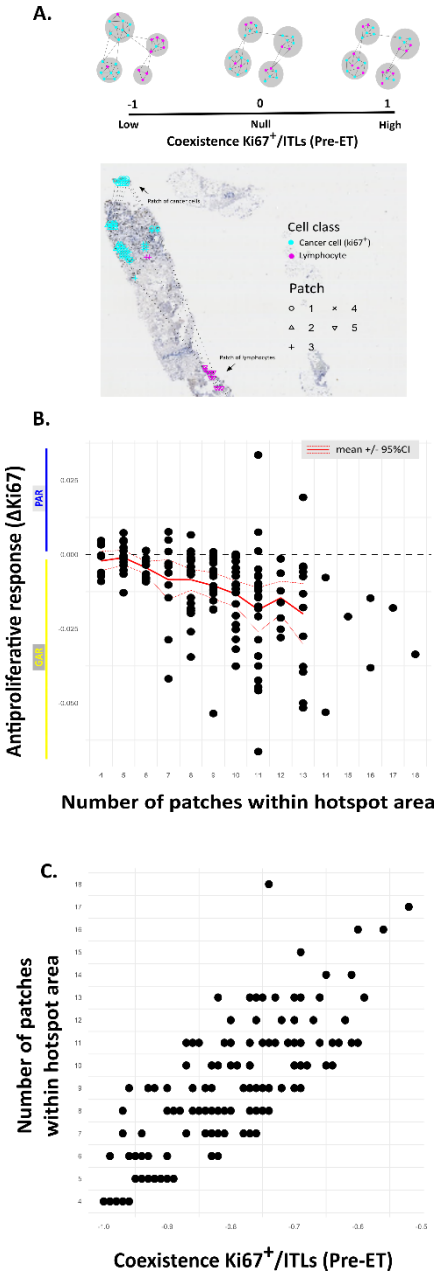

Figure III.3 Fragmented coexistence between cancer and immune cells. (A) Schematic of the possible values of coexistence between Ki67$^+$ (cyan) and ITLs (purple) across patches (grey); and an example image of low coexistence where cell classes do not coexist in the same patch forming mono-class patches (some shown by arrows). (B) PAR and GAR patients' antiproliferative response decreases with the degree of patchiness of communities. From a patchiness value equals to six, average ΔKi67 (solid red line) becomes different from 0. PAR: poor antiproliferative response and GAR: good antiproliferative response. (C) Association between the pre-treatment patchiness and coexistence.

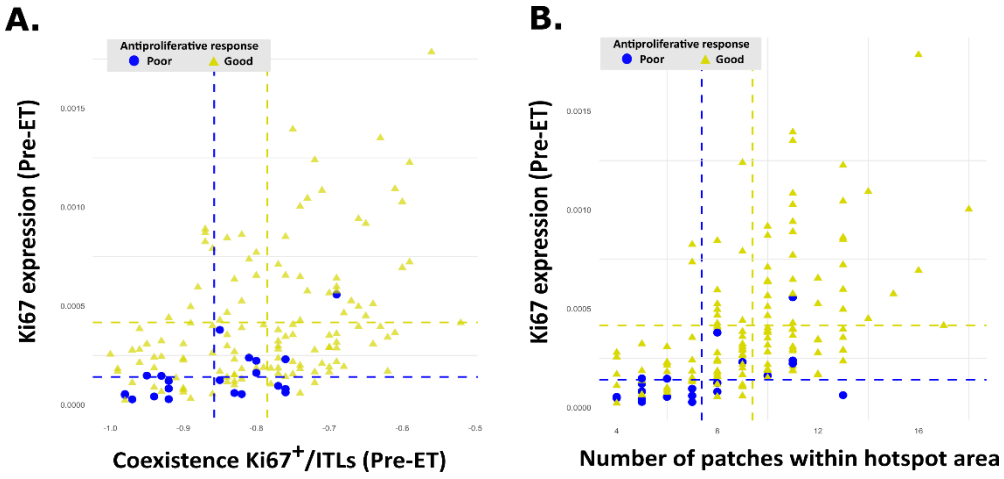

Figure III.4 Ecological differences in antiproliferative response categories. GAR (yellow triangles) and PAR (blue circles) patients differ in their pre-treatment metrics of(A) Ki67 expression, coexistence between immune and cancer cells, and (B) the patchiness of the community.

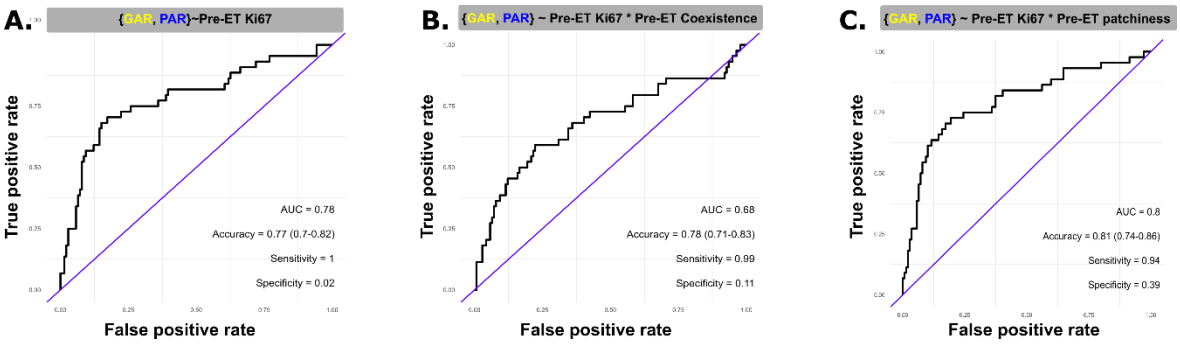

Figure III.5 Prediction of antiproliferative response to POAI in ER^+ breast cancer patients based on pre-treatment ecology. The logistic binomial regression model fitted with 152 patients was used to predict the antiproliferative response of other 184 patients based on (A) pre-ET Ki67 expression, (B) pre-ET ki67 expression and pre-ET spatial coexistence between ki67+ cancer cells and ITLs, and (C) pre-ET ki67 expression and pre-ET patchiness (AUC: area under the curve; Sensitivity: positive predictive value; Specificity: negative predictive value). The positive response for prediction represents a good antiproliferative response and the threshold for prediction after the logistic binomial regression was 0.5.

Supplementary materials

Table III.S1 ANOVA table for the analysis of post-ET Ki67 expression as a function of the pre-ET Ki67 expression and the antiproliferative response group (GAR or PAR).

Term	D.F.	S.S.	M.S.	F	p-value
Ki67 expression	1	$1.48x10^{-05}$	$1.48x10^{-05}$	750.65	$2.81x10^{-92}$
AP response group	2	$5.36x10^{-06}$	$2.68x10^{-06}$	135.68	$2.84x10^{-45}$
Interaction	1	$5.29x10^{-06}$	$5.29x10^{-06}$	267.92	$4.93x10^{-46}$
Residual	383	$7.57x10^{-06}$	$1.98x10^{-08}$		

Table III.S2 ANOVA table for the analysis of |ΔKi67| expression as a function of the pre-ET Ki67 expression and the antiproliferative response group (GAR or PAR).

Term	**Df**	**S.S.**	**M.S.**	**F**	**p-value**
Pre-ET Ki67	1	$3.27x10^{-05}$	$3.27x10^{-05}$	1713.84	$1.81x10^{-143}$
AP response group	2	$8.25x10^{-07}$	$4.13x10^{-07}$	21.65	$1.23x10^{-09}$
Interaction	1	$2.5x10^{-08}$	$2.5x10^{-08}$	1.34	0.25
Residual	383	$7.3x10^{-06}$	$1.9x10^{-08}$		

Chapter IV Emerging Patterns from Organs' Diversity in the Network of Metastatic Cancer Ecosystems

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Abstract

Cancer cells spread between organs in the context of the within-organism ecosystem still a puzzle, where Paget's seed-and-soil hypothesis raised the role of the organ's conditions to explain it. In this contribution, we seek to tackle the complexity of the metastatic process at a coarse-grained scale by defining and quantifying metastasis as a bipartite network between source and acceptor organs of cancer cells. With 8,642 medical records of human metastases from the literature, we quantify the diversity of metastatic incidence among organs, suggesting that local processes may occur at the organ level contributing to the diversity observed in the metastatic spread. This continuum is related to physiological variables, and from it emerges a universal topology, with a nested and modular structure and truncated power-law degree distribution. These results contribute to the development of theory, which summed to the development of evidence support the explanation of local ecology underlying metastatic diversity.

Despite the plethora of lifeforms that invasive species have, there are inherent characteristics to the processes of invasion and colonization, which make the invasion process a global phenomenon. A particular hallmark of invaders is that some species are more invasive than others being the world's worst invasive species (Luque et al. 2013), but also, some habitats harbor more invasive species than others. This variety in the invasion patterns emerges from the interaction between invader's life-history traits, the process of transportation and introduction of individuals (propagule pressure), and the characteristics of the recipient habitat (Turbelin et al. 2017). Cancer ecosystems might not be so different from biological invasions (Chen and Pienta 2011*a*); in particular, when cancer cells migrate from the primary tumor arriving at an acceptor organ where it can succeed in the invasion or perish under local processes that do not allow the invasion.

Notwithstanding, despite many intervening decades of observation and accumulation of evidence, our understanding of metastasis is mostly centered on a small number of organs and tissues (Langley and Fidler 2011; Peinado et al. 2017). These studies indicate that a series of complex processes occur in metastasis (Lambert et al. 2017), beginning with the cell's epithelial-mesenchymal transition (Ganesh et al. 2020), allowing the migration of cancer cells from a source tumor through lymphatic or blood vessels (Pereira et al. 2018). Then, going through numerous intermediate states, habitats, and micro-environmental conditions, and culminating either in spatially distinct tumors within the source organ or in distant organs (Nguyen et al. 2009). Although this sketched sequence is very similar among those cancers that have been studied in detail, it still lacking a broader view, which allows us to identify and quantify why specific organs and tissues (hereafter 'organs') are more commonly the sites of metastasis or with higher invasibility. Also is unclear why some specific primary

tumor sites tend to be associated with one or a few specific metastatic organ sites (Scott et al. 2012); in contrast, others are more generalists having successful invasions in many different organs (Disibio and French 2008), i.e., the continuum of invasiveness and invasibility in metastatic cancer.

After Joseph Récamier coined the term metastasis (1829), two main hypotheses have been treated in the literature to explain why some organs are the target for circulating tumor cells. Under the 'seed-and-soil' hypothesis proposed by Stephen Paget (Paget 1889), cancer cells migrate from established tumors and only create self-sustaining metastatic growth in distant organs if the latter's micro-environmental conditions, defined by local processes, are adequate (Groot et al. 2017); analogous to the Grinnellian niche in ecology (Grinnell 1917). The second hypothesis is associated with James Ewing (Ewing 1924), who suggests that metastatic spread occurs by purely structural factors related to the anatomical connectivity of the vascular system and closeness between source and acceptor organs. Hence, the probability of an organ harboring a metastasis depends in part on the number of cancer cells delivered to it (propagule pressure), which in turn is a function of blood flow and distance to the source organ (Fidler 2002). Whereas there is little support for the single action of the Ewing's hypothesis in explaining observed patterns in metastasis, it is unclear to what extent Paget's and Ewing's hypotheses integrate and contribute to explaining observed patterns of migration (Azevedo et al. 2015).

Nevertheless, how findings related to metastatic incidence generalize across metastatic strategies of both primary and metastatic tumor sites and what macroscopic patterns emerge from the study of the continuum of cancer strategies, if any, remains unknown. However, its unwinding would mean a crucial contribution guiding the research in

metastatic cancers and their underlying causes. In this challenging realm, the application of network science has arisen as an opportunity in medicine (Barabási et al. 2011; Greene et al. 2015). The study of phenomena as networks have to lead to explaining emerging patterns through several mechanisms linked to temporal dynamics and diversity of strategies present in the system (Patterson 1990; Barabási and Bonabeau 2003; Bascompte et al. 2003; Suweis et al. 2013). Here, we exploit a network view on metastasis, carrying out a large-scale statistical analysis by analyzing the association between an organ with a primary tumor and its metastatic sites (Figure IV.1). Inspired by metapopulation theory, we represent organs as habitat patches, potentially harboring tumor cell populations (Keymer and Marquet 2014). In our scheme, organs can be 'source' or 'acceptor' patches. Source patches are those organs where the primary metastatic tumor emerges and from where cancer propagules migrate, and the acceptor patches correspond to those organs that receive these propagules and become colonized. We hypothesize that the idiosyncratic diversity characterizing metastatic cancers is reflected in a quantitative pattern of invasiveness depicted from the connectivity of organs in the network; and that such diversity shapes the metastatic incidence, in source and acceptor organs, with emerging macroscopic patterns related to the metastatic association. With this approach, we aspire to contribute to an ecological theory of cancer, the quantification of invasiveness and invasibility in organs with the resulting macroscopic pattern and the discussion about the role of niche and spatial variables influencing metastasis.

Methods

We studied the metastatic network between organs by constructing a bipartite network based on a matrix representing the number of occurrences that a primary neoplasm in

a source organ generated a metastasis in an acceptor organ. The data were obtained from the literature (Abrams et al. 1950; Patel et al. 1978; Saitoh et al. 1984; Lamovec and Bracko 1991; Bubendorf et al. 2000; Disibio and French 2008; Shinagare et al. 2011). Based on diSibio and French (Disibio and French 2008), 33 anatomical zones (referred here as 'organs') and 21,421 occurrences were initially recorded, maintaining most of the organs present in the original work (Disibio and French 2008). These medical records are from the USA, Switzerland, Germany, and Slovenia, taken between 1885-2009 (view references for particular years and occurrences). Given the nature of the data, we were unable to classify it by gender or other demographic categories, but we decided to exclude organs commonly associated to biological sex (breast, prostate, penis, testicles, uterus, vulva, vagina, and ovaries), and grouping organs as follows to reconcile contrasting terminology between studies: colon/rectum (colon, rectum, anus), intestine (appendix, duodenum, large and small intestines), and neck (branchial cyst, larynx, lip, pharynx, salivary glands, tongue, and tonsil). After this data filtering, a total of 25 organs and 8,642 occurrences were included in the analysis, based on autopsies and tomographies in the case of muscular cancers. In analytical terms, we define the metastatic process as a graph $G = (S, A, E)$ where S and A denote the set of source and acceptor organs, respectively, and E identifies the links or edges connecting them which in this case represents occurrences. An occurrence is tallied if there is at least one record of metastasis from a primary metastatic tumor to an acceptor organ. We studied the weighted network $W = G = (S, A, E)$ of source-acceptor organ interactions $S \times A$ with $S = A = (s_i/a_i, \ldots, N)$, with $i,j: [1,2,\ldots,N]$ and N corresponds to 25 anatomical sites. The network is studied as a matrix where each entry of this matrix represents the metastatic process quantified by $n_{i,j}$ corresponding to the number of occurrences of the metastatic pair source-acceptor, i.e., the number

of reported secondary growth metastases in an acceptor organ *i* that are originated from a primary tumour in a source organ *j*. For network calculations, each cell value $n_{i,j}$ was transformed to a broad sense metastatic incidence (BSI) value calculated as $w_{i,j}=n_{i,j}/T$, with T being the total number of cases (8,642 cases).

Macroscopic network properties

To assess emergent topological properties in the metastatic network as a result of the interaction between the mesoscopic attributes were contrasted observed measures of nestedness (NODF2 and BINMAT) against a set of random null networks (N= 1000) built under a non-sequential algorithm which only preserves the original number of occurrences within the matrix. We evaluate statistical significance by calculating the confidence interval (95% CI) of the mean nestedness of the null models and contrasting that with the observed value of the occurrence matrix (Figure IV.*2*). The degree of each organ allowed us to evaluate the degree distribution for source and acceptor organs, hence if it corresponds to a scale-free network or it does not. Three fits were adjusted to the degree distribution: exponential, power-law, and truncated power-law (R-package bipartite); we choose the best fit according to the Akaike's Information Criterion (AIC). Modularity was estimated with the two algorithms that aim to maximize modularity: QuanBiMo (Dormann and Strauss 2014) and DIRTLPAwb+ (Beckett 2016); both available in the R-package bipartite. These algorithms allow the detection of modules in our bipartite network based on the metastatic incidence patterns. To obtain the observed modularity, we ran 500 iterations following the 'Dormann-Strauss' or the 'Beckett' method, with 100000 steps and tolerance of the difference between Monte Carlo-Markov Chains swaps of 1e-10 in the case of Dormann-Strauss. Both algorithms allow obtaining a final estimation, which

maximizes the likelihood of the modularity; we choose the one with the highest value of likelihood.

Organ level properties and physiological data

Organ level properties correspond to those organ-level metrics resulting from our analyses, including metastatic occurrences and incidence, organ degree (observed number of links), organ's generalism, and physiological metrics obtained from literature associated with metastatic incidence. Firstly, to test the relationship between spatial closeness and source-acceptor seeding, we contrasted metastatic incidence with the spatial closeness between source-acceptor organs based on vascular matrices. Secondly, to evaluate if the likelihood of having primary metastatic tumor is positively associated with the likelihood of secondary growth in acceptor organs, we correlate source-acceptor metrics either for the degree and the number of occurrences. Thirdly, a more in-depth analysis of each organ's role in the metastatic network allows testing if there exists a relationship between occurrences and the level of generalism of connectivity in the network. Finally, aiming to approach to the study of how physiological parameters might account for observed network metrics at organ level, we tested linear correlations (null hypothesis: $\rho=0$) of our metrics against organ-level physiological estimates extracted from the literature: From (Weiss et al. 1980): organ weight, blood volume (ml), blood flow (ml/min), mass-specific blood flow (ml/(min*gr)); From (Sidhu et al. 2011): cardiac output (%); From (Tomasetti and Vogelstein 2015): noncancer cell population number, number of stem cells, number of division of each stem cell per year, number of divisions of each stem cell per lifetime, cumulative divisions of each stem cell per human lifetime; From Richardson, Allan and Le (Richardson et al. 2014): organ turnover (days).

To assess the hypothesis that relates metastatic incidence and spatial distribution of organs, we built vascular incidence matrices based on the main artery or vein conducting blood flow between organs. We identified the main arteries associated with an organ's blood supply: thoracic aorta, abdominal aorta, left gastric coeliac trunk, splenic artery, common hepatic artery, superior mesenteric artery, and internal iliac artery; and those involved in blood drainage: renal veins, inferior phrenic vein, hepatic vein, gastric veins, splenic vein, mesenteric vein, internal iliac vein, and internal jugular vein. To characterize the spatial association emerging from the vascular arrangement, we constructed a binary vascular matrix $M \times M$ with $M = (m_1, m_2, \ldots, M_N)$, with M being the number of organs (number of rows/columns). When two organs shared an artery or a vein, we recorded this co-occurrence as a 1. In contrast, when a pair (m_i, m_j) did not share a vessel, we assigned a 0. For example, the pancreas and liver share blood supply through the abdominal aorta; the 'supply matrix' has a value of 1 associated with the pair 'pancreas, liver'. The following

source/acceptor organs were excluded for this analysis because the available data do not allow the identification of main supply/drainage vessels: bone, lymph nodes, retroperitoneum, omentum, skin, skeletal muscle. Our first approach was to correlate the weighted metastatic matrix (*W*) with both vascular matrices independently, expecting that if spatial organ arrangement plays a role in cancer spread, it will manifest in a significant statistical association.

In order to test the hypothesis which relates the invasiveness of primary metastatic tumors in a source organ with its invasibility, we performed two linear correlations (null hypothesis: $\rho=0$): first between the degree of source (k^S) and acceptor (k^A) organs, and second, between the number of occurrences of primary and secondary-growth metastatic tumors. Then, for each organ, the difference in its degree as acceptor and source was correlated with the difference in its occurrence of primary tumors and metastatic sites. This analysis allowed us to test the association between occurrence and connectivity and to identify two categories of organs: those with higher invasiveness where the occurrence of primary tumor is higher than the occurrence of metastases or $k^S>k^A$; and those organs with higher invasibility where the occurrence of primary tumor is lower than the occurrence of metastases or $k^S<k^A$. Next, we abstract the metastatic process as the establishment of a link between two nodes where two conditions need to be met; first, cancer seeds need to leave a primary tumor in a source organ i with a probability P^s_i, and second, survive the transportation, arrive and establish an invasive diaspora at an acceptor organ j with a probability P^A_j. We considered that the source organ´s degree over the total number of acceptor organs, k^S_i/N^A, may account for P^s_i, and respectively, for acceptor organs that k^A_j/N^S accounts for P^A_j, then we correlated $w_{i,j}$ (BSI for a source-acceptor pair) with the product $P^s_i \times P^A_j$, because for a metastasis to occur cancer propagules need to leave the primary tumor and establish a population in the acceptor organ. The network analysis also allowed us to calculate the generality of each organ using a variation of Schoener's generality as

$G_i = 1 - \frac{L - k_i}{L-1}$, where G_i corresponds to the intensity of generalism of the focal organ i, if such organ is a source organ, then L corresponds to the number of potential acceptor organs, and k_i corresponds to the observed number of connections of the organ i, i.e., its degree. As G_i approaches to 1, an organ is more generalist in terms of its pattern of linking in the bipartite network. Finally, Shannon's entropy was used to calculate a measure of diversity (D_i) of the metastatic recipient or metastatic sources for source and acceptor organs, respectively. For an acceptor organ i its diversity of metastatic sources is calculated as $D_i = \sum_{j=1}^{S} \frac{n_{i,j}}{N} \log\left(\frac{n_{i,j}}{N}\right)$, where $n_{i,j}$ corresponds to the occurrence of metastases in an acceptor organ i from a source organ j and N corresponds to the total number of cases; hence, $n_{i,j}$ over N represents the metastatic incidence.

Simulating the role of spatial arrangement stochastic metastases

To evaluate the importance of source and acceptor statistical traits (degree) in the metastatic process, we simulate metastases in a network of 19 source and acceptor organs. We considered four different degree scenarios that define P^s_i and P^A_j: *empirical*, *nested*, *neutral,* and *variable scenarios.* In the *empirical* scenario, a vector of nodes' degrees was taken from the observed network. In the *nested* situation, a theoretical uniform distribution of P^s and P^A allows getting values for P^s_i, P^A_j*: (0,1)*. For the *neutral* case, all source and acceptor organs have $P^s_i = P^A_j = 0.5$. Finally, in the *variable* case, we combine *nested* and *neutral* cases, with $P^s_i = 0.5$, i:(1, …, 19) and P^A_j with a uniform distribution. The initial simulated matrix was zero-filled, i.e., $n_{i,j} = 0$, for each event a random cell within the matrix is chosen, then if a random number *rd: U(0,1)* is lower than the corresponding product $P^s_i \times P^A_j$ a metastasis from the organ i to the organ j, occurs. Using the same degree cases, we add the supply matrix M to

simulate a spatial constraint to metastasis (where *M* is a binary matrix with organs sharing a proximal artery), in this case, the condition for a simulated metastasis must be $rd<P^{s}_{i}\times P^{A}_{j}\times m_{i,j,}$ with $m_{i,j}$*: {0,1}*. We run each of the eight scenarios 300 times with 10000 metastatic simulations for each replicate, after which we obtain a matrix of metastatic occurrences from which we calculate a simulated matrix of broad sense metastatic incidence to which we compute nestedness and modularity.

All the analyses were performed in R (version 3.6.2), and the BSI data and the vascular incidence matrices are public.

Results

Emerging macroscopic patterns arose from the continuum of invasiveness and invasibility in the network of metastatic cancers. At the network scale, we found that the global architecture is highly structured with a nested subset structure (*Figure IV.2*), a scale-free truncated power-law degree distribution that applies to both source and acceptor organs modules (Figure IV.3A and Table S1), and a modular sub-structure (*Figure IV.3*B). The observed network is statistically more nested than null networks ($NODF2_{obs}$=66.27, $NODF2_{null}$= 63.71±0.14, $BINMAT_{obs}$=9.21, $BINMAT_{null}$=51.23±0.25). Nestedness implies asymmetry, such that there is a core of highly interacting source and acceptor organs and a periphery where specialized organs with a lower degree interact mainly with generalist organs (higher degree) (*Figure IV.2*Figure *IV.2*). The value of nestedness indicates that both source and acceptor organs vary in terms of the number of connections they have (i.e., their 'degree') (*Figure IV.2 - Figure IV.4*). At the organ scale, a gradient in primary tumor invasiveness across source organs underlies the observed continuum; it means that some source organs are connected to more acceptor organs, and/or generate more

migrating cells, and/or these migrating cells are selected for to migration (*Figure IV.4Figure IV.5*). The continuum is formally reflected in a monotonic gradient in invasibility across acceptor organs (i.e., some acceptor organs are more prone to be invaded than others, and/or to receive metastases from more source organs, *Figure IV.3*A). Concerning the evaluation of how spatial closeness between source and acceptor organs might be associated with metastatic incidence, our results show that the network of metastatic incidence is positively associated to the supply network (Kendall's-τ=0.093, p-value=0.047) meaning that the metastatic incidence is higher when source and acceptor organs share an artery. This statistical association does not hold for the drainage network (Kendall's-τ=-0.02, p-value=0.692). A last macroscopic attribute is that the metastatic incidence pattern reveals a modular structure of interacting organs (modularity likelihood$_{obs}$=0.156, *Figure IV.3*), implying that within module connectivity is higher than expected in a random network (Newman and Girvan 2004; Beckett 2016). The modules detected do not include necessarily the heaviest links between source-acceptor within the same module, it is because the available algorithms compute modularity likelihood referred to a maximization for a set of weighted links and not particular paired interactions (Beckett 2016).

About testing how the processes behind generating primary metastatic tumor are associated with receiving metastatic propagules, we did not find a significant association either in the degree ($t_{[16]}$=-0.517, ρ=-0.13, p-value=0.612) nor the occurrences ($t_{[16]}$=-0.485, ρ=-0.12, p-value=0.634), suggesting a decoupling in the events of generating primary metastatic tumors and receiving metastatic propagules (*Figure IV.4*A-B). However, a broader view unveils a significant positive correlation between the difference in occurrences and the difference in the corresponding degree (*Figure IV.4*C; $t_{[16]}$=3.982, ρ=0.64, p-value=0.0006) showing that more connected

organs (acceptor or source) have a higher metastatic incidence compared to less connected organs.

The exploration of the association of some physiological metrics obtained from the literature (see methods) and the network-derived source and acceptor organs' properties, shows interesting patterns that require further research (*Figure IV.5*). For instance, these results reveal evidence of a positive and statistically significant relationship between the occurrence of metastases in acceptor organs and blood flow through those organs (ρ=0.65, p-value=0.03, n=11) and between the occurrence of primary metastatic tumors and cumulative stem cells divisions per lifetime (ρ=0.85, p-value=0.002, n=10). In contrast, the negative and statistically significant relationship between acceptor organ's degree and stem cell divisions per year (ρ=-0.69, p-value =0.019, n=10) shows that organs which have a higher stem cell divisions are less likely to be invaded by metastatic propagules. Other interesting associations are linked to ecological measures such as diversity and generalism. In acceptor organs, their diversity of source is positively associated with their weight (ρ=0.85, p-value=0.001, n=11) and with their cumulative stem cell divisions per lifetime (ρ=0.85, p-value=0.001, n=11). Meanwhile, the diversity of acceptor for source organs is positively associated with its estimated noncancer cell population (ρ=0.73, p-value=0.01, n=11) and the estimation of the blood flow (ρ=0.73, p-value=0.03, n=11). These results come to contribute to the idea of organ-level physiology shapes the likelihood to send or receive metastatic propagules, hence playing an essential role in the ecologies of metastatic seeding.

Finally, the study of the relationship between the establishment of a metastatic link between two organs estimated from their corresponding observed degrees and the corresponding metastatic incidence (BSI) shed light on the underlying mechanisms of

the metastatic process (*Figure IV.6*). We show that metastatic incidence exhibits a threshold behavior as a function of the probability of metastasis ($P^s_i \times P^A_j$) and that there is a positive relationship between BSI and $P^s_i \times P^A_j$ after the threshold limiting metastases. Finally, about the simulation of metastasis as a stochastic process under different degree and spatially-constrained scenarios, we show that variability in source and acceptor organs' degree is a requisite to approach to the observed modular and nested macroscopic pattern (Figure IV.S1). However, it is interesting that a spatial constraint imposed by the observed arterial incidence matrix increases the modularity and the nestedness even beyond the observed value, suggesting that local spatial relationships might not mainly shape the observed macroscopic modular pattern. Overall, either from the observed patterns as from the simulated processes, the results suggest that space and local conditions attributed to organ properties are fundamental to understand the emerging patterns of metastasis.

Discussion

The quantification of the metastatic pattern allows us to expound some mechanisms and refine the hypotheses behind the diversity of metastatic cancers and the metastatic process itself. The observed variability between organs implies that organ level processes, different between organs, account for the spread of cancer between organs. In this context, the macroscopic patterns emerge. Nestedness, scale-free degree distributions, and modular patterns in networks have been suggested to promote diversity, stability, and network robustness to disturbances (Bascompte et al. 2003; Thébault and Fontaine 2010). However, in the context of our study, these network attributes are not related to stability and robustness as they are for ecological communities since organs are not species that can go extinct. Instead, we associate

these macroscopic patterns to the action of several simple mechanisms underlying the observed network structure (Barabási and Bonabeau 2003; Suweis et al. 2013; Leung and Weitz 2016) and related to the organs' attributes that we have shown here.

Mechanisms behind the emerging macroscopic patterns

Nestedness in ecological systems can arise because habitat patches display a gradient in either colonization or extinction probabilities (Patterson 1990). In the case of the metastatic network presented here, both extinction and colonization could influence the observed patterns (*Figure IV.2Figure IV.4Figure IV.6*). Extinction in the context of metastasis corresponds to failed colonization, resulting from either intrinsic unsuitability of specific organs for cancer cell growth or characteristic non-compatibility between certain primary tumor metastatic cells and specific acceptor organ microenvironments. As per colonization, we found positive correlations between blood flow through an organ and the incidence of metastasis (*Figure IV.5*). Blood flow is correlated with the number of propagules that could potentially arrive in a patch or 'propagule pressure' (Blackburn and Duncan 2001), also as has been demonstrated recently, the blood flow rate influences arrest, adhesion, and extravasation of circulating tumor cells (Follain et al. 2018; Onken et al. 2019).

The importance of cellular processes such as stem cells' life history and organ turnover is crucial in understanding the emergence of primary tumors and metastasis (Scott et al. 2013; Tomasetti et al. 2017). Here, we showed that the generalism of a source organ is negatively correlated with cell turnover in that organ (*Figure IV.5*); it implies that source organs with more frequent cell population renewal generate more metastatic links to different acceptor organs with a higher metastatic incidence. These results suggest both a role for propagule pressure, hence colonization, in the observed nested

pattern of the cancer network. Furthermore, the diversity of strategies found in source and acceptor organs (Figs. 2-4) can be influenced by tissue-specific risk factors (Joyce and Pollard 2009; Aktipis et al. 2013), different life-history trade-offs within tumors (Pienta et al. 2013), groups of noncancer cells contributing to metastases (Condeelis and Pollard 2006*b*; Ganesh et al. 2020), and variation in the degree of matching between the quality of the recipient organ and the niche requirements of migrating metastatic cells, all of which drive colonization success (Joyce and Pollard 2009; Pienta et al. 2013; Groot et al. 2017; Ganesh et al. 2020), and some analogous to what is observed in models of metapopulation dynamics (Marquet and Velasco-Hernandez 1997; Keymer et al. 2000*a*).

A scale-free degree distribution (*Figure IV.3*) also is a property shared by different complex networks, where the simplest way to generate such topology is based on the action of two simple generic mechanisms(Barabási and Albert 1999): (i) one that provides for the continuous increase in both the number of nodes and links resulting in the expansion of the network and (ii) one that accounts for an increase in the probability of a site being connected as function of the number of existing connections, known as ‘preferential attachment.’ Two other cases are possible; first, where the truncation phenomenon observed in the degree distribution is likely the result of the small and finite number of nodes (organs) that can potentially be part of the network, and which limits the spread and filling of the distribution (Patterson 1990), and, second, following an optimization framework which can underlie the canonical preferential attachment (D’Souza et al. 2007). In what follows, we go further in the integration of these mechanisms, which can account for the observed architecture of metastasis.

The first mechanism implies that the network structure has changed through time, because of tumor cell migration from primary sites, and/or metastasis to novel acceptor organs. Differences in tissue-level cancer risk may have an evolutionary basis (Davies 2004; Thomas et al. 2016), be associated with the novel, organ-level, environmental conditions such as pre-metastatic conditions (Peinado et al. 2017), or other cellular strategies such as $L1CAM^{+}$ cells (Ganesh et al. 2020). The second mechanism is associated with preferential attachment, which in this context implies that a new primary tumor is more likely to metastasize to an already highly-connected acceptor organ and that a new metastasis is more likely to arise from a primary tumor that has already metastasized to many different organs. This mechanism also can generate nested networks (Medan et al. 2007), whereby specialized (low degree) acceptor organs tend to interact with generalist (high degree) source organs and vice versa (*Figure IV.2*). This preferential attachment and the resulting asymmetric interaction can emerge as results of trade-offs in the metastatic process, where the motile cancer phenotype is selected according to the local conditions behind the probability of success in an acceptor organ (*Figure IV.6*Figure *IV.5*). When, the likelihood of being a successful propagule is maximized (i.e., an increase of the joint probability of migration and establishment, $P^{s}_{i} \times P^{A}_{j}$) the cancer strategy shows an increase in its invasiveness, hence higher metastatic incidence.

Modular networks in ecology have been associated with the presence of species with similar functional traits (Montoya et al. 2015) or subsets of spatial locations with more frequent dispersal (Bascompte et al. 2003). These two mechanisms are plausible in cancer networks. Modularity may reflect a combination of similar traits among some organ groups (due to similar organ environments, shared connectivity and/or functional characteristics (Qin et al. 2016)), and different traits between these groups

and other groups that restrict metastasis between modules. We did not detect a explicit support for spatial proximity within each module besides the overall correlation of matrices, we argue that niche-related characteristics might play a role determining the observed modular arrangement. It remains to be uncovered, which are the traits that source and acceptor organs share within modules in the cancer network, and that may account for their compartimentalization.

The overall observed pattern results from a diversity in the degree distribution, which may be shaped by local mechanisms of selection (Del Genio et al. 2011) understood under the natural selection paradigm operating on the metastatic variants (Hochberg and Noble 2017). In this local selection paradigm, the attributes of organs that help us to understand further the factors that may make an organ more likely to become colonized by cancer cells or defining the number of connections that a given source/acceptor has, have been primarily discussed in the literature. For example, the role of stem cells in tissue renewal might underlie the observed negative correlation between acceptor organ degree and its number of stem cell divisions, implying that organs which on average have fewer or older cells tend to be targeted for metastasis from a more significant number of different primary tumor sources, following Paget's seed and soil hypothesis and cellular processes acting upon the invasion (Merino et al. 2016). In this case, they receive more links because they are inherently more suitable to be colonized, and this is likely one of the mechanisms behind preferential attachment, and hence nestedness, modularity, and truncation power-law (D'Souza et al. 2007; Medan et al. 2007; Leung and Weitz 2016).

The ecological and evolutionary study of cancer is showing promising results for detection, prognosis, and treatment in cancer patients (Greaves and Maley 2012*b*). Our study comes to contribute in that vein showing that: (i) the diversity in the connectivity

patterns and metastatic incidence of organs can recapitulate niche-related processes occurring at the organ level accounting for the diversity of metastatic strategies; (ii) macroscopic patterns contribute to the understanding of potential mechanisms for oncogenesis in source organs and/or incidence of secondary-growth in acceptor organs; (iii) quantitative patterns of invasiveness and invasibility open a preventive framework for metastases detection given a detected primary tumor which undoubtedly requires further research. Nevertheless, despite the clear statistical pattern, the clinical implications must be taken carefully, since the predictive power of models has not reached a mature yet, and causal mechanisms operating locally, not covered here, still waiting to be discovered.

Figures

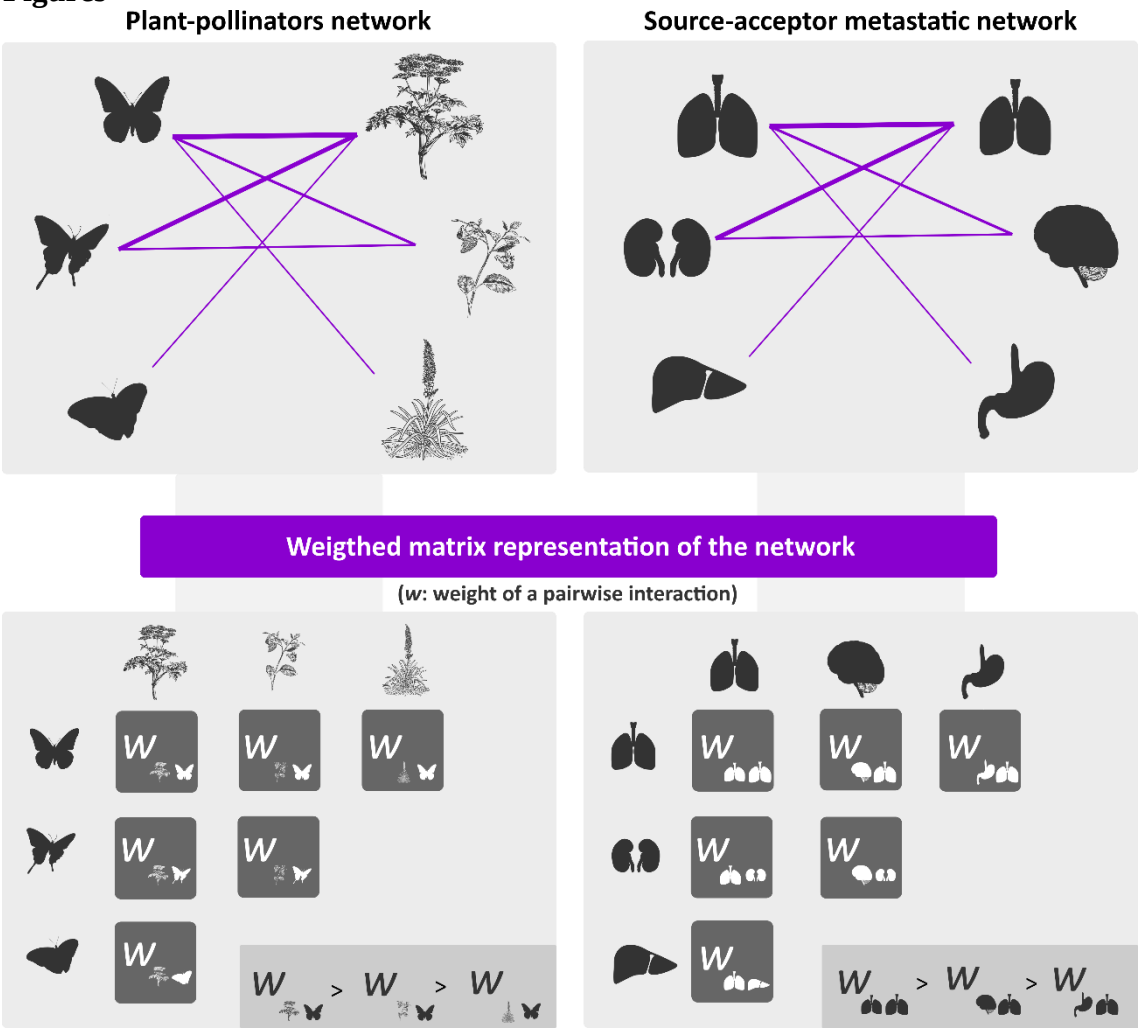

Figure IV.1 Ecological relationships from pollination to metastatic networks. A schematic example of how topological abstract the idiosyncratic differences of different phenomena, a pollination network that relates the interactions between pollinators and plants and a metastatic network that represent the metastatic seeding fro source to acceptor organs. Both networks can be represented through a matrix, where each matrix value (*w*) quantify the strength of the pairwise interaction.

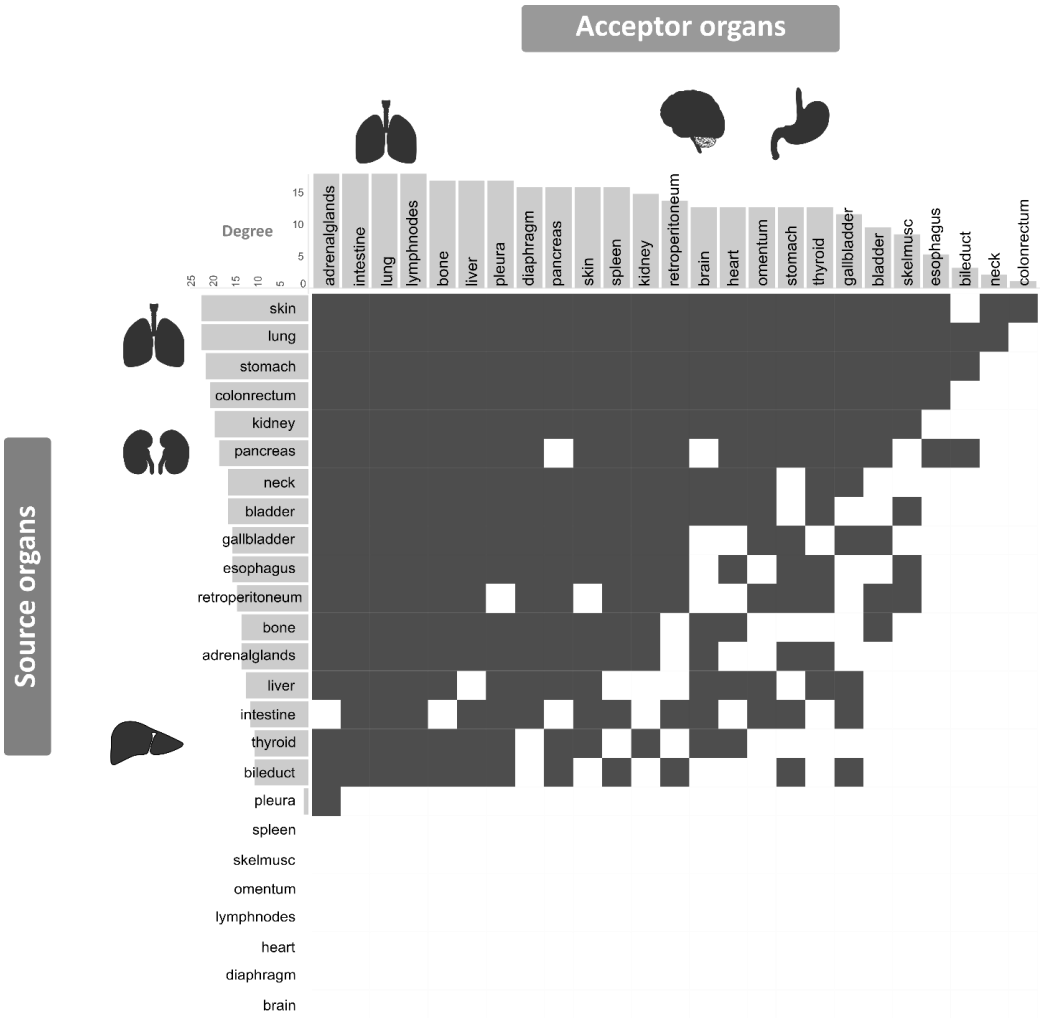

Figure IV.2 Emergent macroscopic properties of the metastatic network; observed nested pattern. For a pair of source and acceptor organs, the cell is shaded if an occurrence in tailed from the data collected from the literature.

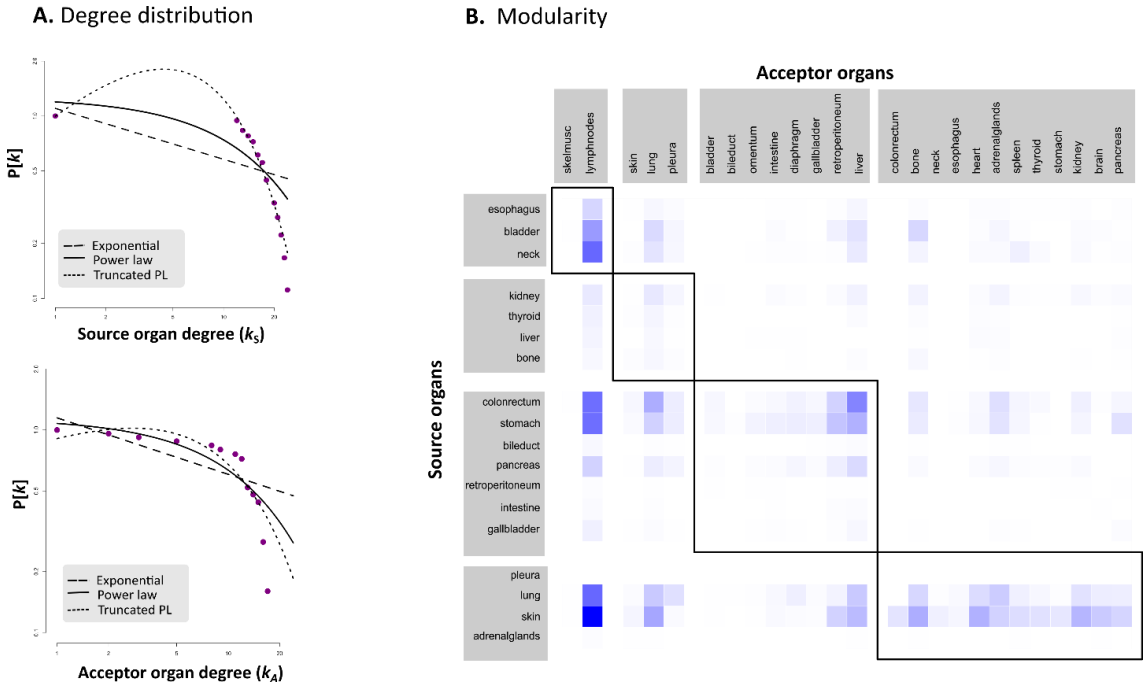

Figure IV.3 Emergent macroscopic properties of the metastatic network; free of scale and modular network. (A) Degree distribution for both groups of organs with three different fits (summary table SIV.1). (B) Modules detection based on the metastatic incidence pattern.

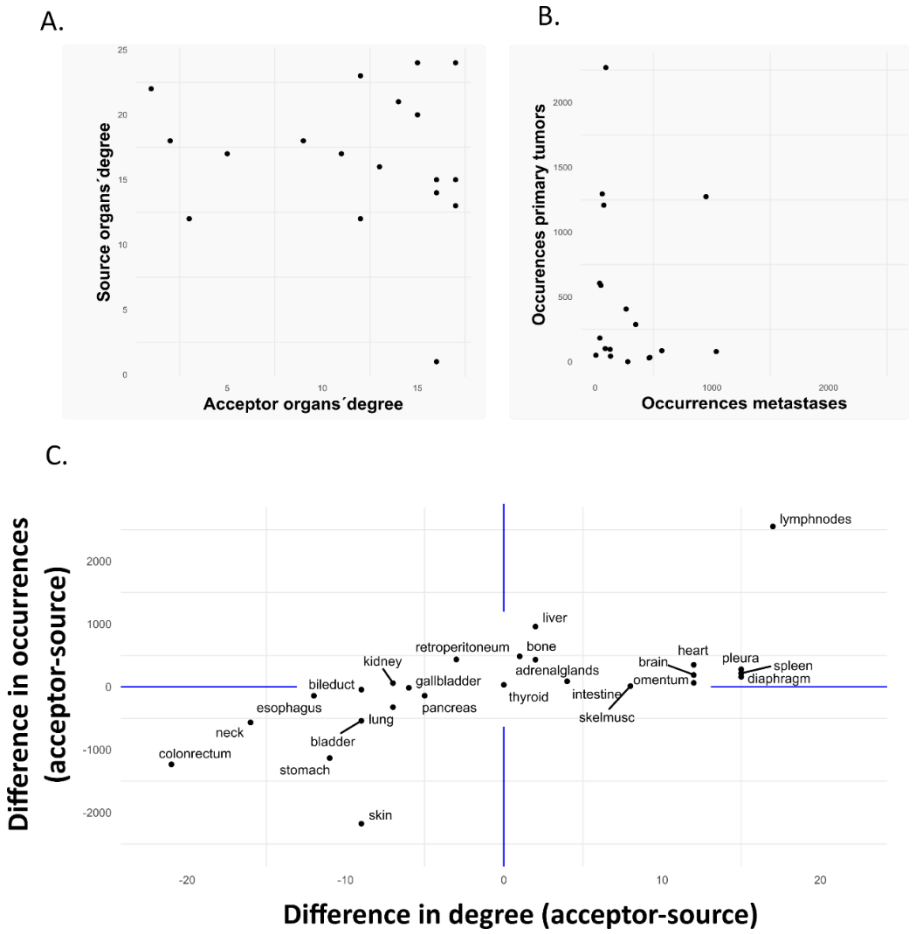

Figure IV.4 Mesoscopic relationship between topology and metastatic occurrence. Testing the association between (A) degree and (B) occurrences for each organ. In (C) a positive relationship between generalism (difference in degree) and invasiveness (difference in occurrences) allows dissecting the continuum of strategies in organs prone to invade (negative differences) and organs prone to be invaded (positive differences).

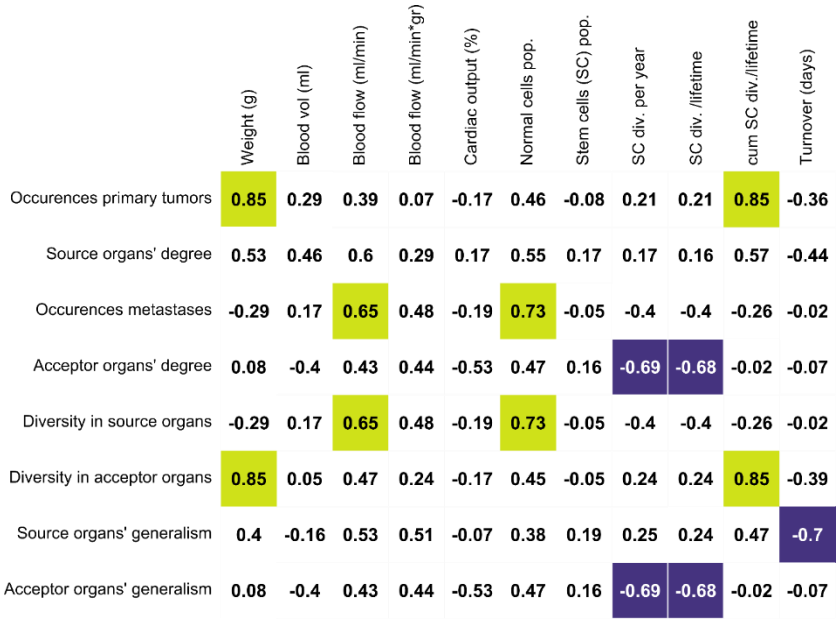

Figure IV.5 Correlation matrix between the organ's physiological estimates and network-derived properties. Each cell shows the correlation coefficient, and coloured cells are the statistically significant correlation (green: positive correlation, purple: negative correlation) corrected by Bonferroni for multiple comparisons. For the origin of the physiological metrics, please, refer to the methods section.

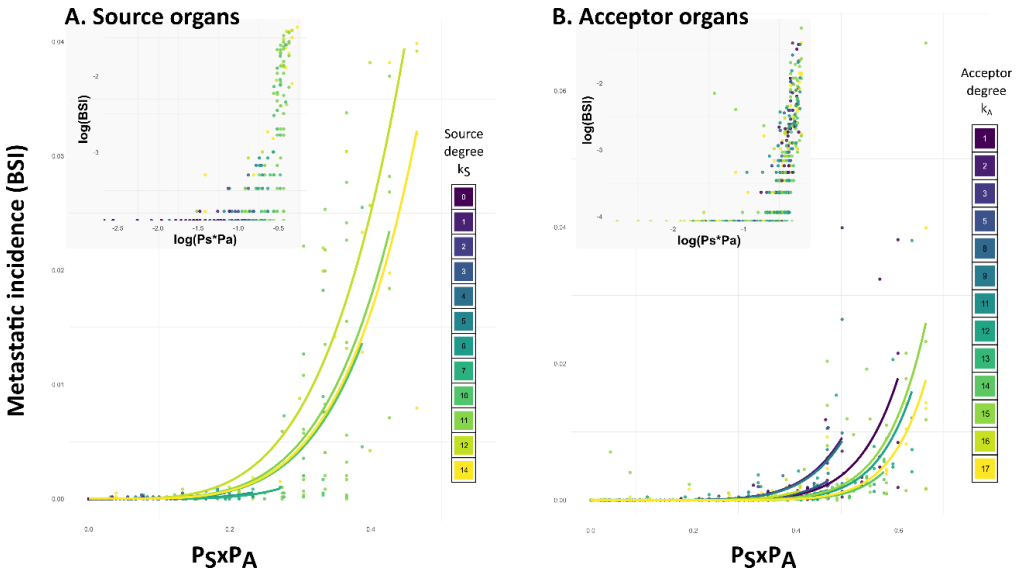

Figure IV.6 Metastatic incidence as a function of the estimated likelihood to leave a source organ and invade an acceptor organ ($P_S \times P_A$). Figures (A) and (B) represent source and acceptor's degree, respectively. A log-log relationship is plotted in the inset.

Supplementary materials

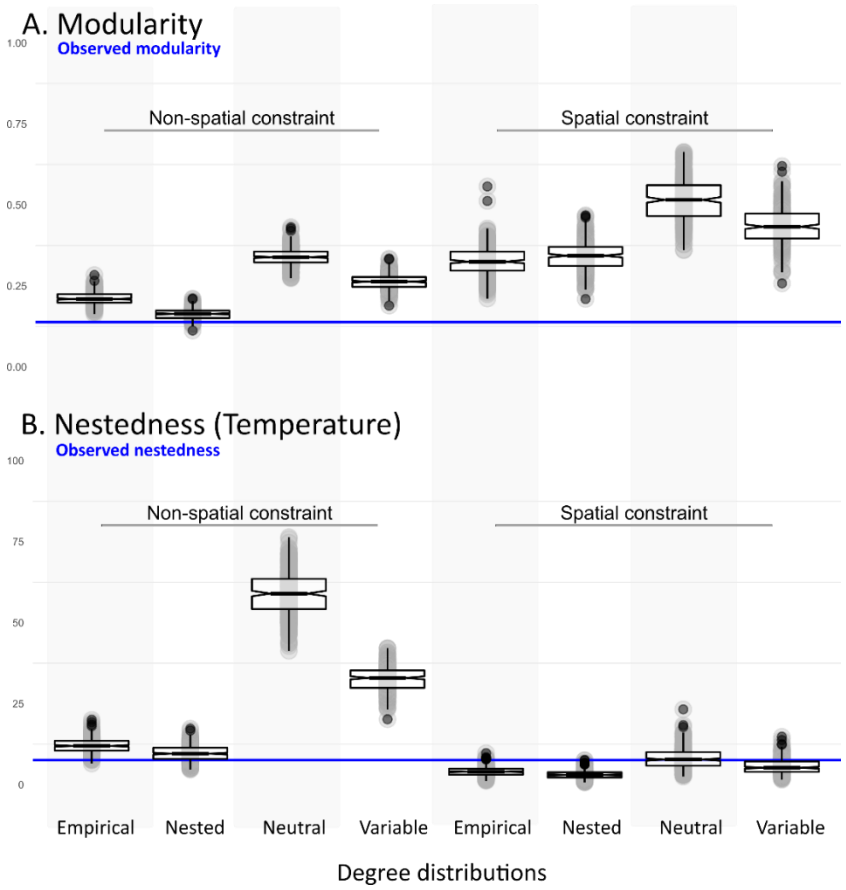

Figure SIV.1 A simulation of the metastatic process (n=300) under four different degree scenarios and considering spatial constraint imposed by the observed supply incidence matrix. The modularity and nestedness of the simulated processes (box plots with median and interquartile range, n=300) are contrasted against the observed value of the empirical network (solid blue line). Non-spatial constraint implies that metastasis depends only on source and acceptor organs degree, while the consideration of the spatial constraint adds that if a pair source-acceptor shares the close artery, the metastasis will occur with a probability $P_S x P_A$, if the two organs do not share a close artery, the metastasis does not occur.

Table SIV.1. Summary statistics associated with the three alternative models fitted to the degree distribution or the probability of observing a source or acceptor organ with k connections to other organs. The truncated power-law fits two coefficients: slope and cut-off; in this table, only the slope estimation is shown. AIC= Akaike´s information criteria.

Source organs' degree					
Fit	Estimate	Std. Error	p-value	R^2	AIC
Exponential	0.126	0.009	0.000	0.984	-31.266
Power law	0.497	0.087	0.000	0.880	-5.913
Truncated power law	-0.229	0.074	0.011	0.992	-38.681
Acceptor organs' degree					
Fit	Estimate	Std. Error	p-value	R^2	AIC
exponential	0.172	0.050	0.018	0.871	0.529
power law	0.840	0.311	0.043	0.787	3.623
truncated power law	-3.121	0.758	0.015	0.981	-10.278

Concluding remarks: Complex Cancer Ecosystems

"Nothing in biology makes sense except in the light of evolution."

T. Dobzhansky

Any biological system has a history. A cumulative evolutionary history, from one generation to another, stored in both the internal and external environment of the system. Also, a contemporary history, where the ecological interactions flourish. The rise of cancer as a novel cellular phenotype in the multicellular organisation reflects the expression of these two temporal dimensions, the evolutionary history since it resembles the transitions from single-cell ancestors to multicellular organisms; and the contemporary history since its evolution, within the Metazoan's life span, depends on the interaction with its biophysical environment.

In this thesis, we worked over some principles from ecology applied to cancer at its different spatiotemporal scales. Although we distinguished different scales of organisation within this complex adaptive ecosystem, the integration between them is needed. However, instead of being sequential and nested integration with bottom-up or top-down causalities, we think that each scale is connected to every other scale. In this section, we will discuss some of these between-scales interactions, our results, and the emerging questions that such interactions bring up.

In the first two chapters, we studied how the cell-environment codetermination shape the cancer phenotype's fitness under the biological phenomenon of ageing. The transformation of the biophysical (internal and external) environment of the cell known as ageing, sets the conditions that cannot support the multicellular organisation (Chapter I, (Davies and Lineweaver 2011; Bussey et al. 2017; Cisneros et al. 2017)). Under this lack of spatial and functional structure, the emerging cellular phenotype interacts with its neighbourhood. There is a myriad of interactions that the cancer cells

establishes with its neighbours; we focus only on competition (Chapter II). However, it can be expanded to other control mechanisms, such as immune cells (Chapter III) (Maley et al. 2015; Nawaz et al. 2015) or even though, to positive interactions (Rak et al. 1996; Xing et al. 2010; Zhang et al. 2011; Man et al. 2013; Wei et al. 2017; Ganesh et al. 2020). Now, if we analyse the context where the cancer phenotype emerges, it is an aged environment where according to our second hypothesis, the cancer cell will face a weaker resident noncancer strategy with a reduced competitive resistance (Chapter II). In the axis of ageing, a simple conceptualisation links the two scales; the individual scale where the cancer phenotype co-emerge with its external environment and the scale of interacting populations, where the cancer strategy competes with noncancer cells. At the same time, these hypotheses catalyse some unresolved questions, for example, whether we can ameliorate the effects of ageing through environmental engineering then reducing the likelihood of oncogenesis and maintaining the superior competitiveness of the resident noncancer cells; or, whether cancer cells emerge as individuals or there is a collective transition mediated by the enacted and competitive environment. The question about collective oncogenetic emergence opens opportunities to study whether it can be catalysed by niche construction, with some initial cancer cells promoting the cancer diversity (Tissot et al. 2016).

On the axis of ecological interactions between cellular strategies, one could wonder how they could help us to explain the macroscopic pattern of metastases (**Error! Reference source not found.**). We could hypothesise that different organs have different competitive resistance or facilitation profiles; hence, how organs age or the distribution of immune cells might explain the cellular mechanisms behind the invasiveness/invasibility continuum. Also, how this continuum of strategies is

spatially organised; can network topological measures (such as the studied in Chapter III) give us some clues about the potential invasiveness of tumours? Moreover, how physical conditions, the quantification of Grinellian niches and biotopes, within the organs can influence these patterns of invasion (Heindl et al. 2016). The spatial dimension is vital to comprehend ecological patterns of interactions and stability (Levin and Paine 1974; Durrett and Levin 1994*b*; Levin 2005; Pascual and Guichard 2005); therefore, its consideration is critical to track the development of the disease (Maley et al. 2015; Nawaz et al. 2015; Nawaz and Yuan 2016; Heindl et al. 2018).

Then, why ecology?

Ecology, coined by Ernst Haeckel in 1866, highlights the study of nature as a whole, where the interaction between organisms and their biophysical environment operates shaping species distribution and abundance over the planet. In the most fundamental level, one could redefine it the light of the codetermination and co-emergence between an individual and its environment, shaping life-history strategies' abundance and distribution. Hence, a broader definition points towards the determining and being determined by their external environment individuals; hence, operational but not functionally enclosed (Darwin 1859; Varela 1979, 1991; Maturana and Varela 1998; Metz 2013; Krakauer et al. 2014; Laland et al. 2016). These interactions will, eventually, drive the organisation of life across scales as they have driven the major transition in life, for example, the emergence of multicellularity (Szathmary and Smith 1995; Michod 2007; Herron and Michod 2008; Du et al. 2015; Szathmáry 2015).

The study of cancer as a life transition and an ecological system has two immediate implications. The first implication is for oncology and the persistent search of the holy grail in genes. It is necessary the acknowledgement that cancer cells at each

spatiotemporal scale of its evolution, like the ones analysed in this thesis, establish a narrow interaction with its biophysical environment, from the determinants behind oncogenesis to the spread of cancer cells between organs. Hence, to understand an emergent population pattern such as the proliferation of a cancer cellular strategy, it is not possible to reduce the whole phenomenon to the additive study of its genetic components. The question about if genetic data is sufficient, or even necessary, to understand an ecological phenomenon such as cancer, is still open (Gatenby 2012); but certainly a shift must be made towards the study of such a complexity which can not be reached by a reductionist approach. The second implication is for ecology, which has limited its view to macro ecosystems, whereas the principles developed in the field may apply and contribute to the understanding of all the scales of the adaptive matter, from cells to the biosphere. The challenge for ecology entails that advances in synthesis and integration by identifying common principles from disparate phenomena; where the epistemological value of ecology is reflected in its approach to understanding complex adaptive systems as a whole, wherein interactions reproduce an emergent phenomenon.

By bringing together experts from different disciplines and fields, we can find solutions for today's complex problems such as cancer, beyond the conventional academic frontiers and paradigms (Anderson and Quaranta 2008; Korolev et al. 2014; Austin 2017; Maley et al. 2017). The field of ecology and evolution of cancer is just emerging with promising ideas, big challenges, and excellent opportunities for integration between disciplines (Merlo et al. 2006; Pienta et al. 2008; Kareva 2011; Korolev et al. 2014; Amend and Pienta 2015; Ducasse et al. 2015; Turajlic and Swanton 2016). If we want to understand and predict the behaviour of complex adaptive systems, such as cancer, then it is necessary to jump from the view of studying

the system as individual parts to its view as a whole systems with emergent properties and patterns. In this endeavour, ecology and evolution offer epistemological and practical tools to reach new frontiers.

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