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MECHANOBIOLOGY OF CANCER CELLS USING ATOMIC FORCE MICROSCOPY

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MECHANOBIOLOGY OF CANCER CELLS USING ATOMIC FORCE MICROSCOPY

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"It was never offered you a wish without giving you the power to make it come true. However, it is possible that you will have to fight for it..."

"Illusions", by Richard Bach

.... to the memory of my father Alípio Brás (who passed away 9 years ago)
and who (together with my mother), prepared me very well for life,
and always encouraged me to start this work,
being one of his most important wishes...
and mine

ABSTRACT

Cell biophysics is an important field in different areas of health, such as inflammation, infection, neurobiology, and cancer. The quantification of mechanical properties on cells and tissues has been reported to constitute a biomarker that allows the differentiation of a healthy from a pathological state. Mechanobiology is defined by the translation of the internal and external forces (sensed by cells) into biochemical signals. The contribution of mechanobiology has been shown as crucial to understanding the appearance, diagnosis, and development of cancer.

In this work, static and dynamic atomic force microscopy was used to assess the mechanical properties of two human cancer cell types: melanoma and colorectal cancer (CRC). In melanoma studies, human neonatal primary melanocytes (HNPMs) from the foreskin, WM793B (human cells established from the vertical growth phase of a primary skin melanoma lesion), and 1205LU (a metastatic cancer cell line in the lung, from WM793B) were used. In CRC the following cells were used: HCT15, HCT116 (cancer cell lines), and SW620 (a metastatic cell line from SW480). Conventional force-distance curves, and z-Step response (static method), and frequency sweep (dynamic assay, within a frequency range of 0 – 100 Hz) were performed. A power-law exponent was fitted of the storage modulus to assess the “fluidity” of the cytosol of the cell. The following parameters were obtained: Young’s (E), storage (G'), loss (G'') moduli, the Power Law exponent (PL) and the loss tangent (LT). Confocal laser scanning microscopy (CLSM) was used to characterize the structure and function of the cells, by staining the nucleus, F-actin of the cytoskeleton, and phosphorylated focal adhesions (p-FAK) in melanoma cells. In CRC cells, nuclei, F-actin, tubulin, vinculin and total focal adhesion were stained. Migration assays were performed, by evaluating the migratory behavior of the two tumor cells. In melanoma cells, regarding mechanical studies the following trend was observed (from highest to lowest): E : WM793B > 1205LU > HNPMs; G' : WM793B > 1205LU > HNPMs; G'' : WM793B > 1205LU > HNPMs; PL: 1205LU < WM793B < HNPMs; LT: 1205LU < WM793B < HNPMs.

E , G' , and G'' moduli followed the same trend. G' presented the same order of magnitude as E , since the contribution of G'' to the apparent Young's modulus was smaller. WM93B were the stiffest and more viscous cells, and HNPMs were softer and less viscous. These results are in accordance with the PL of the G' which, can be correlated with the cytosol

cell “fluidity”, meaning that HNPMs were more “fluid” when compared with the WM793B and 1205LU. Comparing the WM793B with the 1205LU cell line, the WM793B were stiffer and more viscous. CLSM also showed that 1205LU presented more phosphorylated focal adhesions (p-FAK), which can explain the highest migratory behavior compared with the WM793B. p-FAK was not present at WM793B, which only closed 30% of the scratch after 23 h, at the migration assay. 1205LU cell line closed the wound within 23 h. In terms of PL and LT, the WM73B and 1205LU followed the same trend, but not in the same way as expected when correlating these results with the E , G' , and G'' moduli. More studies should be performed on these cells in terms of structure, to evaluate if there are other mechanisms that influence their mechanics.

Regarding the colorectal cancer cell lines, the mechanics results were showing the following trend: E : SW620 > HCT116 > HCT115; G' : SW620 > HCT116 > HCT115; G'' : SW620 > HCT116 > HCT115; PL: SW620 < HCT116 = HCT15 and LT: SW620 < HCT116 < HCT15.

The CRC cells lines followed the same trend in terms of E , G' and G'' moduli, as well as the PL and the LT. The E presented the same order of magnitude of the G' , which can be explained by the fact that the contribution of G'' is smaller in the E . These results were corroborated by the presence of lamellipodia in HCT116 and HCT15 cells, that closed the scratch faster than SW620 cell lines, as well as the presence of several layers of F-actin filaments on SW620 cell, compared with few F-actin filaments in the HCT116 and HCT15. The presence of total FAK was observed in the three cell types, being more predominant in HCT15 cells, which can justify the higher migratory capacity.

Atomic force microscopy (AFM) has proven to be a powerful technique to assess the biomechanics of healthy and tumor cells. The technique has shown the importance of distinguishing the elasticity from the viscosity of cells, as well as the “fluidity” (by frequency sweep), by the application of a power-law exponent of the storage modulus. Mechanical properties can be used as a biomarker of cancer cells, since e.g. in cells it appears to be related with the pathological state of the cell. This technology should be translated to the clinic, and be even present in the surgery room, to be used in real-time, by clinicians to assess the stiffness of healthy and tumor tissues (low values in stiffness correspond to healthy tissues, high values of stiffness correspond to tumor tissues). This

way, it could avoid more than one surgery to the same tumor and to the same patient, avoiding the extra pain in patients and high costs to the healthy system.

RESUMO

A biofísica celular é um campo importante em diferentes áreas da saúde, nomeadamente no cancro. A quantificação das propriedades mecânicas em células e tecidos tem sido relatada como um possível biomarcador que permite diferenciar um estado saudável de um patológico. A mecanobiologia é definida pela tradução das forças internas e externas (sentidas pelas células) em sinais bioquímicos. A contribuição da mecanobiologia tem-se mostrado crucial para a compreensão do aparecimento, diagnóstico e desenvolvimento do cancro.

Neste trabalho, a microscopia de força atômica estática e dinâmica foi usada para quantificar as propriedades mecânicas de dois tipos de células cancerígenas humanas: melanoma e o cancro colorectal (CRC). Nos estudos do melanoma, melanócitos primários neonatais humanos (HNPMs) do prepúcio, WM793B (células humanas estabelecidas a partir da fase de crescimento vertical de uma lesão de melanoma primário da pele) e 1205LU (uma linha celular do tumor metastático no pulmão, da WM793B) foram usados. As linhas celulares HCT15, HCT116 e SW620 (uma linha celular metastática de SW480) foram as celulares tumorais do CRC estudadas. Aquisição de curvas convencionais de força-distância e resposta *z-Step* (métodos estáticos) e curvas de varrimento de frequência ao longo do tempo (ensaio dinâmico, num intervalo de frequências de 0 a 100 Hz) foram adquiridas. Um expoente da lei de potência foi aplicado ao módulo de armazenamento para avaliar a “fluidez” do citosol da célula. Foram obtidos os seguintes parâmetros: Young's (E), armazenamento (G'), perda (G''), lei de potência (PL) e tangente de perda (LT). A microscopia confocal de varrimento a laser (CLSM) foi usada para caracterizar a estrutura e a função das células, por coloração dos núcleos, da F-actina do citoesqueleto e adesão focais fosforiladas (p-FAK) em células de melanoma. Nas células do CRC, F-actina, tubulina, vinculina, e a proteína de adesão focal total foram corados. Ensaio de migração também foram realizados, avaliando o comportamento migratório de todas as células tumorais. Nas células de melanoma, em relação aos estudos mecânicos, observou-se a seguinte tendência (do maior para o menor): E: WM793B > 1205LU > HNPMs; G': WM793B > 1205LU > HNPMs; G'': WM793B > 1205LU > HNPMs; PL: 1205LU < WM793B < HNPMs; LT: 1205LU < WM793B < HNPMs.

Os módulos E, G' e G'' seguiram a mesma tendência. G' apresentou a mesma ordem de grandeza que E, sendo a contribuição de G'', menor. WM93B apresentaram maior rigidez e viscosidade, e as HNPMs foram as menos rígidas e viscosas. Esses resultados estão de acordo com o PL do G' que, pode ser correlacionado com a "fluidez" da célula do citosol, significando que os HNPMs mostraram mais "fluidez" quando comparados com as linhas celulares WM793B e 1205LU. Comparando as WM793B com a linha celular 1205LU, as WM793B apresentaram ser mais rígidas e mais viscosas. O CLSM também mostrou que as linhas celulares 1205LU apresentaram mais adesões focais fosforiladas (p-FAK), o que pode explicar o maior comportamento migratório em comparação com as WM793B. Em termos de migração as WM793B fecharam a abertura apenas 30%, após 23 h. A linha celular 1205LU fechou a abertura antes de 23 h. Em termos de PL e LT, as WM793B e as 1205LU seguiram a mesma tendência, mas não da mesma forma que o esperado ao correlacionar esses resultados com os módulos E, G' e G''. Mais estudos devem ser realizados nessas células em termos de estrutura, para avaliar se existem outros mecanismos que influenciam as suas características mecânicas.

Em relação às linhagens celulares de tumor colorectal, os resultados da quantificação das propriedades mecânicas seguiram a seguinte tendência: E: SW620 > HCT116 > HCT115; G': SW620 > HCT116 > HCT115; G'': SW620 > HCT116 > HCT115; PL: SW620 < HCT116 = HCT15 e LT: SW620 < HCT116 < HCT15. As linhas de células CRC seguiram a mesma tendência em termos de módulos E, G' e G'', bem como o PL e o LT. O E apresentou a mesma ordem de grandeza do G', o que pode ser explicado pelo fato da contribuição do G'' ser menor no E. Estes resultados foram corroborados pela presença de lamelopódios nas células HCT116 e HCT15, podendo contribuir para o fecho da abertura nos ensaios de migração ao contrário das células SW620 que não fecharam a abertura, mesmo ao fim de 144h. As linhas celulares SW620, apresentaram várias camadas de filamentos de F-actina, em comparação com poucos filamentos de F-actina nas células HCT116 e HCT15. A presença de FAK total foi observada nos três tipos celulares, sendo mais predominante nas células HCT15, o que pode justificar a maior capacidade migratória. AFM mostrou ser uma técnica com muito potencial para avaliar a biomecânica de células saudáveis e tumorais. A técnica mostrou a importância de distinguir a elasticidade da viscosidade das células, bem como a fluidez, uma vez que na célula parece estar relacionado com o seu estado patológico. Esta tecnologia, poderia ser introduzida na sala de cirurgia, para

ser utilizada em tempo real, pelos clínicos para avaliar a rigidez de tecidos saudáveis e tumorais (tecidos saudáveis apresentam valores de rigidez baixos, comparados com a elevados valores de rigidez para os tecidos tumorais). Dessa forma, poderá evitar-se mais de uma cirurgia para o mesmo tumor e no mesmo paciente, evitando o sofrimento acrescido dos pacientes e aumento dos custos para o sistema de saúde.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
A2SD	Amplitude square spectral density
ABCD	Asymmetry Borders Color Diameter
ABP	Actin-binding-protein
ACS	Adenocarcinoma sequence
ACTN	Actinin
A _{DC}	Amplitude at DC
AFM	Atomic force microscopy
AIM	At melanoma Foundation
AJ	Adherens junctions
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BEC	Bottom effect correction
BM	Basement membrane
BRAF	v-raf murine sarcoma viral oncogene homolog
BSA	Bovine serum albumin
CAFs	Cancer associated fibroblasts
CAR-1	Chimeric antigen receptor T
Cas	Crk-associated substrate
CCC	Colon cancer cells
CCSA-3	Colon cancer specific antigen-3
CDc42	Control protein 42 homolog
CDH1	Cadherin 1 (protein coding gene)
CDKN2A	Cyclin-dependent kinase inhibitor-2
CEC	Cerebral endothelial cells
CFTR	Cystic fibrosis transmembrane conductance regulator
CLSM	Confocal laser scanning microscopy
CMS	Consensus molecular subtype
CNS	Central nervous system
Coll I	Collagen type-I
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
CSC	Cancer stem cell
CSF-1	Colony-stimulator factor-1
CTCs	Circulating Tumour Cells
CTGF	Connective tissue growth factor
CX	Connexins
d(t)	Deflection time
DAPI	4',6 – diamidino – 2 – phenylindole dihydrochloride
DFC	Dwell force curve
DFC+FS	Frequency Sweep force curve
DFC+S	Step indentation force curve
DMA	Dynamic mechanical analyzer

DMEM	Dulbecco's modified eagle medium
DMT	Derjagin-Müller-Toropov
DNA	Deoxyribonucleic acid
Doc3	Dorsocross3
D-PBS	Dulbecco's phosphate buffer saline
DSc	Desmocollin
DSg	Desmoglein
DTIC	Decarbazine
E	Young's modulus
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ENS	Enteric nervous system
EpCAM	Epithelial cell adhesion molecule
EpEX	Extracellular domains of epithelial cell
EpID	Intracellular domain of epithelial cells
ET-1	Endothelial-1
EVs	Extracellular vesicles
FA	Focal adhesions
FA	Focal adhesion
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FN	Fibronectin
FSS	Fluid shear stress
G	Global
G'	Storage modulus
G''	Loss Modulus
GAGs	Glycosaminoglycans
GLOBOCAN	Global cancer Observatory
GPCR	G-protein coupled receptors
HIF	Hipoxia induced factor
HMGS	Human melanocytes growth supplement
HNPMs	Human neonatal primary melanocytes
HRAS	Harvey rat sarcoma
ICAM	Intercellular cell adhesion molecule
ICAM-1	Intercellular adhesion molecule-1
IFP	Interstitial fluid pressure
ILK	Integrin linked kinase
InvOLVS	Inverse optical levers sensitivity
JKR	Johnson-Kendall-Roberts
k	Cantilever's force constant
K2P	Two-pore domain potassium
K _B	Boltzmann's constant
kc	Spring constant
KCa1.1	Calcium-activated potassium channel subunit alpha-1
KRAS	Kirsten rat sarcoma virus

K _{SHO}	Force constant (single harmonic oscillator)
L	Local
L1 CAM	L1 cell adhesion molecule
LINC	Linker of nucleoskeleton and cytoskeleton complex
LN	Laminin
LN-5 γ 2	Laminin-5 γ 2
LOX	Lysyl Oxidase
LPA	Lysophosphatidic acid
LT	Loss tangent
MCAM	Melanoma cell adhesion molecule
mDIA-1	Mammalian DIA-related formin-1
MelCTCs	Melanocytes circulating tumor cells
MEMS	Micro electromechanical system
MET	Mesenchymal-to-epithelial transition
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinases
MSS	Microsatellite stable
MTOC	Microtubule organizing center
MVP	Microvascular fluid pressure
MYO5a	Myosin Va
η	Viscosity
ND	Not determined
NHM	Normal human melanocytes
NPC	Nuclear pore complex
NRAS	Neuroblastoma rat sarcoma
P2X7	Purinergic type-2 receptor
p53	Protein 53
PAX	Paxillin
PBS	Phosphate buffer saline
PDGFR α	Platelet-derived growth factor receptor α
PDL	Population double level
PDL1	Programmed death ligand 1
PDMS	Polydimethylsiloxane
PDZ	Binding motif (TAZ)
Pen/Strep	Penicillin/ streptomycin
PFA	Paraformaldehyde
p-FAK	Phosphorylated focal adhesion kinase
PI3k	Phosphoinositide 3-kinase
PL	Power law
PMMA	Polymethylmethacrylate
PSD	Power spectrum density
PSDP	Position sensitive photodetector
QCM-D	Quartz crystal microbalance-dissipation
Q-factor	Quality factor
R	Radius of the curvature of the apex of the paraboloid probe
Rac	Ras-related C3 botulinum toxin substance
Ras	Rat sarcoma viral
RFP	Red fluorescent protein

RGD	Arginylglycylaspartic acid
RGP	Radial growth phase
Rho A	Ras homolog A
ROCK	Ros-associated protein kinase
RPMI	Roosevelt Park Memorial Institute medium
RPTP α	Receptor-type tyrosine-protein phosphatase α
RT	Room temperature
SA	Sialic acid
SEM	Scanning electronic microscopy
SFC	Single force-curve
SHO	Single harmonic oscillator
SM4D4	Drosophila mad
SMAD4	Drosophila mad
SphK1	Sphingosine kinase
SPM	Scanning probe microscopy
STM	Scanning tunneling microscopy
SW480E	SW480 Elongated
SW480R	SW480 Round
T	Temperature
TEC	Tumour epithelial cells
TGF- α	Transforming growth factor- α
TGF- β	Transforming growth factor- β
TKR	Tirosine-kinase receptor
TLN	Talin
TME	Tumor microenvironment
TMN	Tumor, Nodes, Metastasis
TNF- α	Tumor necrosis factor- α
TRP	Transient-receptor potential channel
TRPV4	Transient receptor potential vanilloid type-4
TYRP-1	Tyrosine-related protein
UVA	Ultraviolet A
UVB	Ultraviolet B
VCAM	Vascular cell adhesion molecule
VCL	Vinculin
VEGF	Vascular endothelial growth factor
VGP	Vertical growth phase
VWF	van Willebrand factor
Wnt	Wingless and int-1
x	Time average of the vibration amplitude
YAP	Yes-associated protein
YAP/ TAZ	Yes associated protein/ transcriptional co-activator with PDZ-binding motif
z(t)	Piezo displacement time
ZO-1	Zonula occludens-1
ZYX	Zyxin
α	Half-opening angle of a cone of a 4-sided pyramidal
α -MSH	α -melanocyte stimulated hormone
δ	Indentation of the sample

$\delta(t)$	Indentation time
ε	Strain
ν	Poisson coefficient
σ	Stress
τ_l	Loading
τ_d	Dwell
τ_u	unloading
ω_0	Resonance frequency

CHAPTER 1

AIM AND THESIS ORGANIZATION

1.1 Aim and thesis organization

According to the International Agency for Research on Cancer, through the GLOBOCAN 2020, 19.3 million new cancer cases occurred in 2020, worldwide. Female breast cancer has surpassed lung cancer as the most commonly diagnosed cancer, with an estimated 2.3 million new cases (corresponding to 11.7%), followed by lung (11.4%), colorectal (10.0 %), prostate (7.3%), and stomach (5.6%) cancers. Lung cancer remained the leading cause of cancer deaths, with an estimated 1.8 million deaths (18%), followed by colorectal (9.4%), liver (8.3%), stomach (7.7%), and female breast (6.9%) cancers. Europe presents 22.8% of the total types of cancer cases and 19.6% of the cancer deaths, followed by the Americas with 20.9% of incidences and 14.2% of mortality. In Asia, the incidence of cancer is 50% with 58% of deaths. Africa has an incidence of 5.7% and a mortality of 7.2% [1].

Cancer mortality can be reduced if detected and treated early, as cancer responds more effectively to the treatment, resulting in a greater probability of surviving, less morbidity, and less expensive treatments.

Since the 1980s, biophysicists joined medical doctors, chemists, and biologists, using biophysical parameters applied to cancer research to explain biochemical and biological mechanisms. Several biophysical characterization techniques have been used to study cancer cells and tissues. One of these techniques is the atomic force microscopy (AFM), used to study the biophysical parameters of cells and tissues, such as morphometric parameters, mechanical properties, force spectroscopy, and molecular recognition [2]. The current thesis was developed using the AFM technique applied to the study of mechanobiology of colorectal cancer (CRC) and melanoma human cells, providing new insights into the mechanotransduction of these two cancer types. This work can contribute to the use of the AFM as an eventually powerful complementary technique in the surgery room (in real-time), comparing the mechanical properties in the healthy and tumor tissues, since several studies refer that tumor tissues are stiffer (“hard as a rock”) than healthy tissues [3].

This thesis is organized into four chapters:

Chapter 1. Aim and thesis organization and scientific outputs

1.1 Aim and thesis organization

1.2 Scientific outputs

Chapter 2. Introduction

2.1. The importance of biophysics in biological mechanisms

2.2 Mechanotransduction and the importance of nucleus, cytoskeleton and cellular membrane to sense forces and their translation into biochemical signals are highlighted. The importance of the tumor microenvironment (TME), the epithelial-to-mesenchymal transition (EMT) in the cell motion, and the basal membrane (BM) in the cancer invasion are also described.

2.3. A summary of several physical characterization techniques describing if they are suitable of using in cells and/or tissues.

2.4. A brief presentation of atomic force microscope, its components, and characteristics of the atomic force microscope calibration are described, followed by the power spectrum density (PSD) and fitting models. These fitting models include the single harmonic oscillator (SHO) and the Lorentz's.

2.5. Nanoindentation methods (static and dynamic) to determine the mechanical properties in cells are described: conventional force mapping and z-Step response (static methods) and frequency sweep (dynamic method). The fitting algorithms for elasticity and viscoelasticity determination are described: Hertz's, Maxwell's, Kelvin-Voigt's, and Zener's models.

2.6. The application of AFM in cancer research.

Chapter 3. Colorectal cancer (CRC) cells

3.1. It provides a review regarding the mechanobiology of CRC published in **Cancers**, in April of **2022** (doi.org/10.3390/cancers14081945).

3.2. It presents an original manuscript regarding the quantification of mechanical properties of three different CRC cell lines, using AFM. The structure and function of

these cells were studied by CLSM and migration assays. This manuscript was published in **Cancers**, in October of **2022** (doi.org/10.3390/cancers14205053).

Chapter 4. Melanoma cells

4.1. It provides a review regarding the mechanobiology of melanoma, and was published in **Frontiers in Cell and Developmental Biology**, in January of **2020** (doi: 10.3389/fcell.2020.00054).

4.2. It presents an original manuscript on the mechanical properties quantification of human primary neonatal melanocytes (HPNMs) and two melanoma cell lines (WM793B cell line and 1205LU), using AFM. The structure, and function, of these cells were studied by CLSM and migration assays. This manuscript was submitted for publication.

Chapter 5. Conclusion and perspectives

1.2 SCIENTIFIC OUTPUTS

The work performed in the frame of this thesis resulted in the following papers in international peer-reviewed journals

I Brás MM, Radmacher M, Sousa SR and Granja PL. Melanoma in the Eyes of Mechanobiology. *Front. Cell Dev. Biol.* **2020** 8:54. doi: 10.3389/fcell.2020 .

II Brás MM, Sousa SR, Carneiro F, Radmacher M, Granja PL. Mechanobiology of Colorectal Cancer. *Cancers* **2022**, 14, 1945. doi.org/10.3390/cancers14081945.

III Brás MM, Cruz TB, Maia AF, Oliveira MJ, Sousa SR, Granja PL. Radmacher M. Mechanical Properties of Colorectal Cancer Cells Determined by Dynamic Atomic Force Microscopy: A Novel Biomarker. *Cancers* **2022**, 14, 5053. doi.org/10.3390/cancers14205053

IV Brás MM, Sousa A, Cruz TC, Michalewski J, Leite M, Sousa SR, Granja PL, Radmacher M. Microrheological comparison of melanoma cells by atomic force microscopy submitted to Eur. Bioph. J. **2023**

Poster Presentations at International Conferences

I Brás MM. Biomechanics: A biomarker for melanoma diagnosis? 5th i3S Retreat, Speed talk, Póvoa do Varzim, Portuga (November 9 - 10, 2018)

II Brás MM. C Rianna, M Radmacher, JS Sousa, SR Sousa, P Granja. Biomechanical study of healthy and cancer skin cells for melanoma diagnosis. 9th AFM BioMed 2019 Conference, Munster, Germany (September 2 - 6, 2019).

III Brás MM. C Rianna, M Radmacher, SR Sousa, P Granja. Biomechanics: A biomarker for melanoma diagnosis? 8th AFM BioMed 2017 Conference, Krakow, Poland (September 4 -8, 2017).

CHAPTER 2

INTRODUCTION

2. Biophysics of cells and tissues

2.1 The importance of biophysics in biological mechanisms

Biophysics is a science field that uses physical concepts and methodologies to study and explain biological mechanisms [4]. To the question: “What are the major contributions of biophysicists to modern biology?” Zhou provided an answer: “An important contribution of biophysicists is the perspective that biological processes can be understood from the interactions between and within the constituent molecules, so the behavior of biological systems can be predicted from physical principles” [4]. The folding-unfolding proteins [5, 6], the quantification of unbinding forces between molecules [7], the determination of shape, volume, and area of cells [8], and the quantification of mechanical properties of cells and tissues, can be given, in which biophysics play an important role [9-11]. Another relevant question is: “What are the most important directions of biophysics in 21st century?” Again, Zhou proposed: “Biology is becoming more and more quantitative. For example, even a six-fold decrease in Deoxyribonucleic acid (DNA)-binding affinity of a mutant protein may be responsible for a change in phenotype. In the 21st century, early diagnoses and treatments of cancer, Alzheimer’s, arrhythmogenic cardiomyopathies [12], diabetes [13], and other diseases can be anticipated and biophysics will be a central player [4].” Biophysicists are dedicated to solving questions at the molecular level, being focused on studying biological processes at the cellular level.

Cells are the basic building blocks of tissues, being dynamic living structures and having mechanical properties which may change due to the variations of functions and responses to stimuli from the environment [14]. Biological, chemical, and physical processes support the activity of cell machinery. These processes allow cell locomotion, which plays a very important role in biological processes, such as differentiation, growth, invasion, wound healing, remodeling, rebuilding of damaged structures, or formation of metastasis in the case of cancer diseases [15]. During all the previous processes, the cell takes advantage of a variety of shapes and sizes [16].

2.2 Mechanotransduction

Mechanotransduction has received a growing interest. It is defined as the process that translates the external forces sensed by a cell, steaming from the environment such as the ECM stiffness, into biochemical signals and gene expression [17, 18]. It is also mentioned as “the process by which mechanical stimuli are converted into intracellular signals that control cellular physiology in both health and disease” [6]. Those intracellular forces are needed for many cellular functions including cell division, cell motility, muscle contraction, cytokinesis, vesicle, and organelle movement, and cell signaling as well as in cell adhesion, morphology, proliferation, differentiation, and migration [4, 19-21]. It can be classified as being “outside-in” (also called “passive”) or “inside-out” (also called “active”). Figure 1 illustrates passive and active mechanotransduction [22].

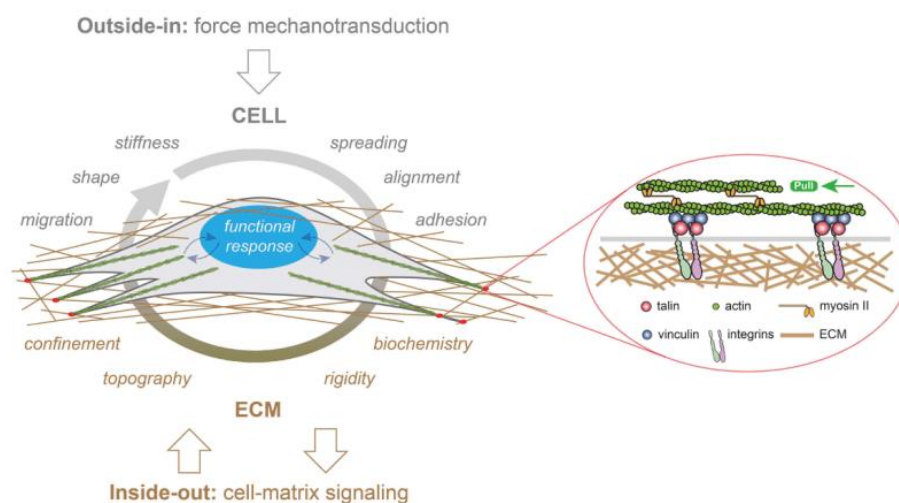


Figure 1. Scheme of “outside-in” (in gray) and “inside-out” (in brown) mechanotransduction. The external forces applied to the cell influence its shape, stiffness, alignment, spreading, migration, and adhesion behavior. The ECM provides multiple cues in terms of confinement, topography, and rigidity. Focal adhesions (in red) are participate in both “outside-in” and “inside-out” mechanotransduction through the integrins located at the cell membrane. Mechanical signals are converted (blue arrows) in biological responses by the nucleus (in blue). Reprinted from Mohammed *et al.* [22].

There are several cellular proteins involved in mechanosensing such as integrins, focal adhesions, and their associated mechanisms (cytoskeleton remodeling, integrin signaling, Ras-homolog (Rho) signaling, and Hippo signaling) [20]. A cell tumor involves alterations in proto-oncogenes and tumor suppressor genes: the proto-oncogenes are activated, while tumor suppressor genes are inactivated [20].

Forces such as tension, compression, shear stress, and hydrostatic pressure are included in the passive mechanotransduction (such as the detection of fluid shear stress by endothelial cells, via a mechanosensory membrane protein complex that bridges the extracellular and intracellular environments) [23].

Another example can be the force from the blood flow passing on endothelial cell and inducing changes in its morphology; cells reorganize and reorient their cytoskeletons in the direction of flow and initiate signaling to neighbor cells, resulting in changes to local vessel elasticity [24]. Active mechanotransduction involves coordinated activity between focal adhesions and retrograde F-actin flow. The change in the stiffness of the microenvironment will need the response of cell physics, adapting the cytoskeleton network in less than 100 ms [25].

Nucleus, cytoskeleton, cell membrane, and ECM matrix have critical roles in mechanotransduction.

2.2.1. Nuclear envelope

The nucleus is connected to the external cell environment through the cytoskeleton, via proteins that cross the cellular membrane. This means that, in terms of mechanotransduction, these connections also provide the basis for mechanosensitivity and force transduction from the external environment to the nucleus [18]. The main mechanical elements exerting forces on nucleus are the nuclear protein, nuclear membrane, membrane proteins, lamina, chromatin, and intranuclear proteins [26]. Figure 2 shows the nucleus proteins and how it is connected to the cytoskeleton through the linker of the nucleoskeleton and cytoskeleton (LINC) [27].

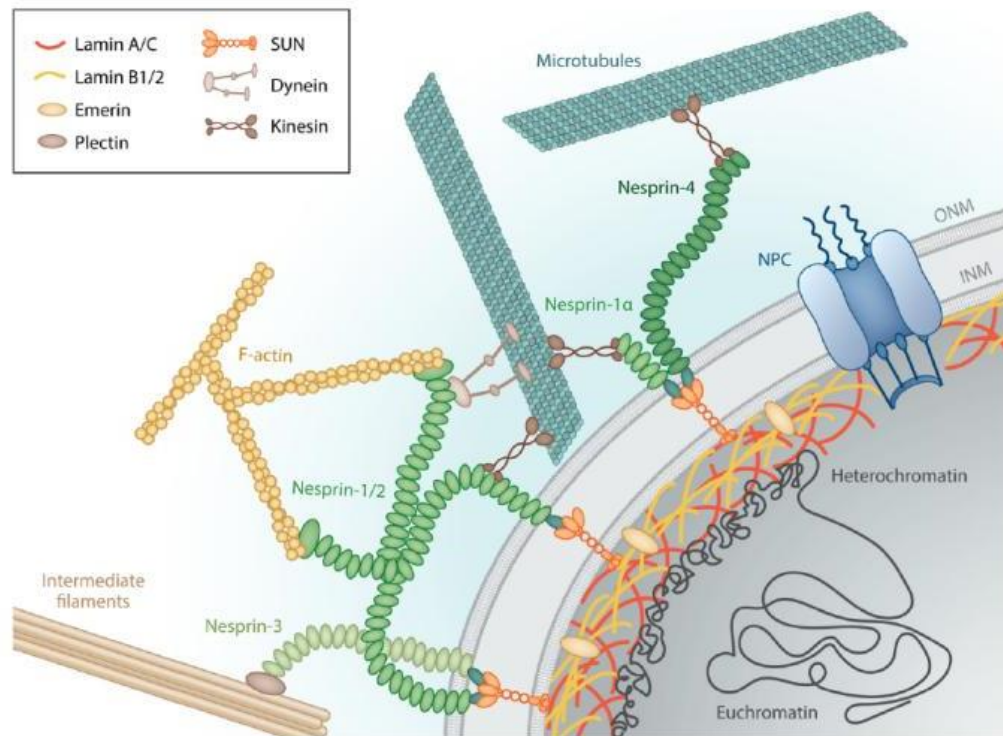


Figure 2. Lamins from the nucleus allow the connection of nucleus to the cytoskeleton, through the LINC, and the nesprin-1,-2,-3 and -4 domains, which interacts with the F-actin, microtubule and intermediate filaments. Lamins A and B stabilize the nucleus and nesprin domains of the LINC complex, which loss, disrupts the cytoskeleton organization within the cell. Reprinted from Vahabikashi *et al.* [27].

The lamina and the nuclear membrane protein forms the inner nuclear envelope. The lamina and the inner nuclear layer are continuous. The lamina is divided in lamina A and lamina B. Lamina A is constituted by lamin A and lamin C [28]. Nuclei that contain more lamin A are stiffer and resist more to deformation. Lamin binds to chromatin and other intranuclear proteins, forming the nucleoskeleton system, resisting to mechanical stress [26]. Nucleus binds to the cytoskeleton through the nesprin, linked to lamin A/ C and cytoskeleton proteins through the linker of nucleoskeleton and cytoskeleton complex (LINC) [26]. This complex is the pathway through which the forces from outside of the cells are transmitted to the inside of the nucleus. The nucleus is also connected to microtubules via kinesin and dynein motor proteins [29]. The network of the nuclear lamina provides support to nuclear size and viscoelasticity of the nucleus when it is subjected to external forces [30, 31]. Weakening of lamin B does not cause any changes in stiffness, meaning that lamin A is dominant to control the stiffness of the

nucleus, which have an elasticity ranging from 0.1 to 10 kPa [31]. Changes in nuclear proteins induce abnormalities in gene expression (such as overexpression of lamin A), signaling, cell division and migration [32].

The nucleus position and cell polarization have an important role in cell migration since the direction of cell migration is given by the polarization of the cell and the position of the nucleus is usually positioned towards the rear of the cell [33]. Generally, in cell migration on 2D substrates the nucleus is positioned behind the centrosome, known as the microtubule organizing centre (MTOC) [18]. The mechanisms responsible for the centrosome orientation relatively to the nucleus are relevant to the cell direction and persistence of migration [18]. It was shown that endothelial cells depleted from nesprin-1, migrated slower, since nesprin-1 depletion resulted in the increase of focal adhesions, cell traction, and nuclear height, and the consequent migration speed decrease [34].

The ability of cells squeezing through constrictions could be limited by the dynamics of the nucleus [35]. For cells passing through constrictions smaller than the nucleus size, the nucleus must deform. Cancer cells associated with lamin mutations have a softer nucleus and seem to pass through small constrictions more easily [35]. Nuclear modulus is also strongly regulated by cytoskeleton behaviour [36]. The study of mechanisms of nucleus mechanotransduction relies on understanding how cytoskeleton-mediated transmission of physical forces from the ECM to the nucleus, which may change nuclear mechanics and architecture, and functions, like gene expression in normal and diseased tissues. Several mechanotransduction pathways join in the nucleus regulating nuclear events, such as DNA replication, transcription, and cell cycle progression. The contribution of the nucleus to mechanotransduction has been very well explained by Pennachio *et al.* [37].

2.2.2. Cytoskeleton

The cytoskeleton is composed of a network of proteins distributed in the cytoplasm, constituted by the actin microfilaments, microtubules, and intermediate filaments. All of them have different mechanical properties, being their function the stabilization of cell

morphology, and definition of its architecture. Actin filaments are the primary force-generating machinery in cells [38]. The cytoskeleton is composed of F-actin, a filamentous polymer, composed of G-actin monomers. This polymerization/depolymerization process contributes to cell motility, regulating several actin-signaling proteins that are part of oncogenic signaling pathways. Usually, epithelial cells have normal differentiation, which is characterized by actin polymerization. Actin depolymerization occurs in the pre-cancerous stages. Cancer progresses *in situ* to an invasive one, co-existing with morphological cell alteration, such as the increase of nuclear-cytoskeleton ratio. Myosin class II motors and myosin classes I, V, VI, X and XVIII are connected to the viscoelastic properties of the cytoskeleton, specially the myosin II, ensuring the bundles formation, with cross-linking proteins, being responsible for cytoskeleton contractibility [21, 39].

The cytoskeleton has also a crucial role in cell mechanotransduction since force transmission through the actin cytoskeleton plays a central role in cell movement, shape change, and internal organization. Figure 3 shows a simple explanation of the mechanism of mechanotransduction through the cytoskeleton [40]. The actin filaments form a kind of gel since the cytoskeleton is a network of cross-linked polymers with permanent links forming a "solid" elastic body. If these links between the polymers have a finite lifetime, this gel has elastic characteristics when deformed briefly ("solid-like" behavior); however, this behavior starts to be defined as "liquid-like", when stresses are applied for a sufficiently long time. This behaviour of the combination of a solid-like and liquid-like state is named viscoelastic. So actin filaments gels are viscoelastic [15]. Microtubules (α - tubulin and β - tubulin) are responsible for the dynamic stability of the cell cytoskeleton [41].

The intermediate filaments (keratins, desmins, vimentin, lamins, nestins, among others) in combination with the microfilaments and microtubules form the structural framework of the cytoskeleton and nucleus, provide mechanical support against cellular stresses and anchor intracellular organelles in place thus enhancing the cell resistance to compression, twisting stretching and bending forces [42].

The intermediate filaments can present several different structural configurations and provide a compliant meshwork in the cell cytoplasm, acting as a "stress absorber" [43].

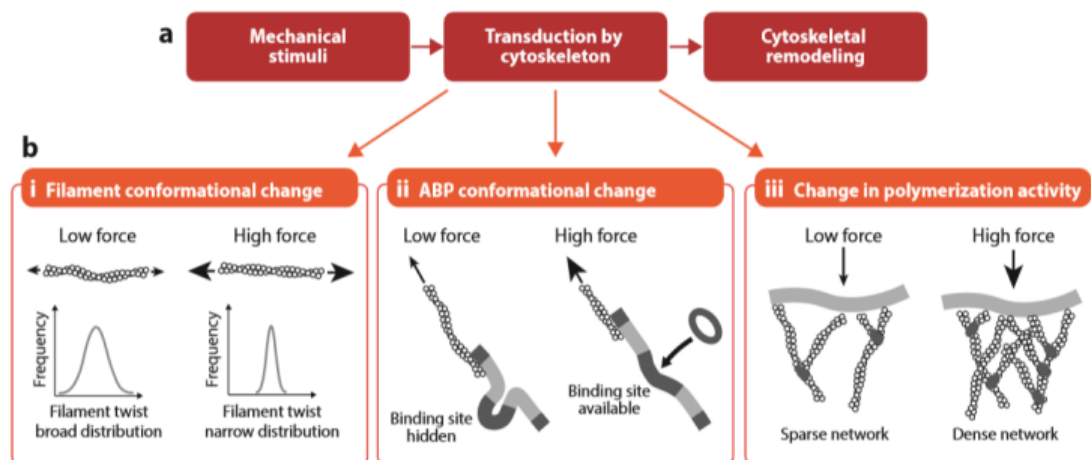


Figure 3. Mechanotransduction and the actin cytoskeleton sensing the forces: a) Cells sense inside the external forces, through the actin cytoskeleton, being translated into biochemical signals. Cytoskeleton remodeling is then induced, promoting the change of the cell shape; b) three main models of mechanical forces transduction by the cytoskeleton are presented: i) change of conformation of actin filaments, under mechanical loadings; ii) proteins binding to actin filaments may change their conformation due to the mechanical loads, contributing to the exposition of binding sites that were not previously available; iii) change of the network density and growth rate, when the actin-binding proteins kinetics polymerization can be influenced by the mechanical load. Reprinted from Harris *et al.* [40].

2.2.3. Cell membrane

Mechanotransduction also influences cellular membrane, once it integrates different proteins, which sense the forces from the neighboring cells and from the ECM. Those proteins (such as the integrins) behave like “bridges” since they sense the forces from the microenvironment to the cytoskeleton. Integrins are transmembrane proteins that are connected to ECM. The main keys regarding of rigidity sense by integrins are: i) the strength of ECM-integrin bond, ii) rates of cell protrusion/ retraction and forces produced during mechanisms and, iii) elasticity associated to mechanosensitive proteins [44]. Adherens junctions are known to be involved in sensing the mechanical microenvironments of cells, similarly to focal adhesions [45]. The cellular membrane also contributes to mechanotransduction process, since there are important transmembrane proteins to transmit the tensions from the outside to inside of the cell, through the focal adhesions (FA) at the cellular membrane [46].

Sens *et al.* referred that tension is known to induce alterations in the cellular membrane, in the regulation of ion channels, playing a crucial role in cell locomotion, shape modification, and volume adjustment [47]. Ren *et al.* recently studied, by AFM, the effect of membrane tension through the use of the standard solid model applied to the cytoskeleton, which allowed the quantification of the viscoelasticity through the force-curve approach as a function of time (stress relaxation) [48]. The decrease in cell stiffness is associated with changes in the cell membrane surface. The authors showed that approximately 5% of the force exerted by the cell on the probe was from the cytoskeleton and the remaining force came from the cell membrane, which allowed hypothesizing that the reduction of the cancer cell stiffness was due to the decrease of the cell membrane tension [49].

Mechanosensitive ion channels in cancer progression are also well documented, especially those involving in Ca^{2+} signaling and their relevant function in tumor physiology [50]. Mechanosensitive channels can be blocked directly by a physical force or downstream signaling cascades. Mechanosensing can be classified as being direct or indirect, as it is very well explained by Petho *et al.* [51]. Briefly, the direct mechanosensing is caused mainly by the physical changes of the plasma membrane and the indirect mechanosensing is a multi-step process that can be mediated through various pathways (Figure 4) [51].

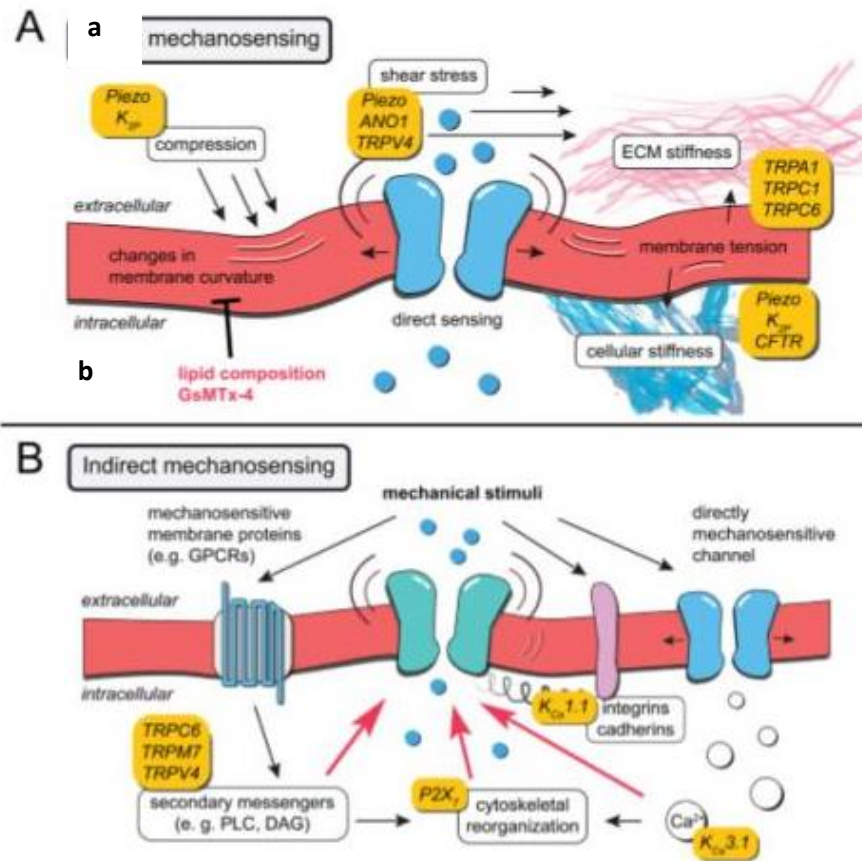


Figure 4. Mechanosensitive channels can be controlled directly by a physical force or downstream signaling cascades. (a) Direct mechanosensing: in tumor tissue, compression causes the physical changes in the plasma membrane, leading to cell membrane curvature, opening some channels (Piezo and Two-pore domain potassium (K2P)). Other molecules such as GsMTx-4 inhibit mechanosensitive channels, caused by physical distortion of membrane. Tumor environment is often associated with fibrosis and increased ECM stiffness, which may cause the increase in membrane tension. Piezo, K2P, cystic fibrosis transmembrane conductance regulator (CFTR) and several transient-receptor potential channel (TRP) channels present on plasma membrane, through the ion fluxes, mediating tumor cell responses. Shear stress sensors (Piezo, anoctamin-1 (ANO1) and transient receptor potential vanilloid-4 (TRPV4)) mediate ion fluxes in healthy and tumor-associated cells. (b) Indirect mechanosensing is a multi-step process that can be mediated through various pathways. Mechanosensitive proteins on the plasma membrane are affected by mechanical stimuli, activate intracellular cascades, leading to the opening of mechanosensitive channels. For instance, stimulation of G-protein coupled receptors (GPCRs) evokes the production of secondary messengers, which in turn activate several transient receptor potential (TRP) channels. Cytoskeletal reorganization and integrin/cadherin signaling may also cause channel opening, as observable in the case of purinergic type 2 receptor family (P2X7) and calcium-activated potassium channel subunit alpha-1 (KCa1.1), respectively. Reprinted from Petho *et al.* [51].

Ren *et al.* used the AFM to measure the force relaxation curves in different cell types (L929 mouse fibroblasts, 4T1 mouse breast cancer cells, and MCF-7 human breast cancer) to measure cell membrane tension, using force indentation experiments. The authors hypothesized that decreasing the cell membrane tension, seems to contribute to explaining why the cancer cells are softer than the normal ones [49].

2.2.4. Tumor microenvironment

The tumor microenvironment (TME) called by the Zhou *et al.*, “the Sanctuary of the devil”, is the physical support for tumor cells to adhere to, to receive nutrients, and avoid the immune system, inducing the appearance and development of tumors [52]. Zhou *et al.* suggest that TME is mainly, divided into two parts: (i) the biochemical microenvironment, and (ii) the mechanical microenvironment. Due to the changes in biochemical components in the TME, the physical properties are dramatically changed [52]. Uncontrolled proliferation of tumor cells constantly compresses adjacent tissues and tumor vasculature, generating internal high-pressure solid stress and further resulting in increased blood/interstitial pressure [52]. The imbalance between pro- and anti-angiogenic factors causes abnormal tumor vasculature that generates high pressure on the TME: this pressure is called fluid stress. Alterations in the composition and density of the ECM occur frequently in tumors [52]. Cancer-associated fibroblasts (CAFs) are constantly remodeling (by depositing or crosslinking) the ECM leading to increased stiffness and altered topology [52]. Stiffness (rigidity) refers to the resistance to deformation in response to applied force, which is measured as Young’s elastic modulus. Topology comprises ECM porosity, fibril orientation, and other fibril characteristics, which wield an important influence on cell polarity and function [52]. Figure 5 shows the complex events around tumor [53].

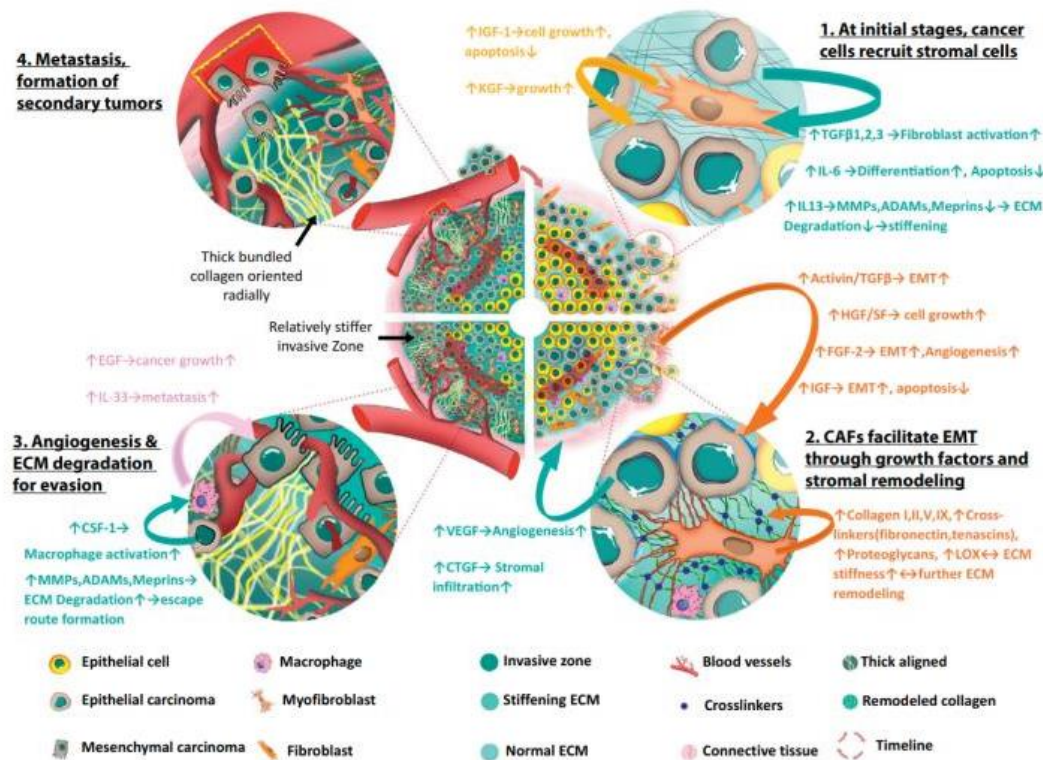


Figure 5. In stage 1, epithelial cancer cells secrete several factors that induce fibroblast activation, decreasing of the ECM degradation by the metalloproteinases (MMPs), and stiffness increase. In stage 2, due to the activation of cancer-associated fibroblasts (CAFs), several growth factors are secreted promoting cell growth and angiogenesis and the remodeling and reinforcement of ECM collagen deposition, increasing stromal stiffness. Due to tumor growth, it becomes hypoxic and some growth factors are segregated such as vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF) supporting the angiogenesis. In stage 3 carcinoma cells, due to EMT, activates several molecules that promote metastasis, and enter in the lymphatic or bloody system. In stage 4, cell migration occurred, adhering far away from the primary tumor, and developing other tumors. Reprinted from Emon *et al.* [53].

The TME is mainly composed of the cellular component (stromal cells, fibroblasts, pericytes, adipocytes, immune cells, etc.), the ECM, and the vasculature (blood and lymphatic vessels) [54]. Several studies were focused on its biochemical cues, such as hypoxia, low pH, inflammation, and immunosuppressive properties, but many aspects must be clarified regarding mechanical cues. Cells at TME are subjected to internal and external forces, which are important in mechanosensing and mechanotransduction [55, 56].

The ECM can be characterized by topography, roughness, and stiffness. The latter can be defined as being the resistance that a material offers to the elastic deformation when

uniaxial stress is applied [57], influencing the cell behaviour and responses. It provides both mechanical and biochemical signaling cues, thus being an indispensable and dynamic structure and directly affecting cellular behavior [57]. Interruptions can occur either in the physical (e.g. porosity), biochemical (e.g. growth factor binding capacity), and/or biomechanical (e.g. stiffness) properties of the ECM [58]. Some of these properties support and regulate cell differentiation, adhesion, survival, migration, and proliferation rates. During cancer progression, a deeper understanding of the ECM compositional changes is crucial to developing better and more targeted treatments [58]. The natural tumor ECM microenvironment has viscoelastic and plastic characteristics, being a dissipative tissue, which characteristics alter cellular response [59]. One cause of tumor stiffness due to variations in the tumor microenvironment is the unbalance between ECM production and its degradation [60]. The ECM stiffness increase has shown the promotion of malignant phenotypes in breast cancer and brain cancer [61, 62].

In 2D models, it is easier to study several physical parameters but not angiogenesis (new vessels formation from pre-existing ones), since it cannot be reproduced in a 2D model. The angiogenesis process is essential when a tissue mass transition from a benign state to a malignant state, in order to avoid tumor hypoxia. 3D cell culture models are an emerging powerful approach for studying ECM mechanics and cellular responses, although they still lack the cell and matrix complexity of natural tissues. Reidy *et al.* described the evolution of 3D models used in CRC studies, as well as their advantages and disadvantages [63]. They showed their utility to predict the response to therapies and resistance. It was shown that an increase in stiffness promoted CRC progression and difficulties in drug delivery, thereby promoting resistance [63]. 3D studies are fruitful since they provide a more similar environment to the native structure (such as 3D geometry, porosity, ECM binding site, ECM heterogeneity, and gradients of biochemical factors, among others). However, it is recognized that there are still several challenges to overcome, namely the difficulties in controlling the environment and in visualizing cell-cell and cell-ECM interactions [49].

Integrin clusters, which morphological changes or distribution, recruit focal adhesion kinase (FAK), sense extracellular alterations in stiffness, tension, or other mechanical

stimuli. Talin, vinculin, paxillin, and adaptor protein p130Cas anchorage to each other and transfer the mechanical cues from integrins to the actin component of the cytoskeleton [21]. In close proximity to the focal adhesion inner core, vasodilator-stimulated phosphoprotein (VASP), zyxin and actinins complex directly regulate actin assembly and dynamics (Figure 6) [21].

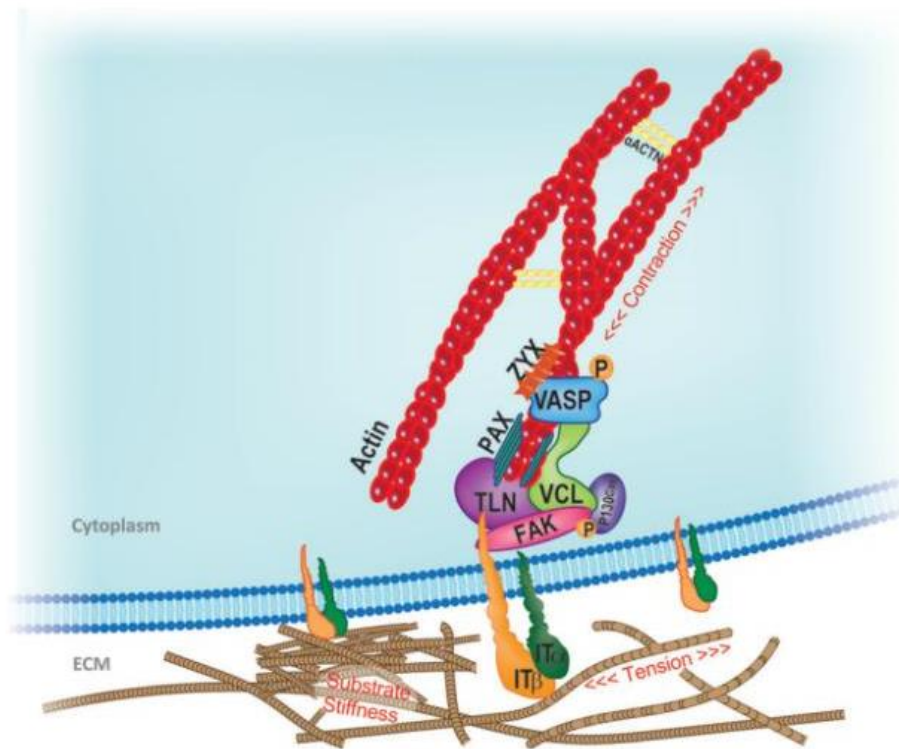


Figure 6. Cell-ECM interaction through the focal adhesions. Integrins clusters sensed the changes in ECM stiffness, tension, or other mechanical stimuli. These morphological changes recruit FAK, talin, vinculin, paxillin, and adaptor p130, transferring the mechanical forces through the integrins to the actin of the cytoskeleton. In close proximity with the FA inner core, VASP, ZYX and actinins complex directly regulate actin assembly and dynamics. ACTN: actinin; FAK: focal adhesion kinase; IT: integrin; PAX: paxillin; TLN: talin; VASP: vasodilator-stimulated phosphoprotein; VCL: vinculin; ZYX: zyxin. Reprinted from Martino *et al.* [21].

Many stress fibres in a cell may induce tension at contact points, such as cell-matrix adhesions and/or cell-cell junctions [46]. Tension in cell-matrix junctions acts through

the FAs (vinculin, talin), having structural and mechanosensing roles [64]. FA proteins connect F-actin to transmembrane receptors known as integrins, which connect to ligands in the ECM. The forces produced by myosin are transmitted through the focal adhesions to the integrin-ECM interface, where the tension forces appear.

The basement membrane (BM) avoids the spreading of cells from the primary tumor, at the stage of carcinoma formation, since it constitutes a barrier in epithelial cancer or carcinomas avoiding the cell invasion into the stroma tissue [65]. This BM is composed of laminin and type IV collagen networks, produced by coordination between the epithelial cells and stromal fibroblasts [66]. It provides structural support to the epithelium, promotes cell adhesion, sustains cell polarity, and it is essential for tissue compartmentalization by separating the epithelium from the stroma [67]. BM avoids the invasion of the carcinoma cells into (intravasation) and out of (extravasation) contributing to the metastization [68]. Cancer cells perforate the BM using MMP-rich protrusions, called invadopodia, and stromal cells can contribute to this process since they also produce matrix proteases [69-71].

In response to applied force, cells will respond with cytoskeleton organization, ECM remodeling, gene expression, differentiation, migration, and apoptosis. Due to contractile forces, cells induce viscoelastic deformation, wrinkle formation, fiber alignment and matrix compaction [47].

2.2.5. Epithelial-to-mesenchymal transition

In the primary tumor niche, the generation of internal forces provides the motility of some cells [72]. The cells lose the connections between them (E-cadherin connections give rise to the N-cadherin) characteristic of the epithelial-to-mesenchymal transition (EMT), in which the epithelial cell phenotype gives rise to mesenchymal cell phenotype [72]. The EMT process has a key role in the acquisition of migratory or invasive phenotypes in several processes (Figure 7) [72].

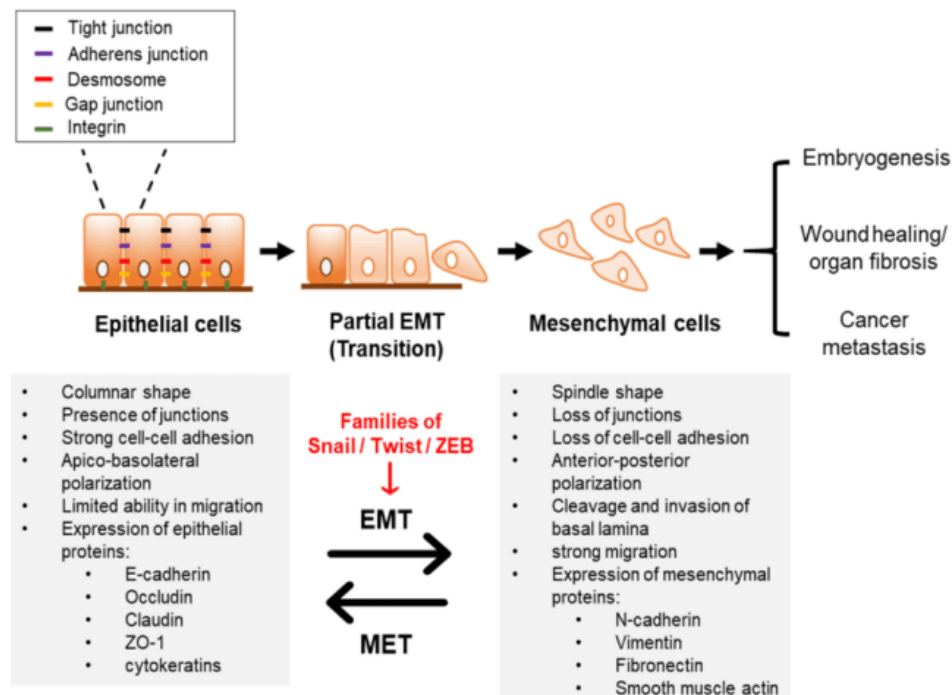


Figure 7. The EMT mechanism: Epithelial cells are attached to the basal membrane. Different cell-cell connections, such as adherens, tight and gap junctions, and desmosomes, maintain the cells connected. The process of EMT causes the loss of epithelial markers (e.g. E-cadherin, claudin, ZO-1), while mesenchymal markers (e.g. N-cadherin, vimentin, and fibronectin) increase, causing changes in the physiology of cells, so cells acquire high motility and invasive properties. This process is regulated by EMT-TFs, such as families of snail, twist, and zinc finger E-box-binding homeobox protein (ZEB). EMT has an important role in physiological processes, such as cancer. Reprinted from Varga *et al.* and Kang *et al.* [72, 73].

2.2.6. Cell motion

Cells are entities characterized by locomotion, which results from the coordination of motions generated by different parts of the cell. Locomotion is the basis for several mechanisms such as extravasation, wound healing, and cell migration [74]. Cell mobility is a process in which the cells are moving without the need for energy. Cell mobility also contributes to the cell movement across the body fluids, like lymph and blood vessels [75]. The internal forces generated due to the active behaviour of the actin cytoskeleton structure, drive cell motility [76]. It involves different steps: a) Protrusion of a thin layer of the cytoskeleton structure, the lamellipodia, at the leading edge of the cell; b) adhesion of the cell on the substrate due to focal adhesions; and c) contraction of the cytoskeleton

[77]. The front of lamellipodia, an actin-rich, ca. 100 nm thick protrusion containing a high network of actin filaments, protrudes due to the forces generated by actin polymerization, forming structures like fingers, the filopodia (presenting actin bundles). They are involved in the disassembly of the actin network at the rear end of a cell, pulling the cell forward. This process has a consumption of energy that is provided by the hydrolysis of adenosine triphosphate (ATP), which drives actin polymerization and depolymerization and the motion of myosin motor proteins [15]. Actomyosin contraction is caused by myosin II molecular motors (mini filaments) interacting with actin filaments. Myosin moves along actin filaments. One or more F-actin filaments can be bound to clusters of myosin motors at the same time. Myosin motors have to walk in different directions too, if two actin filaments have different directions, induce stress on the filaments, promoting the actomyosin contraction [18]. Figure 8 shows the relationship between some proteins such as Rac, Rho and Cdc42 and cell movement, if some of these proteins are inactivated, no lamellipodia are formed [78].

There are two main types of migration: i) Single-cell movement and ii) collective migration of multicellular compartments [35]. The migration of single cells can be divided into ameboid and mesenchymal types of movement [79]. Ameboid movement is characterized by cells moving as round, ellipsoid shape without the involvement of focal adhesions and cell-substrate attachment but with the blebs formation, called the blebby ameboid migration, and is used by embryonic cells, leukocytes moving through the ECM (3D migration) and some cancer cells [80]. The second form of migration is characterized by actin-rich filopodia and weak substrate interactions such as neutrophils and dendritic cells or tumor cells [81, 82]. Mesenchymal migration involves the focal adhesions, strong attachment to the ECM, cytoskeleton contractility, and elongated spindle-like cell bodies as it is shown in Figure 8 [83]. Fibroblasts, sarcoma cells, and highly differentiated tumor cells, which use the EMT transition, use the last mode of motility.

Collective migration is the movement of a cluster of cells through the ECM, preserving the cell-cell junctions [84]. This movement occurs on 2D surfaces, when epithelial sheets travel together across basal membranes (wound healing) [85, 86].

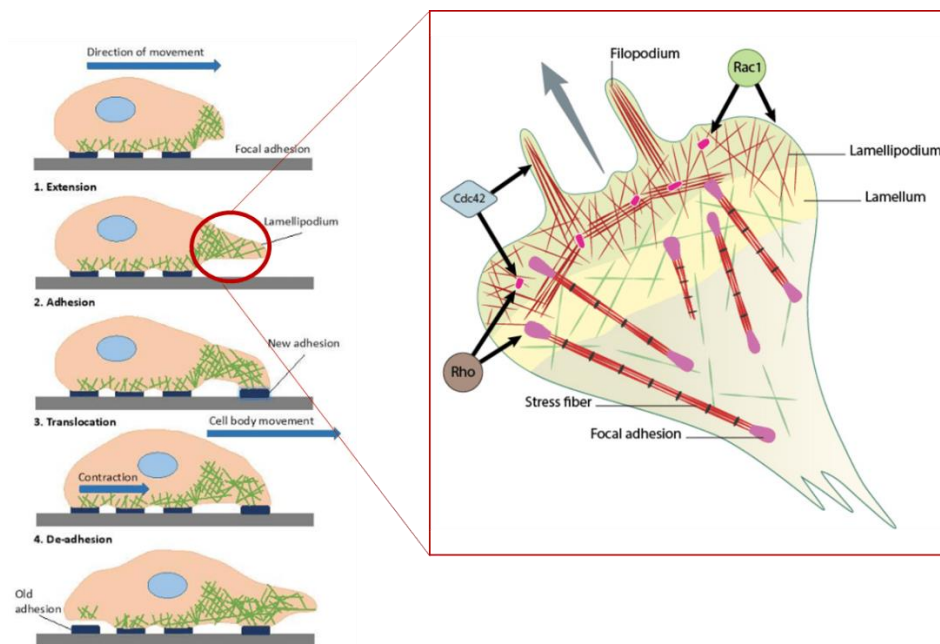


Figure 8. Representative 2D cell migration due to cell polarization, lamellipodia formation and the role of Rac, Rho and Cdc42 proteins. 1) Lamellipodia extension and Cdc42, Rac1 and Rho act at parts of the cell support migration. The cell polarity and filopodia formation are controlled by Cdc42, as well as nascent focal adhesions (shown as yellow dots). Rho is responsible for cell adhesion assembly and maturation, controlling stress fiber formation and contractile activity. Rac1 controls actin assembly and nascent adhesion formation in the lamellipodia 2. New adhesions formation. 3. The translocation of the cell body. 4. The de-adhesion and retraction at the trailing edge. Adapted from Burridge *et al.* [87].

Cell motility has also the contribution of the water flow and the hydrostatic and osmotic pressure [88]. Water can pass through the cell membrane directly or through the aquaporin channels existing in the cell membrane [88]. The direction and magnitude of the water flux depend on the water chemical potential difference across the cell membrane, which is influenced by hydrostatic pressure difference and osmotic pressure [89]. The water flow also plays an important role in the blebbing membrane and in the ameboid mode of cell migration [90, 91]. The extruded blebs are refilled with the cytoskeleton and the cell moves by repeated development of blebs [92-94]. In non-physiological conditions, the osmotic effects can drive microfluidic pumps [95]. All these processes contribute to cell shape and volume changes.

Cells in motion cause the disruption of the tumor mass basement membrane providing the cell invasion through the ECM [68]. The cancer cells adhere to the basement membrane of the vessel, doing the intravasation into the blood or lymph vessels. In these vessels, the cells migrate, and they are subjected to shear flow stress, which is different regarding the fluid type: lymph (64 mPa) and blood (0.1 – 300 Pa) [96]. In some cancer types, like melanoma, the extravasation could be undergoing as a transcellular or paracellular process [9].

Collective migration is the movement of a cluster of cells through the ECM, preserving the cell-cell junctions [84]. This movement occurs on 2D surfaces, when epithelial sheets travel together across basal membranes (wound healing) [85, 86].

In summary, Figure 9 shows the cell mechanical stimulation, inducing mechanism of cell response like differentiation, proliferation, apoptosis etc.

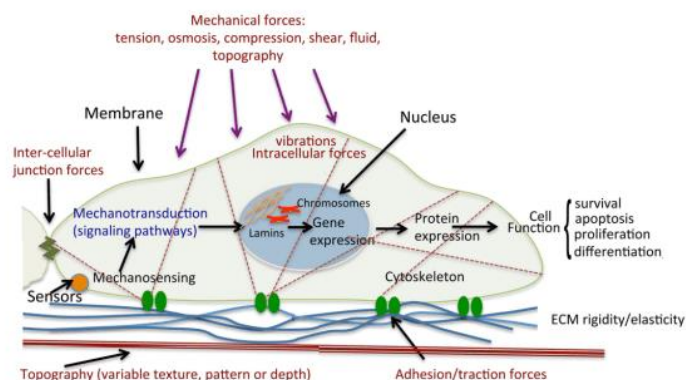


Figure 9. Cell mechanical stimulation. Mechanosensors of the cells, such as integrins, are activated due to external forces, such as ECM rigidity/elasticity, topography, tension, osmosis, compression, shear, fluid, and contact with neighboring cells. Inside the cells, the cytoskeleton generated tractile forces due to actomyosin contraction, microtubule polymerization, and depolymerization. The sensors activate several biochemical cascades, resulting in the modulation of protein expression and cellular functions, such as survival, proliferation and differentiation. Reprinted from Tsimbouri *et al.* [97].

Figure 10 shows a representative scheme about mechanotransduction pathways related to polarized migration of tumor cells. Forces are transmitted from the TME to the intracellular environment through the mechanosensors activation existing at the cell

membrane (such as integrins, FA and caveolin-1). Forces are sensed at the cytoskeleton or other signaling pathways, such as integrin-FAK, Ras homolog (Rho), and Hippo signaling. The nucleus responds to mechanical forces from the TME through the LINC complexes and lamins (changing the chromosomal organization and gene expression). All the processes contribute to tumor migration [52].

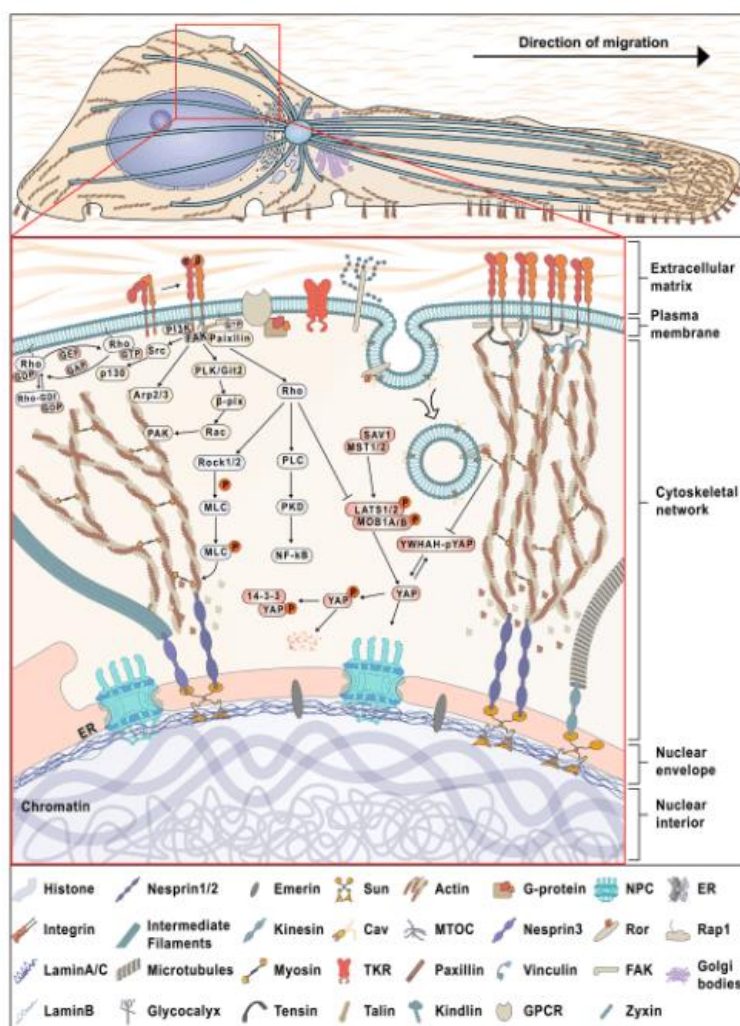


Figure 10. Mechanotransduction pathways related to polarized migrating tumor cells. Forces induce the activation of mechanosensors (integrins, focal adhesions and caveolin-1), transmit the forces in the TME to intracellular locations through the cytoskeleton, which is directly linked to abundant mechanosensors or other signaling pathways, such as integrin-FAK, Rho and Hippo signaling. Cytoskeleton transmits the forces to nucleus through the LINC complexes and lamins, changing the chromosomal organization and gene expression, promoting cell tumor migration. ER - Endoplasmic reticulum; FA - Focal adhesion; FAK - Focal adhesion kinase; GPCRs - G-coupled receptors; LINC, - Linker of nucleoskeleton and cytoskeleton; MTOC - Microtubule organizing center; NPC - Nuclear pore complex; TME - Tumor microenvironment; TKR - Tyrosine kinase receptor. Reprinted from Zhou *et al* [52].

In conclusion, if a cell does not translate well a mechanical cue or finds itself in an abnormal environment, its normal functioning will be disrupted, and disease will be installed [4]. Several pathological diseases, such as asthma [98], osteoporosis [99], deafness [100], atherosclerosis [101], glaucoma [102], muscular dystrophy [103], and cancer [62] can be directly caused by irregular cellular or tissue mechanics [4].

2.3. Mechanical characterization techniques of cells and tissues

Different characterization techniques can be used for the quantification of mechanical properties of cells and tissues, depending on the type of cell or tissue, sample properties (such as size, shape, composition, etc.), and experimental design and requirements. This section will address the mechanical characterization techniques suitable for cells and tissues, which tend to be ultra-soft and viscoelastic. In the case of cells, the most relevant factor is whether the method requires them to be adherent or allows them to be suspended in solution. The former is essential when tackling the mechanical properties in adherent cells, tissues, and artificial matrix environment and the latter is appropriate for cells from the bloodstream. The measurements may represent globally the sample (e.g. the average mechanical properties of a suspended cell, as in pipette suction), or whether it uses a local probe, as in AFM, being able to distinguish different areas of a cell. Regarding the force or deformation applied, it can be performed in (quasi-)static or dynamic conditions. Strictly speaking, every method will allow varying force or deformation. Here, methods will be considered dynamic only when the rate of deformation can be varied over several orders of magnitudes, over a range of frequencies.

In general, there are two different types of methodologies used for cell mechanics studies: force-application (Figure 11) and force-sensing techniques (Figure 12) [104].

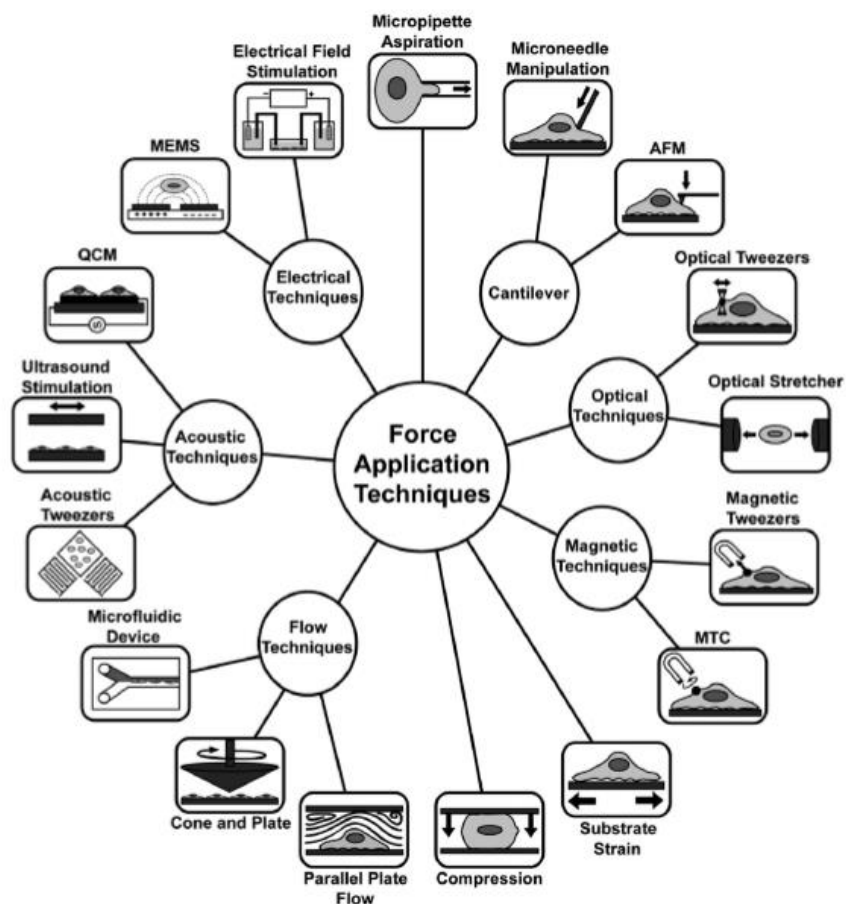


Figure 11. Force application techniques. Reprinted from Rodriguez *et al.* [104].



Figure 12. Force sensing techniques. Reprinted from Rodriguez *et al.* [104].

Generally, all of the above methods either apply a force and measure the resulting deformation, or apply a deformation and measure or infer the applied force, the design and physics, behind each instrument, may be very different. In a stretching device, the sample is plated or mounted on a soft and stretchable thin film, which is then elongated [104]. In micropipette aspiration, a cell is sucked into the opening of a small pipette by hydrodynamic pressure or rather suction [52]. Shear flow applies a liquid flow along the surface of cells or tissues and thus applies a shearing fluid force [105]. Dynamic mechanical analyzer (DMA) is suitable to study the viscoelastic properties of polymers, applying sinusoidal stress and measuring the strain of the sample, as a function of temperature and frequency, being performed in air or in liquid [106]. A micro-indenter, using a micrometer-sized stylus or tip, will indent cells or tissues [107]. With the rheometer, the sample is located between two plates allowing the application of a shearing (rotating) force at various frequencies, thus measuring the mechanical response as a function of frequency [108]. In an optical tweezer, a particle (often a micrometer-sized bead) is trapped in a laser focus, moved around, and used to apply a force, locally [104, 109]. In the optical stretcher, the optical force directly deforms cells; often two opposing laser beams are used, in order not to move the cell, but only to deform it [104, 110]. Atomic force microscope is similar to micro indenters. However, the atomic force microscope tip can be smaller and of arbitrary shape (often pyramidal or spherical), the force resolution is improved and there is the possibility to position the tip on the sample, combined with the ability to achieve topographical images [111]. Magnetic twisting offers the possibility for a cytometer using small magnetic beads bound to the surface of cells where rotating forces can be applied [112]. Quartz crystal balance with dissipation (QCM-D) is based on the changes of resonance frequency and energy dissipation on an acoustic wave, being possible to obtain information about the mass changes of the adsorbed sample [113]. Elastography is based on the distortion of the tissue, by pushing/deforming or vibrating the surface of the human body or organ. With a probe or a tool, it measures the degree of torsion, which will depend on the stiffness of the tissue [114]. Microelectromechanical systems (MEMS) can use a probe, and a micro flexible beam, acting as a force sensor, both being made of crystalline silicon or other materials. The piezoelectric material holds the sensor base (silicon chip). The probe can be coated with a protein, which simulates the ECM-cell adhesion. There are focal

adhesions formed between the cell and the protein. The cells are stretched by moving the piezoelectric in the opposite direction of the cell and which will apply a force on the probe that, in turn, is measured by the deflection beam [115]. Fluidic techniques include: a) constricted geometry with the construction of structures on a microfluidic platform, deforming single cells (the time of cell passage and cell's velocity are measured) [104]; b) aspiration-induced, imply the use of microfluidics targeting the cell centre between two converging streamlines, characterizing the deformation of the cell [4, 115, 116]; c) electrically-induced is also a mechanical manipulation, which principle is based in the movement of any particle through an orifice along with the electric current, producing a change in the impedance, due to the movement of electrolytes caused by the particle motion. The effective voltage that crosses the cell membrane is a function of cell diameter, so a cell with a higher diameter, when exposed to a higher voltage, would increase its permeability [115]; d) micro cytometer contains several micro channels which are functionalized with an antibody that is specific for antigens of living cells under certain regulated hydraulic pressures. It deforms cells, analyzing the cell size, deformation/stiffness, and levels of surface receptors for a single living cell [104]. Dynamic mechanical analyzer (DMA) was included [117] as well as the rheometer [118] for completeness, although these instruments only allow the investigation of macroscopic samples and their global properties, being thus only suitable for tissues but not applicable for one cell.

Table 1 presents the characterization techniques that were classified considering the following features: a) static or dynamic; b) local or global; c) used in adherent or non-adherent cells or tissues.

All the techniques described allow the determination of mechanical properties. DMA, rheometry, nanoindentometry, and elastography are suitable for analysis at the macroscale, while the AFM at the nanoscale, allowing also assessing the contribution of the cells and ECM in several pathologies.

Table 1. Characterization techniques for the mechanical properties quantification on biological samples.

Method	Instrument	Static (S) or dynamic (D)	Local (L) or global (G)	Cells / tissue	Refs.
Optical	Optical stretcher	S	G	Non-adherent cells	[104, 119, 120]
	Optical tweezer	S/D	L/G		[104, 121, 122]
	Traction force microscopy	S	G	Adherent cells	[104, 123]
	Optical coherence elastography	D	G	Tissues	[124]
Local probing	AFM	S/D	L	Adherent cells/tissues	[11, 125, 126]
	Microneedle manipulation	S	L	Adherent cells	[104]
Acoustic	Acoustic tweezers	S	G		[104, 127]
	Ultrasound stimulation	D	G		[104, 127]
	Quartz Crystal Microbalance – Dissipation (QCM-D)	D	G		[104, 128]
Electrical	Electrical field stimulation	S	G		[104]
	MEMS	S	G		[104]
Magnetic	Magnetic tweezers	S	G		[104, 129]
	Magnetic twisting cytometer	S/D	L		[104, 130]
Fluidic	Parallel plate flow chamber	S	G	Non-adherent cells	[104, 131]
	Cone-and-plate	S	G		[104]
	Shear flow	S/D	G	Non-adherent cells/tissues	[104]
Others	Stretching device	S	G	Non-adherent cells	[104]
	Micropipette aspiration	S	G		[104, 132]
	Dynamic Mechanical Analyzer (DMA)	S/D	G	Tissues	[117]
	Nanoindenter	S/D	G		
	Micro post arrays	S	G	Adherent cells	[133]
	Rheometer	S/D	G	Tissues	[134]

Note: Local (L) – Use of an instrument to study a specific part of the cell or tissue;

Global (G) - Use of an instrument to study the entire sample.

Some non-invasive characterization techniques have been used to measure the mechanical properties on the skin (natural and artificial) to evaluate the elasticity of this organ, such as: pipette suction, twistometry, levarometry, indentometry, acoustic waves,

and elastography [135-137]. Some of these techniques (indentometry) helped researchers to distinguish between young and old age skin [138].

From all mechanical techniques discussed here, AFM seems to be the most promising one given its wide range of applicability, from the analysis of isolated live cells to tissues in their physiological state, allowing to dynamically following the response. However, for non-adherent cells, e.g. those circulating in fluids like blood, other methods, such as shear flow or optical tweezers, are more appropriate.

2.4. Atomic force microscopy (AFM)

AFM belongs to the scanning probe microscopy (SPM) techniques invented in the 1980s. AFM was invented in 1986 by Binnig, Quate and Gerber inspired in the scanning tunnelling microscope (STM) [139]. In spite of the STM achieves resolutions of atomic size, the AFM came to overcome one limitation of the STM: it was only possible to use it on conductive samples. AFM is a surface characterization technique using a probe attached to a cantilever, which scans the surface. The surface scanning is performed like an “ancient record player”, with the difference that the equipment incorporates a huge number of improvements in order to achieve the atomic-scale resolution [140]. Basically, the equipment is constituted by (i) a scanner made of a piezoelectric ceramic material (it has the ability to increase or decrease the size in all three dimensions, according to the applied voltage) [141]; (ii) a holder that is positioned inside of the scanner hole; (iii) a cantilever that is fixed to the holder; (iv) and a laser beam aligned on the back of a cantilever, in the direction where the tip is. During scanning, the laser beam is reflected to a position sensitive photodiode, to measure the cantilever deflection. The whole system is controlled by an electronic part called a “feedback loop”, that maintains the force constant, between the sample and the tip. When the sample is approached to the cantilever tip, contact forces (electrostatic, van der Waals and most general quantum mechanical repulsion) are generated between the tip and the sample, pushing the tip into contact with the sample, inducing a cantilever deflection, which is often kept constant during the scanning [140]. The deflection signal (measured in V) of the cantilever needs to be calibrated to convert in a deflection (measured in nm) by multiplying it with deflection sensitivity, or more accurately with the inverse optical

lever sensitivity (InvOLVS), measured in nm/V. Multiplying the deflection with the force constant of the cantilever will give you the loading force (in N). The photodiode is divided into four quadrants where the difference between the upper and lower two segments gives the deflection signal, whereas the sum of all four segments measure the entire intensity, usually called sum signal. [140]. These parameters must be adjusted according to the mode and experiments performed. Figure 13 shows a schematic representation of the AFM principle [142].

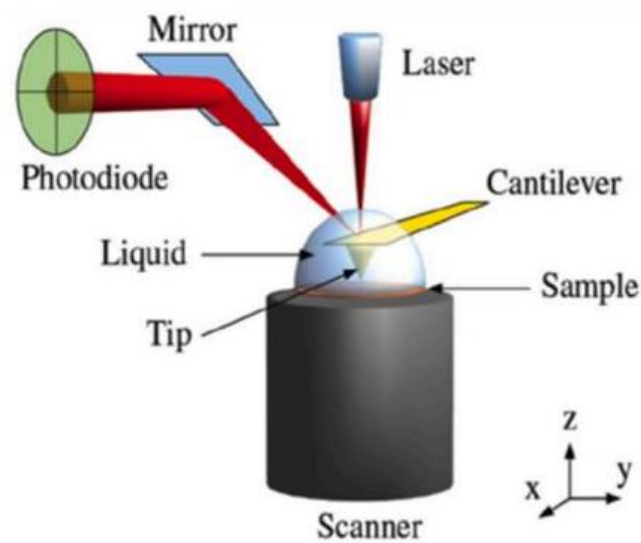


Figure 13. AFM working principle. A laser beam is aligned on the back of a cantilever, where a spherical or pyramidal tip is on the opposite side. When the tip scans the surface, the cantilever will bend with a certain deflection. The deflection of the cantilever is reflected in the photodetector (or photodiode). Through the application of Hooke's law, the force applied to the cantilever during scanning is calculated, by multiplying the spring constant (k) with the cantilever deflection. Reprinted from Hammond *et al.* [142].

The AFM technique presents several advantages when compared with other microscopic techniques. The sample is analyzed without any type of preparation, almost all type of samples can be analyzed in physiological conditions, with high resolution (given that it can use very sharp tips, which apex radius can go down to 2 nm). Particles in suspension, cannot be analyzed since they compromise the stability of the tip [140]. AFM can be used as a static or dynamic technique. An example of AFM used as a

dynamic technique is the characterization of mechanical properties using the frequency sweep method [10].

Cantilevers are a key element of the AFM, being its mechanical properties responsible for the performance of this equipment. Cantilevers are made of silicon or silicon nitride, covered with a native oxide layer of 1-2 nm thickness [143]. Some of them are coated with a gold layer on the back to increase the reflection capacity when used in liquid. Cantilevers are made by nanofabrication processes and are characterized by the following parameters: material composition, shape, length, width, thickness, height, resonance frequency, Q-factor and spring constant (or stiffness) [143]. Tip cantilever assembly is performed using a technology similar to integrated circuit fabrication, allowing good reproducibility. The most important parameter is the sharpness of the apex, measured by the radius of curvature (spherical approximation) (Figure 14) [140]. The tip can also be spherical, which is suitable for nanomechanics assays in soft samples (cells and tissues). However, it is not appropriate for imaging, given that image formation is due to the effects of the interaction forces between the tip and surface, during the scanning over the sample.

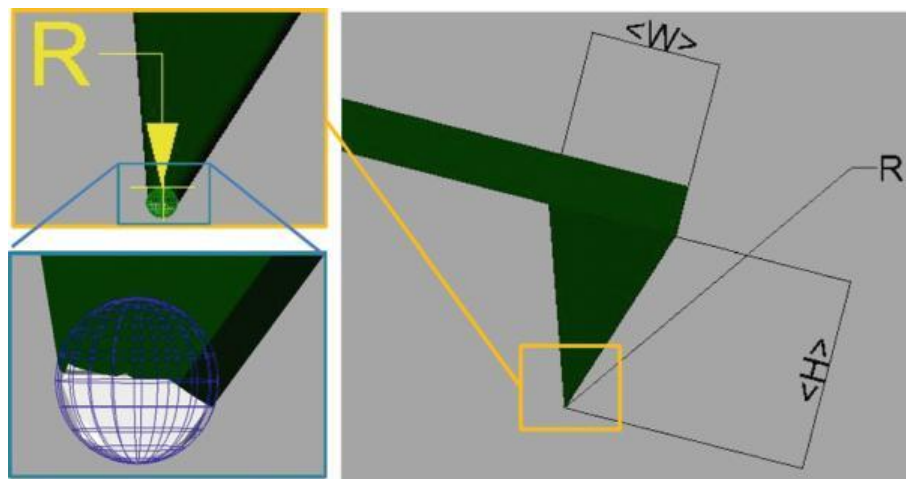


Figure 14. The essential parameters in a tip are the radius of curvature (R) and the aspect ratio (ratio of H to W). Reprinted from Torre *et al* [140].

A good cantilever must have a high sensitivity, which is achieved with a low spring constant, and a large deflection at small forces, being long and thin[140]. Figure 15 shows some soft v-shaped cantilevers [140]. The k_c of the cantilever is influenced by the length

and the thickness, where for a simple rectangular cantilever k_c will be inversely proportional to the square of the length and directly proportional to the thickness cubed [144].

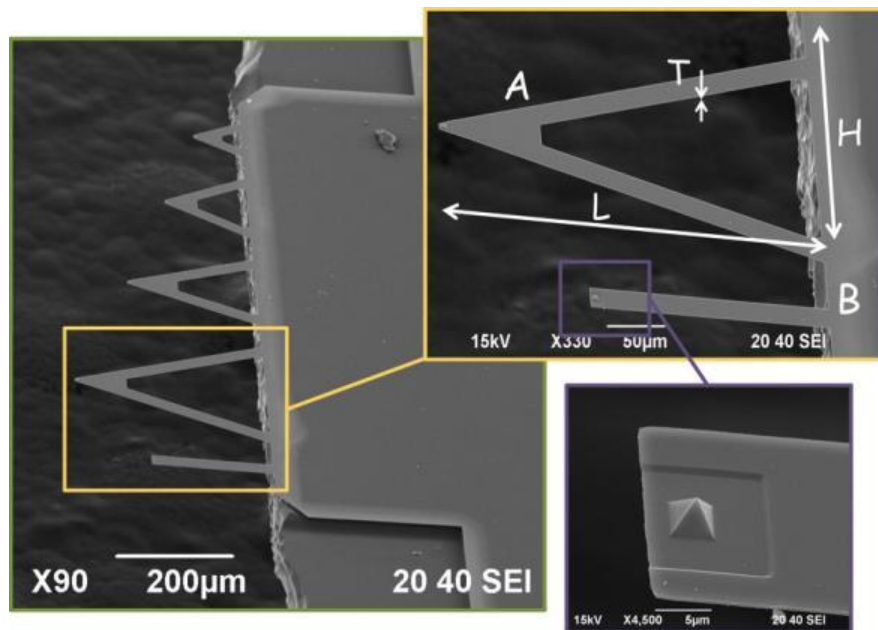


Figure 15. SEM images show triangular (A) and rectangular (B) MCLT cantilevers made of silicon nitride (from Bruker). On the lower right, there is an image of a pyramidal tip. Reprinted from Torre *et al.* [140].

Mechanical properties, such as stiffness (spring constant) and resonant frequency of a cantilever depend on its width (W), length (L), and thickness (T) [140]. The quality factor (Q -factor) is a dimensionless parameter that describes the dampening an oscillator or resonator is [140]. The Q -factor of a cantilever is related to the oscillation amplitude peak of the lever. The narrower the amplitude peak is, the higher the Q -factor is. The Q -factor is seen in the amplitude versus resonance frequency oscillation graphic when the cantilever is oscillating freely (often called thermal noise tune) [140].

Smaller cantilever lengths result in higher resonant frequencies. The soft cantilevers with low resonance frequency are more appropriate for imaging in contact, nanoindentation, and force spectroscopy assays of soft samples, such as biological samples in liquid [140]. The forces range used for nanoindentation assays are in the order of magnitude of nN and for spectroscopy and molecular recognition assays are in the order of pN [140]. The hard cantilevers are more suitable for hard samples imaging in air, in oscillating mode [140]. The lateral resolution of an atomic force microscope depends on the tip sharpness.

The sharper the tip is, the better lateral resolution is achieved, being the lateral resolution defined as the less space between the centres of two features, which allows for seeing both separately.

Accurate calibration of the cantilever is one of the most important procedures to obtain reproducible results in nanomechanics, force spectroscopy and molecular recognition assays as the determination of binding forces between single molecules, cell-cell adhesion, protein unfolding, etc. [140]. Calibration of a cantilever (pyramidal or spherical), through the calculation of the invOLS or deflection sensitivity, allows the determination of the cantilever spring constant (k_c) and the force applied on the sample [145].

The main purpose of the invOLVS calculation is to obtain a conversion factor that converts the signal voltage (V) read by the sensor in deflection sensitivity (presented in nm/V). This conversion factor can be subject to errors due to the contamination of the tip or substrate, mainly if the analyses are performed on biological samples [146].

Suppliers sell the cantilevers with the k_c values in an interval range, but the values are not accurate because of the microfabrication wafer, due to the thickness and length variations. If the user intends to use a cantilever with an accurate k_c it can be determined using an interferometer [146]. However, calibrated cantilevers are very expensive, so the operator may wish to calibrate the cantilevers by him/herself. In the literature, more than one method are mentioned to calibrate the force constant of an AFM cantilever the added-mass method [147]; the use of reference cantilever [148]; and the thermal noise method [141, 149]. While the added mass method is destructive and the reference cantilever is not practical, make thermal K noise the most used method [149].

2.4.1. Calibration of AFM deflection sensitivity

The elastic modulus of a material corresponds to the slope of its stress-strain curve in the elastic deformation region [150]. To calibrate the deflection sensitivity, typically force-distance curve in air, acquired with a pyramidal cantilever tip, is performed [140]. The curve represents the deflection of the cantilever versus the extension of the piezoelectric scanner. These curves are acquired in air, on a rigid and clean surface, like mica or glass substrates [140]. When using soft cantilevers, an adhesion region is observed due to the

capillary forces, causing non-linearities in the deflection signal due to saturation and/or friction [140]. The curve in Figure 16 shows the approach and retraction, performed in air, showing the unspecific adhesion in the area defined by B, C, and D [140].

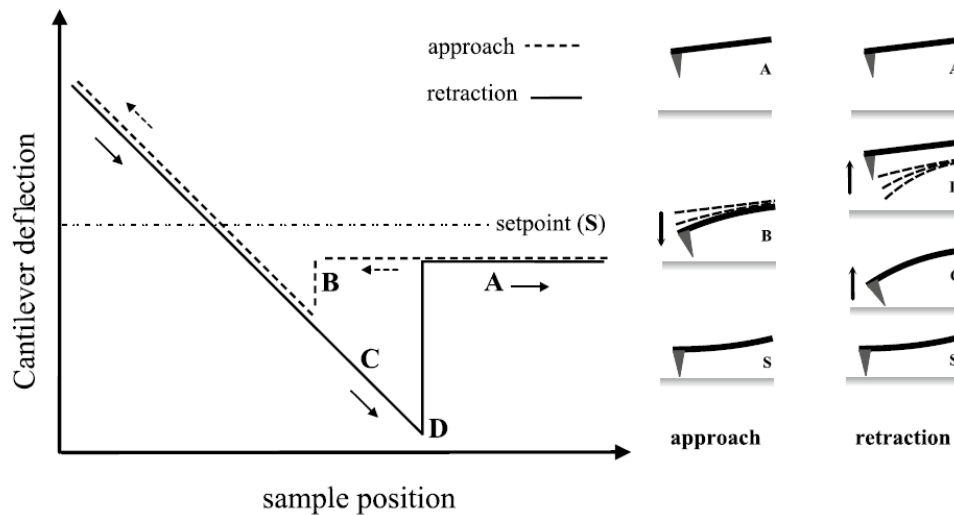


Figure 16. A typical force-distance curve with the approach and retraction, performed in air, showing adhesion in the area defined by B, C, and D. From A to B positions the tip is approaching the surface. On B, the contact with the tip and the surface is done due to attractive forces (van der Waals forces). After B, the cantilever deflects as the piezo continues to push it down on the surface until it reaches the force limit that was defined. On C, since the piezo moves away from the surface, the tip starts to withdraw. The region between C and D is due to capillary forces of the water layer on the surface but it is also due to adhesive surface properties. At some point, depending on the thickness of the water layer, the scanner retracts enough that the tip stays free (in D). Between D and A, the cantilever returns to the original position, being ready to start another measurement. Reprinted from Torre *et al.* [140].

If the sample is analyzed in liquids, the force-distance calibration curve must be performed in liquids as well (Figure 17). In this case, capillary forces do not exist anymore, and the large adhesion area is not present in the force-distance curve [146]. There may be still some remnant adhesion due to van der Waals forces; however, this does not distort the force curve, even for very soft cantilevers.

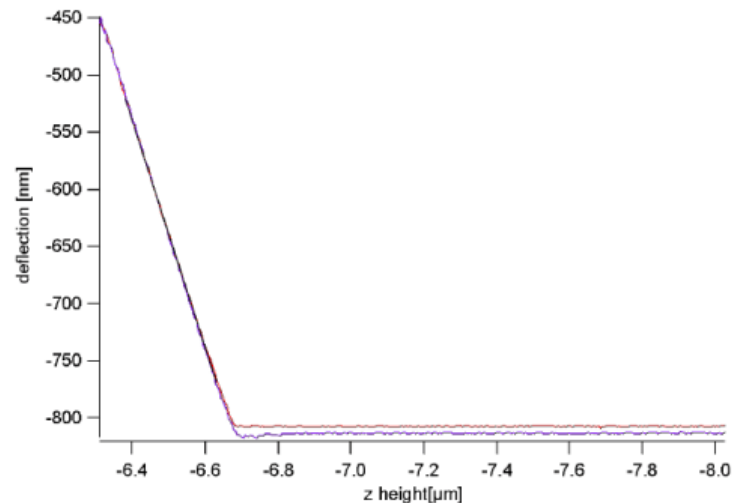


Figure 17. The force-distance curve obtained in water does not show adhesion and there is no difference between the approach and retraction in the contact region. Reprinted from Schillers *et al.* [146].

Two examples of approach force curves taken with a pyramidal and a spherical tip are presented in Figure 18. Different slopes can be found in different parts of the curves. If the slope is calculated close to the contact point, variations can be found: 7% for pyramidal and 10% for colloidal tips. Hence, there is a slight difference between a pyramidal and a spherical tip [146]. According to Schillers *et al.*, this deviation in the slope is caused by systematic errors, namely non-linearity in the deflection's sensor, or the piezoelectric scanner, or due to the friction and interaction forces between the tip and the sample [146]. Since these are systematic errors, no improvements will be found if more force curves are averaged and thus a suitable analysis range (in deflection) should be sought [146].

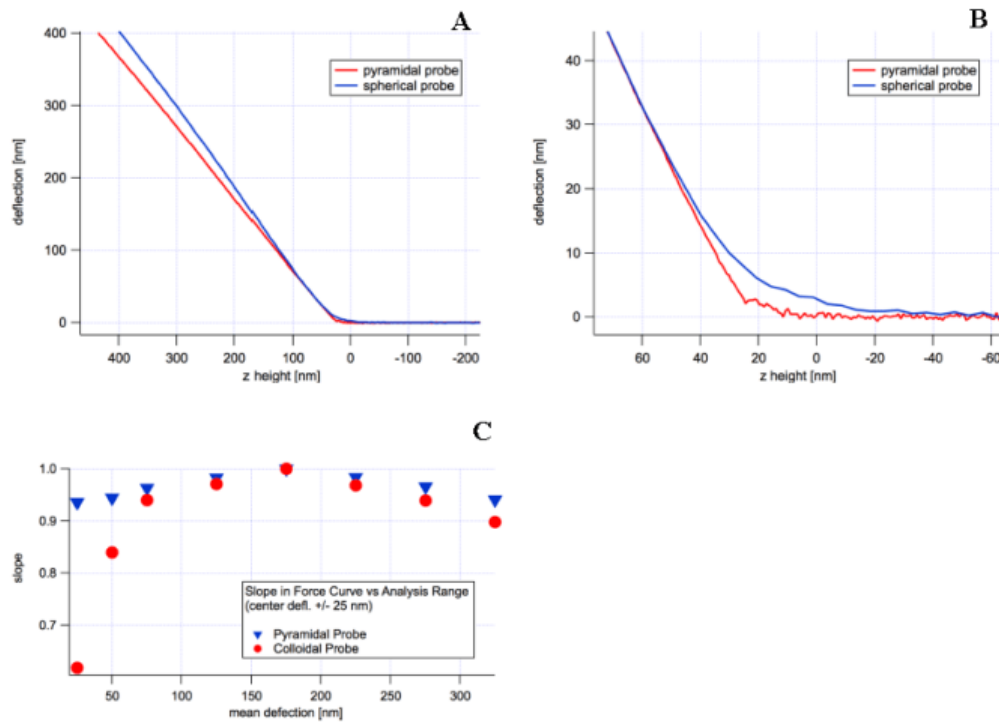


Figure 18. Approach force-distance curves acquired with a pyramidal tip (red in A and B) and with a spherical one (blue in A and B). Panel B is a zoom-in of the entire dataset A, showing that close to the contact point we get deviations from a perfect force curve. B corresponds to an enhancement of the contact region of A, showing the deviation of a perfect distance curve. In pyramidal tips, this is due to electrostatic repulsive forces, while in the spherical tip it is due to a contaminated layer. In C the slope values calculated in sliding windows of 50 nm width calculated from the force data of A, are shown. Even when disregarding the extreme values at 25 nm close to the contact point, the variation in slope is 7 % for the pyramidal probe and 10 % for the colloidal. Reprinted from Schillers *et al.* [146].

2.4.2. Deflection sensitivity of a vibrating cantilever

2.4.2.1 Power spectra density of single harmonic oscillator (SHO)

According to Boltzmann's equipartition theorem a cantilever will always show thermal fluctuation by oscillating at its resonance frequencies, which says that each degree of freedom of any system has average energy according to $\frac{1}{2} k_B T$:

$$\frac{1}{2} k_B \langle x^2 \rangle = \frac{1}{2} k_B T \quad (\text{Eq. 1})$$

Where:

k is the cantilever's force constant,

x is the time average of the vibration amplitude (i.e. the deflection of the cantilever),

k_B is Boltzmann's constant

T is the absolute temperature.

The angle brackets denote the time average of the amplitude vibration. AFM cantilevers are not single harmonic oscillators (SHO) with one resonance frequency, but have different vibrational modes and each mode has $\frac{1}{2} k_B T$ of energy [151]. Each mode has a different resonance frequency, making it possible to separate the modes in the frequency domain. Consequently, the AFM usually records the fluctuations of cantilevers, plotting the amplitude squared spectral density (A2SD). It is needed to multiply k (the force constant divide it by time and then Fourier transform, to get the Power Spectral Density (PSD). Since the k is unknown, it is needed to calculate the A2SD, to calculate the force constant. A2SD is proportional to PSD.

The PSD refers to the spectral energy distribution that would be found per unit of time, since the total energy of such a signal overall time would generally be infinite [151].

The equipartition theorem addresses the energy that is shared equally amongst all energetically accessible degrees of freedom of a system so that a system will generally try to maximize its entropy (i.e. "spread out" the energy that is in the system) by distributing the available energy evenly amongst all the accessible modes of motion [151].

The vibrational modes of a cantilever are different for a free cantilever (used in the thermal method) and a cantilever when it is in contact with the sample, meaning that the sensitivity factor used to convert the photodiode signal (measured in V) to cantilever deflection (measured in nm) is also different in both cases [151]. The amplitude sensitivity of a free cantilever cannot be easily calibrated in AFM, so a correction factor κ is used to calculate the amplitude signal from the measured deflection signal [146].

In the literature, a slightly differently defined correction factor $e = 1/\kappa^2$ can also be found [152]. Depending on the instrument, the deflection signal is directly corrected by the κ

factor as described above or the result of the power spectral density analysis is corrected accordingly [152].

The mean square displacement of one vibration mode could either be calculated by averaging over the deflection signal as a function of time, or by integrating the PSD over a specified bandwidth. Alternatively, and often more accurate, a model function is fitted to the PSD and then the integration is done analytically over the model function to yield the mean square displacement [140]. An obvious choice of a model function is a single harmonic oscillator, which will give the following result for the force constant :

$$k_{SHO} = \frac{2k_B T}{\pi \omega_0 Q A_{DC}^2} \quad (\text{Eq. 2})$$

Where:

Q is the quality factor

A_{DC} is the amplitude at DC (at frequency 0)

ω_0 is the resonance frequency, respectively.

2.4.2.2. Power spectra density of a Lorentzian oscillator

In liquids like water, the cantilever damping is increased but, more importantly, the effective mass is also increased, since water is dragged along with the cantilever [153]. This results in a decrease of resonance frequency and in a widening of the resonance peak, which aggravates the recording of good thermal spectra in water. Since the soft cantilevers are highly damped in liquids (low Q), it has been suggested to use the Lorentzian model [152]. To determine the deflection sensitivity, it is necessary to obtain the thermal of the free cantilever in liquid.

Figure 19 shows the PSD of a soft cantilever, in liquid. It is observed that the Lorentz's method (blue line) fits better the thermal data (red line) [154].

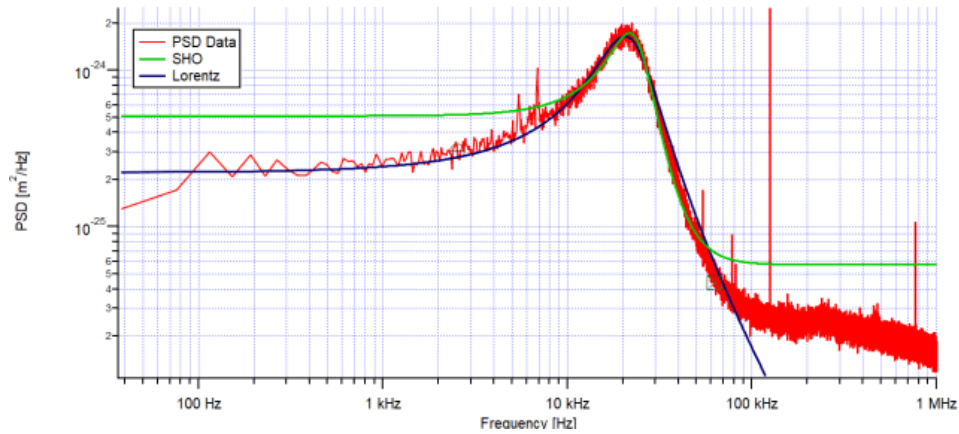


Figure 19. Power spectrum density of thermal noise of a soft cantilever obtained in water. Data were fitted with the single harmonic oscillator (SHO) model and Lorentz's model to calculate the force constant. As it can be seen, the Lorentz's model (blue line) fits better the thermal data of the cantilever in liquid (red line) than the SHO (green line). Reprinted from Brás *et al.* [154].

The main question in the PSD data and force constant calculation is to decrease sources of error. In the PSD there could be two sources of error:

- (i) The noise, depending on the frequency of the instrument, will add to the PSD, resulting in a force constant that is not the true value, being lower than it should be. In AFM instruments, some noise filters (low pass filters) existing in the equipment, reduce the high-frequency noise (cutting the frequencies of 1 MHz or higher), which are not relevant for soft cantilevers [146]. Low-frequency noise is the most important in the case of soft cantilevers. The source of this noise can result from the thermal drift of the instrument or from the environment, such as building vibrations, being more serious for the soft cantilevers, mainly if the cantilever is oscillating in liquids, where there is a drop to 1 kHz in terms of resonance [146]. In some equipment, there is the presence of high pass filters to cut-off low-frequency noise. This will result in substantial errors in thermals [146];
- (ii) The force constant will be (both in air and in liquid), proportional to the inverse of the deflection signal squared, meaning that, if the deflection signal has not been calibrated correctly, the force constant will not be correct. The deflection errors will enter quadratically to the force constant value, being a very serious error [146].

2.5. Nanoindentation to assess mechanical properties

2.5.1. Single force maps with single force distance-curves

Using AFM, force maps are recorded to obtain values of the mechanical properties of samples, through the acquisition of single force-distance curves (approach and retraction) [154]. Briefly, there is a grid constituted by $N \times N$ pixels and, in each pixel, a force-distance curve is recorded. These force-distance curves are obtained in several distinct places of the sample. If the sample is a cell, these grids are always recorded in the nuclear region of the cell to avoid the contribution of the substrate in thinner areas of the cell. This method allows obtaining the mapping of the electrostatic forces between the tip and the sample in different regions [125]. The force-distance curves are fitted with a suitable model considering the sample type, the tip used and the existence or not of adhesion between the tip and the sample. With this method, it is possible to obtain images of low resolution correlated to the physical properties of the sample surface. It means that, if the approach portion of each curve after contact is fit using an indentation theory, a stiffness map is obtained and the stiffness is calculated, through the calculation of the apparent Young's modulus and deformation (indentation) [125].

2.5.2. Force maps with z-Step response

The z-Step response methodology is a static method to determine the viscoelasticity (elastic modulus and dynamic viscosity) of biological samples [155, 156]. Rianna *et al.* and Babu *et al.* used this methodology to study the mechanical properties of normal and cancerous thyroid cells seeded in hydrogels substrates with different stiffness values and normal, scar and fibroblasts in Dupuytren's disease, respectively [155, 156].

Briefly, a conventional force-distance curve is performed on the sample. When the approach ramp achieves the maximum deflection, the z-height is kept constant for 2.0 seconds, except a small z-Step is applied for 0.5 seconds. With the application of the standard linear solid model (or Zener's model), it was possible to determine the true elastic modulus and the dynamic viscosity of the cells [155]. In this model, a Zener's element with a spring k_1 working in parallel with a Maxwell's model (which consists of

a spring constant k_2 and a viscous damping element with coefficient f plus the cantilever spring constant k_c). Z-height motion results in the deflection of cantilever d , which is fitted with an exponential function and indentation δ of the sample with the mathematical equation $z = d + \delta$ [155]. Despite the existence of other models, this is a simple one for measuring relaxation times (Figure 20) and the viscoelastic properties of the sample can be quantified [146]. The fitting of the conventional force-distance curve is performed in the approach line, using the Hertz's model, which equation is always necessary since it includes the tip geometry, as well as the Poisson's ratio of the sample and the apparent Young's modulus [145].

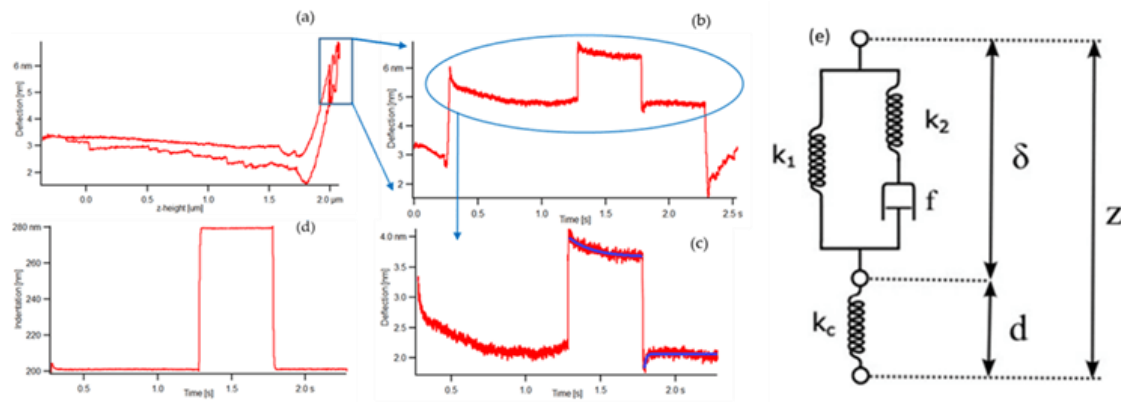


Figure 20. A representative z-Step response methodology applied on cells: (a) Conventional force-distance curve (cantilever deflection vs. z-height of the piezo scanner); (b) End of the approach curve, with constant z-step in cantilever deflection, applied for 2 s, where it is possible to see the creep of the cell; (c) Second step in force is applied and the fit of the step is performed (blue line) to subtract the creep in the analyzes; (d) Indentation vs. time of the cells; (e) Standard linear solid model. The sample is modelled by a Zener's element, where a spring k_1 is parallel to a Maxwell's element, consisting of a spring k_2 and a viscous damping element f . The cantilever is characterized by its spring constant k_c . Viscous (hydrodynamic) damping of the cantilever is neglected here, due to the slow stress relaxation of the sample, which is the predominant contribution. Reprinted from Rianna *et al.* [155].

2.5.3. Frequency Sweep

Frequency sweep is a dynamic method used in microrheology, where the sample is oscillated at various frequencies [10]. Oscillatory microrheology determines the glassy transition frequency of cells [157]. During cell indentation by the cantilever, a sinusoidal excitation signal is applied to the z piezo. The cantilever experiences in-phase and out-of-phase deflection signals, which are used to determine the complex shear modulus, the creep compliance, and the stress relaxation, as described by Flormann *et al.* [126]. After performing the conventional approach force-distance curve, the tip stays in contact with the sample for a given time (dwell time), while the tip oscillates at increasing frequencies, e.g. from 1 Hz to 1 kHz, allowing the determination of the viscoelasticity at different frequencies [10]. A stiff surface of the sample will result in the tip oscillation amplitude being higher, compared with a soft surface, in which the tip oscillation amplitude is lower. de Sousa *et al.* presented four time-dependent functions: simple force curve, dwell force curve, step-indentation force curve, and frequency sweep force curve (Figure 21) [10].

The colored arrows show the contact point of each curve. The loading (τ_l), dwell (τ_d), and unloading (τ_u) times are also shown. This methodology allows the determination of microrheological properties (with frequency variations) such as: storage modulus (G') (elasticity component), loss modulus (G'') (viscous component), and loss tangent (described by the ratio between G'' and G' , providing information if the sample is more solid-like or liquid-like). In terms of mechanobiology, cell behaviour has viscoelastic relaxation properties. This method also allows the explanation of the cellular mechanical behaviour, quantifying a power-law exponent of the storage modulus, which provides information about the “fluidity” of the cytosol of the cell [10].

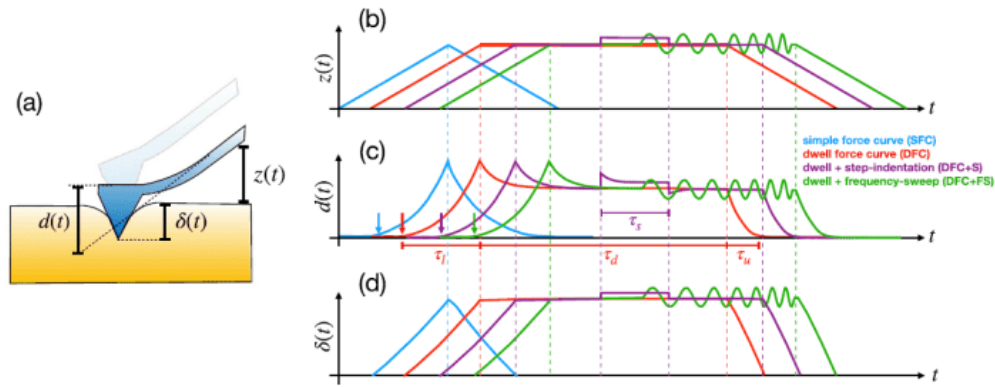


Figure 21. (a) Indentation of a soft material using an AFM pyramidal tip, showing the piezo displacement $z(t)$, cantilever deflection $d(t)$, and indentation depth $\delta(t)$. Panels (b), (c) and (d) displayed the time-dependent functions respectively, for different AFM-based nanoindentation experiments: Simple force curve (SFC, in blue), dwell force curve (DFC, in red), step-indentation force curve (DFC+S, in purple), and frequency-sweep force curve (DFC+FS, in green). For better visualization, the curves were shifted horizontally. The contact points are highlighted by colored arrows. The loading (τ_l), dwell (τ_d), and unloading (τ_u) times are also shown. Reprinted from de Sousa *et al.* [10]

2.5.4. Models for elasticity and viscoelasticity fitting

There are several models, from contact mechanics, used for fitting force-distances curves, allowing the determination of the elastic properties of a sample. The choice of the model must be performed according to several parameters, such as sample type (biological, polymeric film, polymeric material, etc.), presence or not of adhesion forces between the tip and the sample, and tip geometry. Johnson-Kendall-Roberts (JKR) model is used for large tips on soft samples when large adhesion forces are present [143]. The Derjaguin-Müller-Toropov (DMT) model (an alternative model for adhesive contact), the contact profile remains the same as in Hertzian contact but with additional attractive interactions outside the area of contact [10]. This model is used with small tips, stiff samples, and with small adhesion [143]. The Oliver and Pharr model is used to analyze the unloading of the force-distance curves, to obtain the relation between an applied load and the penetration depth obtained [158].

2.5.4.1 Elasticity determination (Hertz's model)

Heinrich Hertz derived a contact mechanics model for two touching elastic spheres. This model can be used and applied to the case of one sphere touching a flat surface, which is relatively close to the case of AFM studies of cells and other soft surfaces. Sneddon has done an extension of this hertzian contact model describing the deformation of a flat surface by a conical probe. Later Rico modified this model for the case of an n-sided pyramid, describing better the relevant case of pyramidal probes in AFM. Subtleties often ignored are that the Hertz model for a sphere, should only be applied as long as the indentation is smaller than the radius of curvature of the tip. The conical model of Sneddon is often applied to pyramidal tips, and in some cases, it has even been called, erroneously, pyramidal model. The real pyramidal tips are blunted (i.e. they have a spherical cap at their end, which is often ignored) [159].

All these Hertzian models applied to AFM share the following facts and thus will be discussed here together: the sample (cell) is assumed to be homogeneous, isotropic infinitely thick (ignoring the bottom effect) deformations are small, thus the sample is in its linear regime [145, 160]. Strictly speaking, cells do not have these characteristics; nevertheless, it is a good starting point to gain knowledge. In addition cells are viscoelastic, however, since the cells are indented slowly so that the contribution of viscous properties will be small, and the response of the cell will come predominantly from the elastic behavior [145].

More important is the bottom effect: when the sample gets thinner the underlying stiff support can be sensed, and the numbers derived from the simple Hertzian models will be wrong. Since the mechanical data are recorded in the highest parts of the cells (the nuclear region), the effect of the bottom parts is neglected, so the use of bottom effect corrections (BEC) is not used. As a rule of thumb, no need for BEC is assumed to be needed when the indentation is smaller than 0.1 to 0.2 for pyramidal probes. For spherical probes the criterium has to be somewhat more strict [160].

The expression of the Hertzian models, according to the geometry of the tip are the following:

$$F_{paraboloid} = \frac{4}{3} * \frac{E}{1-\nu^2} * \sqrt{R} * \delta^{3/2} \quad (\text{Eq. 3})$$

$$F_{cone} = \frac{2}{\pi} * \frac{E}{(1-\nu^2)} * \tan(\alpha) * \delta^2 \quad (\text{Eq. 4})$$

$$F_{pyramid} = \frac{1}{\sqrt{2}} * \frac{E}{(1-\nu^2)} \tan(\alpha) * \delta^2 \quad (\text{Eq. 5})$$

Where:

E is the Young's modulus;

ν is the Poisson's ratio of the sample;

R is the radius of the curvature at the apex of the paraboloid (equivalent to the radius of spherical probes, when a spherical tip is used);

δ is the indentation of the sample;

α is the half-opening angle of a cone or a 4-sided pyramid.

If the four angles of the pyramid are not identical, which often is the case for AFM tips, the best way is to use the average of all angles, i.e. θ or even better, $\tan(\theta)$.

Figures 22 and 23 show the interaction between the spherical tip and the surface, respectively [161].

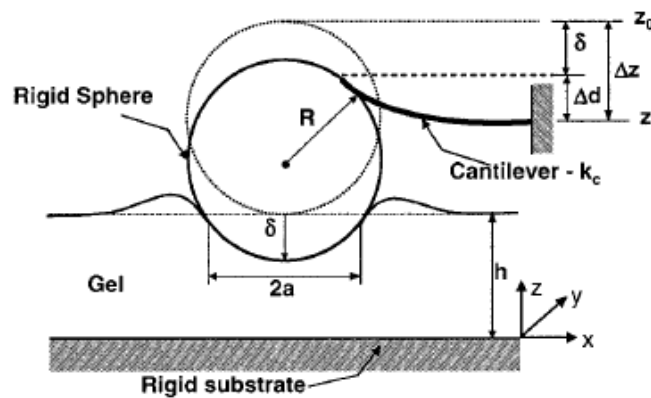


Figure 22. Hertz's model representation. Reprinted from Dimitris *et al.* [161].

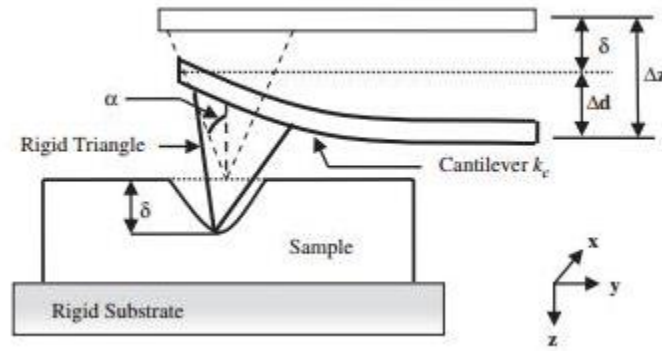


Figure 23. Sneddon's model representation. Reprinted from Bornschlogl *et al.* [5].

2.5.4.2. Viscoelasticity determination

Viscoelastic materials have two distinct characteristics: the viscous part (they deform gradually when subjected to external forces) and the elastic part (they deform instantaneously and will recover to their original shape after the external force is removed) [59]. The mechanical quantification of materials is performed in the form of their stress-strain (or load-deformation) response, in contrast to pure viscous fluids, in which there is a deformation permanently followed by a reorganization of the molecules in the fluid [59]. In this case, there is no elastic behaviour. In purely elastic materials, the curves of stress and strain (the loading and the unloading curve) are identical. In inelastic materials, there is a hysteresis between the loading and the unloading curves, since energy is dissipated by the material [59]. In summary, viscoelastic materials are characterized by three key points: stress relaxation (the stress decreases with the time, being the response of viscoelastic material to a constant strain step), creep compliance (constant stress with increasing strain as a function of time) and hysteresis (the difference between the loading and unloading processes) [59].

To determine the viscoelastic properties of a sample, it is necessary to conjugate the elastic models from above with viscosity models. The latter uses a spring combined with a damping element (dashpot component). This way, it is possible to separate the elastic component from the viscous component. There are several models used in AFM data analysis, such as the Maxwell's model, the Kelvin-Voigt's model and the standard linear

solid model (also known as Zener's model) [162]. For viscoelasticity studies it is important to consider the creep compliance and the stress relaxation of the sample, as a function of time [162]. Diverse transitions exist for different materials, although the glassy transition is the main type of deformation for viscoelastic polymeric materials [162]. The viscoelasticity of materials can be addressed through the relaxation modulus or the dynamic modulus. In polymers, both depend on temperature, which can be transferred into a time or frequency equivalency [59]. Several viscoelastic models are being used to quantify the viscoelasticity properties of materials.

Figure 24 shows the viscous (due to the use of a dashpot element), elastic (due to the use of a spring element), viscoelasticity in biological samples (using the Maxwell's model and the Kelvin-Voigt's model) and the viscoelasticity model of biological samples (using a standard linear solid model, also named Zener's model). The viscoelasticity models in biological samples show the variation of the strain as time-dependent (creep) and the stress as time-dependent (stress relaxation) [162].

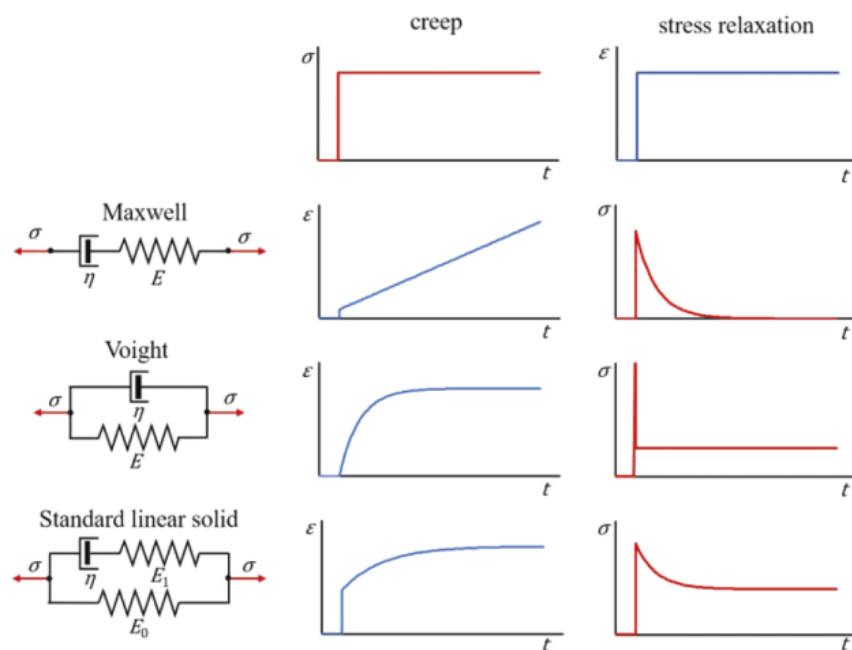


Figure 24. Creep and stress relaxation in spring–dashpot models of viscoelastic materials. Left: Commonly used models consisting of simple combinations of springs with elastic modulus, E , and dashpots with viscosity, η . Centre: Strain (ϵ) in response to a stress step (σ). Right: Stress in response to a strain step (stress relaxation). η : dashpot viscosity, E : spring constant [162].

Table 2 presents a brief comparison between the Maxwell's and the Kelvin-Voigt's models.

Table 2. Comparison between the Maxwell's and the Kelvin-Voigt's models.

Maxwell's model	Kelvin-Voigt's model
Good for predicting stress relaxation	Good for predicting creep
Poor at predicting creep	Not accurate in predicting stress relaxation
Used for soft solids	Used when the load is not too high as for organic polymers, rubbers, wood

2.6. AFM in cancer research

At present, it can be envisioned that AFM could be used as a diagnostic tool in cancer disease, due to its powerful application to probe the morphology of live cells, as well as to quantify and spatially map cell mechanics and physical properties, in physiological conditions (in fluid environments), in real-time (Figure 25) [163].

AFM can be combined with many optical and advanced microscopy techniques, which facilitate the analysis of certain parts of biological samples [129]. AFM can be used to assess the nanomechanical fingerprints of solid tumors. This nanomechanical fingerprints may constitute a biomarker, providing new insights and a novel approach for cancer diagnosis, and contributing to the development of therapeutic strategies. AFM is proposed as an adjuvant technique to standard biopsy procedures, offering an objective, novel, and quantitative diagnostics [57, 164]. A large number of AFM mechanical studies has already been conducted on several cancer cell types, such as brain [165, 166], thyroid [155, 167], breast [168, 169], lungs [170, 171], liver [172], stomach [173], pancreas [174, 175], intestine [4, 133, 176-179], kidney [167, 180], urinary tract [181-185], skin [184, 186-188], liver [172], colorectal [133], kidney [10], pancreas [174], bone [8], lymph [106], ovary [189], bladder [190], and cartilage [191].

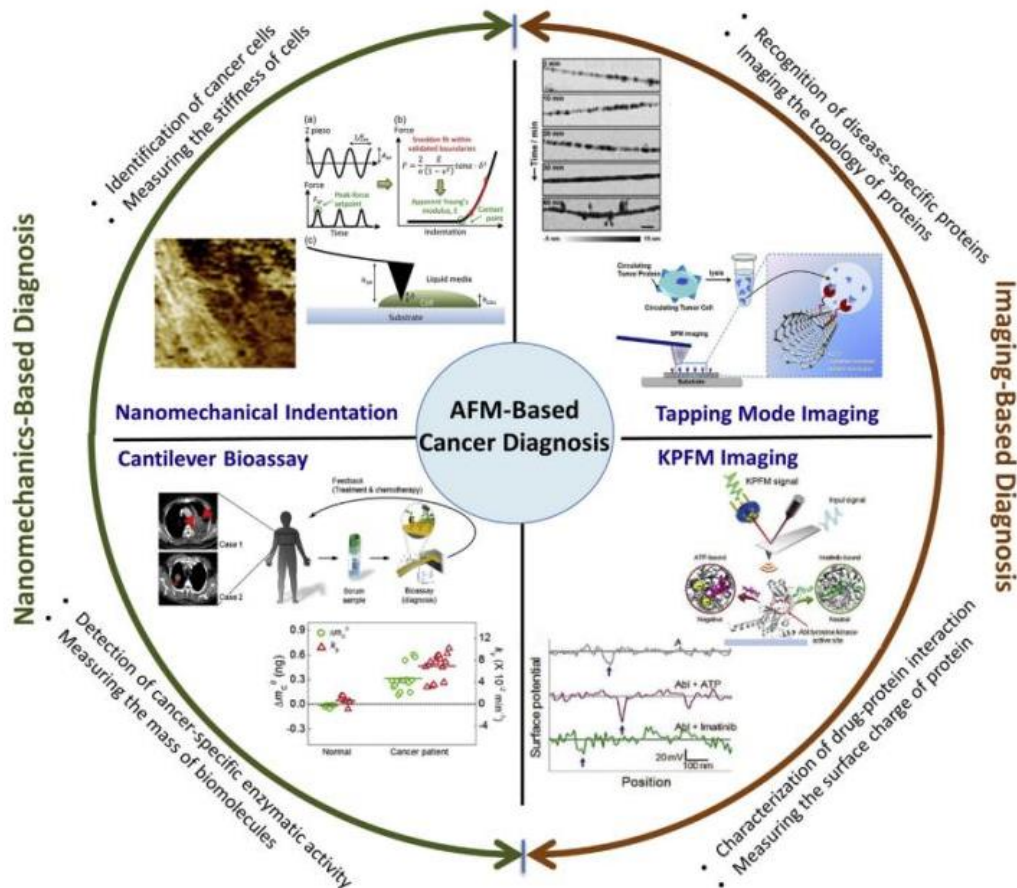


Figure 25. Schematic representation of AFM-based diagnosis, which can be divided into AFM imaging-based diagnosis and AFM nanomechanics-based diagnosis. Through imaging, cancer-specific biomolecules charges using Kelvin probe force microscopy can be identified. With nanomechanics such as AFM mapping, it is possible to identify the pathological state of cells and tissues. Reprinted from Know *et al.* [163].

Some examples showed that AFM was used to compare mechanical properties among cancerous cells from the liver, lungs, skin, and breast [170], and gastric cells [173]. The majority of the liver cancer cell population showed protuberant enlarged nuclei with a lack of cytoskeleton to support the cell structure [170]. Cross *et al.* also compared the stiffness of lung, breast, pancreatic, and liver cancer cells with their normal counterparts and found an apparent Young's modulus around four times lower than for the latter ones [174]. Bu *et al.* compared the viscoelasticity of human normal liver (L02), hepatic cancer (HepG2), hepatic stellate (LX2), and gastric cancer (NCI-N87) with human normal gastric (GES-1) and gastric cancer (SGC7901) [192]. The first four types of cell lines behaved as linear viscoelastic materials, in opposition to the last two gastric cells, which

behaved as nonlinear due to failing in satisfying the verification relation for linear viscoelastic theory [192].

Cross *et al.* reported the stiffness, measured by AFM, of live metastatic cancer cells collected from the pleural fluids of patients with suspected pancreas, lung, and breast cancer. The metastatic cancer cells were 70% softer than benign cells [174].

From all the previous works, cancer cells were usually softer than normal cells, but their stiffness changed according to the stiffness of the environment because of its mechanosensitivity [176]. Cells need to remodel their cytoskeleton to adhere to a certain substrate, meaning that this will alter the rheological properties inside, which could also contribute to the change of their deformation capacity, in the case of a metastatic cell that needs to move across the surrounding tissue [176]. Only a few studies showed that cancer cells are stiffer than normal cells [181, 188].

Studies of mechanical properties of cancer cells are also suitable and very helpful to see the effect of the administration of drugs. Mechanical studies were performed to evaluate the side effects of anticancer drugs on cardiomyocytes [4, 192]. AFM was also used in mechanobiology analysis, as a new perspective for cancer immunotherapy research [2].

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CHAPTER 3

COLORECTAL CANCER CELLS

3.1 Mechanobiology of Colorectal Cancer

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Simple Summary: It is well documented that colorectal cancer (CRC) is the third most common cancer type, responsible for high mortality in developed countries, resulting in a high socio-economic impact. Several biochemical and gene expression pathways explaining the manifestation of this cancer in humans have already been identified. However, explanations for some of the related biophysical mechanisms and their influence on CRC remain elusive. In CRC, biophysics and medical research have already revealed the importance of studying the effects of the stiffness and viscoelasticity of the substrate on cells, as well as the effect of the shear stress of blood and lymphatic vessels on the behavior of cells and tissues. A deeper understanding of the relationship between the biophysical cues and biochemical signals could be advantageous to develop new diagnostic techniques and therapeutic strategies. Being a disease with a high mortality rate, it becomes crucial to dedicate efforts to finding effective, alternative therapeutic strategies.

Abstract: In this review, the mechanobiology of colorectal cancer (CRC) are discussed. Mechanotransduction of CRC is addressed considering the relationship of several biophysical cues and biochemical pathways. Mechanobiology is focused on considering how it may influence epithelial cells in terms of motility, morphometric changes, intravasation, circulation, extravasation, and metastization in CRC development. The roles of the tumor microenvironment, ECM, and stroma are also discussed, taking into account the influence of alterations and surface modifications on mechanical properties and their impact on epithelial cells and CRC progression. The role of cancer-associated fibroblasts and the impact of flow shear stress is addressed in terms of how it affects CRC metastization. Finally, some insights concerning how the knowledge of biophysical mechanisms may contribute to the development of new therapeutic strategies and targeting molecules and how mechanical changes of the microenvironment play a role in CRC disease are presented.

Keywords: colorectal cancer; biophysical cues; biochemical pathways; mechanobiology; atomic force microscopy.

1. A Brief Introduction to Colorectal Cancer (CRC)

Sung *et al.* presented the cancer incidence and mortality provided by the International Agency for Research on Cancer (IARC) at GLOBOCAN 2020. Worldwide, breast cancers present the highest number of new cases (11.7%), followed by lung (11.4%) and CRC (10.0%). Lung cancer was the leading cause of cancer death (18%), followed by CRC (10%). In summary, CRC is the third in terms of incidence but the second in terms of mortality [1,2]. The risk of CRC has been growing over the past few years due to environmental factors, a sedentary lifestyle, and diet [3]. Shussman and Wexner referred that multiple, cumulative genetic alterations lead to the formation of a neoplastic process, normally explained taking into consideration molecular biology and biochemical phenomena, and frequently not through biophysics, in which the forces exerted by the cells and the ECM are translated into biochemical signals (mechanotransduction) [4]. A deeper understanding of biophysical mechanisms behind biochemical pathways can lead to the development of new therapeutic strategies. For that purpose, it is important to understand the influence of several physical cues, such as: (i) internal forces of the cells; (ii) forces produced by the neighboring cells; (iii) forces coming from interstitial spaces (such as mechanical tension, compression, and hydrostatic pressure); (iv) forces resulting from the alterations of ECM stiffness; (v) shear stress (the parallel force per unit area applied to cell walls) from the fluid flow (such as blood); and (vi) forces from adhesion molecules [5]. Understanding the role of biophysical cues makes it possible to evaluate which biomechanical response will be activated. Each response will induce changes in CRC cell behavior (increased motility, shape changes, stiffness changes) which can promote the CRC cell motility, migration, invasion, extravasation, and metastasization. The origin of adenocarcinomas, as well as the large intestine tissue with its several different cell types and functions has already been well described in the literature [3,4,6–9].

Not every CRC shares similar driving mutations, making it extremely difficult to design a general molecular therapy [10]. Surgery is the initial approach in terms of treatment if an early diagnosis is performed. However, if it has already been metastasized, this approach is not effective anymore. These patients also develop drug resistance, and the recurrence of cancer is very common [11].

1.1. From Crypt Dysfunction to Polyp Formation

CRC begins at the epithelial cells, starting with adenoma (polyps with potential for carcinogenesis) and evolving to adenocarcinoma, existing at the mucosa layer that lines the colon and rectum [3,12,13]. The epithelial cells are connected between them through tight junctions (claudin and occludin) which, in turn, are connected to the cytoskeleton (myosin and F-actin filaments) through the myosin light-chain kinase (MLCK) and zonula occludins (ZO), such as ZO-1 [14]. The function and constitution of tight junctions have also been previously well described by Balda *et al.* [14]. The adherens junctions are formed by E-cadherin, β -catenin, α -catenin 1 and δ 1-catenin (also known as p120 catenin), and the desmosomes, formed by the desmoglein and desmocollin. These two proteins bind to keratin through desmoplakin, and then keratin binds to the cell nucleus (Figure 1). The expression dysregulation of some tight junctions, such as ZO-1 and ZO-2, may lead to some types of cancer, such as breast cancer [14,15].

Lechuga *et al.* reported recently that epithelial junctions are exposed to constant protrusive, tensile, and contractile forces which are generated by two cytoskeletal motors: actin filament polymerization and myosin II-dependent contractility [16]. The activity of these motors is controlled by biochemical and mechanical properties of the environment. Altered mechanical properties of tissues in different diseases should not be assumed to be modeled by our understanding of the junction-associated actomyosin. Ex vivo primary epithelial cell organoids and animal models with epithelial-specific knockouts of different actin regulators should be introduced as innovative models, providing novel critical insights to the understanding of the roles and mechanisms of cytoskeletal regulation of epithelial barriers during mucosal inflammation [16].

Cellular attachment to the underlying basal lamina induces proliferation in the epithelium, thereby generating stress in it, causing its buckling, thus inducing a displacement and initiating crypt budding and fission. Although still lined by a single layer of epithelial cells, adenomatous crypts exhibit multiple branching events and are typically elongated and deformed [17,18]. Van Leeuwen *et al.* explained that an increase in cell proliferation, accompanied by a reduction in cell-cell adhesion, may lead to crypt fission[19].

The buckling of a colorectal crypt is described by models developed for this purpose. One of the models describes a cross-section of a colorectal crypt when it is unfolded and modeled as

a beam connected to the tissue by a series of viscoelastic springs. The authors hypothesized that buckling was driven by growth of the epithelium and explored the consequences of altered proliferation and stromal adhesion. They predicted that an increase in proliferation could initiate buckling if there was a sufficiently flexible layer [20].

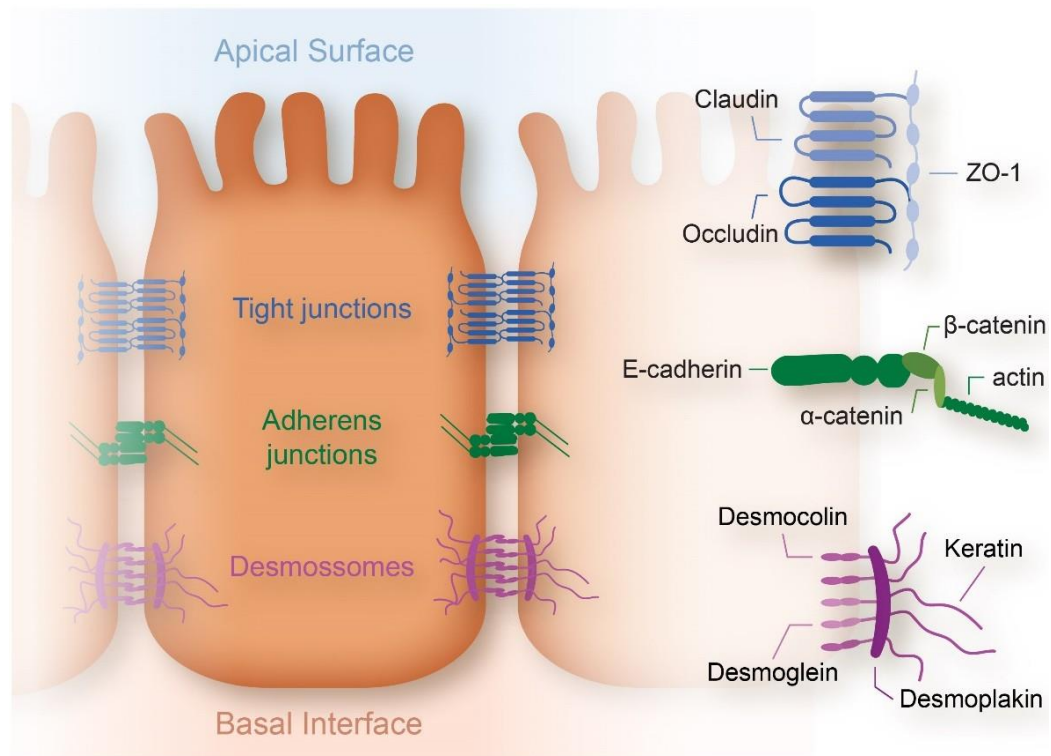


Figure 1. Epithelial cells at human colon intestine, with the adherens junctions, tight junctions and desmosomes. The adherens junctions are complexes that assemble and disassemble, allowing cells to respond to forces, biomechanical signals, and structural changes in their microenvironment. The tight junctions (occludins and claudins) are intercellular adhesion complexes, that constitute a paracellular diffusion semipermeable barrier, being charge and size selective. The desmosomes are strong cell-cell adhesion molecules, mainly present in tissues that sense intense mechanical stress. Adapted from Balda *et al.* [14].

Different epithelial folding, such as bending (related with myosin differentials) [21], buckling (related with myosin compression), growth, and pumping (related with osmotic pressure), were studied in the framework of the mechanobiology of intestinal organoids [22–24]. The old, damaged, or no longer needed colon epithelial cells are replaced every 3–5 days by new healthy cells through several events of mechanical functions, such as division, migration, and extrusion [25]. Figure 2 shows the large intestine tissue with its

several different cell types and thenormal regulation mechanisms of migration and cell differentiation on the crypt. The growth and division of new cells are usually regulated, although sometimes new cells grow and divide before they are required, thus originating a polyp. These polyps result from a failure or inability in relation to the proliferation, differentiation, or apoptosis of the epithelial cells at the mucosa. Polyps are classified according to the following parameters: macroscopic appearance (flat or sessile and with a stalk or pedunculated), size, number, anatomic distribution, and histology [4].

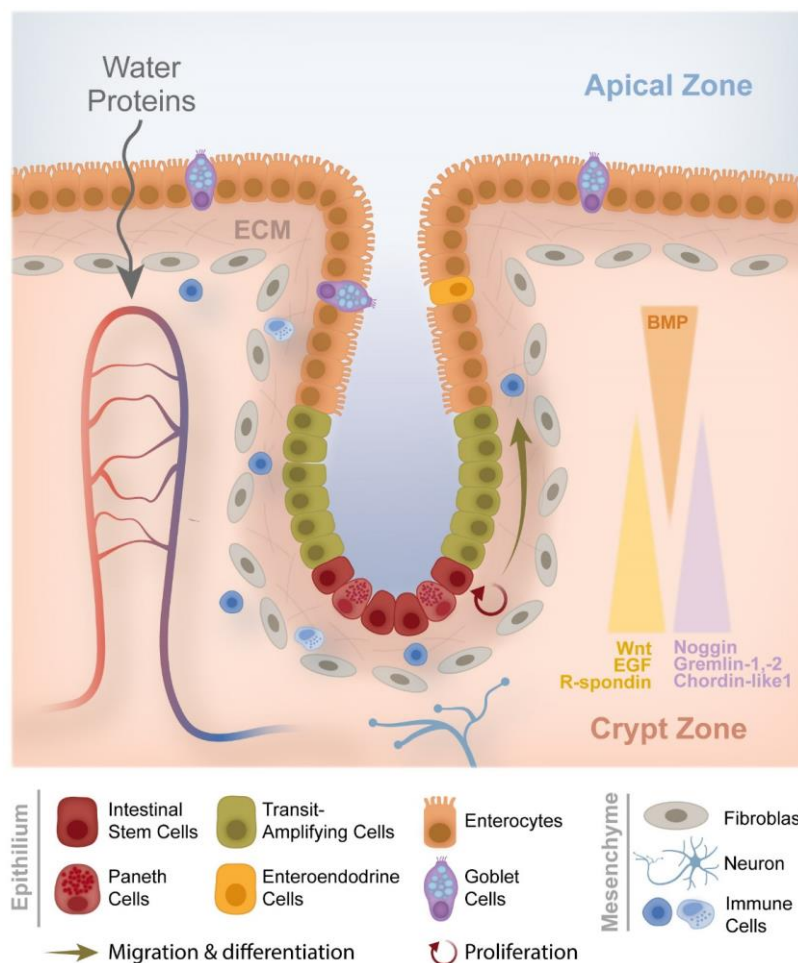


Figure 2. The intestinal stem cells are at the bottom of the crypt. These cells give rise to transit amplifying cells. During their differentiation, these cells migrate along the crypt, reaching the surface. Intestine stem cells regulation, as well as the proliferation and differentiation processes, are mediated by signals which come from the surrounding mesenchymal cells. At the bottom of the crypt, are expressed genes (Wnt, R-spondin, EGF) that regulate the cell cycle, and the intestine stem cells and progenitors increase, despite the genes inducing apoptosis being downregulated. The bone morphogenetic protein (BMP) pathways are expressed on the top of the crypt. In the meantime, BMP antagonists (noggin, gremlin, and chordin-like 1) are expressed, at the bottom of the crypt, by stromal cells. R-spondin is a protein that promotes the canonical Wnt/ β -catenin pathway signaling. Adapted from Audrey [6,15,26]. ECM—extracellular matrix; EGF—Epidermal growth factor; Wnt—Wingless and Int-1.

1.2 CRC Formation and Development

In terms of microscopical anatomy, the colon is composed by different layers, with specific cell types and functions, which are well described by several authors [27–30].

CRC can be classified according to 11 stages using the tumor, nodes, and metastasis (TNM) system, which is based on three key pieces of information, according to the American Cancer Society: tumor (extent of the tumor), nodes (assessment if cancer spread to nearby lymph nodes), and metastasis (assessment if cancer spread to distant lymph nodes or distant organs, such as the liver or lungs) [31]. Cancer cells metastasize to the lymph nodes via lymphatic vessels and to distant organs (liver, lungs, etc.) via blood vessels [3].

The beginning of CRC is related to a deficiency in cell migration from the crypt, which depends on the adenomatous polyposis coli (APC) protein. If there is a loss in this protein, then cell migration is stopped, leading to the accumulation of cells in the amplified zone of the crypt. The amount of these cells is then increased, all of them accumulating mutations, thus resulting in the formation of a tumor. The epithelium is very sensitive to mutation and carcinogenesis, since the cell replication rate in the epithelium of the colon and rectum is high, with a replication rate of 10¹⁰ cells every day [32]. In CRC, the epithelial-to-mesenchymal transition (EMT) drives cellular migration, characterized by the acquisition of a mesenchymal phenotype through the dissolution of tight junctions, disruption of apical-basal polarity, and reorganization of the cytoskeletal architecture [33].

2. CRC Mechanotransduction

Cancer mechanics is the study of forces existing between tumor cells and the extracellular environment. Sawminathan *et al.* mentioned the biophysical stimuli that cells sense from the microenvironment. Forces are produced by the neighboring cells and by the confined interstitial spaces, such as tension, compression, and hydrostatic pressure. Alterations in the ECM stiffness and shear stress from fluids (such as blood) are also sensed by the cells. The response to these mechanical stimuli will be different regarding the cell and the tissue type [34]. Mechanotransduction use several ways to respond to biophysical cues:

(i) mechanosensors in the cell membrane (transmembrane receptors, growth factor receptors and proteins that mediate cell-cell adhesion and cell-ECM interactions),

(ii) mechanosensitive ion channels, (iii) ECM proteins, (iv) cytoskeleton components, and

(v) nuclear structures (involving the chromatin) [5]. Proteins, such as α -catenin, β -catenin, integrins, von Willebrand factor (vWf), talin, vinculin, p130 Crk-associated substrate (Cas), focal adhesion kinase (FAK), and Src kinases, are included in the focal adhesions and adherens junctions [5].

Mechanical stimuli also change due to: (i) cell polarity, motility, shape, geometry, topography, and roughness changes [35]; and (ii) ECM stiffness and tissue geometry changes [36]. Mechanical features inform cells which biochemical pathways need to be up- or downregulated to maintain tissue homeostasis [37]. In the next sections, the importance of the nucleus envelope, cytoskeleton, cell membrane, stroma, and ECM will be addressed. Some examples of cell studies will be given to correlate the physical parameters in the CRC cell behavior and impact on mechanical properties, and the influence of the environment, such as the stroma and ECM, will be addressed in cells mechanotransduction.

2.1. Contributors for Mechanotransduction

2.1.1. Nuclear Envelope

The main components of a cell are nucleus, cytoplasm, and cellular membrane and they have important functions in cellular biophysics. The main nucleus mechanics components are the nuclear membrane, membrane proteins, lamina, chromatin, and intranuclear proteins. The nuclear membrane protein and the lamin (protein network) form the inner nuclear envelope. The inner nuclear layer and the lamina are continuous. Lamina is divided into lamina A (lamin A and C) and type B lamina. Lamina supports the nucleus and is defined by the shape and size of it. Nuclei that contain more lamin A are stiffer and more resistant to deformation. Lamin binds to chromatin and other intranuclear proteins, forming a nucleoskeleton system, resisting mechanical stress, and being a scaffold in transcriptional regulation. The nucleus is coupled to the cytoskeleton through the nesprin. This binds to

lamin A/C and cytoskeleton proteins bind through the linker of nucleoskeleton and cytoskeleton complex (LINC) [38]. This complex is the connection through which the forces from outside the cells are sensed inside the nucleus.

Several studies reported that nuclear membrane proteins are associated with the progression of cancers and thus can be used as biomarkers, such as colon cancer-specific antigen-3 (CCSA-3) for colon cancer [39]. The mesh network of the nuclear lamina provides support to nuclear size and viscoelastic behavior of the nucleus when subjected to external forces [40,41]. For instance, the depletion of lamin B does not cause any change in stiffness but causes additional blebbing in the nucleus [42]. This means that lamin A is the dominant factor in controlling nuclear stiffness [43], which has a value ranging from 0.1 to 10 kPa. The viscosity of the lamina is controlled by lamin A, enabling the nucleus to support the applied force [44]. Changes in nuclear proteins induce malformation in cell division, migration, signaling, and gene expression, such as the overexpression of lamin A. This stimulates the reconstruction of the cytoskeleton via the upregulation of actin-binding protein plastin-3 and downregulation of E-cadherin in colon cancer cells (CCCs), thus resulting in the increase of migration and invasiveness of cancer cells [44]. Pennacchio *et al.* explained the contribution of the nucleus in mechanotransduction in a comprehensive manner [45].

2.1.2. Cytoskeleton

The cytoskeleton is composed of a network of proteins distributed in the cytoplasm, constituted by the actin microfilaments, intermediate microfilaments, and microtubules. All of them have different mechanical properties, their function being the stabilization of cell morphology and definition of its architecture. Actin remodeling is associated with different cancer phenotypes as well as mechanical property changes. The cytoskeleton is composed by F-actin, a filamentous polymer, composed of G-actin monomers. This polymerization/depolymerization process contributes to cell motility, regulating several actin signaling proteins that constitute part of the oncogenic signaling pathways, which will be discussed in Section 2.4. Usually, epithelial cells have normal differentiation, which is characterized by actin polymerization. Actin depolymerization occurs in the pre-cancerous stages. Cancer progresses in situ to become invasive, co-existing with morphological cell alteration, such as the increase of nuclear-cytoskeleton ratio. Myosin-II is a motor protein

that is connected to the viscoelastic properties of the cytoskeleton. The cytoskeleton also has an influence on focal adhesions and adherens junctions [38,46].

Podosomes through the secretion of matrix metalloproteinases (MMPs) and invadopodia (actin-rich protrusions of the plasma membrane) degrade the ECM, thereby promoting invasion of other tissues. Protrusions of lamellipodia and filopodia promote cancer cell invasion, generating traction forces that are needed for mesenchymal-mode migration. Blebs are formed when a stimulus weakens the F-actin cortex, whose disruption is driven by the increase of hydrostatic pressure generated inside the cytoplasm. Bleb expansion occurs simultaneously with the contraction caused by bleb neck formation. In turn, bleb retraction occurs through F-actin assembly at the bleb cortex and due to Ras homologous A (RhoA), Rho-associated coiled-coil kinases (ROCK), and myosin, producing traction forces that help the cell to move forward. In some types of cancer, blebs are used to invade [47]. The blebs are retracted by actomyosin contraction and induce the appearance of traction forces that move the cells forward. De Nicola *et al.* described that membrane blebbing is associated with apoptotic cell death [47–49]. The aggressive cancer types do not respond to antitumor drug-induced apoptosis, which may be affected by actin cytoskeleton structures [50]. Stress fibers, lamellipodia, and filopodia are considered to promote the survival of cancer cells. Actomyosin contraction is essential for sensing the mechanical environments surrounding cells [50]. EMT promotes cell invasion and metastasis, since this process is associated with the decrease of E-cadherin and the increase of N-cadherin expression, given that the latter is weaker than E-cadherin and weaken cell–cell adhesions [50].

Pachenari *et al.* described the role of actin microfilaments and microtubules proportions in different grade I and grade IV CCCs, in which microtubules have an effective role in the reorganization of cytoskeleton in the transition from non-aggressive to malignant phenotypes [51]. According to them, when cells are forced to migrate through narrow pores in the ECM, high levels of actin-myosin contractile forces are transmitted to the nucleus, which could cause the DNA-repair factors to become mislocalized and ultimately increase the amount of DNA mutations in the cell [52]. This mechanically induced DNA damage may contribute to the cancer cell heterogeneity seen in tumor populations [37]. Biber *et al.* recently described actin regulators, such as diaphanous (Dia), and their role in CRC progression and metastasis [53]. The authors showed the abnormal expression of

mammalian DIA-related formin-1 (mDia1) in colon carcinoma tissues in comparison with normal tissue [53]. Knockdown of mDia1 in CCC lines resulted in a compromised ability to perform adhesion, invasion, and migration in vitro. Furthermore, on mDia1-depleted cells, the ability of CCCs to invade adjacent tissues and extravasate from blood vessels was impaired. Interestingly, early cell adhesion defects observed in mDia1-depleted CCCs were due to microtubule dysfunction and not to actin dynamics under non-stimulatory conditions [54]. The injection of mDia1-knockdown cells resulted in significantly lower metastatic lesions in the lungs of animals [54]. Figure 3 shows the invasion of cells due to invadopodia, filopodia, and respective protrusions [53].

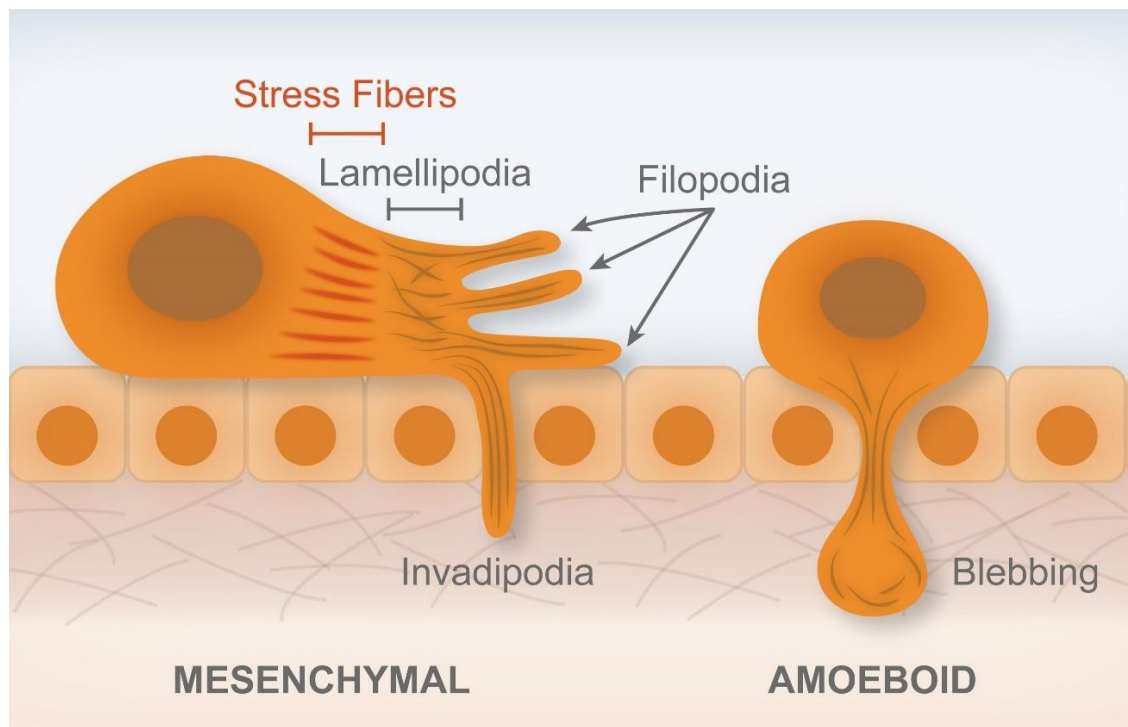


Figure 3. Cell migration through the epithelium and basal membrane (invadopodia) and through the reorganization of the cortex (blebbing). The cells are pushed in a certain direction due to the lamellopodia and filopodia forces produced at the leading edge. After receiving microenvironmental cues (such as growth factors, cell-cell contacts and extracellular matrix signals), invadopodia invade the neighboring tissues. Reorganization of cell cortex can induce blebbing, which facilitates amoeboid-like migration. Adapted from Biber *et al.* [53].

Kumar and Weaver reported that, during cancer progression, proteins of the cytoskeleton induced changes of mechanical properties in cancer cells regarding contraction, stretchability, deformability, and viscoelastic parameters in general [55]. Microtubules,

microfilaments, and intermediate filaments are cell structures that regulate these processes [56]. A good biomarker of cell state could be cytoskeleton reorganization during cancer progression, providing crucial information for new developments in cancer diagnostics, preventive actions, new therapies, and better drug efficiency [57]. Studies of single-cell stiffness designed to investigate the contribution of cytoskeleton biopolymers (e.g., from microtubules and microfilaments) to the mechanical properties of cancer cells are fundamental [58]. Janmey *et al.* reported that the highest cell resistance to deformation was provided by F-actin up to a critical strain value [59]. This characteristic indicated that actin networks at the cell cortex enabled cell locomotion. They are also known to react quickly to external forces, having an important effect on the formation of leading-edge protrusions during cell motility [60]. In fact, microtubules are the second major constituent of the cytoskeleton acting with the other filaments, in the stabilization of cell structure in compression loading. In ovarian cancer, metastatic cells have shown less distinct F-actin structures, with thin stress fibers and less defined and disorganized microtubules [61].

2.1.3 Cell Membrane

The cell membrane also influences cellular mechanotransduction, since it is composed of several proteins which sense the forces from the neighboring cells and from the ECM. They are the “bridges” sensing the forces from the microenvironment to the cytoskeleton, once the forces are transmitted from the mechanosensor membrane proteins, such as integrins. For example, tension is known to induce alterations in the cellular membrane, in the regulation of ion channels, playing a crucial role in cell locomotion, shape modification, and volume adjustment [46]. Ren *et al.* recently studied the effect of membrane tension, by AFM, through the use of the standard solid model applied to the cytoskeleton, which allowed for the quantification of the viscoelasticity through the force-curve approach as a function of time (time relaxation) [62]. The decrease in cell stiffness is associated with changes in the cell membrane surface. The authors showed that approximately 5% of the force exerted by the cell on the probe was from the cytoskeleton and the remaining force was coming from the cell membrane. Hence, it can be hypothesized that the reduction of the cancer cell stiffness can be due to the decrease of the cell membrane tension [62].

Integrins are transmembrane proteins that are connected to the ECM. Laminin-5 γ 2 (LN-5 γ 2) plays an important role in the tumor budding of CRC, due to the interaction between LN-5 γ 2 and integrin β 1, which promotes it via the activation of FAK and yes associated protein-1 (YAP). Cucurbitacin B can block the interaction interface between LN-5 γ 2 and integrin β 1, substantially inhibiting tumor budding [63]. Adherens junctions are known to be involved in sensing the mechanical microenvironments of cells, similarly to focal adhesions [64]. EMT has been described as a cooperation of a complex network and it includes factors classified in three groups: EMT inducers (extracellular cues), EMT core (transcription factors arranging the EMT program), and EMT effectors (effector molecules executing the EMT-related cellular transformation) [65]. The best characterized external inducers are the transforming growth factor- β (TGF- β) signaling, rat sarcoma viral (RAS), and the Wnt/ β -catenin pathway [66]. The overexpression of genes encoding for proteins linked to a mesenchymal phenotype, such as vimentin, fibronectin, α -smooth muscle actin, and N-cadherin, and the down-regulation of epithelial markers, such as E-cadherin, claudins, and occludins, constitute the final effects of EMT regulators [67]. Bellovin *et al.* mentioned that studies in breast and colon cancers indicate that the cytosolic expression of p120 controls the invasive phenotype of E-cadherin negative cells [57]. Ieda *et al.* reported that red fluorescent protein (RFP) expression is driven by mesenchymal vimentin promoter. An inflammatory environment induced RFP expression in association with the EMT phenotype in CRC cells, demonstrating the distribution of RFP-positive CRC cells in rectal and metastatic tumors [68].

According to Boesch *et al.*, the epithelial cell adhesion molecule (EpCAM) is present in the surface membrane of healthy epithelial cells [69]. EpCAM plays an important role in the EMT process. It is a transmembranous glycoprotein, consisting in an extracellular domain (EpEX) and an intracellular one (EpICD). It has been shown that the activation of the β -catenin/c-Myc pathway is induced by the shedding of EpICD to the nucleus, thus resulting in tumor proliferation. The overexpression of EpCAM is associated with cancer progression and a poor outcome [70]. In healthy tissues, the expression of EpCAM is limited to the basolateral membrane of epithelial cells and presents adhesions facilitated by cadherins, being absent in the tight junctions and desmosomes [71]. Winter *et al.* show that the shift of adhesions from strong to weak is caused by EpCAM modulation of cadherin-mediated contacts by antagonizing E-cadherin [72]. EpCAM can also interact with

the cytoskeleton, binding to actin filaments. EpCAM is involved in the disruption of the interaction between actin filaments and α -catenin. Defects in cell-to-cell adhesion promote proliferation, migration, and differentiation, with EpCAM being a pro-metastatic molecule. EMT in tumor cells causes a downregulation of EpCAM, which is believed to induce motility and cell migration [73]. The overexpression of EpCAM is also associated to resistance to anticancer drugs in CRC [74]. EpICD can interact with α -actinin. The existence of cancer stem cells (CSC) (tumor cells that, through the EMT, adopt migratory potential) and EMT is a strong combination for metastatic CRC progression. CSCs detach from the primary tumor and intravasate into nearby vessels, to survive in circulation and extravasate to form metastasis in organs, such as the liver and lungs. It seems that CSCs are more efficient in metastasis formation since EMT cells first need to undergo the reverse process, i.e., mesenchymal-to-epithelial transition (MET), before engrafting. As a consequence, CRC progression is driven by CSC (EpCAM positive) and EMT (EpCAM negative) and also by the EpCAM expression [69].

2.1.4 Stroma and ECM

The stroma is composed by an insoluble, often highly crosslinked, complex network of fibrous proteins (collagen, elastin, fibronectin, and laminin), glycoproteins, and proteoglycans (chondroitin, heparin, keratin sulfates, and hyaluronic acid) surrounding tissue cells [37]. When compared with cancer tissue, there is an increase of several proteins and cells, such as fibroblasts, immune cells, epithelial cells, ECM, and other proteins (e.g., fibronectin). Basically, the cancer niche is composed by the cancer cells in the middle, the basement membrane (BM) around, the stroma, and the ECM. If the BM membrane is disrupted, the cancer cells migrate through the stroma and ECM. Breaking the BM of the organ, cancer cells will intravasate into the blood or lymphatic node system.

Hence, the BM constitutes a physical barrier for avoiding the spread of primary tumor to adjacent tissues at the stage of carcinoma formation [75]. BM is composed of laminin and type IV collagen networks, produced by coordination between the epithelial cells and stromal fibroblasts [76]. It provides structural support to the epithelium, promotes cell adhesion, sustains cell polarity, and it is essential for tissue compartmentalization by separating the epithelium from the stroma [77]. Cancer cells perforate the BM using

MMP-rich protrusions, called invadopodia, and stromal cells can also contribute to this process since they also produce matrix proteases [78–80]. CRC-specific matrix-associated molecules include intestinal receptors, proteases, mucins, and lectins, all contributing to the tissue stiffness [81].

The ECM can be characterized by its topography, roughness, and stiffness. The latter can be defined as being the resistance that a material offers to the elastic deformation when a uniaxial stress is applied [82], while the elastic modulus of a material corresponds to the slope of its stress–strain curve in the elastic deformation region [83]. In response to applied force, cells will respond with cytoskeleton organization, ECM remodeling, gene expression, differentiation, migration, and apoptosis. Due to contractile forces, cells induce viscoelastic deformation, wrinkle formation, fiber alignment, and matrix compaction (Figure 4) [84].

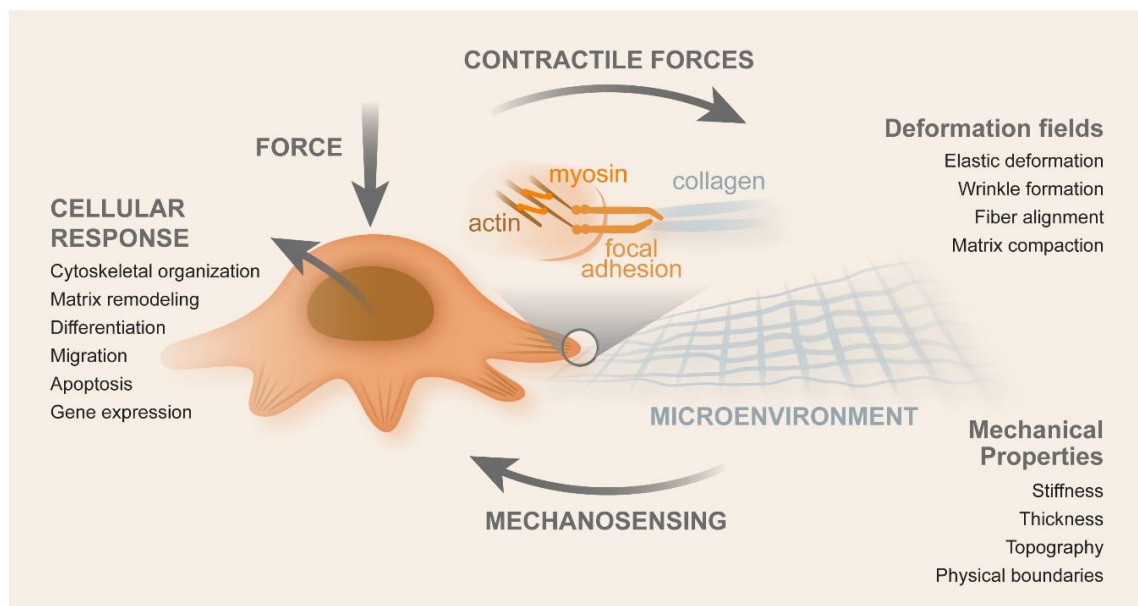


Figure 4. The feedback loop of force-deformation in cells and microenvironment. The cells sense the influence of the ECM microenvironment, such as stiffness, thickness, topography, and the physical boundaries with other cells (mechanosensing). These forces are transmitted to the interior of the cell through molecules that are bound to the cytoskeleton. The cytoskeleton transmits those forces to the nucleus, which will initiate the mechanics of cell response due to the activation of several gene expression pathways, that, in turn, will promote cytoskeleton organization, matrix remodeling, differentiation, migration, and apoptosis. The cytoskeleton organization and migration will produce the polymerization of F-actin filaments and the contraction of myosin motors, promoting cell motion, elastic deformation, and fiber alignment. Adapted from Mohammadi and McCulloch [84].

The ECM provides both mechanical and biochemical signaling cues, thus being an indispensable and dynamic structure and directly affecting cellular behavior. Interruptions can occur either in the physical (e.g., porosity), biochemical (e.g., growth factor binding capacity), and/or biomechanical (e.g., stiffness) properties of the ECM. Some of these properties support and regulate cell differentiation, adhesion, survival, migration, and proliferation rates. During cancer progression, a deeper understanding of the ECM compositional changes is crucial to develop better and targeted treatments [85]. Mohammadi and Sahari used hydrogels to study how they affected the phenotype and gene expression of cancer cells [86]. Tang *et al.* indicated that increasing substrate stiffness promoted the metastatic phenotype in colon carcinoma cells [87]. CCCs were characterized by metastatic hallmarks, such as weak attachment to substrates and cells (after culturing on stiff substrates for seven days) [88]. This suggested that substrate stiffness may be a crucial parameter in the pathogenesis of cancer invasion. An increase in stiffness has been associated to the cell spreading area and size of focal adhesions [84], highlighting the importance of single-cell measurements to define the relationship between cell mechanics and the formation of cell adhesions. This process could be important to understand how cancer cells interact with ECM of varying stiffness values. In ECM stiffness, the elastic and inelastic behavior must be taken into account, which may modify mechanosensation by cells [84]. A possible approach for therapeutic intervention seems to be preventing or reversing tumor stiffening since the stiffness of the tumor microenvironment contributes to tumor growth and metastasis [86].

Pinto *et al.* reported macrophages as additional key players in cancer progression, with the capacity to modulate cancer cell migration, invasion, and metastasis [89,90], with these immune cells being highly plastic, possibly adopting numerous and distinct polarization phenotypes, namely either pro- or anti-inflammatory. Along with these cells, decellularized ECM has been described as having relevance in cancer progression. Human CRC and non-neoplastic mucosa were efficiently decellularized. Before repopulation with primary human monocytes, they were studied regarding their viscoelastic properties using rheometry. The tumor decellularized matrices presented higher stiffness than normal decellularized matrices [89]. They further showed that normal and tumor decellularized matrices constitute excellent scaffolds for recreating microenvironmental features, leading to a better understanding of

mechanisms of disease and therapeutic resistance [89].

Several distinct studies also reported that cell properties depend on substrate stiffness [87,91–94]. CCCs modify their mechanical properties to resist intravasation, shear stress associated with circulation, and extravasation [95]. Mechanical cues can also play an important role in the early phases of metastases by adapting to the tumor microenvironment. The stiffness of ECM obtained through the crosslinking of collagen was achieved by modeling integrin's expression and resulted in the induction of malignant behavior in mammalian epithelial cells [96]. One cause of tumor stiffness due to variations in the tumor microenvironment is the unbalance between ECM production and its degradation. According to Peddareddigari *et al.*, in CRC, there is the production of MMP-1, -2, -3, -7, -9, and -13. When an increase in MMP-3 production occurred, CRC presented microsatellite instability (with changes in small portions of chromosomes) with a poor prognosis [66,81]. Meanwhile, when an increase of MMP-12 production was observed, there was a good prognostic of survival since this molecule had an inhibitory effect on angiogenesis [81].

In the process of strain stiffening the resistance of crosslinked ECM increases with increasing matrix deformation, through which the ECM undergoes cell-induced deformation. The rate and magnitude of matrix deformation determine the amount of plastic deformation. Slow rates of force-mediated collagen remodeling cause a stable alignment of its fibers, which is related to poor outcomes [97]. Proteolytic cleavage by MMP-14 can indirectly counteract the effect of crosslinking by decreasing matrix stiffness [98]. It is likely that cycles of proteolytic degradation, new synthesis, and new crosslinking, enable permanent matrix remodeling which is mainly elastic over long-time scales [86].

Nebuloni *et al.* investigated the biochemical and physical diversity of ECM in healthy, perilesional, and CRC samples. The authors observed that ECM from perilesional and CRC samples withstood the proliferation and invasion of tumor cells. From healthy ECM to perilesional and CRC, a progressive linearization and organization of fibrils was observed, associated with an increased collagen crosslinking and consequent increased stiffness. In perilesional ECM, these modifications corresponded to an increase in vascularization, whereas in neoplastic ECM they were associated with the altered

modulation of some proteins [99]. This study has shown that the increase in stiffness and crosslinking of perilesional ECM favors a suitable environment for CRC invasion associated to the vascularization process. The stiffness increase of colon tissues may be used as a marker of desmoplastic tissue predisposing to invasion, which can be used as a new potential application for follow-up of adenoma with invasive potential [99].

In 2D models, it is easier to study these parameters but not angiogenesis (new vessels formation from pre-existing ones), since it cannot be reproduced in a 2D model. The angiogenesis process is an essential step when a tissue mass transitions from a benign state to a malignant state, meaning that if the oxygen is missing the tumor becomes hypoxic.

Three-dimensional (3D) cell culture models are an emerging powerful approach for studying ECM mechanics and cellular responses, although they still lack the cell and matrix complexity of natural tissues. Reidy *et al.* described the evolution of 3D models used in CRC studies, as well as their advantages and disadvantages. They showed their utility to predict the response to therapies and resistance. It was shown that an increase in stiffness promoted CRC progression and difficulties in drug delivery, thereby promoting resistance [100]. Moreover, 3D studies are fruitful since they provide a more similar environment to the native structure (such as 3D geometry, porosity, ECM binding site, ECM heterogeneity, and gradients of biochemical factors, among others), although they recognize that several challenges remain to be overcome, namely the difficulties in controlling the environment and in visualizing cell–cell and cell–ECM interactions, as well as in quantifying results without specific imaging technology [101]. Lysyl oxidase (LOX) has already been used to increase the collagen matrix stiffness in order to promote relevant physiological conditions. Hydrogels are known to have poor mechanical properties. However, when methacrylamide and methacrylate groups are incorporated, stiffness increases significantly, mimicking the CRC microenvironment [100].

2.2 Mechanics of CRC Cells

Cells respond to internal force generation, as well as to the change of mechanical properties of their surroundings. Internal forces contribute to cell motility, stiffness, focal adhesion, and shape changes. Cells respond to external stimuli transmitted by the ECM (through stiffness) by changing their Young's moduli, maturation, and focal adhesion

number. The responses mentioned previously were observed through a range of mechanisms, namely integrin activation and reinforcement as well as the activation of putative force sensors, stretch-activated channels, protein unfolding, and activation of signaling pathways [102,103]. Cell surface roughness, shape, actin organization, stiffness, and adhesion are modulated by CRC cells to better accomplish their specific tasks in cancer growth and invasion [104].

In cell biophysics, cell morphology, shape, and roughness can also provide significant information since these parameters may affect the mechanical properties in disease. Boccaccio *et al.* used nanoindentation by atomic force microscopy (AFM) to study the effect of surface roughness and cell shape in mechanical properties variation of SW480 and SW620 cell lines on colon cancer and lymph-node-derived metastasis, respectively [105]. The authors found differences relative to roughness properties for the SW480 (round, R shape and elongated, E shape), showing that rounded cells (SW480R and SW620) possessed comparable elastic parameters, which were different from those of elongated SW480E cells. This conclusion allowed them to formulate the hypothesis that the properties of rounded cells are intrinsically different from those of the elongated ones [105]. This could also contribute to the hypothesis that the role of subcellular components is different from one cell to another. Palmieri *et al.* also observed that SW480 populations have specific functions when proliferating in the primary site (SW480R) and metastasis (SW480E), being reflected in cell shape and mechanical properties. The R-type cells showed a decrease in stiffness due to the actin anisotropy, as opposed to E-type cells [104]. The SW620 cell type, with its round shape, took advantage of its high adhesion capacity (shown between cells and AFM tip) to metastasize, by better adhering to the blood vessel walls. Regarding shape and topography, studies developed by Ball *et al.* in osteoblast-like cells showed the shape factor changes with respect to substrate morphology where the cells were seeded [106]. In terms of the quantification of cell elongation, a shape factor of 1 corresponds to circular cells, and a factor of 0 corresponds to elongated cells.

Tang *et al.* studied three different human CCC lines (HCT-8, HCT-116, and HT-29), all of them having a low metastatic potential and an epithelial elongated phenotype (E-type) [93]. When these cells were seeded on a stiff substrate, they adhered, spread, and proliferated, forming a monolayer with E-cadherin-mediated junctions. A very rare, small number of

round-shaped cells (R-type) was observed on top of these monolayers. However, if the HCT-8, HCT-116, and HT-29 cells were seeded on a soft substrate, 50–70% of these E-type cells transitioned to R-type. This confirmed that the stiffness of substrate has implications in cancer cell metastatization [93].

Tang *et al.* showed that when cultured on soft substrates (21 kPa), human colon carcinoma cell line HCT-8 changed from low to high metastatic state and thus lost mechanosensitivity as they underwent E-to-R transition [93,107]. Cell spread area, proliferation, and migration of HCT-8 R cells were not sensitive to substrate stiffness changes in contrast to E cells. R cells acquired autonomy and anchorage independence, being more invasive than E cells [108]. Lammermann *et al.* recently described the different amoeboid phenotype based on the force relationship of the three major forces in cell migration, i.e., adhesion, contraction, and polymer network expansion [109]. Panzetta *et al.* showed the inherent amoeboid appearance of the S174T colon carcinoma cell line [110]. Duchalais *et al.* demonstrated, by AFM, that tumor epithelial cells (TEC) have stronger adhesion forces to enteric nervous structures than to mesenchymal cells [111]. TEC adhesion to non-myelinated intrinsic enteric nervous system (ENS) involves direct interactions with enteric neurons. The removal of enteric neurons from ENS cultures resulted in a significant decrease in tumor cell adhesion. ENS significantly promoted TECs migration when compared with migration through mesenchymal cells. TECs migration followed faithfully the trajectory of the ENS structures. The enteric neuron used the neural cell adhesion molecule L1 (L1CAM) and N-cadherin to bind to CRC. The authors hypothesized that CRC cells bind to the L1CAM of ENS using an integrin since the binding of L1CAM to $\alpha 5\beta 1$ -integrin was reported in other organs. The authors also mentioned the presence of N-cadherin expression in both TECs and ENS, suggesting homophilic binding. A possible local path for CRC spreading may be the dense network of local enteric neurons in the CRC microenvironment [111].

Figure 5 shows the cells involved in the adhesion and migration of CRC cells through the local enteric neurons network [111].

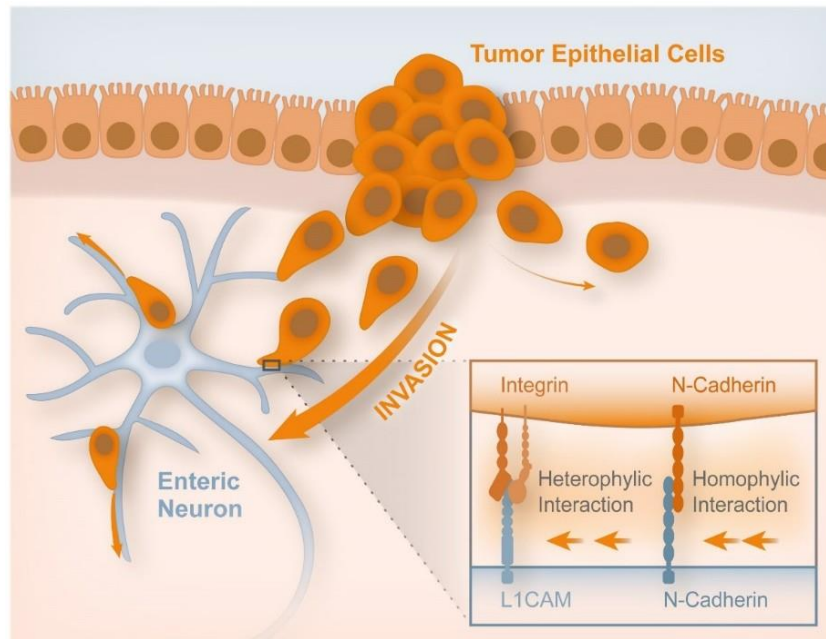


Figure 5. Cells involved in the adhesion and migration of CRC cells through the local enteric neurons network. It is speculated that tumor epithelial cells migration is performed along the neuronal fibers, through the heterophilic bond of L1CAM—integrin and suggesting the existence of homophilic bond of N-cadherin. These physical process guides the migration of CRC cells. Adapted from Duchalais *et al.* [111].

2.3 Techniques to characterize mechanotransduction

For mechanobiology and mechanotransduction studies, several techniques can be used, such as AFM, microindentation, shear rheometry, shear wave and magnetic resonance elastography, micropipette aspiration, optical stretching, and magnetic twisting cytometry, which have been developed to evaluate the mechanical properties of cells and tissues [82]. Table 1 presents different techniques used in mechanotransduction studies, including a brief summary on each of them. The characterization techniques mentioned below allow the quantification of biophysical parameters, from cells up to solid and irregular biological samples, in opposition to the histopathology and immunohistochemistry assays, which are the standard methods still in use but result in the loss of mechanical properties after tissue fixation.

Table 1. Characterization techniques to study the mechanobiology and mechanotransduction.

Technique	Principle	Refs.
Atomic force microscopy (AFM)	Mechanical characterization of samples in physiological conditions; allows quantification of mechanical properties of cells and tissue samples and also quantification of molecules and cells interactions by force spectroscopy, using dynamic conditions.	[82,112–114]
Microindentation	Quantification of micro-mechanical properties of biomaterials, hydrogels and biological samples down to cell-length scale, in physiological and dynamic conditions	[115]
Rheometry	Quantification of mechanical properties of soft tissues, thus being a promising technique for cancer diagnosis, as well as for assessment of effectiveness of anticancer treatments, in dynamic conditions	[116]
Shear wave elastography and magnetic resonance elastography	Characterization of healthy and diseased tissues in a non-invasive manner, with the generation of cross-sectional images, showing the stiffness of the tissue.	[117,118]
Elastography	Quantification of stiffness of the tissue is evaluated by the tissue response to an externally applied mechanical stimulus, leading to a measurable tissue deformation.	[119]
Micropipette aspiration	Allows study of a whole cell which, under the suction pressure, undergoes a deformation process, thereby allowing measurement of geometry of the cell inside the capillar.	[120]
Optical stretching	Formation of an optical trap, resulting from the two opposing laser beams, presenting a Gaussian intensity distribution, able to capture the cell that is in suspension and stretching it in situ; it acts on the entire cell membrane, making it possible to measure its viscoelastic properties.	[121]
Magnetic twisting cytometry	It is based in the application of a twisting field, generating rotational shear stresses in several directions; it allows the attachment of specific beads to the cytoskeleton through the membrane mechanoreceptors, applying local mechanical stress to live cells.	[122]
Terahertz waves	It is used in cancer diagnosis, not through mechanical properties but using imaging and spectroscopy (in a range of 0.1 and 10 THz).	[123,124]

Recently, Deptula *et al.* used AFM and shear rheometry as potential techniques for cancer diagnosis by studying the mechanical properties of human colon cancer fresh tissues, as possible mechanomarkers in the oncological clinical field for predicting the stage of disease [112].

AFM was also used by Cross *et al.* to study the morphological changes and nanomechanics of adenocarcinoma cells (from breast and pancreas), lung carcinoma cells, metastatic cells found in the body pleural fluid, and compared them with the mesothelial cells (cells that line the body cavity). This work showed that the stiffness of carcinoma cells was lower than that of mesothelial cells, in spite of them having similar shapes. This work also showed that the nanomechanics analysis fitted well with the results of the immunohistochemistry used for cancer detection, demonstrating that mechanical quantification can be a biomarker in cells removed from body fluids and not only for solid tumors [125].

2.4 Biophysical Cues vs. Biochemical Signaling

Thanki *et al.* referred the classification of CRC according to the consensus molecular subtypes (CMS): CMS1 is immunogenic and hypermutated, CMS2 has the highest overall survival and is activated by the Wnt/ β -catenin pathway, CMS3 presents a metabolic phenotype, while CMS4 cancers present a stromal gene signature and the worst survival [126].

Each CMS is characterized by the activation of different signal transduction pathways. The CMS classification provides information to clinicians about prognosis, therapeutic response, and new insights for the development of therapeutic strategies [126]. Singh *et al.* also referred two different molecular classifications of CRC through the candidate cancer gene (CAN) [2].

In the case of CRC, Pandurangan *et al.* and Smit *et al.* mentioned that the main signaling pathways affected in CRC epithelial cells are: the APC, KRAS, transforming growth factor- β (TGF- β /Sma genes and the Drosophila Mad (SMAD4), protein 53 (P53), Wingless-related integration site (Wnt)/ β -catenin, YAP/Transcriptional co-activator with PDZ-binding motif (TAZ) hippo signaling, Rho-family, Tumor necrosis factor- α (TNF- α), and phosphoinositide 3-kinase (PI3k) pathways, which are known by the adenoma-carcinoma sequence (ACS) [3,127]. It seems that there is an optimal balance between mechanical and genetic cues and that plays a key role in the genesis and development of malignancies [56,128,129]. As a consequence, a balance between the biomechanical and biochemical properties of cellular components and ECM alters cellular behavior, stimulating biochemical pathways which will be drivers of cell proliferation, migration, invasion, survival, and metastization [130]. Some biophysical stimuli activate more than one biochemical pathway response. Some activation pathways induce the activation of other sequence pathways, which means that in CRC there is a co-existence of several pathways, almost simultaneously.

The epithelial proliferation is associated to the mutation of APC gene, a key tumor suppressor gene. Loss of APC function leads to colorectal tumors [95]. Whitehead *et al.* applied mechanical strain (deformation, in z-direction, 20 min with a load of 800 Pa) on colon explants from healthy and APC-deficient mice. Tissues showed shape

changes at the cellular level, which were proved to be coincident with the expression of two oncogenes (Myc and Twist1) in tissue explants with APC deficiency, but not in the wild-type colon explants. This can be explained by the fact that with the mechanical deformation, the APC deficient tissue was associated to an increased number of β -catenin positive nuclei per crypt, but this fact was not detected in wild-type mice colon epithelium [131]. This mechanical strain can be associated to the intestinal transit and, when APC is downregulated, can favor the presence of polyps and tumor growth [132]. It is known that APC can also be overexpressed and cause CRC, which means that APC has a dual role in the CRC cancer. It is crucial to know more about these two opposite mechanisms to develop suitable therapeutic strategies [133,134]. The expression of APC promotes cell asymmetry due to the longer cellular protrusion formation [135].

Zanconato *et al.* also corroborated that most CRC cases carried mutations leading to the Wnt signaling pathway, among which were mutations inactivating the APC tumor suppressor [36]. Wnt pathway was mentioned by Ciasca *et al.*, reporting the mechanical cues that may contribute to early phases of tumor initiation, since that regulator is one of the most important in cell proliferation [136]. The same authors described well the two stages of the Wnt pathway (on and off) leading to cell proliferation [95]. In a study with 224 CRC cases, in 94% of them, a mutation in one or more members of the Wnt signaling pathway was detected [137]. Lee *et al.* corroborated that the Wnt/ β -catenin cascade plays the dominant role in controlling the fate of epithelial cells in the intestine and colon, as well as a mutation in the APC. Additional mutations, such as the human gene that encodes a protein called B-Raf (BRAF) protein pathway, resulted in the growth of a small adenoma with clinically significant size (>1 cm) [13,137]. Additional mutations in p53 drive the malignant transformation to adenocarcinoma. Stages of CRC development and the extent of disease were associated with the activations of cyclooxygenase-2 (Cox-2), EGF, and VEGF [13].

The p53 mutation was found in ca. 60% of CRCs [138]. In patients with CRC, the apoptosis of p53 proteins is downregulated [139]. This mutation is sensitive to ECM stiffness. In integrin signaling pathways, the downregulation of molecules

suppressed cancer progression due to p53 wild-type. Ebata *et al.*, on the contrary, documented that the molecules upregulated by p53 mutants were $\alpha 5$, $\beta 1$, and $\beta 4$ integrins and fibronectin. It has been revealed that, by regulating the expression of E- and N-cadherins, p53 prevents EMT [50,140].

In CRC, KRAS is the most frequent isoform mutation, accounting for about 20% of all human cancers, while neuroblastoma rat sarcoma (NRAS) and Harvey rat sarcoma (HRAS) mutations are found in about 8% and 3% of cancers, respectively [66]. The involvement of RAS in cellular plasticity was demonstrated by morphological and molecular correlations. Invasive and migration properties of malignant cells influence cellular plasticity. The initiation of EMT in CRC involves RAS signaling, leading to tumor spreading. According to the adenoma-to-carcinoma sequence, KRAS and p53 genes are regularly mutated. Mutations of KRAS were able to promote the development of CRC, while NRAS mutations were not, as investigated in an APC-deficient mouse model. The RAS function has been well described by Maffeis *et al.* [66].

Hippo signaling can be ON or OFF [50,141]. If Hippo are ON, this is corresponding to YAP/TAZ being inactivated. If Hippo signaling are OFF, this corresponds to YAP/TAZ being activated [142]. YAP/TAZ is misexpressed in several solid cancers such as CRC. High expression of nuclear YAP is related with tumor progression and survival decrease. However, a dual role of YAP has been recognized both as an oncogenic and as a tumor suppressor in differential phases of CRC progression [143]. Ma *et al.* reported that hypoxia-inducible factor (HIF) was present at the intestine and HIF-2 α was essential for CRC growth and progression. The authors found that HIF-2 α activated YAP1 was crucial for cell growth during hypoxic stress in colon-derived cell lines [144]. However, Ou *et al.* described the dual role of YAP, i.e., both the oncogenic and tumor suppressor. To understand these two mechanisms is important for the development of CRC therapeutic strategies [143]. At cell-ECM adhesion sites, where the cell geometry and matrix stiffness are noticed, the YAP plays an important role in signaling activity [145]. In animals, the Hippo signaling pathway is considered the main transcriptional effector, being identified as a key mechanotransducer, performed by nuclear tips of mechanical stimuli [146]. Cellular proliferation and inhibition of apoptotic signals, loss of contact inhibition and tumor

growth, and increment in cell size and organ growth were associated with YAP/TAZ accumulation and its activation in the nucleus [146]. Elosegui-Artola *et al.* highlighted that ECM–nuclear mechanical coupling translocates YAP in response to substrate rigidity [147]. The authors explained that, using soft substrates, the forces applied directly to the nucleus induced YAP nuclear translocation while decreasing mechanical restrictions to molecular transport from nuclear pores. When cells were exposed to a stiff environment, they established mechanical connections through the forces exerted by focal adhesions from the cytoskeleton to the nucleus [147].

TNF- α has been described as another signaling pathway to be taken into account in CRC. Liu *et al.* used AFM to study the mechanical properties of EMT in HCT116 human CCC lines, in the presence and absence of TNF- α treatment, since chronic inflammation is considered the seventh hallmark of cancer [148]. TNF- α is widely recognized as among the main mediators of cancer-related inflammation, mediating all steps of tumorigenesis, such as proliferation, invasion, angiogenesis, and metastasis formation, by accelerating tumor invasion and metastasis through EMT. TNF- α induced morphological changes consistent with EMT in HCT116 cells, since cells with no TNF- α treatment adhered to each other, while after treatment, cells exhibited morphological changes and intercellular spaces [148]. These findings suggest that it is crucial to investigate medical substances to avoid morphological changes in CCCs consistent with the EMT, thus avoiding the existence of (or at least part of) conditions to metastization. It is not well known which biophysical cues can activate this pathway.

Zessner-Spitzenberg *et al.* investigated the role of TGF- β in shifting the epithelial cancer cells towards a pro-migratory phenotype via stromal signaling and, consequently, invasiveness [138]. A poor prognosis subset of CRC was identified in some studies, which was characterized by high stromal activity and elevated levels of TGF- β [149]. This protein has been recognized as a main driver of CRC metastasis [138]. Tsuji *et al.* reported differences in terms of microvessel growth in the tumor tissues, in relation to amounts, diameter, and spatial direction in carcinomas with metastasis and without metastasis [150]. The authors also found significant differences related to the presence of VEGF and reported a higher VEGF-positive cell count in tumor tissues when compared to normal tissues. With tumor progression, microvessel

diameter showed a significant increase and microvessels counts a decrease, which can be partially explained by the VEGF expression. In the first step of metastasis, the microvessel diameter seems to be the predominant parameter responsible for intravasation cell cancer [150].

Ebata *et al.* reported the importance of Rho family activation in cancer metastasis [50]. Rho GTPases, such as Rho, Ras-related C3 botulinum toxin substance 1 (Rac), and cell division control protein 42 homolog (Cdc42) regulate the activation of actin regulators, contributing to the formation of filopodia, lamellipodia, and stress fibers, respectively [141]. The formation of actin-mediated structures, such as lamellipodia, filopodia, podosomes, and invadopodia, is associated with the invasion of cancer cells [140,141]. Rho signaling is related to the ECM stiffness and the ECM proteins have an important role in the activation of several biochemical pathways. Gagné *et al.* recently demonstrated that integrin-linked kinase (ILK) specifically induced the initiation of fibronectin, fibrillogenesis, during cell spreading, promoting the Rho/ROCK-dependent cell contractility and maturation of the integrin-actin structures [151]. According to them, fibrillogenesis and its effect in Rho signaling, cell contractility, and spreading did not depend on ILK in epithelial crypt cells in the human intestine [151]. Fibronectin interacts with an ECM glycoprotein named tenascin-C, participating in cell migration and organogenesis. Tenascin-C is crucial in the activity of growth factors, proteases, and protease inhibitors during cell spreading. This protein also induces Rho A/ROCK-dependent cell contractility and the integrin-actin axis structures [152]. Fibrillogenesis, cell contractility, and spreading are independent of integrin and kinase in epithelial cells crypt in the human intestine [151]. Krndija *et al.* mentioned that the Rho family also mediated the contractibility, number, and size of adhesion sites, as well as the increase of stress fibers [153]. The generation and response forces were identified as key elements in cell migration. The authors reported that the expression of receptor-type tyrosine-protein phosphatase alpha (RPTP α) in human SW480 CCC line was related to cell contractibility, a process in which there is an increase of that protein, thus increasing the number and size of adhesion sites and stress fibers (bundles of F-actin and myosin II held together by crosslinking proteins, thus ensuring the cytoskeletal contractility) [154].

RPTP α influenced cell spreading on low-rigidity surfaces, suggesting that force-responsive proteins can influence cancer cell behavior and identify potential novel targets for cancer therapy [153]. PI3k signaling is related to cell growth, differentiation, cell motility, intercellular trafficking, and cellular survival. This signaling pathway is not activated directly through a specific biophysical stimulus. It is activated as a consequence of other activations of biochemical pathways [95,155].

Table 2 shows some examples of putative genes which are influenced by biophysical cues, inducing the activation of biochemical pathways.

Table 2. Biophysical cues and its relation with biochemical pathways, their influence on CRC behavior, prognosis, and possible therapeutic strategies.

Biophysical Cues	Biochemical Pathway Activation	Cell Process Activation	Prognosis	Therapeutic Target	Refs.
Cell stress (UV, hypoxia) ECM stiffness	p53	Loss of cell adhesion promoting EMT Stroma enriched in CAFs DNA damage Filopodia formation	Good	Promote the wild-type p53 Avoid CAFs differentiation Avoid the ECM stiffness Regulating the expression of E- and N-cadherins, p53 prevents EMT	[39,66,156,157]
ECM stiffness	APC (loss) APC (overexpression)	Decrease of microtubule stability Shape changes in cells Cell deformation Loss of cell adhesion Induce cell protrusions Alter cell shape Cellular asymmetry by inducing longer cellular protrusion	Good	Validating the Wnt pathway as a therapeutic target	[13,132,135,158]
Cell friction forces ECM rigidity Fluid shear stress Forces applied on the nucleus	Hippo ON (YAP/TAZ off) Hippo ON (YAP/TAZ off) Hippo OFF (YAP/TAZ on)	Cell size increment Cell size increment MMP-7 increasing (break the BM) Cell stretching and density increasing B-catenin inside the nucleus More research must be performed	Poor	The therapy must be applied according to the CMS	[39,142,143,147,159–164]
ECM stiffness	KRAS	EMT Cellular plasticity PI3K activation Cytoskeletal deformability	Poor	Targeting EMT	[66]
ECM via interaction with fibronectin through tenascin-C ECM stiffness	Rho-family	Filopodia formation Lamellipodia formation Podosomes formation Contractability Activation of actin regulation Stress fibers increase Loss of cell polarity	Poor	Target the integrins and ECM connections; target the tenascin-C Targeting VEGF and EGFR	[39,50,141,153]
ECM stiffness Shear stress Compression Cell adhesion	Wnt (on) Wnt (off)	APC mutation activation Nucleus with β -catenin inside More studies are need	Good		[95]

Note: APC, Hippo, and Wnt signaling pathways have a dual role in CRC cells (two colors were used, corresponding to each case: blue—if the biochemical pathway is downregulated, loss or off; red—if the biochemical pathway is overexpressed or on).

These biochemical pathways, in turn, will influence the cell behavior. Prognoses were also able to take into consideration the CMS classification. Some possible therapies were indicated. The signaling pathways that have a dual role (APC, YAP/TAZ, and Wnt) in CRC are highlighted with different colors (blue corresponding to under expression or loss and red corresponds to overexpression of the pathway).

2.5 Role of Cancer-Associated Fibroblasts (CAFs)

Fibroblasts produce the ECM and the collagen fibers. Fibrosis is defined as a normal reaction in the wound healing process, in which there is an excessive accumulation of ECM proteins. In cancer, it is expected that condensed collagen works as a barrier to avoid the spread of the cancer cells [165]. Bremnes *et al.* described how the fibroblasts are activated to CAFs in tumor disease [152]. CAFs, being the major constituent of the tumor stroma, play an important role in cancer progression [152]. CAFs activity is regulated by various cancer-associated growth factors, connected to the metabolism, proliferation, and metastization of cancer cells [166,167]. Shin *et al.* evaluated cancer invasiveness and the reactions of abnormal cells and their relationship with CAFs in long-term in CRC patients [165]. The authors concluded that the percentage of mature CAFs in the intra-tumoral stroma and the invasive front were 57.6% and 60.3%, respectively. The mature CAFs in the invasive front presented a significantly higher amount of epidermal growth factor receptor (EGFR), when compared with the immature CAFs. Hanahan *et al.* described that the increase in the number of mature fibroblasts implicated the increase of lymphatic invasion in the intra-tumoral stroma. With the progression of the tumor, the surrounding microenvironment became enriched in CAFs, cells from the immune system, and ECM [168]. Gaggioli *et al.* studied cell invasion in an animal model and showed that CAFs were responsible for cancer cell invasion through the type I collagen/matrigel hydrogels [169]. Cancer cells sense the physical forces exerted by CAFs, through the heterotypical cell-cell interactions, which stimulated their invasion [170]. Glentis *et al.* explained that in carcinoma in situ, the BM segregated tumor cells from the stroma [171]. For tumor dissemination to the adjacent tissues, this barrier must be broken, which may be achieved by proteolysis. The authors have shown that CAFs promoted cancer cell

invasion through the BM, independently of the activity of MMPs. The BM is also affected by CAFs, since BM is pulled, expanded, and softened by them. The formation of gaps contributes to cancer cell migration. These gaps are increased by the BM alteration, since CAFs modify the organization and the physical properties of the BM, allowing cancer invasion [77].

Belli *et al.* reported that the ECM stiffness of primary tumors may be affected by CAFs since it improves cancer cell invasion, inducing EMT, thus supporting the spreading of metastasis [77]. CAFs also express intercellular adhesion molecule 1 (ICAM1), with its functions of regulating immune response, programming cell death protein ligand (PDL)1 and PDL2, and mediating immunosuppressive functions [77]. Mierke recently presented a correlation between cells and their microenvironment, including CAFs and type I collagen fiber scaffolds [172]. The synthesis and deposition of collagen and released LOX and transglutaminase-2 exosome are influenced by CAFs, increasing the collagen stiffness [172]. Peng *et al.* recently reported CAFs resulting from the activation of fibroblasts, which were activated by integrin $\alpha\beta6$ on CCCs [173]. Normal fibroblasts showed less parallel organization of fibronectin when compared with CAF-derived matrices [173]. Platelet-derived growth factor receptor- α (PDGFR α) and myosin-II-driven traction forces transduced by integrin $\alpha5\beta1$ contribute to CAFs matrix organization [174,175].

Attieh *et al.* also referred that the CAFs, being the most abundant cells in the tumor stroma, have the capacity to contract the matrix and induce invasion [176]. The authors reported that CAFs contributed to fibronectin assembly and induced invasion through integrin $\alpha\beta3$. If there was no fibronectin present, the contractibility of the matrix induced by CAFs was maintained but the ability to induce invasion was eliminated, meaning that CAFs retained the capacity to induce invasion in a fibronectin-dependent manner. CAFs produce a rich fibronectin ECM, with anisotropic orientation, which promotes cancer cell migration in certain directions [174]. New hallmarks of CAFs promoting tumor invasion can be defined in relation to the fibronectin assembly and integrin $\alpha\beta3$ expression [176]. Periostin is a ligand for integrins $\alpha\beta3$ and $\alpha\beta5$, also being the CAF-released matricellular protein that

induces angiogenesis. In several cancers, such as oral, ovarian, breast, and colon, periostin is believed to induce an improvement of cell survival growth, proliferation, migration, invasion, and angiogenesis, since the previous two integrins facilitate signaling pathways [175,177]. Periostatin also promotes cell adhesion and migration. On colon cancer cells, only integrin $\alpha\text{v}\beta 3$ interacts with periostin, facilitating metastatic growth and angiogenesis [52,175,178]. Staudacher *et al.* reported that, in CRC patients, the worse prognosis can be associated to the combination of activin-A and TGF- β expression. TGF- β increases the epithelial and stromal activin-A secretion, with migration being dependent on it [179]. Metastasis is also promoted by the upregulation of MMPs by activin-A. MMP-7 induction by activin-A is a requirement for dissolving BM and other ECM components to allow the metastatic spread [180]. According to Storm *et al.*, CAFs may exert a significant force on the ECM which is generated by ROCK-dependent actomyosin, through integrin-mediated adhesions [181]. Calvo *et al.* reported that the stiffness of the ECM was caused by the tension forces due to actomyosin contractility, increasing the collagen tension at the tumor microenvironment [182]. Madsen *et al.* reported that the contractility of CAFs was reduced under hypoxic conditions [183]. Zhou *et al.* mentioned that tumor budding, being the existence of a single cell or clusters of up to five cancer cells, is an independent prognosis factor for CRC [184]. This process is still not clear and the drugs that could inhibit this process are still in a very early research stage [165,184]. Tumor budding was visualized in both mature and immature stroma samples, occurring frequently in infiltrating type tumors. EGFR overexpression, maturation of CAFs, and infiltrating tumor growth configurations conducting to potential tumor were associated to well-connected islands (Figure 6) [37,175].

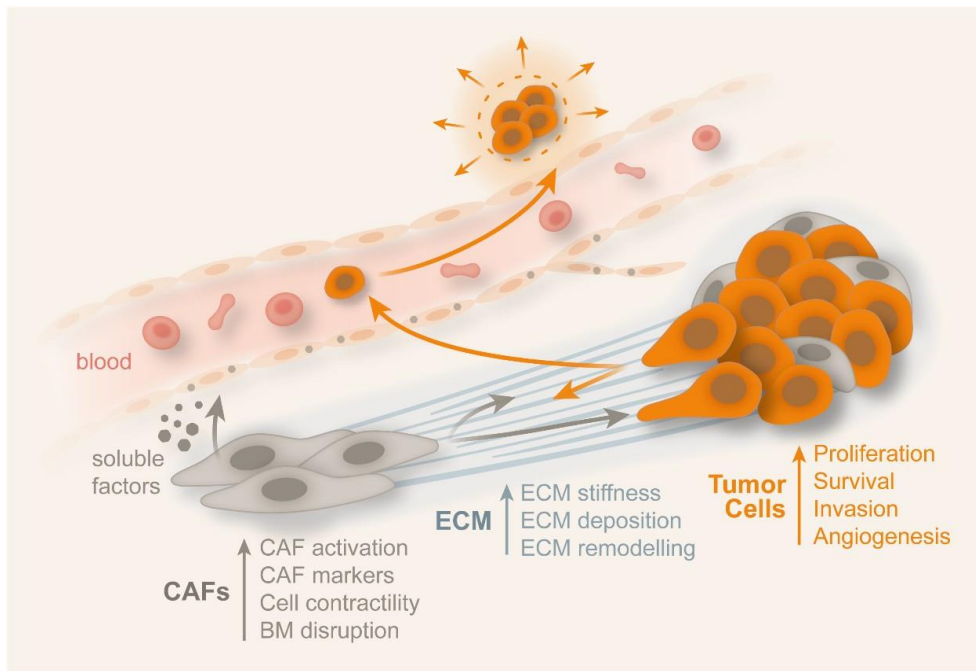


Figure 6. The stiffening and angiogenesis resulting from the remodeling of the ECM, is associated with the presence of cancer-associated fibroblasts (CAFs). Cancer cells at the leading edge of the tumor will assume epithelial-to-mesenchymal transition (EMT) and travel along collagen and fibronectin fibrils towards the vasculature. Due to CAFs, fibrils and collagenase are aligned at the edge of the tumor to promote invasion. The cell cytoskeleton will reorganize due to the shear forces sensed, eventually promoting adherence to the blood vessel wall. CAFs also contribute the disruption of the basement membrane. Adherent cells that survive will extravasate to a distant tissue. Cancer cells will not undertake mesenchymal-to-epithelial transition (MET), if the tissue is considerably stiff, and therefore the cells become dormant. MET will occur only if the elastic modulus is low and a secondary tumor will then be formed. With tumor progression, the matrix stiffness will increase. Adapted from Libring *et al.* [37] and Jang *et al.* [175].

2.6 Impact of Shear Stress on Metastization

To survive the mechanical stress associated with intravasation, circulation, and extravasation, the metastatic cells modify their morphology and mechanical properties, such as adhesion and stiffness. These metastatic cells result from the physical stimuli involved in the differentiation of non-invasive cells [95]. The surface of cells senses mechanical forces generated by fluid shear stress in the adhesion points through integrins 1 and 3, being decoded in changes of gene expression. Endothelial cells were used in most studies [95]. In CRC, progression of cell cycle is

regulated by mechanical forces due to fluid shear flow [185]. Since cancers of intestinal tract grow into the gut lumen, they are wide-open to different degrees of fluid shear stress [186]. In CRC, it is controlled by mechanical forces, which are promoted by fluid shear flow. A fluid flow of $0.4\text{--}3.5 \times 10^{-4} \text{ N/cm}^2$ is the normal range found in the intestinal epithelium during peristalsis of the gut. Avvisato *et al.* exposed SW480 cells to a flow shear stress of $1.5 \times 10^{-4} \text{ N/cm}^2$ and found that the expression of β -catenin decreased by shear stress, inhibiting the Wnt-pathway activity [185]. However, the expression of other genes, namely those responsible for laminin, were increased by shear stress [185]. After being exposed to a shear stress of $2.5 \times 10^{-4} \text{ N/cm}^2$, the SW480 cell line (which does not express E-cadherin) showed a decrease in β -catenin signaling, in which the $\alpha 6 \beta 4$ integrin functions as a mechanosensor for fluid stress modulation. The authors also found that the cell cycle on SW480 cell line was blocked in G1 by the fluid shear stress [185]. The importance of the actin and microtubules in cancer cell adhesion was reported by Suresh *et al.* [56]. The stable adhesion of cancer cells to the walls of small blood vessels influenced metastatic formation. Focal adhesions are involved in the cell signaling modulated by cyto-disruption, during in vitro flow conditions. These data led to hypothesize that changes in the cell stiffness and voracity of the cell adhesion molecules (due to disruption of the cytoskeleton) were very important for adhesion interactions in vivo [56].

Delon *et al.* recently studied the influence of a fluid shear stress using $0\text{--}9.93 \times 10^{-5} \text{ N/cm}^2$ on Caco-2 cell line monolayers with a microfluidic device [187]. Caco-2 cells experienced significant phenotypical and functional changes when exposed to fluid shear stress, when compared with cells studied in static conditions. The authors mentioned that fluid shear stress exposure altered the mucus production, tight junctions expression, cytoskeleton organization, microvilli formation, activity of mitochondria, and cytochrome P450 expression [187]. They highlighted that, while a minimum fluid shear stress was a requirement to promote the formation of tight junctions, the cell-cell tight junctions as well as the monolayer barrier functions decreased, due to the application of high mechanical forces on those monolayers [187].

3 Mechanobiology in CRC Therapeutics

Conventional therapies, such as chemotherapy and radiotherapy, have been developed based on biological and biochemical knowledge of cancer cells. However, physical properties have emerged as playing an important role to fight cancer. These conventional treatments have the disadvantages of affecting not only tumor cells, but inducing undesirable collateral effects in healthy cells and tissues. Cell membranes and the cytoskeleton of cancer cells are targeted by chemotherapeutic agents, inducing cytotoxicity and altering the adherence process. The effects of chemotherapy usually rely on altered cell mechanics and may lead to vascular complications. The causes of those complications are related to mechanical obstructions of blood vessels from the main organs. The obstruction is caused by the increase of circulating leukemia cells, as well as the increase of cytoadherence and stiffness [56]. The only advantage of conventional treatments is that they show fast results (independently if they were efficient or not) and, if they are not efficient, clinicians may quickly change the drug (or combinations of drugs).

The biophysical effects (elastic changes) of chemotherapy were studied on acute lymphoblastic leukemia and acute myeloid leukemia cells (removed from peripheral blood or bone marrow of 8–78-year-old patients) [188]. Both cell types were treated with dexamethasone and daunorubicin. Nanoindentation assays by AFM showed that leukemia cells exhibited doubled stiffness values during cell death [188]. Cells taken from the patients but not exposed to chemotherapeutic drugs did not show significant changes in their elastic moduli. The stiffness increase seems to result from the reorganization of the actin microfilaments, due to the polymerization and depolymerization, during apoptosis [189,190].

Immunotherapy has the advantage of reinforcing the immunological system based on antibodies, vaccines, and adoptive therapies. The disadvantage is that results are not so quick as those obtained with chemotherapy and/or radiotherapy. Some cancers do not respond to immunotherapy [126]. The combination of immunology with biophysical studies has led to mechanobiology [191]. Chimeric antigen receptor-T cell (CAR-T) constitutes a promising method to recruit T-cells to fight cancer cells. The CAR-T is separated in the CAR-T intracellular domain and its plasma

membrane, due to mechanical forces produced by membrane motions, which activate signaling processes. The CAR-T has been mentioned to be helpful when joined to hematology malignancies. This molecule is also interesting to be used in solid tumors, such as CRC. The objective is to promote the binding of T-cells to different antigens of the CRC cells [192]. AFM could be a suitable methodology to study the specific bindings of CAR-T cells and the tumor molecules. AFM single-cell force spectroscopy can be used to study the binding of the CAR-T and eventual antigens that could exist in CRC cells. Kristi *et al.* used AFM to characterize the molecular interaction forces between the immune cells system and the antigen-presenting cell [191]. Lysophosphatidic acid (LPA) is a ligand for cell surface receptors. CRC cells demonstrate an aberrant expression of LPA receptors, contributing to tumorigenesis through the increase of inflammatory responses [193]. High levels of LPA2 are expressed by human CRC tissues compared with corresponding healthy colonic tissues. In adenocarcinomas of the colon, the LPA1 expression is lower [194]. With the LPA receptors being very important in colon cancer, they should be one of the targets in terms of the development of new therapies, i.e., new antibodies that could block the LPA binding receptor or new therapeutics that stop the gene pathway responsible for the LPA expression. AFM is a powerful tool, since the probe can be functionalized with molecules such as antibodies to study the specific target receptors.

Cortes *et al.* using AFM nanoindentation (through measurement of elasticity), found that when a specific drug was used to treat pancreatic ductal adenocarcinoma, cell stiffness was reduced, thereby suppressing cell invasion [195].

CTCs provide information about physical (size, density) or biological (tumor markers) parameters found in the patient's blood [196–198].

The SW480 cell line treated with fullerenol was studied by Liu *et al.* using AFM to demonstrate the morphological and mechanical changes [199,200]. The Young's modulus was used together with the adhesion force to infer the resistance of cells to the administered drug. The multinuclear cells, treated and untreated with two different concentrations of the drug, showed similar characteristics regarding their height, length, width, and roughness. For mononuclear cells, the distribution of those

parameters was significantly different. The authors observed that multinuclear cells are more resistant to the fulleranol when compared with the mononuclear ones [201].

Pachenari *et al.* studied the effects of albendazole, a microtubule-targeted drug, on CCCs [51]. The authors reported the difference in grade I and grade IV CCCs, in terms of the proportion of microtubules and actin filaments. Micropipette aspiration was used to assess the viscoelastic behavior of grade I and IV of SW48 CCC line (more aggressive) compared to HT29 CCC line (less aggressive). Using relative fluorescence revealed that the ratio of actin filaments to microtubules in SW48 cells was equal to 1.33 ± 0.45 , which was significantly lower than that of HT29 cells of 2.48 ± 0.85 . The volume of the SW48 changes was significantly higher (about 3.5 times), after insertion of the cell body into the micropipette, when compared with HCT29 cells. SW48 cells have shown to be softer than the primary grade of CCCs, meaning that the decrease in cell elastic modulus was associated with their higher invasiveness. This property seemed to help the invasion of the cells through the microvasculature and cell spreading [51]. Extracellular vesicles (EVs) produced by the CRC cells must be a target, since according to Serrati *et al.* they contribute to the CRC dissemination into peritoneal cavities due to the EVs released, inducing the alteration of peritoneal mesothelial cells [202].

In terms of translation to the clinic, the AFM can be a promising tool in the surgery room, based on the concept that the CRC tissues are stiffer than healthy ones. In practice, after removing the tumor mass, the elasticity properties of tissues would be measured from the center to the periphery. When the stiffness values on the periphery of the tumor would be similar to the values of the healthy tissues around it, this would mean that it would not be necessary to extend the tumor area to be removed. Although this procedure is always carried out afterwards by anatomic pathology analysis, carrying out this analysis *in situ* would be faster and would avoid the eventual repetition of the surgery. A similar approach using Raman confocal microscopy has already been implemented in surgery of the head and neck in the Netherlands [203].

It was shown that, in CRC, using different approaches and techniques, bringing together biologists, physicists, biochemists, and medical specialists, is very

important to deepen our knowledge and to develop novel drugs and treatments for CRC.

4 Conclusions and Assessment

Mechanotransduction has been shown to modulate cell functions in tumorigenesis and metastasis. Since the stiffness of the tumor microenvironment contributes to tumor growth and metastasis, trying to prevent or reverse the tumor stiffening could be an approach for new therapeutic strategies. Targeting the mechanosensing mechanisms, through which cancer cells sense the stiffening of the tumor microenvironment, could be an option for developing anti-tumorigenic therapies. In CRC, the cytoskeleton reorganization of cells can be a good biomarker of cell state during cancer progression, providing crucial information for new developments in cancer diagnostics, therapies, and drug efficiency. Integrins must be explored since these molecules are crucial to transfer forces from the ECM through the cell membrane and cytoskeleton.

Several biomechanical and biochemical pathways were reviewed, showing how biophysical cell and ECM parameters have an impact on biochemical cell signaling. Wnt/ β -catenin, YAP/TAZ, TNF- α , TGF- β , APC, Rho family, RAS, p53, and P13k are known pathways activated in CRC. Mechanical stimuli, such as matrix stiffness, matrix elongation, and cell density, influence the previous signaling pathways. The fate of epithelial cells in the intestine and colon is controlled by a dominant force coming from the Wnt/ β -catenin cascade, as well as the mutation in the APC. Hippo signaling, through the YAP/TAZ, is related to cell-ECM adhesion sites, where it senses cell geometry and detects matrix rigidity. ECM-nuclear mechanical coupling translocates YAP in response to substrate rigidity. TNF- α is recognized as one of the major mediators of cancer-related inflammation, mediating all steps of tumorigenesis, such as proliferation, invasion, angiogenesis, and metastasis formation by accelerating tumor invasion and metastasis through EMT. Rho family activation is also related with cancer metastasis. The activation of these actin nucleators induces the formation of stress fibers, lamellipodia and filopodia. The invasion of cancer cells is associated with the formation of several actin-mediated structures, including lamellipodia, filopodia, podosomes, and invadopodia. RAS is

implicated in ECM topography, stiffness, density, and porosity changes. The family of apoptosis-stimulating proteins of p53 is dysregulated in CRC patients. P13k dysregulation is related to the cryptbuckling tissue and ECM alterations. TGF- β promotes the excess of ECM production and mediates the crosslinking of ECM through the upregulation of lysyl oxidase. The crosslinking of ECM increases its resistance when forces are applied, being translated in the increase of elasticity.

CAFs are among the cells that promote angiogenesis in CRC. In addition, they promote the disruption of the BM, facilitating the invasiveness of the CRC. CAFs also produce several MMPs, among which some are responsible for angiogenesis. CAFs inactivation should be further investigated. EMT is also an important process in CRC, being crucial to increase efforts in the investigation of substances to stop EMT.

Regarding intravasation into blood vessels, CRC cells are exposed to shear forces exerted by blood flow, which facilitates the interaction of cancer cells with endothelial cells, therefore promoting extravasation. New therapies should target the interaction between CRC and endothelial cells from vessel walls, impairing the bindings between the adhesion molecules of cancer cells and the vessel site receptors, contributing to decrease in extravasation and metastization.

High internal solid and fluid pressures of the tumor microenvironment contribute mostly to restricted drug delivery to tumor cells. Hypoxia contributes to solid tumors due to the compression of blood and lymphatic vessels and induces resistance to chemo-radio-, and immunotherapies. Accordingly, therapeutic strategies that target the sources of high solid or fluid pressure in the tumor vasculature can greatly improve the main route of drug access through drug delivery to the tumor site.

It is expected that liquid biopsy techniques with circulating tumor cells from blood will become common practice to be used as a diagnostic routine for CRC as well as other cancers. Additionally, 3D studies should become more widely used since they provide a similar environment to the native structure, thus providing a better and more meaningful translation of research to the clinics.

The translation of AFM to the clinical could be a huge advantage in the diagnosis of cancer for the distinction of healthy and tumor tissues, in situ during the surgery,

through the determination of nanomechanics.

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3.2 Mechanical Properties of Colorectal Cancer Cells Determined by Dynamic Atomic Force Microscopy: A Novel Biomarker

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Simple Summary: Colorectal cancer (CRC) is presently the third-most abundant and the second-most lethal cancer worldwide. Thus, there is a real and urgent need to investigate the processes behind the appearance, development, and proliferation of CRC cells. Several biochemical pathways have been investigated to understand their role in oncogene activation and tumor-suppressor gene inhibition. Despite the research increase in biochemistry, there is still a need to better understand the biophysical cues that drive the activation of signaling pathways relevant to mechanotransduction and cell transformation. The elucidation of these biological processes may help to hinder oncogenic mechanisms and to find biomarkers that could be used to design more personalized therapeutic strategies.

Abstract: Colorectal cancer (CRC) has been addressed in the framework of molecular, cellular biology, and biochemical traits. A new approach to studying CRC is focused on the relationship between biochemical pathways and biophysical cues, which may contribute to disease understanding and therapy development. Herein, we investigated the mechanical properties of CRC cells, namely, HCT116, HCT15, and SW620, using static and dynamic methodologies by atomic force microscopy (AFM). The static method quantifies Young's modulus; the dynamic method allows the determination of elasticity, viscosity, and fluidity. AFM results were correlated with confocal laser scanning microscopy and cell migration assay data. The SW620 metastatic cells presented the highest Young's and storage moduli, with a defined cortical actin ring with distributed F-actin filaments, scarce vinculin expression, abundant total focal adhesions (FAK), and no filopodia formation, which could explain the lessened migratory behavior. In contrast, HCT15 cells presented lower Young's and storage moduli, high cortical tubulin, less cortical F-actin and less FAK, and more filopodia formation, probably explaining the higher migratory behavior. HCT116 cells presented Young's and storage moduli values in between the other cell lines, high cortical F-actin expression, intermediate levels of total FAK, and abundant filopodia formation, possibly explaining the highest migratory behavior.

Keywords: colorectal cancer (CRC); atomic force microscopy (AFM); frequency sweep;

viscoelasticity; creep; stress relaxation; power-law exponent; focal adhesions

1. Introduction

According to the Global Cancer Observatory report (GLOBOCAN 2020 report) published by Sung *et al.*, breast cancers are the most frequent tumors (11.7%), followed by lung (11.4%) and colorectal cancers (CRC; 10.0%) [1]. Interestingly, although CRC ranks as third in terms of incidence, it is second in terms of mortality [2].

The development of CRC is related to a deficiency in cell migration from the crypt, which depends on the adenomatous polyposis coli (APC) protein, leading to the accumulation of cells in the amplified zone of the colonic crypt. The amount of these cells may increase exponentially by accumulating mutations, thus resulting in the formation of a tumor [3]. In CRC, the epithelial-to-mesenchymal (EMT) transition drives cellular migration and is characterized by the acquisition of a mesenchymal phenotype through tight junction dissolution, disruption of apical-basal polarity, and reorganization of the cytoskeletal architecture [4]. The neoplastic process relies not only on the referred molecular biology and biochemistry phenomena but also on internal and external forces exerted by the cells that are translated into biochemical signals (mechanotransduction) [3]. A deeper understanding of the biophysical mechanisms behind biochemical pathways can help to clarify cell motility, invasion, migration, extravasation, and metastization processes and thus assist in the development of new and improved therapeutic strategies [5].

To perform mechanobiology and mechanotransduction studies, several techniques can be used, namely, atomic force microscopy (AFM), microindentation, shear rheometry, shear wave, magnetic resonance elastography, micropipette aspiration, optical stretching and/or magnetic twisting cytometry, all of which have been developed to evaluate the mechanical properties of cells and tissues [6]. AFM can be carried out in physiological conditions and hence allows not only the determination of mechanical characterization of samples, including cells [6,7] but also measurement directly on tissues [8]. For instance, Boccaccio *et al.* used nanoindentation by AFM to study the effect of surface roughness and cell shape in mechanical properties variation of SW480 and SW620 cell lines on colon cancer and lymph-node-derived metastases [9].

Previous research reported the quantification of cells' mechanical properties considering that they are isotropic, purely elastic, and semi-infinite materials, presenting the determination of the apparent Young's modulus since the viscous component cannot be separated from the elastic one [10]. However, cells are structures that also show a viscous response. The cytoskeletal filaments and the cytosol determine the elastic and viscous properties of cells. The polymerization of monomeric G-actin to filamentous F-actin affects the elastic properties of cells. Viscosity seems to depend on the complexity of the cytosol due to the concentration of several components and the presence of a polymeric network and organelles. The power-law exponent of the storage modulus is often interpreted as an expression of cell fluidity and the solid-like or liquid-like state of cells [11]. Hence, we used AFM frequency sweep to quantify the viscoelasticity of cells based on the determination of the frequency-dependent storage and loss moduli. A power-law exponent was applied to the storage modulus, which, if often correlated with the fluidity of the cell cytosol, as well as a liquid-like state, facilitates the shape changes and cell deformation, thereby promoting the invasion of the basal lamina, underlying stroma and vascular and lymphatic spaces [12]. Furthermore, this methodology can also provide information about creep and stress relaxation as a function of time [11,13]. Stress-relaxation is a time-dependent component, decreasing under a constant strain, and creep is defined as constant stress with decreasing strain as a function of time [14]. Herein, we aimed to unveil the mechanical properties of different CRC cell lines by different atomic force methods and cross-analyzing such results with cell-migration features, namely, cytoskeleton protein expression and localization and gap-closure abilities. Hence, we quantified the mechanical properties using dynamic AFM on the three CRC cell lines: HCT15, HCT116, and SW620. Frequency sweep results showed that SW620 was the stiffest, also presenting a lessened migratory behavior. HCT15 cells were the softest, exhibiting abundant focal adhesions and presenting a rapid migratory behavior. HCT116 cells, which in terms of stiffness exhibited an intermediate level, presented filopodia formation and the highest migratory behavior. In summary, our study elucidates the mechanical properties of CRC cells and can potentially be used as a biomarker for their motility and migratory

behaviors

2. Materials and Methods

2.1. CRC Cell Cultures

The culture of the CRC cell lines HCT116 and HCT15 (derived from a primary colorectal cancer) and SW620 (derived from lymph-nodes metastasis) was carried out in T25 flasks. HCT116 and HCT15 cells were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 (Pan-BioTech, Aidenbach, Germany) supplemented with 10% (*v/v*) fetal bovine serum (FBS; Gibco, Grand Island, NE, USA) and 1% (*v/v*) penicillin/streptomycin (pen/strep; Merck, Darmstadt, Germany). The SW620 cell line was cultured in Dulbecco's modified Eagle medium (DMEM; Pan-Biotech, Aidenbach, Germany) supplemented with 10% (*v/v*) FBS and 1% (*v/v*) pen/strep. Before medium supplementation, FBS was inactivated at 56 °C for 30 min and filtered with a 0.22 µm filter system. After cell confluence, the monolayer was washed with 5 mL of phosphate-buffered saline (PBS). The PBS was removed, and 1 mL of a mixture of 0.05% (*v/v*) trypsin/0.53 mM ethylenediamine tetra acetic acid (EDTA; Merck, Darmstadt, Germany) was added. The cells were incubated in 5% CO₂ for 3 min. After confirming the microscope detachment of all cells, 5 mL of each medium (RPMI1640 or DMEM, depending on cell type) was added. Cells were then centrifuged at 1300 rpm for 5 min. Then, 5×10^4 and 7.5×10^4 cells were seeded in round Petri dishes 55 mm in diameter (TPP, Darmstadt, Germany). The two densities were used to determine the best condition for mechanical quantifications using the AFM. Cells were kept inside the CO₂ incubator for 24 or 48 h before AFM analysis. In the case of SW620 cells, the measurements were performed after 4 days since shorter periods did not promote adequate cell attachment to the Petri dish. The CRC cell lines were observed in cell clusters.

2.2. Assessment of Cell Mechanics Using Static and Dynamic AFM Methodologies

An MFP-3D atomic force microscope (Asylum Research, Santa Barbara, CA, USA),

mounted on an inverted optical microscope (Axiovert 135TV, Zeiss, Aalen, Germany), was used to quantify the mechanical properties of CRC cells. The optical microscope combined with the atomic force microscope provided adequate observation of the tips and cells. The Petri dish with cells was fixed to an aluminum holder with vacuum grease and mounted on the atomic force stage with 2 magnets. The atomic force microscope head, including the sample, was enclosed in custom-built polymethylmethacrylate (PMMA) box to inject and maintain a 5% CO₂ environment. Force–distance curves were recorded above the nuclear region of the cells, typically at a scan rate of 2 Hz, corresponding to a maximum loading rate of 1 nN/s. The trigger threshold was set to 3 nm (maximum deflection of the cantilever); however, due to tilt in the force curve (in the part off the surface), the maximum deflection was often up to 7 nm, which corresponds to a maximum loading force of 1 nN. The mechanical properties (Young's modulus) were obtained by fitting the data between 0.5 nm and 3 nm in deflection (corresponding to 0.1 nN to 0.4 nN). Conventional force–distance curves were recorded in at least 70 cell clusters for each CRC cell. Force mappings of 6 × 6 force–distance curves were acquired over a cell area of 5 × 5 μm². The measurement of single force–distance curve maps and frequency sweep maps were obtained following this order for all cell lines. The atomic force microscope measurements were always performed after the cells' second passage.

All data analysis and processing were performed using the package IGOR Pro 7 (Wavemetrics, Tigard, OR, USA) to quantify the mechanical properties of cells. Hertz's model for spherical tips was used to calculate Young's modulus for each force–distance curve within a force map [15]. Hertz's model equation considers the geometry parameters of the cantilever tip (regarding the shape and cantilever tip radius). The median of these values (rather than the mean, due to the asymmetry of the distribution of the values) was considered as a representative modulus of a force volume, thereby of each cell cluster region. The parameters used in Hertz's fit window were the following: spring constant of the pre-calibrated cantilever provided by the supplier (k); the fit model was a sphere; the tip radius used was 75 nm; fit range was set from 500 pm to 2 or 3 nm deflection; tilt correction of the force–distances curves were performed; Poisson's ratio used was 0.5. The deflection

sensitivity was obtained by fitting a thermal to the thermal data.

2.2.1. Calibration Procedure

Pre-calibrated peak force cantilevers (model PFQNM-LC-A-CAL; Bruker, Billerica, MA, USA) with a pre-defined nominal spring constant were used. A thermal of the cantilever was captured in deionized water to obtain the power spectral density (PSD) of the deflection data. The thermal was fitted with the single harmonic oscillator (SHO) or the Lorentzian model. The selected model was the Lorentzian one since it better fit the thermal acquired in liquids. For instrumental reasons, the data were captured with a deflection sensitivity based on an SHO fit, which was then corrected before analysis based on a Lorentzian fit (see Supplementary Materials, Figure S1) [16]. The Petri dishes with deionized water were fixed to an aluminum holder with vacuum grease and mounted on the atomic force microscope stage with two magnets. The inverse optical lever sensitivity (InvOLVS) of the cantilever was calculated using the no-touching calibration method. For frequency sweep, the hydrodynamic correction factor (b-hydrodynamics factor) of the cantilever was measured in each culture medium used following the procedure by Alcaraz *et al.* [17].

2.2.2. Single Force–Distance Curve Maps

The force–distance curves were performed on HCT15, HCT116, and SW620 cells. The measurements were carried out while always keeping constant the maximum applied force, meaning that the value of indentation was variable since cells showed variable elastic modulus values. Conventional force–distance curves were recorded, measured in at least 70 cell clusters, meaning that the value of cell cluster indentation was variable. As before, force mappings of 6 x 6 force–distance curves were acquired over an area of $5 \times 5 \mu\text{m}^2$. In this method, the apparent Young's modulus was calculated by applying Hertz's model fitting to the approach curve [18]. Figure 1 presents an example of a conventional force–distance curve obtained for each pixel of each force map. Figure 1A shows the approach curve (cantilever deflection versus the height piezo), and the

deflection approach curve fitting, using Hertz's model. Figure 1B shows Hertz's fitting of the force versus the indentation of the sample.

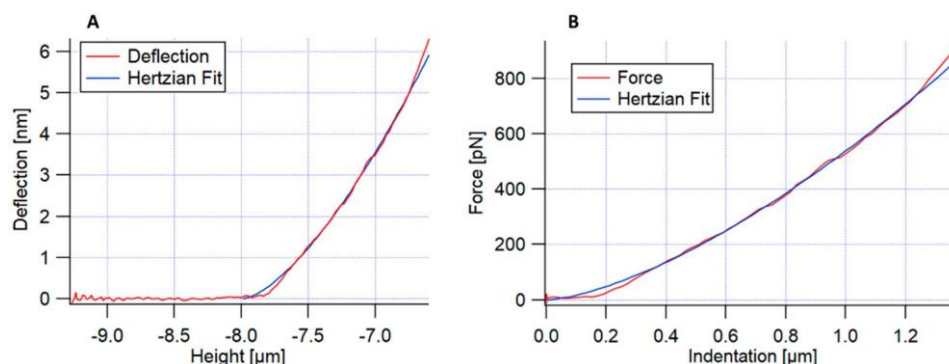


Figure 1. Representation of an example of the approach of a conventional force–distance curve. (A) Approach curve (cantilever deflection versus the height piezo), with Hertz's fitting; (B) Hertz's fitting of the force versus indentation of the sample.

2.2.3. Frequency Sweep

Quantification of dynamic viscoelastic properties was performed in CRC cells using frequency sweep. During cell indentation by the cantilever, a sinusoidal excitation signal is applied to the z-piezo transducers. The cantilever experienced in-phase and out-of-phase deflection signals, which are used to determine the complex shear modulus, creep compliance, and stress relaxation, as described well by de Sousa *et al.* [11]. In the present work, at the end of a conventional force–distance curve, a frequency sweep between 1 and 100 Hz, for 8.7 s, was applied with a trigger point of 3 nm and an amplitude of 80 nm [11,13]. At least 70 cell clusters were measured, with a dwell time of 8.7 s. Maps of $5 \times 5 \mu\text{m}^2$ were measured, with 16 force–distance curves using the frequency sweep method, which allowed the measurement of the power-law exponent (storage modulus as a function of frequency). It was also possible to observe the creep and stress relaxation. An example of a frequency sweep curve is presented in Figure 2, presenting the initial creep and stress relaxation of the sample, although the initial creep and stress relaxation were not analyzed.

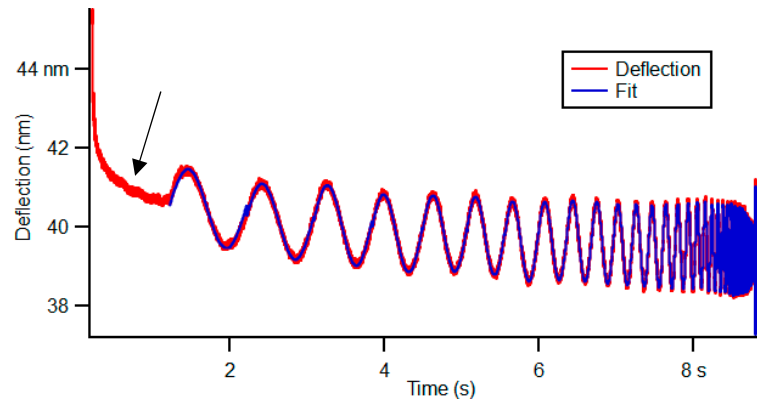


Figure 2. A representative of the deflection signal versus time during dwell, i.e., while the tip is in contact for 8.7 s, during frequency sweep. The first region of red curve (identified by the black arrow) presents the sample creep after loading the cell during the approach. This stress relaxation of the cell was not analyzed. The analysis of the curve starts with the curve fitting (blue color).

Basically, using frequency sweep, the cantilever oscillated around a given indentation with a certain amplitude at a given frequency. When the cantilever contacts the cell, the force increases quickly until the trigger point (maximum cantilever deflection defined) is reached. The cantilever is excited to sinusoidal oscillations with frequencies from 1 to 100 Hz for the 8.7 s in contact. We used 50 frequencies, which were spaced in a geometric series (1 Hz, 1.15 Hz, 1.32 Hz, 1000 Hz), such that we had a constant density of values on a logarithmic frequency scale. If the cantilever touches a hard or elastic sample and its excitation is harmonic but off-resonance on the basis, then the deflection signal and the sinusoidal excitation signal are in-phase. If the cantilever touches a viscous sample with the excitation in the same way, then it exhibits its maximum deflection when moving fast through the fluid. In the present case, there is the inflection point of the sinusoidal excitation, meaning that in the viscous part of the sample, the deflection and excitation are 90° out-of-phase. In summary, if the sample behaves more solid-like or more liquid-like, it can be expressed by the phase shift between the two signals, with values between 0° and 180° , respectively [19].

Cells can present creep and stress relaxation as a function of time [11]. Figure 2 shows an example of the frequency sweep curve, where it can be observed that when the tip indents the sample in the first seconds, the sample presents creep and stress relaxation

as a function of time. However, although viscoelastic materials such as cells present creep and stress relaxation, these parameters were not analyzed in this work [20].

To analyze the data, several parameters were used: spring constant k of the pre-calibrated cantilever provided by the supplier, Poisson's ratio of 0.5, sphere tip radius of 75 nm, analysis range from 500 pm to 3 nm deflection, the correction factor obtained in the PSD corresponding to the fit thermal with the Lorentz's model, and finally, the b-hydrodynamic factor of 7.43×10^{-7} .

For each frequency, we obtained the loss and the storage moduli, as presented in Figure 3.

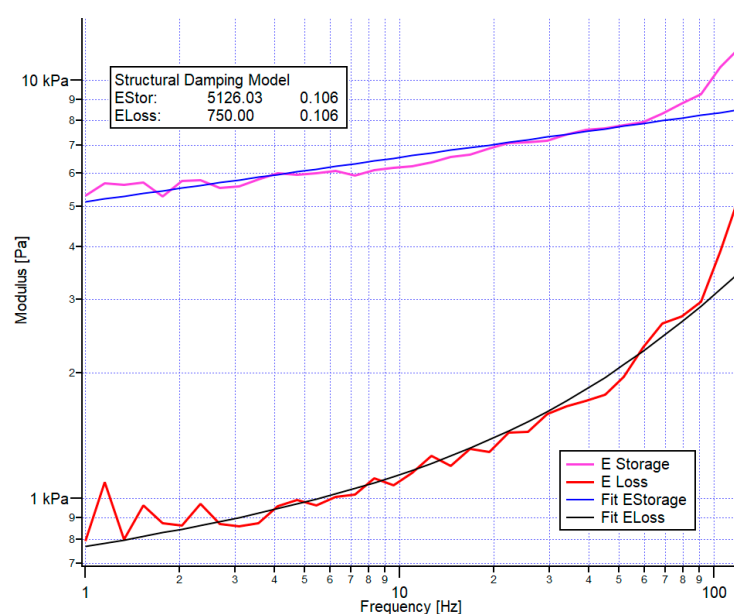


Figure 3. An example of the loss and storage modulus versus frequency obtained by the frequency sweep. A power-law fit is applied to the storage modulus, whereas the structural damping model is used to fit the loss modulus. Both fit procedures result in a storage and loss modulus value for the lowest frequency (here, 1 Hz). Presently, 5126 Pa and 750 Pa are shown, respectively, and a power-law exponent of 0.106.

Since the storage modulus follows a power law, we characterized the storage modulus by its value at the lowest frequency (here, 1 Hz) and the power law in the example of Figure 3. The storage modulus and its power law were 5126 Pa and 0.106, respectively. Despite applying a correction for the hydrodynamic drag of the cantilever, the apparent loss modulus showed, at a higher frequency, additional viscous damping, which was attributed to the viscosity of the medium. Thus, we applied a double power-law fit to the data (lower exponent determined from the elastic modulus, plus a second

one corresponding to hydrodynamic damping Exponent 1).

2.2.4. b-Hydrodynamic Cantilever Factor Determination

Hydrodynamic correction has been performed according to Alcaraz *et al.* [17]. In short, we have recorded the response of the cantilever at various distances from the sample (0, 200, 300, 600, 1100, 2100, 3600, and 5100 nm), while the z-height of the sample is modulated at several amplitudes (50, 100, 200, and 500 nm) in DMEM or RPMI 1640 culture media; the viscosity of these media may be different, as well as by the viscous component of the cantilever. Following the procedure of Alcaraz *et al.*, we extrapolated the b-hydrodynamic correction factor to a distance of 0 nm from the sample and used this correction when fitting frequency sweep data on cells [17].

2.3. Immunohistochemistry

Cells were seeded onto UV-sterilized glass coverslips at a concentration of 2×10^5 cells $\times$ per well of a 24-well plate. After 48 h of incubation, the medium was removed, and cells were washed once with PBS and fixed with 4% (*v/v*) paraformaldehyde (PFA; Electronic Sciences Microscopy, Hatfield, PA, USA) for 20 min at room temperature (RT). Cells were then washed 3 times with PBS for 5 min and permeabilized with 0.25% (*v/v*) triton X-100 (ITW, Camdenton, MO, USA) for 5 min at RT. Samples were washed 3 times with PBS and incubated with 5% (*w/v*) bovine serum albumin (BSA; VWR International, Radnor, PA, USA) for 1 h at RT. Subsequently, samples were incubated with the primary antibodies against total FAK polyclonal antibody (1:100; Thermofisher Scientific, Waltham, MA, USA), vinculin Ab(42H89L44) ABfinity™ rabbit MAb (1:200; Thermofisher Scientific, Waltham MA, USA), and tubulin (1:4000, Sigma-Aldrich, Albucho, Germany) at 4 °C overnight. Secondary antibody goat anti-rabbit alexa fluor 488 F(ab')₂ fragment (1:100; Thermofisher Scientific, Waltham, MA, USA) was added and incubated for 1 h at RT. After 3 washing steps, the conjugated probe phalloidin alexa fluor 568 (1:40 dilution; Molecular Probes-Invitrogen,

Eugene, OR, USA) was added for 30 min at RT. The nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:500; Sigma-Aldrich, St. Louis, MI, USA), and coverslips were then mounted in vectashield (Vector Laboratories, Newark, CA, USA). Samples were analyzed by confocal laser scanning microscopy (CLSM; model SP5 microscope; Leica, Wetzlar, Germany). The imaging data were treated with ImageJ/Fiji (version 1.53C) [21].

2.4. Cell Migration Assays

Cell migration assays of the three CRC cells were performed in 3 independent experiments, each with 3 technical replicates per cell type. Culture inserts to wells for self-insertion (Ibidi, Graefelfing, Germany) were plated in 12-well plates (Falcon, Nürburgring, Germany). The cell culture in the inserts was used according to the manufacturer's protocol. Each insert has two parts with outer dimensions of 8.4 mm x 8.4 mm x 5 mm. The growth area per well was 0.22 cm². The coating area per well was 0.82 cm², the volume per well was 70 µL, and the width of the cell-free gap was 500 µm 100 µm. For the migration assay, 7.5 10⁴ cells were used in each half of the insert. The migration was performed with an imaging system equipped with a fully automated microscope (model IN Cell Analyzer 2000; GE Healthcare, Chicago, IL, USA), with 5% CO₂ and temperature controlled at 37 °C for 144 h. Fifteen fields of view (FOV) with 15% overlap were acquired with a Nikon 10 /0.45 Plan Apo objective spanning the entire gap, each 4 h during the first 72 h and then each 24 h until the end of the experiment. The final images were then reconstructed with the IN Cell Developer Toolbox software (GE Healthcare, Chicago, IL, USA). Quantification of the gap closure area over time was performed with an ImageJ/Fiji macro (Wound-Healing Area v1.0) available at the "Search Engine for BioImage Analysis" repository (<https://se4bia.i3s.up.pt>, accessed on 25 August 2022). Averages of 2 regions of interest (ROI) per technical replicate per cell line were calculated, and the average of the 3 independent experiments has been calculated and plotted with Prism GraphPad.

2.5. Statistics

For the AFM analysis, the median values of Young's modulus were determined from 3 independent experiments for each cell type. For comparing experiments, the median and the 25% and 75% percentiles to visualize differences were used; for quantifying significance of differences, the Wilcoxon Rank, which does not assume normally distributed data but rather looks at the order of data points, was used. (***) Denotes that the difference between groups of distinct CRC cells was statistically significant at a p -value below 0.001.

3. Results

3.1. Cells' Mechanical Properties Determination by AFM

To assess the mechanical properties of the CRC cell lines, cell clusters of adherent HCT15, HCT116, and SW620 cells were chosen (Figure 4). The quantification of mechanical properties was performed using conventional force–distance curve acquisition and frequency sweep.

Mechanical properties of cells were measured by AFM through the acquisition of conventional force–distance curves and force–distance curves with frequency sweep as described above. From the approach part of the force curve, we calculated Young's modulus, which we shall call here apparent Young's modulus since it reflects the elastic properties of the cell mainly with an additional contribution of the viscosity of the cell due to the motion of the cantilever. Frequency sweep allows separating the elastic and viscous response unambiguously, thus resulting in the storage and loss modulus as a function of frequency. The apparent Young's modulus from the force curve ramped at typically 1 force curve per second is similar to the storage modulus of a sweep at the lowest frequency applied (1 Hz) and theoretically slightly larger.

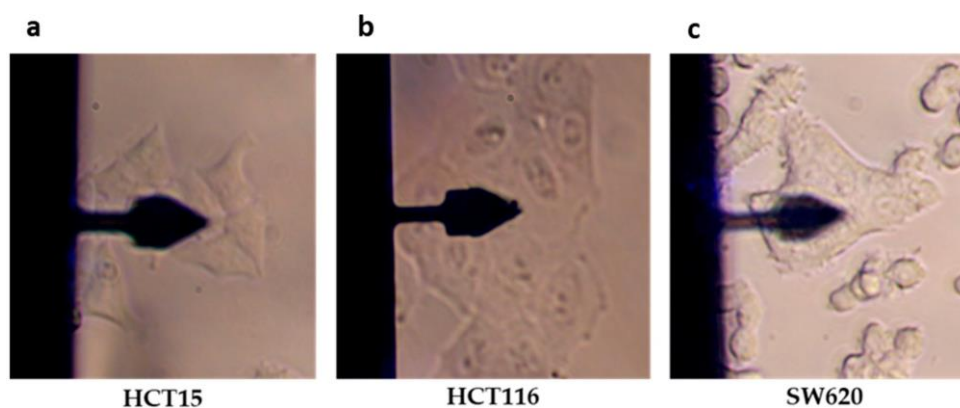


Figure 4. Representative images of cell types used for quantification of their biomechanical properties by AFM, using a model PFQNM-LC-A-CAL cantilever. (a) HCT15, (b) HCT16, and (c) SW620 cells.

Figure 5 shows the histograms of mechanical quantification of the three cell types, with a boxplot displaying the median and the respective percentiles (25th and 75th).

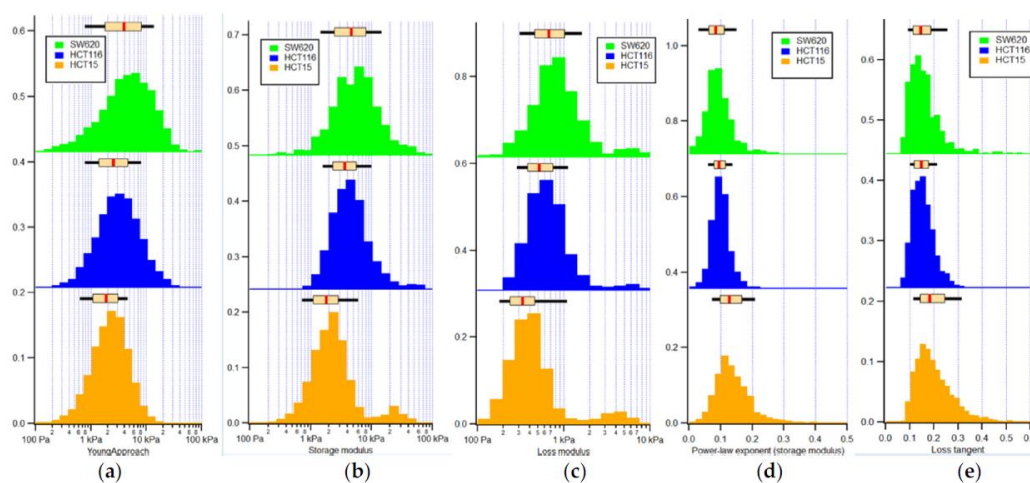


Figure 5. Histograms of mechanical properties quantification of the three CRC cell lines: (a) apparent Young's modulus; (b) storage modulus; (c) loss modulus; (d) power-law exponent of the storage modulus; and (e) loss tangent. The boxplot above the histograms presents the median (red line) and the 25th and 75th percentiles (extent of rectangle in cream color), as well as the 10th and 90th percentiles (black lines extending beyond the box), respectively. Orange: HCT15, blue: HCT116, and green: SW620 cells.

Tables 1 and 2 present the median with the 25th and 75th percentiles along with the p values for the apparent Young's, storage, loss moduli, power-law exponent applied to

the storage modulus, and loss tangent of HCT15, HCT116, and SW620 cell lines.

Table 1. Values of median and percentiles of Young's modulus, storage modulus, loss modulus, power-law exponent applied to the storage modulus, and loss tangent quantification determined by AFM of HCT15, HT116, and SW620 cell lines.

Mechanical Property	Cell Line								
	HCT15			HCT116			SW620		
	25th	Median	75th	25th	Median	75th	25th	Median	75th
Apparent Young's modulus (kPa)	1.70	1.87	2.62	1.94	2.61	3.67	2.86	4.29	5.78
Storage modulus (kPa)	1.39	1.91	2.38	2.76	3.64	4.8	3.8	4.66	6.78
Loss modulus (kPa)	0.25	0.34	0.41	0.42	0.51	0.63	0.59	0.71	0.84
Power-law exponent of the storage modulus	0.11	0.12	0.15	0.08	0.09	0.11	0.07	0.08	0.10
Loss tangent	0.15	0.18	0.21	0.13	0.15	0.16	0.12	0.14	0.17

Table 2. Statistical data treatment of HCT15, HCT116, and SW620. The Wilcoxon Rank's test was used. (***) Statistically significant differences between the distinct CRC cell lines ($p < 0.001$).

Cell Line	Apparent Young's Modulus (kPa)	Storage Modulus (kPa)	Loss Modulus (kPa)	Power-Law Exponent of the Storage Modulus	Loss Tangent
HCT15/HCT116 ***	0	0	0	0	7.21×10^{-12}
HCT15/SW620 ***	0	0	0	0	3.15×10^{-7}
HCT116/SW620	0 ***	0.004 ***	1.7×10^{-5} ***	0.004	0.82

When analyzing Table 2, significant differences can be seen regarding HCT15 and HCT116, and HCT115 and SW620 in all mechanical parameters. Comparing HCT116 and SW620 cell lines, the storage modulus, power-law exponent of the storage modulus, and the loss tangent did not present significant differences.

3.2. Cancer Cells' Morphology and Motility Structures

To unveil the potential relations of CRC cell lines' migratory features with the mechanical results, the expression and localization of cytoskeleton proteins were

analyzed. Figures 6–9 represent data obtained by CLSM, in which staining of nuclei, tubulin, F-actin filaments, vinculin, and the total FAK was performed. Our detailed motility analysis (Figures 6–9) revealed that HCT15 cells exhibited an elongated shape, with the absence of cortical F-actin ring but well-defined cortical tubulin cytoskeletal organization, accompanied by reduced vinculin and total FAK staining at the focal contact points, but abundant small filopodia projections, characteristic of greater migratory behavior. On the contrary, HCT116 cells exhibited an elongated shape, less evident cortical tubulin with a well-defined cortical F-actin ring, abundant cell-cell contact areas, exhibiting vinculin and evident FAK staining, in comparison with the other cell lines, distributed throughout the whole cytoplasm. Cell margins presented abundant small filopodia, suggesting an intermediate migratory behavior. Interestingly, SW620 cells showed a rounded shape, with a well-defined cortical F-actin, particularly evident at cell-cell contact points. These cells did not express vinculin but displayed abundant FAK, distributed throughout the cytoplasm with evident focal contact points, accompanied by the absence of filopodia, suggestive of lessened migratory behavior, as has been already suggested by Li *et al.* [22].

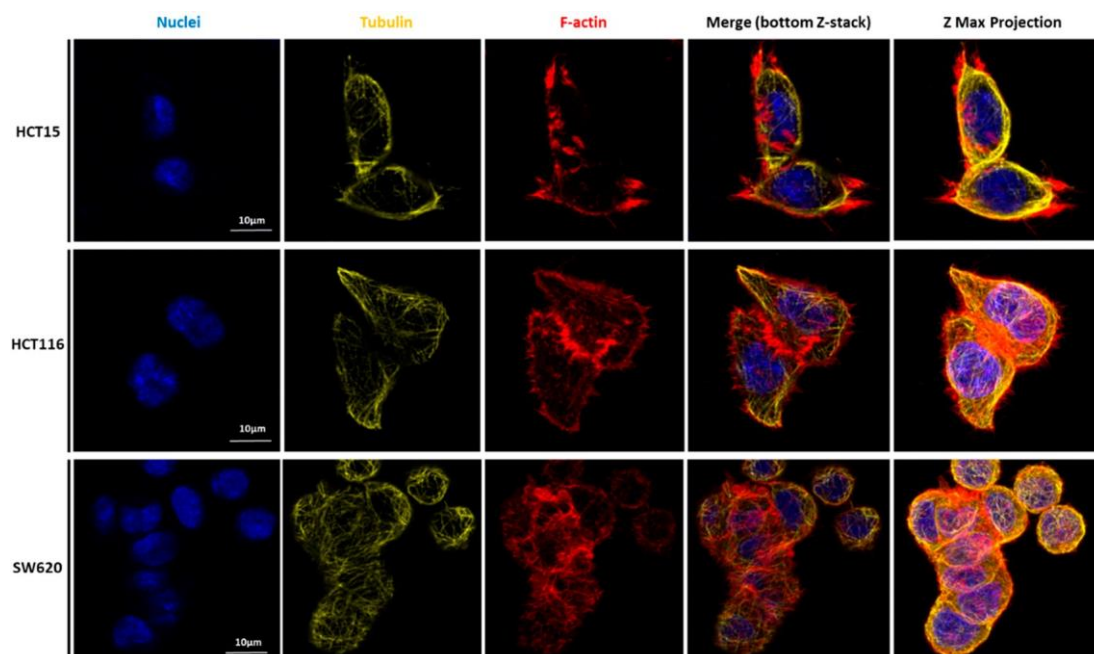


Figure 6. Expression of tubulin and F-actin in CRC cells. Staining of HCT15 (**top**), HCT116 (**middle**), and SW620 (**lower images**) cells for DAPI to localize the nuclei (blue), tubulin (yellow), and phalloidin to detect F-actin filaments (red). The bottom Z-stack is shown, and a Z-maximum projection of each condition is also depicted (**right panels**). Scale bars: 10 μm.

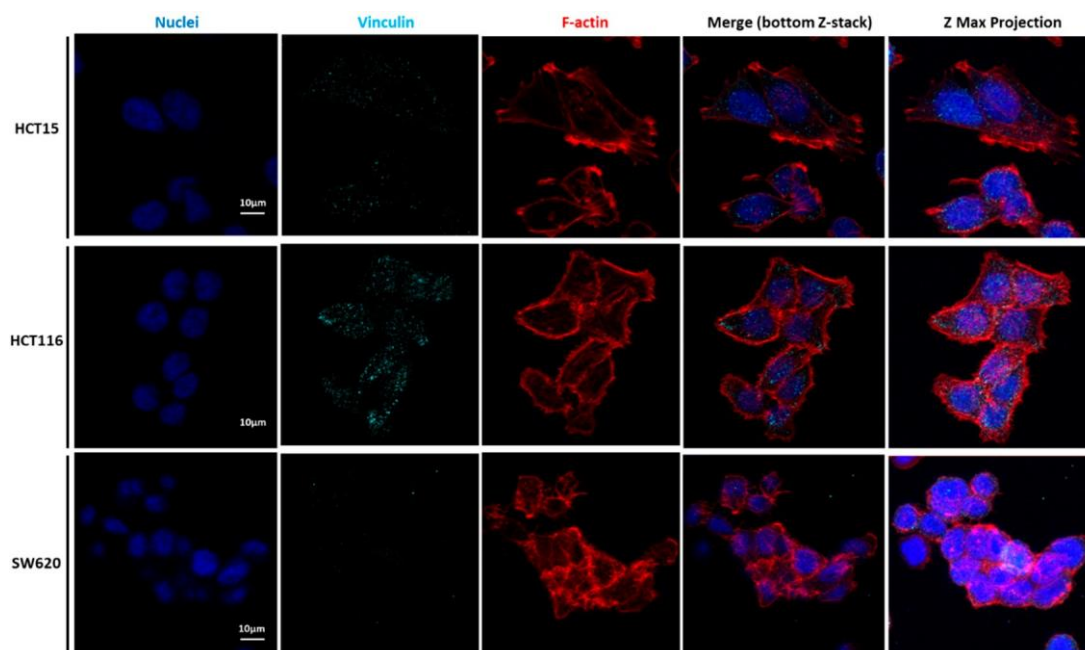


Figure 7. Vinculin expression in CRC cell lines HCT15 (**top**), HCT116 (**middle**), and SW620 (**lower images**) immunostained for DAPI to illustrate the nuclei (blue), vinculin (cyan), and phalloidin for F-actin filaments (red). The bottom Z-stack micrograph is shown, and a Z-maximum projection showing the overall stains is also represented (**right panels**). Scale bars: 10 μ m.

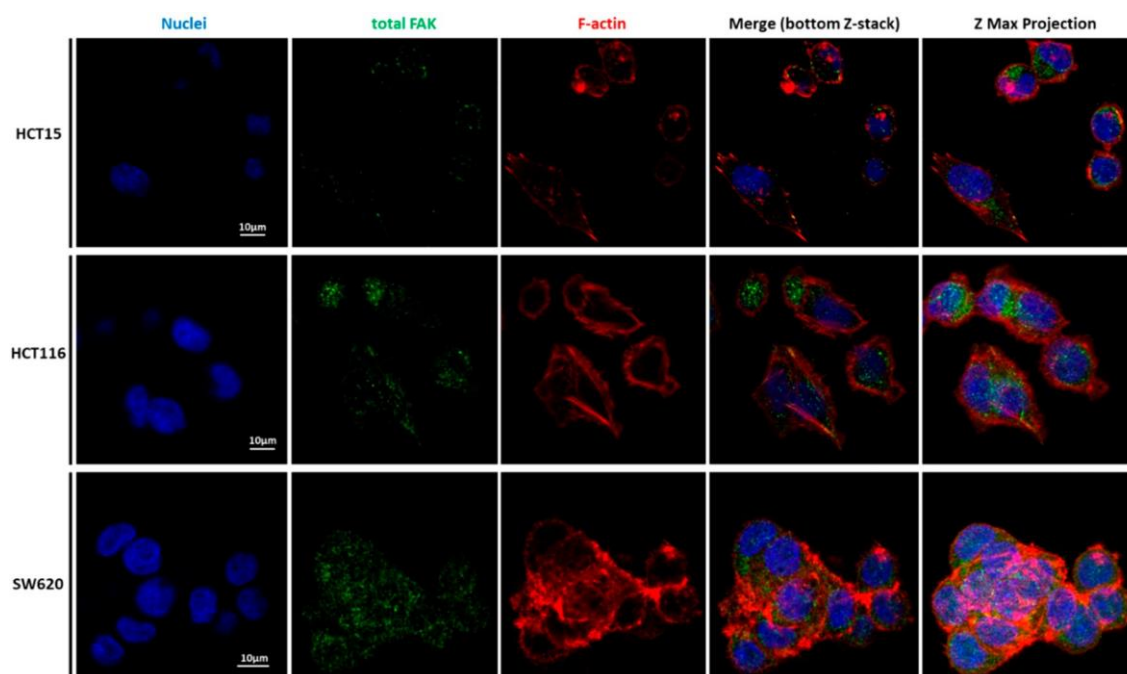


Figure 8. Immunolocalization of FAK in CRC cells. Staining of HCT15 (**top**), HCT116 (**middle**), and SW620 (**lower images**) cells with DAPI to identify the nuclei (blue), phosphorylated FAK (green), and phalloidin for F-actin filaments (red). Shown are the bottom Z-stack and the Z-maximum projection of the imaged area. Scale bars: 10 μ m

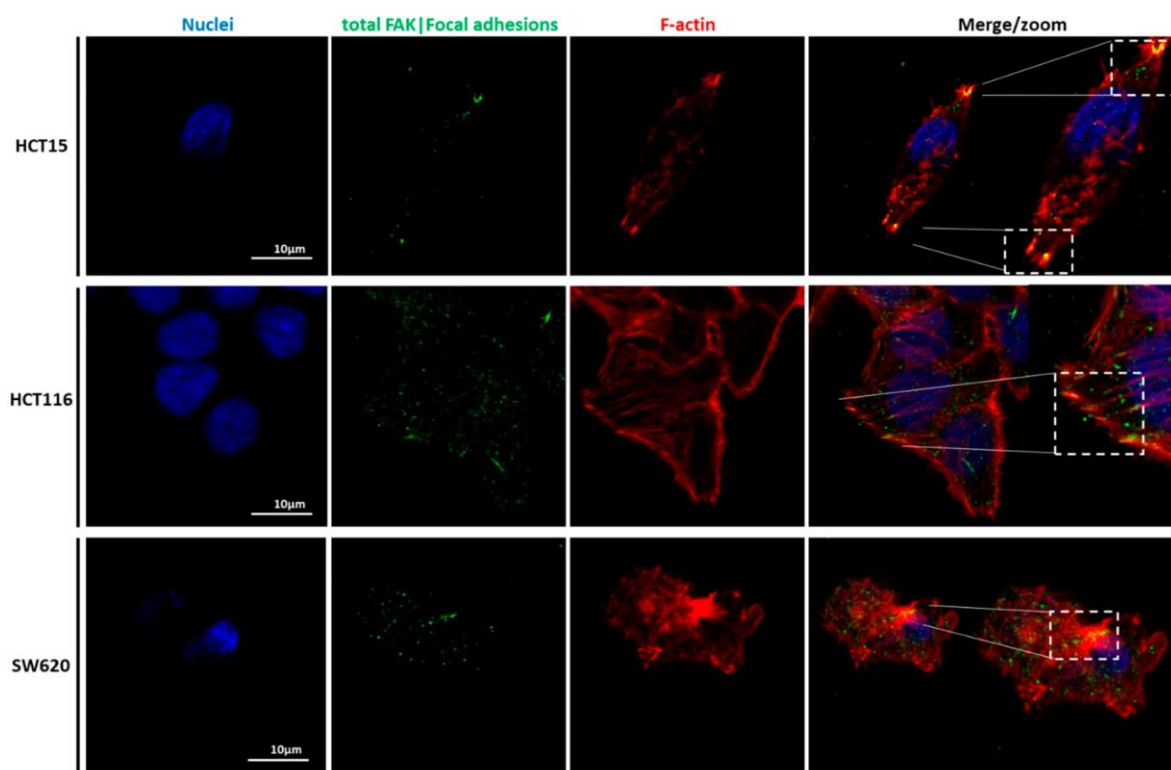


Figure 9. Illustration of the focal adhesion sites of CRC cells seeded in 2D. Immunolocalization of nuclei (blue), phosphorylated FAK (green), and F-actin filaments (red) in HCT15 (**top**), HCT116 (**middle**), and SW620 (**lower images**) cell lines. The zoomed areas indicate the co-localization of FAK with the F-actin filaments at the focal adhesion complexes located at the bottom z-stack. Scale bars: 10 μm .

1.1. Cell Migration Assays

To evaluate the functional impact of the observed biomechanical properties, cell migration assays were performed, and the graphic illustrating the gap closure as a function of time (from 0 to 144 h) was obtained for each cell line (see Supplementary Materials, Figure S2). Interestingly, our results demonstrated that SW620, the stiffest cell line, although displaying abundant FAK distributed throughout the cytoplasm, presented no filopodia motility structures and was indeed the less migratory cell line. In contrast, HCT15 cells, which had the softest biomechanical properties, exhibited abundant focal adhesions, abundant small filopodia projections, and experienced faster migratory behavior. Importantly, HCT116 cells, which in terms of stiffness exhibited an intermediate state, presented abundant filopodia at the cell margins and displayed the highest migratory behavior.

Table 3 presents a summary of the results obtained in AFM, CLSM, and cell migration assays for the CRC cell lines analyzed, namely, HCT15, HCT116, and SW620.

Table 3. Summary of results obtained from AFM, CLSM, and cell migration assays of the three CRC cell lines

Analyzed Parameters	Cell Lines		
	HCT15	HCT116	SW620
Storage modulus (kPa)	1.91	3.64	4.66
Loss modulus (kPa)	0.34	0.51	0.71
Power-law exponent of the storage modulus	0.12	0.09	0.08
Loss tangent	0.18	0.15	0.14
Cortical actin	Low amount (+)	Very well distributed (+++)	Defined (++)
Cortical tubulin	(+++)	(+)	(+)
Vinculin	(+)	Distributed in whole cytoplasm (+++)	(-)
Total FAK	At focal contact points (+)	Abundant and distributed through whole cytoplasm (++)	Abundant and distributed through whole cytoplasm and at focal contact points (+++)
Filopodia formation	(+++)	(+++)	(-)
Cell migration	Gap closed at 96 h (++)	Gap closed at 72 h (+++)	Did not close the gap at 144 h (-)

Legend: (-) absence; (+) low; (++) intermediate; (+++) high.

2. Discussion

The main objective of this study was to compare the mechanical properties of three CRC cell lines, correlating their biomechanical properties with cell structure and migratory behavior.

The selection of the cells was based according to the consensus molecular subtype (CMS) classification, which included several hallmarks, such as the presence of microsatellite instability of chromosomes, mutation pathways, the inflammatory profile of the tumors, and their prognosis [2].

Accordingly, the selected CRC cell lines had one mutation in common—the Kirsten rat sarcoma (K-RAS). According to CMS classification, the HCT15 and HCT116 cells belong to the CMS 1 subtype (immune, due to the infiltration of cells from the immune system), are highly microsatellite unstable (MSI), and cancers with those cells generally present a poor prognosis [23]. The SW620 belongs to the CMS 2 subtype (canonical, ErbB, hippo-Wnt), microsatellite stable (MSS), and cancers with those

cells have a good prognosis [23]. The HCT15 and HCT116 cell lines were derived from a colorectal primary tumor. They were both positive for G13D, wild-type for B-RAF, and harboring PI3Kinase (PI3K) mutations (H1047R for HCT116 and E545K; D549N for HCT15). However, while HCT116 is wild-type for TP53, HCT15 exhibits a mutation in this gene. The SW620 (metastasis at lymph node from the SW480 primary tumor cell line) cells are positive for G12V, wild-type for B-RAF and PI3K mutation, and harbor a TP53 mutation (R273H; P309S) [24].

We observed during cell culture seeding that the three cell types grew in clusters, in agreement with others, namely, for HCT15 [25], HCT116 [26], and SW620 cells [27].

We started to quantify the mechanical properties through conventional force–distance curves by calculating the apparent Young’s modulus, followed by quantification of storage modulus (elasticity) and loss modulus using frequency sweep. The apparent Young’s modulus does not allow the distinction between elasticity and viscosity since the fitting of the approach curves are fitted with Hertz’s model, which just takes into consideration the geometry and shape of the probe and the indentation, assuming that cells are isotropic materials, semi-infinite elements, and have pure elastic features [10]. One experimental way to disentangle the elastic and viscous response of cells is applying a sinusoidal modulation to the z sample height while in contact. Here, we swept the frequency of this modulation to acquire the storage modulus (G') and the loss modulus (G'') as a function of frequency.

From frequency-dependent data, we could acquire the power-law exponent in a certain frequency range (here, from 1 to 100 Hz), which several authors correlated with the cytosol cell fluidity [13] and is known to determine the ability of cells to change their shape and contract during motile processes [28,29].

The quantification of the viscoelastic properties from frequency sweep data corresponds to the slow response behavior of the cell. In this work, only one power-law exponent was measured, obtained from the frequency sweep.

The mechanical properties of the cells were also correlated with cell shape, cytoskeleton structures, the presence of focal adhesions, as well as with their migratory capability. Through analysis of Table 3, the mechanical properties of cells present the following order for the three cell types:

- Apparent Young's modulus: $SW620 > HCT116 > HCT115$;
- Storage modulus: $SW620 > HCT116 > HCT115$;
- Loss modulus: $SW620 > HCT116 > HCT115$;
- Power-law exponent of the storage modulus: $SW620 < HCT116 = HCT15$;
- Loss tangent: $SW620 < HCT116 < HCT15$.

The CRC cell lines followed the same trend in terms of apparent Young's, storage, and loss moduli, as well as the power-law exponent of the storage modulus and the loss tangent. The apparent Young's modulus has the same order of magnitude as the storage modulus, which can be explained due to the fact that the contribution of the storage modulus is much larger than the loss, hence the contribution of viscous effects to the response of the cell during loading in a force curve is dominated by the elastic properties and only marginally affected by the viscous properties of the cell. Both storage and loss moduli will, in a simplified picture, be related to the amount and resistance that the cytoskeleton F-actin filaments offer to deformation. The more actin filaments (including crosslinks between or bundling of actin filaments), the higher the elastic modulus will be and the larger the friction of the cytosol. When the cell is deformed by the AFM tip, the cytosol will have to be moved through the network of actin filaments. Hence, storage modulus and loss modulus will follow the same trends. Nevertheless, there are subtle differences between different cell types, which will become apparent when comparing the loss tangent (the ratio between loss and storage modulus) and power law exponent of the storage modulus, which both point to how liquid-like or how fluidic cells are. These results are corroborated by the presence of filopodia in the HCT15 and HCT116 cells, which closed the gap before the SW620 cell lines.

The studies available in the literature regarding CRC cell lines quantified the apparent Young's modulus only. Palmieri *et al.* reported a value for the apparent Young's modulus of SW620 cells of 0.480 kPa, which is below about 10 orders of magnitude of our value [30]. It can be hypothesized that the difference observed may be due to our quantification being carried out in SW620 cell clusters, where layers of F-actin filaments

were observed on top of each other. Liu *et al.* also quantified the apparent Young's modulus for HCT116 cells, which was in the range of 6.3 to 7.1 kPa, which is approximately three orders of magnitude higher than those reported here [26]. Nematbakhsh *et al.* quantified the viscoelasticity of adherent malignant breast cancer cells, namely, non-invasive MCF-7 cancer cell lines and the highly invasive MDA-MB-231 cancer cell line. The authors found an apparent Young's modulus for MCF-7 and MDA-MB-231 cancer cell lines that is in an interval of 0.30 to 0.70 kPa and 0.10 to 0.65 kPa, respectively. Comparing the MDA-MB-231 cancer cell line, which is highly invasive, with our SW620 (the metastatic version of the SW480 primary tumor cell line), our apparent Young's modulus is significantly higher compared with the breast cancer cell lines. These results could be explained as due to our measurements being performed in SW620 cell clusters, which were shown to be stiffer [30].

CLSM was used to visualize the differences in terms of cellular structures and function of the three cell types. Nuclei, actin filaments, tubulin, vinculin, total Fak, and focal adhesions were labeled (Figures 6–9). Total FAK includes the free FAK distributed inside the cell and the p-FAK (also called the activated FAK) that integrates the focal adhesion complex [31]. In our work, analyzing Figure 9, it is possible to observe the presence of FAK superimposed with some parts of the cytoskeleton in the HCT15 cells, which may be part of the binding complex of the cells to the substrate, forming the focal adhesion. Tubulin is a protein that polymerizes into long chains that form microtubules. These structures can present different formations, which avoid the cells' division in terms of mitosis, promoting cell death. The normal colon expresses little or no tubulin β II [32]. Ruksha *et al.* observed β II-tubulin expression in patients with CRC cancer. Those who presented more β II-tubulin amount had a poor prognosis [33]. Kaverina and Straube mentioned that microtubule-dependent alteration of cell migration implied changes in microtubule dynamics [34]. HCT15 presented considerably more cortical tubulin than HCT116 and SW620, which could be related to the migratory ability of such cells. However, different tubulin isotypes were not stained, so it is difficult to draw more conclusions in terms of mechanical and migratory properties.

Li *et al.* mentioned that vinculin expression was significantly downregulated in highly metastatic CRC cell lines, expressing EMT indicators. This loss of vinculin can be used as a prognostic factor for CRC [35]. Focal adhesions (FAs) are protein complexes that bind the cytoskeleton to the ECM or substrate via the integrin receptors present at the cellular membrane. Several proteins constitute the FAs, such as FAK and talin, both found at the end of stress fibers. The function of FAs is to transmit the force internally generated by the cytoskeleton to the ECM or substrate, and vice versa, via adhesion receptors [36]. FAK is one of the key signaling molecules regulating cell adhesion, migration, and survival [37]. It is localized in the focal adhesion complexes formed at the cytoplasmic side of the cell attachment to the ECM or substrates, being activated after force generation via actomyosin fibers attached to this complex [37].

SW620 cells presented more F-actin, which could be explained by their layer-by-layer growth, i.e., F-actin filaments in multiple cells were piled and hence superimposed. This observation is most likely responsible for the highest elastic modulus values in the SW620 cell line when compared to the HCT15 and HCT116 cell lines. Xu *et al.* compared different cancer cells, including HCT116, in terms of expression of p-FAK, through the inhibition of sphingosine kinase 1 (SphK1) since this enzyme plays an important role in CRC metastasis [38]. They observed that the inhibition of SphK1 reduced p-FAK expression, thus decreasing cell mobility. In Figures 8 and 9, FAK can be observed and co-localized with F-actin, mainly in HCT116, which could indicate the presence of FAs and, together with the filopodia formation, as well as the presence of vinculin, explain the migratory behavior of HCT116. The filopodia formation and vinculin were not present in the SW620 cell line, which can support the hypothesis of lower migration of the SW620 cell lines. In the case of vinculin, Humphries *et al.* mentioned that it could regulate integrin dynamics, regulating the mechanotransduction force machinery, contributing to binding sites inducing a great focal adhesion growth through the direct interaction with actin and talin [39]. Albuquerque-González *et al.* studied the effect of migrastatin and imipramine inhibition of lamellipodia protrusion in HCT116 cells [40]. In the case of present HCT116 cells, their migratory behavior can thus be possibly attributed to lamellipodia formation.

Regarding cell migration (see Supplementary Material, Figure S2), only the HCT116 cells closed the gap completely, while it took 96 h for the HCT15 cells to completely close the gap. The SW620 metastatic cells did not close the gap, not even after 144 h (6 days). In fact, SW620 cells could only close the gap by 50% at 120 h. The results obtained for SW620 cells are in accordance with AFM and CSLM assays. The SW620 metastatic cell line is the stiffest compared with the HCT15 and HCT116 cell lines. In the CLSM images, the SW620 cell line did not present vinculin. The more metastatic these cells are, the less vinculin expression they present. Vinculin contributes to the stabilization of focal adhesion under high forces through the regulation of contractile stress generation, influencing cell migration speed [41,42]. The cells deficient in vinculin do not form lamellipodia and accumulate stress fibers or extend over the substrate; only filopodia is formed [43]. As in ovarian cancer, the loss of vinculin expression in ovarian cancer cells predicts a poor prognosis for this cancer type [44]. Targeting FAK is considered promising to avoid cancer progression, inhibiting mechanisms such as cancer migration and invasion and enhancing therapeutic strategies for CRC [45].

Some works have been published using the AFM as a powerful tool to distinguish healthy states from different pathological states in cells and tissues [8,46]. This technique could have a practical application in the surgery room to help the surgical team to distinguish tumor tissues from healthy ones through the measurement in loco of the stiffness. This procedure could avoid more surgeries on the same patient/the same tumor, avoiding physical and psychological pain and extra costs to the health system.

4. Conclusions

This work had the objective of comparing three CRC cell lines, namely, HCT116, HCT15, and SW610, in terms of their mechanical properties, structural features, and cell migration. All cells were significantly different from each other in terms of apparent Young's, storage, and loss moduli. In terms of power-law exponent (correlated with fluidity) and loss tangent, the HCT116 and SW620 cells were shown to not be significantly different. SW620 metastatic cell lines presented the highest

elasticity (storage modulus), cortical and distributed F-actin filaments in several layers, fewer focal contacts, the absence of vinculin, and lessened migratory behavior. HCT15 cell lines presented a lower elasticity (storage modulus), high cortical tubulin, less cortical F-actin, less vinculin, and more focal adhesion contact points presenting filopodia, which could explain the migratory behavior. HCT116 cell lines presented elasticity (storage modulus) in between the SW620 and the HCT115 cell lines, with high cortical F-actin, a high amount of vinculin, less tubulin, and intermediate levels of total FAK, abundant filopodia formation, which is likely associated with the greatest migratory behavior. This study also contributed to the hypothesis that there may be mechanisms other than mechanical properties that must be evaluated, mainly in the case of the HCT15 cell line. In the present work, distinct types of tubulin were not stained and thus do not allow distinction between them, which could provide more detailed information to understand how and which tubulin filaments contribute to mechanical and migratory properties.

Supplementary Materials: The following supporting information can be downloaded at: [https:// www.mdpi.com/article/10.3390/cancers14205053/s1](https://www.mdpi.com/article/10.3390/cancers14205053/s1), Figure S1: Example of a power spectral density (PSD) with the thermal fits using single harmonic oscillator (SHO) and Lorentz's algorithms, for the cantilever model PFQNM-LC-A-CAL. For both algorithms, the corrections factor was calculated. The thermal data are shown in red, in which the maximum amplitude peak corresponds to 20 kHz. The fit of the thermal with the SHO algorithm is represented by the green line. The Lorentzian fit of the PSD is presented by the blue line. It was observed that the Lorentz's algorithm fitted better the thermal data when compared with the SHO fitting, as can be seen when analyzing at the noise level away from the peak, e.g., below 3 or above 50 kHz. Figure S2: Cell migration assay of HCT15, HCT116 and SW620 cell lines. (a) Time-lapse images of the gaps after 0, 24, 48, 72, 96, 120, and 144 h. Scale bars correspond to 120 μm . (b) Cell migration assay quantification. Graph showing the cell-free gap closure over time. Dashed lines represent the standard error of the mean of three independent.

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A.F.M.; investigation, M.M.B., T.B.C. and A.F.M.; resources: P.L.G. and S.R.S.; writing—original draft preparation, M.M.B.; writing—review and editing, M.M.B., T.B.C., A.F.M., M.J.O., S.R.S. and P.L.G.; supervision, S.R.S., P.L.G. and M.R.; funding acquisition S.R.S. and M.R. All authors have read and agreed to the published version of the manuscript.

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Supplementary materials

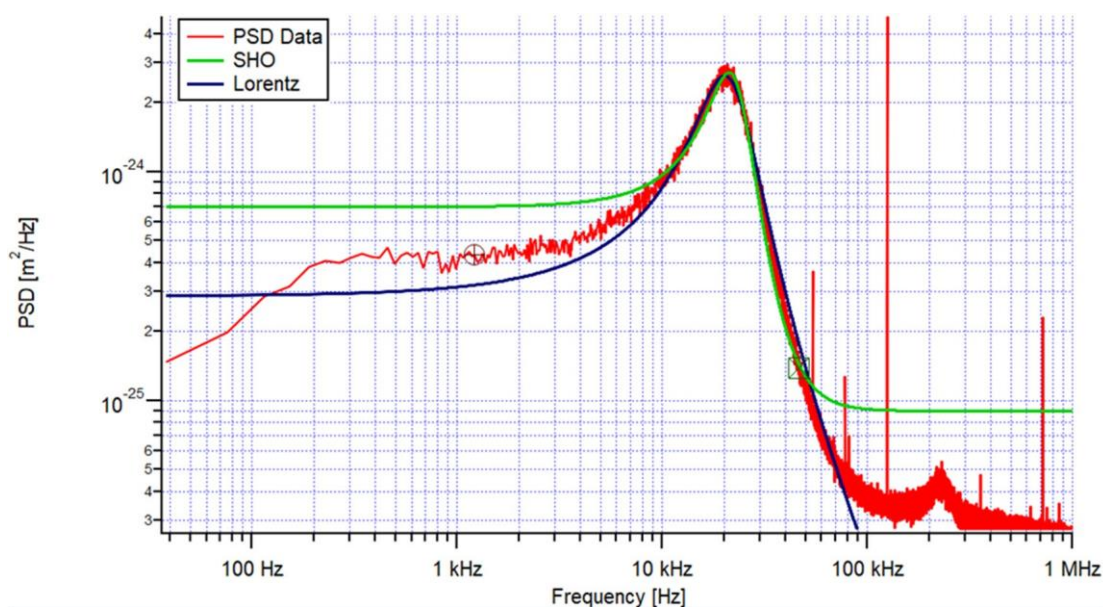


Figure S1. Example of a power spectral density (PSD) with the thermal fits using single harmonic oscillator (SHO) and Lorentz's algorithms, for the cantilever model PFQNM-LC-A-CAL. For both algorithms, the corrections factor was calculated. The thermal data are shown in red, in which the maximum amplitude peak corresponds to 20 kHz. The fit of the thermal with the SHO algorithm is represented by the green line. The Lorentzian fit of the PSD is presented by the blue line. It was observed that the Lorentz's algorithm fitted better the thermal data when compared with the SHO fitting, as can be seen when analyzing at the noise level away from the peak, e.g. below 3 or above 50 kHz.

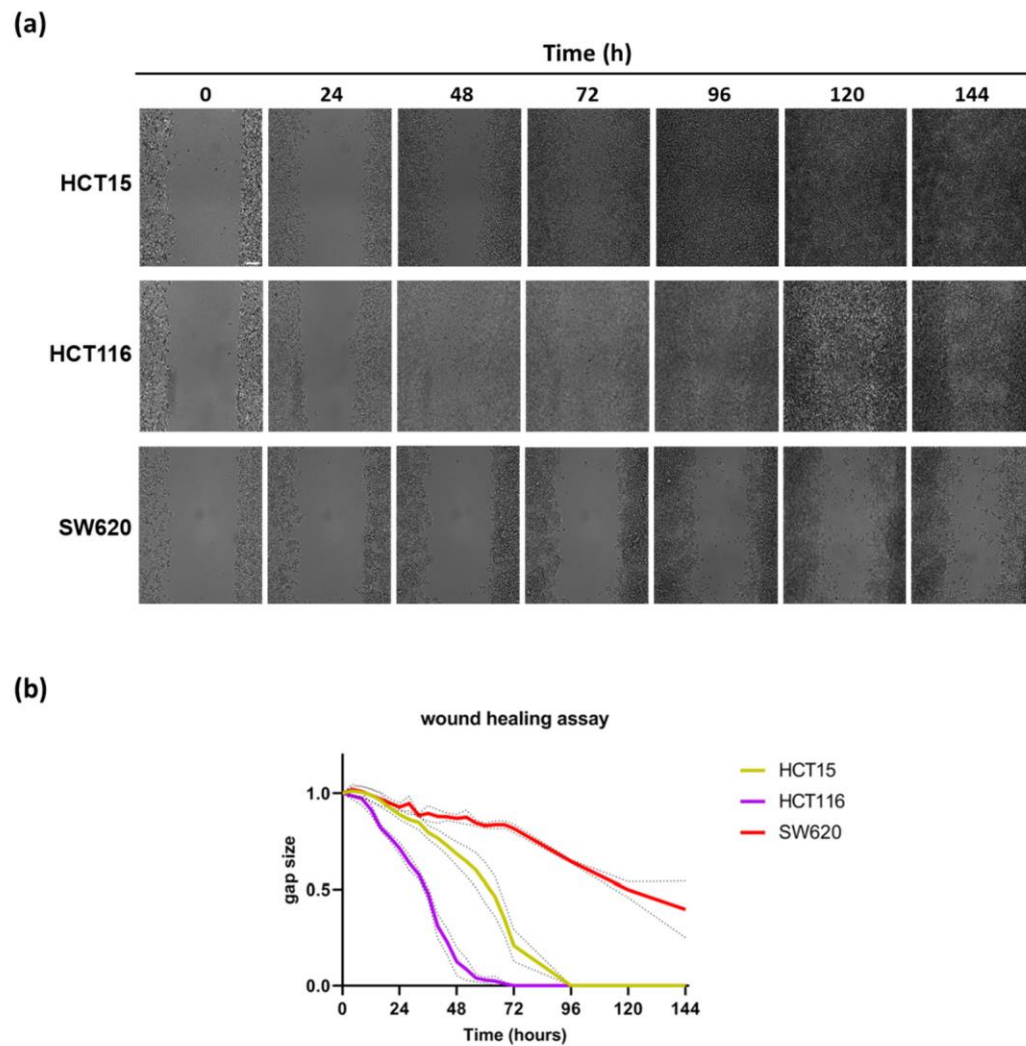


Figure S2. Cell migration assay of HCT15, HCT116 and SW620 cell lines. (a) Time-lapse images of the gaps after 0, 24, 48, 72, 96, 120, and 144 h. Scale bars correspond to 120 μm . (b) Cell migration assay quantification. Graph showing the cell-free gap closure over time. Dashed lines represent the standard error of the mean of three independent.

CHAPTER 4

MELANOMA CELLS

4.1 Melanoma in the eyes of mechanobiology

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Skin is the largest organ of the human body with several important functions that can be impaired by injury, genetic or chronic diseases. Among all skin diseases, melanoma is one of the most severe, which can lead to death, due to metastization. Mechanotransduction has a crucial role for motility, invasion, adhesion and metastization processes, since it deals with the response of cells to physical forces. Signaling pathways are important to understand how physical cues produced or mediated by the Extracellular Matrix (ECM), affect healthy and tumor cells. During these processes, several molecules in the nucleus and cytoplasm are activated. Melanocytes, keratinocytes, fibroblasts and the ECM, play a crucial role in melanoma formation. This manuscript will address the synergy among melanocytes, keratinocytes, fibroblasts cells and the ECM considering their mechanical contribution and relevance in this disease. Mechanical properties of melanoma cells can also be influenced by pigmentation, which can be associated with changes in stiffness. Mechanical changes can be related with the adhesion, migration, or invasiveness potential of melanoma cells promoting a high metastization capacity of this cancer. Mechanosensing, mechanotransduction, and mechanoreponse will be highlighted with respect to the motility, invasion, adhesion and metastization in melanoma cancer.

Keywords: melanoma, mechanobiology, melanocytes, keratinocytes, invasion, adhesion, migration, metastization

MELANOMA

Melanoma is one of the most severe skin cancers in humans with a mortality rate of 80% (Hofschroer *et al.*, 2017), due to the high resistance of this tumor to radiotherapy and chemotherapy (Uong and Zon, 2010). The resistance to those treatments is due to high number of mutations (approx. 75%) affecting specific genes, which lead to an increasing proliferation and survival (Kozar *et al.*, 2019). Immunotherapy is being exploited and investigated, and can be grouped in five types: (i) targeted antibodies (Sanlorenzo *et al.*, 2014; Herzberg and Fisher, 2016); (ii) adoptive cell therapy (Sanlorenzo *et al.*, 2014); (iii) oncolytic virus therapy (Franklin *et al.*, 2017); (iv) cancer vaccines (Tuettenberg *et al.*, 2007; Rodriguez-Cerdeira *et al.*, 2017); and (v) immunomodulators (Johnson *et al.*, 2014; Faries, 2016; Cancer Research Institute, 2019).

Chemotherapy and immunotherapy, can be combined (biochemotherapy) regarding the stage disease (Sanlorenzo *et al.*, 2014; Kozar *et al.*, 2019). The disease appears due to the malignant transformation of melanocytes located in the stratum basale of the epidermis although it is not clear how a melanocyte becomes malignant (Uong and Zon, 2010). Bandarchi *et al.* (2010) suggested that a combination of up and down regulation of factors in several molecular pathways, seem to be the mechanism of transformation of normal into malignant melanocytes. If the disease is detected in an early stage, there is a high probability of disease control, however, when metastization occurs, the 5 years life expectancy drops to 14%. Some epidemiological evidence related to melanoma incidences in white and dark skinned population (Bandarchi *et al.*, 2013) was presented by the authors whereas Bradford (2009) presented epidemiological data about the total numbers of melanoma incidences in the world. Dark skin results in a lower incidence of skin cancer due to the protection of the increased epidermal melanin, which filters UV light twice as good as white skin of Caucasian people (Bradford, 2009). Malignant melanoma are diagnosed by the ABCD rule, where A stands for asymmetry, B for borders irregularity, C for color variation and D for diameter higher than 6 mm (Bandarchi *et al.*, 2010). Melanoma shows three perceptible steps in tumor progression: (i) one only affecting the epidermis; (ii) a second one affecting both the epidermis and the superficial papillary of the dermis; and (iii) a third one affecting the epidermis, dermis and hypodermis (vertical growth melanoma) (Bandarchi *et al.*, 2010). Haass *et al.* (2005) divided the process of melanoma development in the following steps: (i) the damage of adhesion between keratinocytes by down-regulation of E-cadherin, P-cadherin, desmoglein, and connexins, which is influenced by growth factors produced by fibroblasts or keratinocytes; (ii) the interaction of melanoma with fibroblasts and/or melanoma with melanoma cells normally not found in melanocytes, is up-regulated by MCAM and N-cadherin; and (iii) alteration of integrin expression, inducing the loss of basement membrane anchorage (Haass *et al.*, 2005). According to the At Melanoma Foundation (AIM), melanoma develops in a similar way than other cancers: the DNA of a gene that controls cell division and proliferation is mutated. This damaged gene results in a lack of control of cell division and growth. This DNA mutation is passed to the daughter cell during division. Melanoma is originated from a melanocyte that experienced too many mutations growing in an abnormal way. In the case of

melanoma, this DNA mutation is caused by an overexposure to UV radiation, affecting the melanocytes (At Melanoma Foundation, 2014). When there is DNA damage, several genes may be affected, resulting in encoding errors of several molecules hampering their function (At Melanoma Foundation, 2014; Kunz, 2014) and there is a risk for a melanocyte to generate a melanoma. If the melanoma is not suppressed or treated, it will spread along the epidermis, after penetrating the dermis and hypodermis, getting in contact with the lymph and the blood vessels (At Melanoma Foundation, 2014). The main function of melanocytes is to produce melanin (responsible for skin pigmentation) that absorb UV light to avoid keratinocytes' DNA damage (Hirobe, 2014). Keratinocytes will distribute melanin to the upper parts of the skin layer (Wang *et al.*, 2017). Wang *et al.* (2017) also mentioned that hormonal factors, family history and cosmetics are the three main triggering and aggravating factors for melanoma development. When a primary melanoma is formed, it activates some signals, which will provide conditions that make the primary tumor strong such as: (i) attachment to the wall of the blood and/or lymphatic vessels, enabling the movement through a new organ; (ii) enough nutrients to grow; and (iii) it is resistant to the immune system. With the three conditions mentioned before the primary tumor has the capacity to promote the metastization (At Melanoma Foundation, 2014). Why can melanoma develop in some body parts that are not exposed to sunlight? It was suggested that there are different changes occurring in genes of tissue cells exposed, compared with the changes of genes of tissue cells not exposed to UV light. These mutations are transmitted into the next generations. Some genes appear during melanoma development, being not genetic. The transformation of melanocytes into melanoma cells is a very complex process, which involves several signaling pathways reviewed by Paluncic *et al.* (2016). Briefly, melanocytes are formed from their precursors: melanoblasts. Melanoblasts differentiate into melanocytes, which get mature and start melanin production on melanosomes, which will be transferred to keratinocytes. Melanomagenesis depends on the microenvironment, genetic and environmental factors. Although genetic factors are crucial, they are not sufficient to induce melanomagenesis (Paluncic *et al.*, 2016). Melanoma tumors are different from patient to patient. The environment, where melanoma tumors grow, is very complex depending on the interactions with ECM, microvasculature, fibroblastic cells and changing concentration of growth factor,

cytokines, and nutrients like glucose, oxygen, etc (Paluncic *et al.*, 2016). Some signaling pathways are more involved in tumor development, whereas other signals are more crucial in the metastization process. The description of biochemical pathways signaling is beyond of this review, however the readers can find more information in different sources (Dissanayake *et al.*, 2007; Straussman *et al.*, 2012; Moriceau *et al.*, 2015; Stark *et al.*, 2015; Wang *et al.*, 2016; Ahmed and Haass, 2018; Kodet *et al.*, 2018). Melanoma can be pigmented or not pigmented. The color of melanoma is due to the existence of melanin, secreted and stored in melanosomes in the cytoplasm of melanocytes. There are two types of melanin: eumelanin (has a black color) and pheomelanin (has a red/yellow color). Normal melanocytes may or may not secrete either type of melanin (Lassalle *et al.*, 2003). Mechanical properties and cell pigmentation are correlated (Sarna *et al.*, 2013, 2014). As melanoma needs a high amount of iron and copper, the development of substances that target these ions could be another methodology (Gorodetsky *et al.*, 1986).

MECHANOBIOLOGY OF SKIN CELLS

Currently, mechanobiology brings together biologists and biophysicists in order to better understand the role of mechanical forces in cell motility, adhesion and invasion. For developing therapies that avoid the invasiveness of cancer cells, it is crucial to understand the mechanisms of tumor invasion. The mechanical stiffness of tumors is correlated with its invasiveness, which was demonstrated by a study with cancer ovarian cells: more invasion corresponds to softer cells resulting in deformation and shape (2011). Makale (2007) raised three hypotheses correlating cancer and cell mechanobiology: (i) the cell biology of tumor invasion is a key to understand cancer treatment; (ii) cancer cells activate physiological mechanisms and migratory processes that are used by cells usually during embryo morphogenesis; and (iii) forces that are sensed by the tissue and cells trigger the differentiation and spreading of tumors. In fact, mechanical forces are generated by tissues, being sensed by cells, during embryogenesis and tumor expansion (Makale, 2007). In melanoma the Epithelial to Mesenchymal Transition (EMT) switching, and forces behind this process (Wei *et al.*, 2015) are crucial processes regarding the loss of connections between melanocyte and keratinocytes; melanocytes acquired the embryonic phenotype, promoting cells invasion, their intravasation in body fluids and the extravasation to other organs.

Arriving at their target destination, tumor cells are reverted to their original phenotype known as Mesenchymal to Epithelial Transition (MET), establishing secondary tumors. The production of metalloproteinases (MMP) will induce ECM degradation, favoring metastization (Paluncic *et al.*, 2016). Migration of collective cells interact with the extracellular environment via integrins (Hegerfeldt *et al.*, 2002). When normal cells are compressed because of a tumor, these forces will be sensed by cell body, through membrane molecules, the integrins, which are connected to the cytoskeleton, and to the nucleus. All cells respond to these external forces organizing cellular components in the cell and generating biochemical signals that will help them to adapt to external physical pressure. The normal ECM produced by fibroblasts also induces pressure on tumor cells via its cytoskeleton, resulting in cell and nucleus deformation. A consequence of nucleus compression could be the modulation of gene expression that may induce changes in cell viability, promoting locomotion, due to the adoption of a more malignant phenotype. In spite of high tumor stroma stiffness, tumor cells are deformable and have some degree of plasticity (Makale, 2007). In melanoma, the relation among melanocytes, keratinocytes, fibroblasts and the ECM mediate and control this disease. In the next paragraphs, mechanobiology of these cell types and ECM contribution to melanoma will be addressed.

The Role of Melanocytes

Melanocytes are located in the basal layer of the epidermis, eyes, ears, hair and meninges (Bandarchi *et al.*, 2013). Melanocytes have a dendritic shape (Figure 1). They are originated from migratory embryonic cells, called neural crest cells, which have a high capacity of migration (Uong and Zon, 2010). Melanocytes are the cells responsible for skin pigmentation, through the production of melanin, also named melanogenesis (Lassalle *et al.*, 2003). They are present in the epidermis and exhibit long dendritic protrusions, which are extended through the epidermal keratinocytes. Melanocytes synthesize melanosomes and transport them to the keratinocytes (Zhang *et al.*, 2004). Melanocytes adhere to keratinocytes through E-cadherins, desmoglein (DSg)/desmocollin (DSc) and connexins (CX) molecules (Li *et al.*, 2003) and to the basement membrane through integrins (Wang *et al.*, 2016). The ratio of melanocytes to keratinocytes is around 1:36/1:40. 8 to 12% of malignant melanoma are associated with

mutations on CDKN2A (cyclin dependent kinase Inhibitor 2) gene (Bandarchi *et al.*, 2013). Uong and Zon (2010) also mentioned that the pathways involved in melanocyte development and proliferation start to be subverted in melanoma formation. BRAF and NRAS (a gene of the RAS family identified for the first time at human neuroblastoma) (Pugh *et al.*, 2013) overactive mutations are found in 65 and 20% of melanomas, respectively. CDKN2A is lacking by melanoma cells, consequently the encoding suppressor genes (INK4a and ARF) are not active (Bandarchi *et al.*, 2010). Malignant melanocytes are characterized by changes of biophysical properties (like shape, elasticity and stiffness) which will contribute to the cell locomotion, invasion and metastization in other organs. In this case, the plasticity of melanocytes is very important, since they need to be highly deformable. Since the mechanical properties of melanoma cells may be affected by the production of melanin, this has to be considered (Sarna *et al.*, 2013). Melanin and melanogenesis may act in two different directions: melanin act as a protection shield to radiation, however it also attenuates the effectiveness of radiotherapy, chemotherapy and phototherapy in the case of melanotic melanoma. This characteristic can provide tumor growth and progression (Slominski *et al.*, 2015). AFM was used to observe the shape of human epidermis melanocytes in culture and to observe the melanosome transfer in vivo. Filopodia originated from the dendrite tips of the cell body of melanocytes, which contained melanosomes have been observed (Zhang *et al.*, 2004). Van Den Bossche *et al.* (2006) hypothesized different mechanisms for melanin transfer: (i) keratinocytes phagocyte the melanocyte dendrites; (ii) the melanosomes release melanin into the extracellular space by melanosome exocytosis, being internalized by the keratinocytes; (iii) melanosomes are transported via membrane nanotubes; and (iv) the melanocytes enriched of melanosomes are phagocytosed by the keratinocytes (Van Den Bossche *et al.*, 2006). Wu *et al.* (2012) studied melanocytes of mice lacking myosin Va (Myo5a). This protein is involved in the accumulation of melanosomes in the melanocyte dendrites. When myosin Va is present, melanosomes stay concentrated in the centre of cancerous melanocytes reducing melanosome transfer to keratinocytes (Wu *et al.*, 2012). Wäster *et al.* (2016) studied the relation between UV skin radiation and skin pigmentation response. They concluded that when pigmented skin is exposed to UVA, there is a spillage of extracellular vesicles from the melanocyte's plasma membrane. This does not happen when pigmented skin is exposed to UVB

(Wäster *et al.*, 2016).

AFM was used to study the morphological changes of melanocytes exposed to the melanocyte stimulated hormone α -MSH, which induces melanization (Shin *et al.*, 2014). The authors found that the roughness inside the pore-like structures on dendrites increased and MSH produced an increase in the size and density of the melanosomes. However, regarding the response to the presence of α -MSH, more studies are needed to correlate morphological shape alterations with the movement of melanosomes (Shin *et al.*, 2014). Human melanocyte's pigmentation in melanoma cells are also important in regulation of elastic properties according to Choi *et al.* (2014).

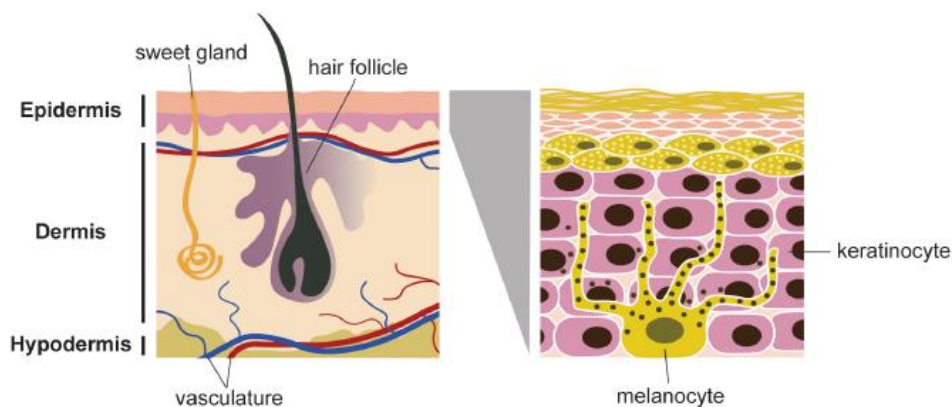


FIGURE 1 | Schematic representation of skin and cell population. Melanocytes are located in the basal layer of epidermis. Each melanocyte is connected to several keratinocytes. Melanosomes (black spots) are distributed in the dendrites, produce and store melanin. The dendrites allow the transfer of the melanin to keratinocytes, which will be transferred to the stratum corneum layer of the epidermis.

The authors studied the effect of substrates with different stiffness on the pigmentation, using normal human melanocytes (NHM) and melanoma cells (MNT1) cultured on polydimethylsiloxane (PDMS) coated with laminin. They observed a reduction of dendrite formation in both cell type as well as a difference on the transference rate of melanosomes for both cell type on softer substrates. Cells cultured on stiffer substrates showed lower migration capacity once traction forces formation are more evident through via focal adhesion integrin complexes (Choi *et al.*, 2014). Based on some studies of cell elasticity, cancer cells typically show a decrease in stiffness relatively to normal cells. Sarna *et al.* (2013) described a study of elastic properties comparing melanoma cells and healthy melanocytes. Basically, the authors used AFM to determine the stiffness of

human melanoma cells SKMEL-188 with different stages of pigmentation and compared with SKMEL-188 cells not pigmented and with NHMs healthy human melanocytes (Sarna *et al.*, 2013). They found that healthy melanocytes were softer than cancer cells. This feature could be related with the amount of melanin present in melanoma cells, resulting in some limitations for cancer diagnosis using the stiffness. Lazova and Pawelek (2009) mentioned that melanoma cells do not excrete the pigment like healthy melanocytes do, which may result in pigment accumulation in melanoma cells in relation to melanocytes. This could explain the higher stiffness of cancer cells, since they are often highly pigmented (Lazova and Pawelek, 2009). In conclusion, by AFM nanoindentation assays it is not possible to distinguish melanoma cells from melanocytes. AFM can be used as a complementary technique, however, this study was useful in understanding better pigmented cell elasticity. Sarna *et al.* (2014) also mentioned the need to understand if there is a correlation between the pigmentation of melanoma cells and the metastization phenotype process. In this process, malignant cells must pass the epithelial barrier, having the capacity to deform their cell body, including the nucleus to migrate through tissue. Cells with low Young's moduli exhibit a great potential to invade tissue (Sarna *et al.*, 2014), so this is why biophysical properties, as cell elasticity, must be taken into account. The authors showed that melanin granules inhibit the transmigration potentialities of melanoma cells. For non-pigmented melanoma cells, the stiffness was lower, and the transmigration efficiency was higher. This study contributed with new insights about the relationship between pigmentation and elasticity of melanoma cells in terms of migration and invasion. Some substances are responsible for the retraction of melanocyte dendrites. Ito *et al.* (2006) have studied the effect of dendrite retraction using a flavonoid substance. In this case, TYRP-1 signaling pathway is interrupted, once this molecule is important for melanosome migration. Rho signaling molecule, when activated induces dendritic retraction, reducing the melanosome transfer to the keratinocytes but does not affect the amount of melanin production. However, this melanin will not be transferred due to the dendrite's retraction. The mechanism of dendrite retraction via RhoA involves changes in cytoskeleton and microtubules organization due to actin and tubulin-b disassembly. These changes will induce stress fiber formation promoting actin polymerization and reorganization of microtubules (Ito *et al.*, 2006). Rac is involved in melanocyte dendrite

growing, inducing the appearing of lamellipodia (Scott, 2002). The dendrite tips of melanocytes are also stimulated by the α -MSH and endothelin – 1 proteins from keratinocytes that are surrounding the melanocytes and the ECM produced by the keratinocyte (Kippenberger *et al.*, 1998). Kippenberger *et al.* (1998) demonstrated that co-cultures of melanocytes and keratinocytes in the presence of high concentration of calcium, showed sheets of keratinocytes formation, surrounding by melanocytes with polar dendrites extended around the differentiated keratinocytes. Differentiated keratinocytes will have stable areas of contact with melanocytes due to the calcium gradient. The normal melanocytes that lose their dendrites soon will be in an apoptosis state (Kippenberger *et al.*, 1998). The senescence of primary melanocytes could also be important in the melanoma appearance (Feuerer *et al.*, 2019), however, there are no studies that correlate melanocytes' senescence with mechanical properties measured by AFM. Some studies showed a correlation between the melanocytes aging and senescence biomarkers (Wang and Dreesen, 2018) and exposition of melanocytes to light (Bennet *et al.*, 2017). AFM is a powerful tool to assess the morphological and elastic properties regarding the interface between primary melanocytes and primary melanoma with and without senescence. In opposition to the melanoma cell lines that are immortalized and thus, submitted to several passages, the primary melanocytes and primary melanoma cells should be assessed in order to evaluate what passage the senescence will not interfere with mechanical properties. The quantification of senescence level can be performed using the quantification of the Population Double Level (PDL) method (Kruse and Patterson, 1973).

The Role of Keratinocytes

Keratinocytes are the predominant cell type in the epidermis, most of them (90%) being located at the outmost epidermis layer (McGrath *et al.*, 2004). Keratinocytes are connected to each other through actin cell junctions (Hsu *et al.*, 2018).

The main function of keratinocytes is to form a barrier against pathogenic microorganisms, heat, UV radiation and water loss. They also produce cytokines that attract leukocytes to the location of pathogen invasion (Hall *et al.*, 2014). Keratinocytes also produce different keratins. Keratins (type 1, 5, 10, and 14) are the main structural protein of their cytoskeleton, all of which play an important role in the mechanics of

these cells (Ramms *et al.*, 2013). Keratins become altered when responding to stresses, resulting in its modification and reorganization (Karantza, 2011). Keratinocytes become corneocytes, when they are completely differentiated, losing their nucleus and cytoplasmic organelles (Mi, 2009). Differentiation of keratinocytes is driven by a calcium gradient, which is established between the interior of the cell and the ECM (Proksch *et al.*, 2008) and regulated by vitamin D3 (Bikle, 2004), cathepsin E (Kawakubo *et al.*, 2011), TALE homeodomain transcription factors (Jackson *et al.*, 2011), and hydrocortisone (Rheinwald and Green, 1975). Keratinocytes are affected by senescence, losing their biological function with age (Orioli and Dellambra, 2018). Keratin, actin filaments and microtubules form the cytoskeleton network of keratinocytes, providing shape and structure of cells allowing transmission of mechanical loads (Silver and Siperko, 2003). For keratinocytes migration one of the most important parameters is the substrate stiffness (Ramms *et al.*, 2013), since it is expected that the keratinocytes must change their shape during migration through the epidermis until they reach the outmost layer and become corneocytes (Zarkoob *et al.*, 2015). This change in shape will affect also the components existing inside the cytoplasm, namely the cytoskeleton, in which keratin fibers are crucial (Ramms *et al.*, 2013). Ramms *et al.* (2013) used AFM to probe keratinocytes (in the nuclear region and in the cell body) deficient in keratins finding that they are softer than healthy ones. In addition, with magnetic tweezers, the viscous contribution to the displacement of magnetic beads increased, corroborating the results achieved by AFM. These authors also found that the softening was reversible and could depend on the re-expression of two types of Keratins (K4 and K15) only (Ramms *et al.*, 2013). Bar *et al.* (2014) reported the skin fragility in mice and hampered desmosomes adhesion when there is a lack of all keratins Bar *et al.*, 2014. AFM showed a reduction of adhesive forces and membrane stability in murine keratinocytes when lacking keratin filaments (Vielmuth *et al.*, 2018). Adherent junctions (AJ) have functions in mechanosensing and transducing mechanical forces between the plasma membrane and the actomyosin cytoskeleton. Desmosomes and intermediate filaments promote the mechanical stability to maintain tissue architecture and integrity when they are submitted to mechanical stress. Both (AJ and desmosomes) connect the actin and keratin filament network of adjacent cells. Desmosomes are important for intercellular cohesion, where keratins determine cell mechanics but are not involved in tension

generation (Hatzfeld *et al.*, 2017). Zarkoob *et al.* (2015) investigated the role of substrate stiffness on keratinocyte colony formation (in vitro) during epithelial formation, due to calcium exchange during keratinocyte cell culture. Keratinocytes cultured on soft polyacrylamide substrates ($E = 1.2$ kPa) presented small contact areas, an increase in migration velocity and in colony formation compared to those cultured on stiff polyacrylamide gels ($E = 24$ kPa) (Zarkoob *et al.*, 2015). How melanoma cells escape the keratinocyte's control invading other tissues and form metastasis, is still unclear. In a healthy tissue, there is a homeostasis maintained by each cell. In this normal tissue, keratinocytes are the "surveyors" of melanocytes, controlling their growth and behavior through complex paracrine growth factors and cell-cell adhesion molecules. When alterations occur to this homeostatic balance, cell-cell adhesion and cell communication molecules change and induce melanoma development resulting in the damage of the epidermal melanin unit, inducing the continuous melanocyte proliferation, and eventually leading to melanoma development (Haass *et al.*, 2005). Chung *et al.* (2011) suggested keratinocytes-derived ECM factors may act as regulators of melanocytes. The authors showed that laminin-332, a component of basement membrane, plays a crucial role in the adhesion and migration of melanocytes and melanoma. It is known that keratinocytes regulate the behavior of melanocytes such as proliferation, melanin synthesis and dendrite formation and the ECM also regulates different cell behaviors (Chung *et al.*, 2011).

The Role of Fibroblasts and the ECM

Fibroblasts are produced in the bone marrow. They synthesize the components of the ECM, for instance collagen, glycosaminoglycans and elastic fibers. Fibroblasts also produce a structural network called "stroma" in animal tissues (connective tissue), playing an important role in cancer and in wound healing (Desjardins-Park *et al.*, 2018). Kwa *et al.* (2019) mentioned that cancer is associated with fibroblasts and how these fibroblasts affect metastization. Fibroblasts produce high amounts of ECM molecules, cytokines and growth factors contributing to the stroma, cancer progression and metastization due to several pathways. These pathways could be a target for new cancer treatment strategies to be developed. However, it is difficult to characterize this subtype of fibroblasts and to identify specific molecules involved in this process (Kwa *et al.*, 2019). Mechanosensing has a large impact in Cancer-Associated Fibroblasts

(CAFs). Fibroblasts sense mechanical stresses and activate mechanosignaling processes, which use mechanoreceptors located in the cell membrane, the cytoskeleton and transcription factors. The ECM stiffness is determinant in the mechanosignaling, which by itself can activate the mechanosensing pathways in CAFs, leading to ECM production and stiffness increasing, supporting different CAFs differentiation (Kwa *et al.*, 2019).

The study of mechanosignaling and fibroblasts response to the ECM stiffness was conducted using hydrogels, to mimic pathologically the ECM of tissues. Viji Babu *et al.* (2018) have used the AFM to study the stiffness and viscoelasticity of normal, scar and Dupuytren's disease fibroblasts, growing on top of soft (1 kPa) and stiff (50 kPa) hydrogels. Basically, a MLCT-Bio pyramidal cantilever (nominal spring constant of 0.01 N/m, Bruker, United States) was used to indent the fibroblasts, using the z-step response methodology, in which the elasticity and viscosity was assessed (**Figure 2**). The authors wanted to evaluate the effect of TGF- α in the fibroblast's elasticity. The authors concluded that Dupuytren's fibroblasts increased their elastic moduli and became stiffer in TGF- β 1 presence, whereas they did not find significant changes in the elastic moduli of scar and normal fibroblasts, before and after addition of TGF- β 1. Dupuytren's fibroblasts presented a wide number of well-organized stress fibers and bundles of stress fibers were even thicker in presence of TGF- β 1 (Viji Babu *et al.*, 2018).

RhoA signaling is the key for the fibroblast's reaction to ECM stiffness. RhoA is activated by a stiff matrix, causing cell contraction and actin polymerization, inducing the differentiation of fibroblasts in myofibroblasts. Therefore, CAFs need the activation of this pathway, since it is required for their contractile and invasive properties. It has been mentioned that there are several CAF subtypes, however, there is a lack of information about their plasticity, which could be important for the progression and metastization of cancer (Kwa *et al.*, 2019). AFM could give new insights regarding the characterization of fibroblasts subtype based in their morphometric characteristics (shape factor, volume and area) and mechanical properties. As far as we know, there is not any specific study in literature, which could be useful for the development of therapies directed to a specific fibroblast subtype related with melanoma. This study could also promote new insights to develop therapeutic strategies to other cancer types.

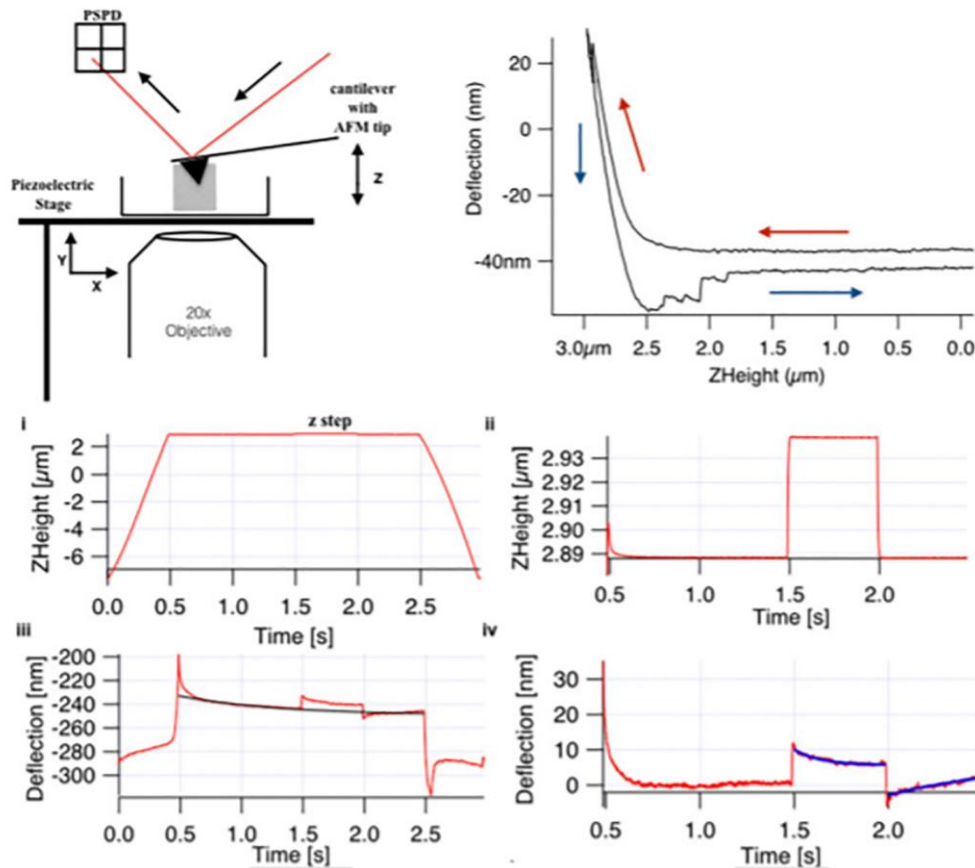


FIGURE 2 | Basic components of (A) AFM and (B) force curve with (C) viscoelastic creep response measurement. (A) The basic four components of AFM (i) a laser diode, (ii) a cantilever of 0.01 N/m spring constant with 30 nm radius AFM pyramidal tip, (iii) a position-sensitive photo detector (PSPD), and (iv) xyz-piezo stage. (B) Sample indentation by the AFM tip obtains the force curve that gives the approach (red arrow) and retract (blue arrow) curve on deflection vs. Z-height graph, and apparent Young modulus was calculated by applying Hertz model to the approach curve. In creep response curve (C), the Z-height profile (i) shows the approach and retract ramp toward the cell for 3 s, and in-between, there is a z step, which is applied at $t = 1.5$, which is enlarged in panel (ii). (iii) The deflection data show global creep of the cell, which includes the creep after loading and unloading step, which is enlarged in panel (iv), and global creep was determined by the exponential fit (black curve) and was subtracted for qualitative analysis (reproduced with permission from John Wiley and Sons with Copyright Clearance Center's Right, Journal of Molecular Recognition, 2018) (Viji Babu *et al.*, 2018).

The ECM is composed by (i) the structural proteins collagen and elastin; (ii) adhesion proteins such as fibrillin, fibronectin, vitronectin and laminin; and (iii) the proteoglycans (which have a protein core bound to glycosaminoglycans) (Makale, 2007). The ECM is responsible for cellular support. Variations in its structure and composition alter the mechanical properties of the ECM. Generally, the external forces applied to cell-matrix complexes are transmitted to the interior of the cell by integrins according to three steps (Roca-Cusachs *et al.*, 2009): (i) integrins binding to ECM molecules; (ii) transmission of forces to the interior of the cells, through integrins, where they are transformed into biochemical signals (mechanotransduction); and (iii) integrins binding

to cytoskeleton, transmitting the forces all over the cell, and reinforcing their adhesion properties to resist the force. The integrins inside of cell recruit several other molecules, which bind to the cytoskeleton and/or signaling proteins to form what is called “focal adhesion” to resist to the external force (Roca-Cusachs *et al.*, 2009).

What are the mechanical cues sensed by cells when forces are applied from the outside? Integrin-associated complexes (IACs) in cellular membranes establish the connection between the ECM and the cytoskeleton of the cell, via actin filaments (Haase *et al.*, 2016). Haase *et al.* (2016) found that physical forces stemming from the ECM have a huge impact on gene regulation; it has been hypothesized that gene expression is affected by nucleus deformation, which was observed in 3T3 fibroblasts. The actin and microtubule network generates nuclear deformation. The deformation of the nucleus will be anisotropic being observed along the shorter axis and is intrinsic to the nucleus. This behavior is due to the chromatin organization and lamin-A expression (Haase *et al.*, 2016). The ECM uses the concept of “tensegrity,” which employs tensile forces and rigid support structures to distribute and manage the loads mediated by the cellular cytoskeleton complex. In the cytoskeleton microfilaments and microtubules, which are connected to the ECM, there are forces in between (Makale, 2007). This means that the pressure exerted on the ECM will influence the cell’s behavior in terms of differentiation, motility, adhesion, invasion, and metastasis (Makale, 2007). ECM mechanical properties are directly related with elastin fibers, collagens, glycosaminoglycans (GAGs) and proteoglycans. Elastin fibers contribute to cyclic stretching during life; collagen fibers contribute to the tissue stiffness and mechanical strength of tissues and GAGs affect ECM viscoelasticity, forming bridges between the collagen fibers and contributing to the compressive tissue stiffness. Cells sense the forces exerted by the ECM, converting these stimuli into intracellular signal pathway, which further downstream regulate transcriptional changes (Hsu *et al.*, 2018). Several mechanosensors are identified in cells: glycocalyx, lipid raft/caveolin-1, cell adhesion structures (integrin, hemi-desmosomes and focal adhesion), ion channels (calcium-sensing receptor), transient receptor potential channels, connexins, intercellular complexes (desmosomes or cadherin) and the cytoskeleton (particularly the actin filaments) (Hsu *et al.*, 2018). Any abnormality that causes changes in the ECM mechanical properties, cell interactions, or cellular response

to mechanical stimuli will induce tensional homeostasis, contributing to several diseases like cancer and fibrosis (Hsu *et al.*, 2018). Integrins (transmembrane proteins) are responsible for transmission of forces between ECM and the interior of the cell. Tumor cells expressed the $\alpha_5\beta_3$ integrin and the invasiveness of melanoma cells depends on the expression of β_3 and β_1 integrins, which are related with their aggressive properties (Petitclercet *et al.*, 1999; Hegerfeldt *et al.*, 2002). Therefore, one therapeutic strategy could contribute to the reduction of these integrins expression (Makale, 2007). Stiffness of tumors is important since it activates biochemical pathways regarding the cell cycle, EMT, cell motility and leads to compression in neighboring healthy cells. These healthy cells may then activate tumorigenic pathways (Broders-Bondon *et al.*, 2018).

Cell spreading depends on substrate stiffness, which, in turn, depends on the complex adhesion type. A7 melanoma cells were seeded in polyacrylamide gels laminated with collagen type I (Coll I), fibronectin (FN) and a mixture of Coll I and FN (Winer *et al.*, 2011). **Figure 3** shows the stiffening and spreading of filamin A-expressing A7 human melanoma cells and is compared with different stiffnesses substrates coated with FN or Coll I. Stiffening but not spreading of A7 melanoma cells depends very strongly on whether integrins specific for FN or Coll I are engaged. When A7 cells are plated on gels coated with saturating amounts of either FN or Coll I, they spread to approximately the same extent (**Figure 3B**), but the cells on Coll I are much stiffer than those adherent to FN (**Figure 3A**). When both FN and Coll I are present, allowing both α_1 and β_3 integrins to bind, adherent area increases, but cell stiffness reaches an intermediate value between those found on FN or Coll I alone (Kadow *et al.*, 2007).

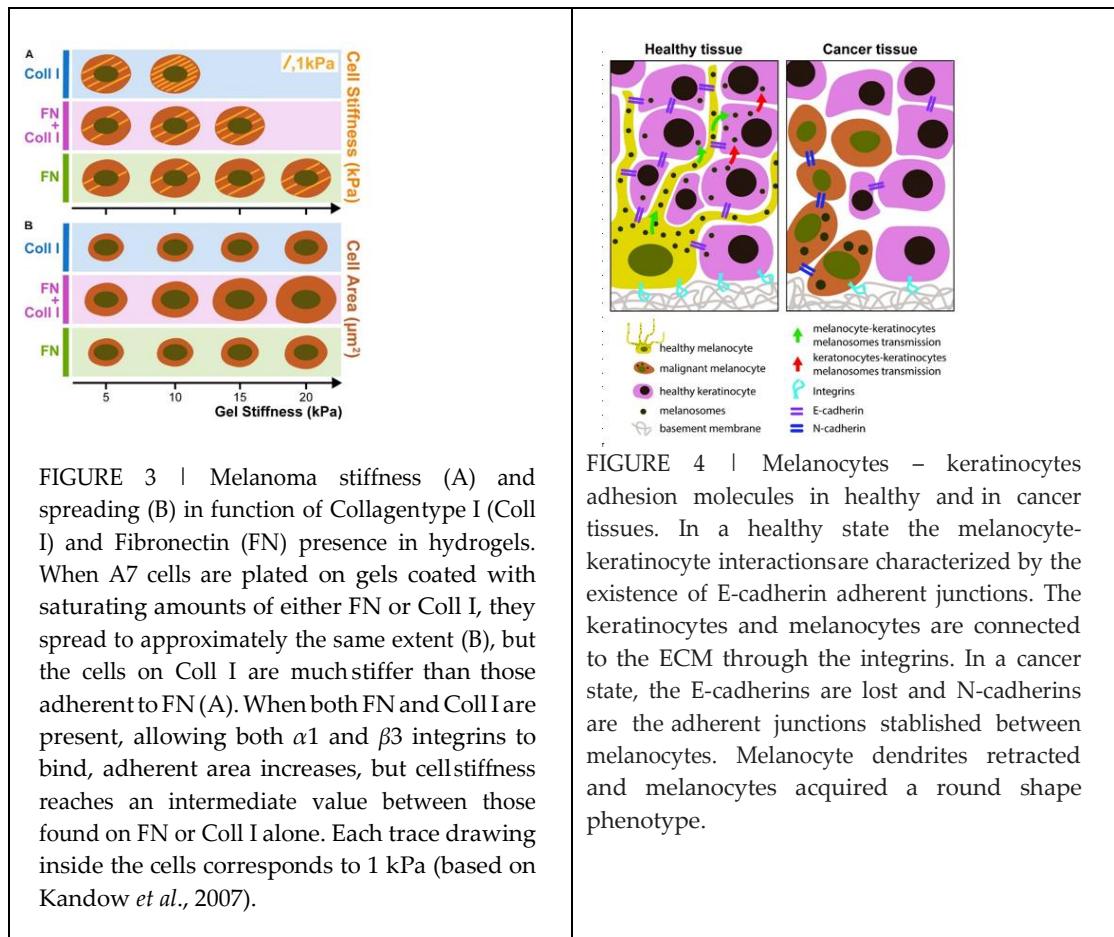
CELL MIGRATION AND PROGRESSION IN MELANOMA

Motility

Melanoma starts with the lost binding intercellular connections between melanocytes and keratinocytes (**Figure 4**).

In the process of melanoma development, due to EMT, cells acquired a mesenchymal stem cell phenotype, contributing to cell migration (Bandarchi *et al.*, 2010). The EMT model is very well explained by Nieto *et al.* (2016) and Brabletz *et al.* (2018), the forces involved in EMT and its relation with cancer metastasis are described by Wei

et al. (2015). After losing their connections, melanocytes move due to a motility process. Motility is the capacity of a living system to move independently, using metabolic energy to perform mechanical work and should not be confused with mobility (Allen, 1981; **Figure 5**).



For cell migration, there is the contribution of the internal forces (which are generated by some structures present inside each cell, such as the cytoskeletal filaments) and the external forces (the shear flow of blood or lymphatic fluids contributions). The motility of cells through tissues is a process mediated by internal forces, which are related with the polarization of the cell body within the ECM. This motion depends on shape changes, cell-substrate interaction, and the generation of forces from the cells mediating through the tissue. The internal forces responsible for cell motility are composed by the following steps: (i) cytoskeletal polarization, forming extensions of actin filaments located in the leading edge; (ii) cell protrusions connect to the ECM scaffold by cell surface adhesion receptors; (iii) contraction of actomyosin motors that are connected

with the ECM substrates exerting traction forces; (iv) bipolar tension, which is transmitted through the substrate, allows the retraction of the cell rear completing the movement of the cell; and (v) when cells move, they produce ECM degrading proteases and the transmigrated ECM is remodeled by proteolysis (Tao *et al.*, 2017). Tao *et al.* (2017) also refers that the actin cytoskeleton exhibits viscous and elastic responses, which can be characterized by the relaxation time observed in a creep response after applying a stress. The relaxation corresponds to the time scale where the cell cytoskeleton can flow and behave in an elastic way.

Cytoplasm is more than cytoskeleton: it has a lot of water content (around 70%), which also plays a role in cell motion, since water flows in between the cytoplasm organelles. The external forces are related with the water flow and osmotic pressure, as well to the fluid shear stress of the blood and lymph. The water can pass through the cell membrane via aquaporin in both directions, so the cell can change its volume, either forced by external cues like the osmotic pressure difference between inside and outside of the cell or actively controlled by ion pumps (Tao *et al.*, 2017).

Cytoskeleton rearrangement occurs through the arrangement of three proteins: Rho, Rac and Cdc42. The Rho GTPase family since they influence the appearance and structure of filopodia, stress fibers and adhesion plaques of melanoma cells (Tao *et al.*, 2017) encourage actin retraction; Rac was treated to encourage lamellipodia extension but block actin depolymerization; Cdc42 was assumed to encourage filopodia elongation and block actin depolymerization (Tao *et al.*, 2017). Motility is resulting from what is calling the mechanotransduction “inside-out” combining with a water flow in and out.

In human melanoma, by micropore filtration, it was found that vimentin and keratin intermediate filament expression results in a more migratory and invasive phenotype (Gao, 2014). The authors also found that damage of actin filaments and microtubules increases the deformability contributing to motility and metastization (Gao, 2014). Cell motion requires that the cytoplasm viscosity change in the cell periphery through the transformation of the gel state to a fluidic one. This mechanism also induces formation of protrusions that facilitate cell locomotion. Actin filaments are bonded to the plasma membrane by a glycoprotein. In the presence of this glycoprotein cells are stable, the protrusions are well organized, occurring cell movement. If this glycoprotein is not

present, as in the case of certain human melanoma, locomotion is impaired, and the plasma membrane shows blebbing (because of the cytoskeleton instability). If the presence of the glycoprotein is re-established, blebbing disappears and cell movement is recovered.

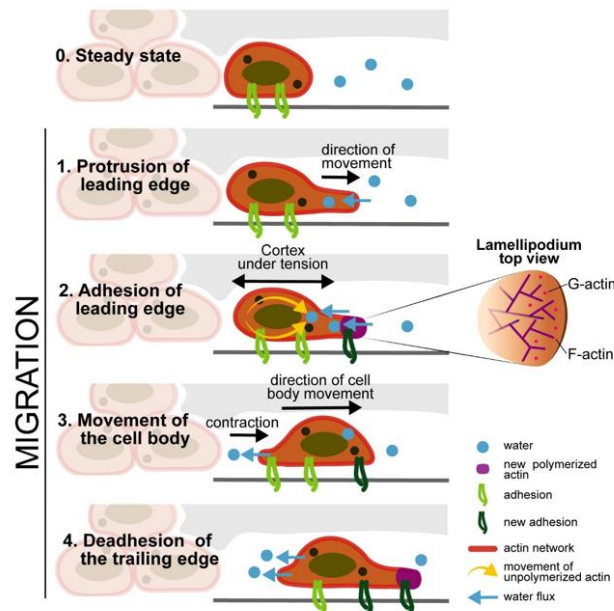


FIGURE 5 | Cell motility: Stage 0 (Steady state) -Cells maintain the connections to the ECM through the integrins; Stage 1 (Protrusion of the leading edge) -internal forces induce the F-actin polymerization and the water flow enters through the cell membrane; the actin polymerization induce the formation of protrusions; Stage 2 (Adhesion of leading edge) - the cortex under tension, accumulates new actin, through the movement of unpolymerized actin to the protrusion edge, and new adhesion molecules appear in the protrusion part. Stage 3 (Movement of the cell body) - there is the contraction of the cell in the same direction of the cell movement, and the water flow exits from the cell in the opposite direction, contributing to cell motility; Stage 4 (Deadhesion of the trailing edge) - Deadhesion on the cell back edge: the integrins in the back of the cell decreases, and new adhesion molecules appeared in the front of the cell in the same direction of cell movement based on Tao *et al.* (2017).

Actin filaments are bonded to the plasma membrane by a glycoprotein. In the presence of this glycoprotein, cells are stable, the protrusions are well organized, occurring cell movement. If this glycoprotein is not present, as in the case of certain human melanoma, locomotion is impaired, and the plasma membrane shows blebbing (because of the cytoskeleton instability). If the presence of the glycoprotein is re-established, blebbing disappears and cell movement is recovered. This glycoprotein also increases the stiffness of normal cells, meaning that in melanoma cancer the lack of this protein increases the deformability and contributes to the efficiency of the migration process of tumor cells (Gao, 2014). The whole cell, including the nucleus,

must present a certain plasticity. Inside the nucleus, there is chromatin together with lamins in coordination with the cytoskeleton, maintaining shape and mechanical stability of the nucleus. Chromatin works like an elastic spring and is responsible for the force response to small deformations of the nucleus. Lamin A deforms easily for small extensions and gives stiffness to resist large nuclear deformations (Stephens *et al.*, 2019). Microtubules in the cell cytoskeleton exert forces generated by dynein that can deform or even cause the rupture of the nucleus. Vimentin protects the nucleus giving it stability and perinuclear stiffness, which hinders 3D motility. Actin also stabilizes the nucleus shape together with microtubules (Stephens *et al.*, 2019). Alterations to chromatin due to mechanical or physical properties, which could alter the nuclear shape, may be a biomarker of disease. Mechanotransduction pathways that sense and respond to ECM forces will regulate the shape of the nucleus. Physical properties of nuclear components also regulate cellular functions and could give rise to abnormal nuclear shape or other diseases (Stephens *et al.*, 2019). In these cases, nuclear disruption may occur originating inflammation, senescence or cancer.

Wen *et al.* (2017) studied the morphological differences in four cell types: a human healthy melanocyte and melanoma cell types M14, A375 and MV3. Transwell migration assays were performed and filopodia, lamellipodia, stress fiber and adhesion plaque were characterized. Melanocytes did not show stress fibers or adhesion plaques. In contrast, the three melanoma cell types showed thin and short filopodia and stress fibers. The transwell test showed that melanocytes have no ability to migrate through the transmembrane, in contrast to the other three cell types (Wen *et al.*, 2017). Rho GTPases family members are involved in several cellular activities (like stress fibers and adhesion plaques) through the signal transduction, such as cytoskeleton rearrangement, cell proliferation, cell polarization and cell chemotaxis, being reduced in melanoma cells. The overexpression of Rho can inhibit the expression of other factors like P21 (a tumor-inhibitor factor). Thus, Rho may be crucial for the inhibition of melanoma cells. Rac1 is responsible for microtubule stabilization, being increased in melanocytes (Wen *et al.*, 2017).

Invasion

Weder *et al.* (2014) also demonstrated, by AFM studies, that the transformation of Radial Growth Phase (RGP) to Vertical Growth Phase (VGP) contribute to the

melanoma progression. The transformation of RGP to VGP cells is associated with decrease of cell stiffness, which promotes the cell deformation and invasion into the surrounding stroma and the intravasation (Weder *et al.*, 2014). Invasion is the capacity of cell penetration into neighboring tissues. This concept also includes the extravasation, however, this concept will be described in the section “Metastization.” The invasion of melanoma cells occurs through the blood or the lymphatic system. Primary melanoma will grow and spread horizontally on the top of skin layer (epidermis). When the melanoma cells go deeply into the dermis (vertical growth) they can reach the lymph nodes or blood vessels (intravasation). Some cells may get detached from the primary tumor and spread along the vessels, forming metastases, which eventually spread to regional lymph nodes (the cervical, the axillary and the inguinal basin). If the melanoma cells will be confined to these sentinel nodes, they can be removed by surgery. In the lymphatic vessels, there are the lymphocytes B and T and the Natural Killer cells. Melanoma cells must have a certain plasticity to cross not only the lymph vessels (endothelium tissue) but also the smooth muscle that is in the external part of the lymphatic vessels (this smooth muscle tissue avoids that the lymphatic fluid goes in the opposite direction as well as the valves that are inside of these vessels). The vessels are porous, allowing the entrance of other cells such as cancer cells (At Melanoma Foundation, 2014). The Circulating Tumor Cells (CTCs) are a subject that is transversal to several cancer types. Studying this cell type could be beneficial for developing new therapeutic strategies against metastization (Wang *et al.*, 2018). CTCs traveling across the blood need to be strong enough to withstand the blood shear stress (Huang *et al.*, 2018). **Figure 6** illustrates different steps of melanoma progression, from invasion until the metastization process.

The mechanical forces exerted by the tumor components are named “solid stress.” These forces drive the tumor tissue and deforms the surrounding normal tissue. The normal tissue, in turn, resists tumor expansion. Solid stress affects tumor pathophysiology in two manners: directly by compressing cancer and stromal cells and indirectly by deforming blood and lymphatic vessels. Cell compression induces alterations in gene expression, cancer cell proliferation, apoptosis and invasiveness, stromal cell function, and the organization of extracellular matrix and its synthesis. Blood and lymphatic vessel compression reduces the delivery of oxygen, nutrients and

drugs, creating a hypoxic and acidic microenvironment, compromising therapeutic outcomes (Padera *et al.*, 2004; Stylianopoulos and Jain, 2013; Jain *et al.*, 2014). The forces exerted by the fluid components in the tumor are called “fluid stress.” These forces include the microvascular and interstitial fluid pressures (MVP and IFP, respectively), as well as the shear stress exerted by blood and lymphatic flow on the vessel wall and by interstitial flow on cancer and stromal cells as well as extracellular matrix (Koumoutsakos *et al.*, 2013). Fluid stresses are a combination of the effect of structure of tumor vessels and blood and lymphatic vessels compression. The compression of blood vessels reduces the cross section of vessel, increasing the resistance to the blood flow, which can affect the MVP, shear stress and perfusion (Jain, 1988). The compression of lymphatic vessels avoid the drainage of excessive interstitial liquids, resulting in accumulation of fluids in the interstitial space and formation edema (Jain and Baxter, 1988; Baxter and Jain, 1989). The hyper-permeability of the tumor vessels, formed during the process of angiogenesis, usually lead to increased fluid flux from the vascular to the interstitial space. This fluid leakage reduces tumor perfusion and, along with the loss of functional lymphatics through physical compression, elevates IFP. The elevated IFP reduces perfusion, because the upstream microvascular pressure is communicated to the extravascular space and then to downstream vessels, minimizing the pressure drop between upstream and downstream segments of vessels leading to reduced flow. The elevated IFP and MVC will contribute to a barrier to drug delivery therapies (Jain *et al.*, 2014).

In the eyes of mechanotransduction, fluid shear stress activates YAP1 protein (yes-associated protein 1) which starts a step cascade that promotes cancer cell motility, since it is a potent oncogene present in several cancer types (Huang *et al.*, 2005). Lee *et al.* (2017) showed that mechanical forces exerted by the fluid flow regulate cell behavior, being crucial for the metastasis process (Lee *et al.*, 2017). Chivukula *et al.* (2015) also demonstrated that cancer prostate cells may be able to adapt to the fluid shear stress (FSS). Micropipette aspiration was used to measure the Young's modulus of prostate epithelial and prostate cancer cells. The Young's modulus was determined before and after the exposition (low and high) to FSS. Normal prostate cells

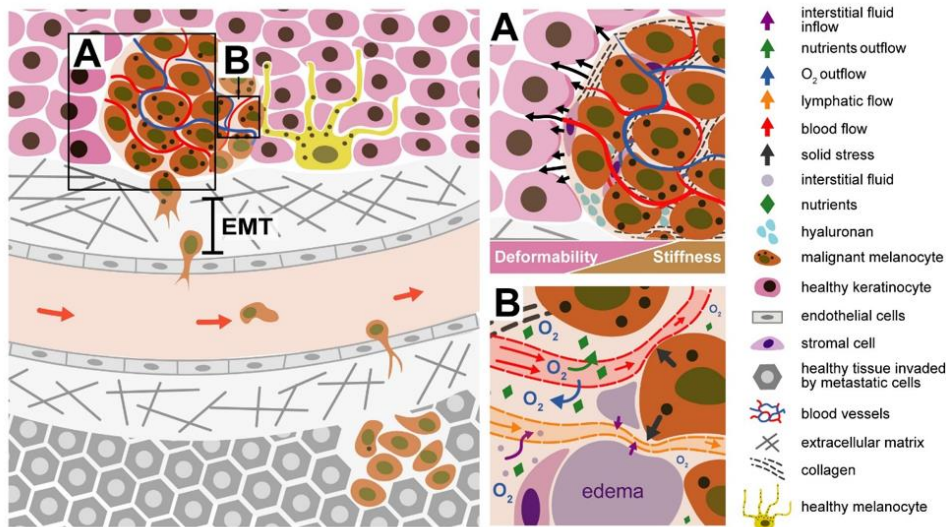


FIGURE 6 | Illustration of a malignant melanocyte invasion to a new organ. (A) Tumor microenvironment - the tumor tissue becomes stiffer comparing with the surrounding tissue microenvironment; the generation forces (solid stress) by the tumor components contribute to displacement of the surrounding normal tissue. The EMT process contributes to cell movement. The malignant melanocyte, due to internal forces, is detached from the primary tumor through the ECM, passing through the endothelial cell of the blood or lymph vessel (intravasation). The tumor environment is stiffer than the healthy cells, being not so deformable, when compared with the tissue around. (B) Liquid stress is related to the forces exerted by the blood, lymphatic and interstitial vessels. The tumor compresses the vessels, which are squeezing; the O₂ comes outside the vessels and the tumor is deprived of oxygenation, must develop conditions to grow in hypoxia. The compression of lymphatic vessels avoids the drainage of excessive interstitial liquids, resulting in accumulation of fluids in the interstitial space and formation of edema.

were 140% stiffer than cancer cells not exposed to FSS. After exposing cancer cells to low and high FSS, the authors obtained only 47 and 77% of the initial stiffness, respectively. The Young's modulus of normal cells did not change significantly when in contact with FSS. Blood vessels are very diverse in terms of size, porosity, structure, cellular components, mechanical properties, depending on their location in the body and the specific function of the local environment. Endothelial cells can sense the strength of shear flow stresses and respond to it with the production of nitric oxide and prostacyclin to relax vascular smooth muscle cells, increasing the diameter of the vessel and thus decreasing the flow shear stress (Huang *et al.*, 2018). When melanoma cells move through the blood or lymph, they are subjected to shear forces caused by liquid flow. How melanoma cells react to the shear stress of the circulating fluid? For blood fluid characterization, the following parameters must be taken into account: the pressure, the fluid shear stress, the viscosity, the blood vessels stiffness and the circulating blood cells. All maintain the mechanical equilibrium in a healthy state. If this equilibrium is lost, it

will contribute to the appearance of hematological or vascular diseases (Qiu *et al.*, 2019). Several techniques can be used to measure the mechanical properties of blood fluid, blood cells, and tissues in contact with blood and adherent cells to blood vessels. AFM (Kasas and Dietler, 2013), optical tweezers (Tan *et al.*, 2012) and micropipette aspiration (Daza *et al.*, 2019) are used to measure the mechanical properties of cells. AFM is used to measure the mechanical properties of tissues (Lekka *et al.*, 2012); the micro-viscometer is used to measure the viscosity of blood (Kim *et al.*, 2017) and traction force microscopy is suitable to measure focal adhesion forces exerted by adherent cells to the ECM or other substrates (Checa *et al.*, 2015). A detailed description of these techniques is beyond the scope of this manuscript.

Marsavela *et al.* (2018) mentioned the importance of the Melanoma Circulating Tumor Cells (MelCTCs) as a benefit and challenge for clinical applications. The MelCTCs cells are clones of primary and/or circulating metastatic cells in blood. MelCTCs can pass through the blood flow using a passive intravasation, which requires low energy. The melanoma cell detached from the tumor due to the increased blood flow and/or the existing low levels of CDH1 (tumor suppressor gene). Activated intravasation occurs when some melanoma metastatic proteins (NEDD9 and DOC-3) are expressed by the cell, inducing the Rac18 activation, causing changes in actin assembly in the cell causing the migration into vessels. The intravasation by active process means better metastatic ability, since the melanoma cell survives in the blood stream (Mumford and Robertson, 2014).

In spite the MelCTCs being in low concentration in the periphery of blood system, to collect these cells is an advantage, mainly when the biopsy of the tumor is difficult to perform due to its localization. EpCAM, are found in cancers such as breast and prostate cancers but, are not present in melanoma cells, once the breast and prostate cancer cells are original from epithelia tissue and the melanocytes are derived from neural crest. Other limitation is that MelCTCs are a very heterogeneous population (Marsavela *et al.*, 2018). There are several isolation techniques for MelCTCs, from the use of simple surface markers until new methodologies based in the physical characterization of MelCTCs. MelCTCs have a size range between 13 and 21 μm , what confirm the molecular and phenotype heterogeneity (Aya-Bonilla *et al.*, 2017). More studies are needed to be performed in the MelCTCs to know more regarding its

phenotype and know their behavior when subjected to some drug treatment. The authors hypothesized that AFM could have a significant contribution in this field regarding the study of morphometric parameters when the MelCTCs are subjected to specific drugs. Force spectroscopy molecular recognition studies could be helpful to improve the knowledge about the receptors of MelCTCs through the utilization of polyclonal antibodies in order to find receptors expressed in these cells. These results could lead to the development of markers and detection strategies helping in the isolation of these cells. The new findings could be promising to find new drugs in immunotherapy fields.

Adhesion

Melanoma cell adhesion molecule (MCAM) is a cell-surface adhesion molecule expressed by melanocytes in over 70% of metastatic melanoma cells, not being expressed in normal melanocytes *in vivo* (Yoshioka *et al.*, 2003). Endothelin-1 (ET-1) contributes to the up-regulation of MCAM that will contribute for the melanoma progression (Mangahas *et al.*, 2004). Chen *et al.* (2019) mentioned that the MCAM is a driving force for the dissemination of melanoma cells through specific binding in original skin lesions (Chen *et al.*, 2019). There are antigens shared between melanoma and vascular endothelial cells. The intravasation of the melanoma cells is based in melanoma adhesion molecules and the interaction with surface molecules on endothelial cells. L1-CAM from melanoma cells bind to integrin $\alpha v \beta 3$ inducing intravasation. N-cadherin also bind to endothelial cell (Braeuer *et al.*, 2013). Klemke *et al.* (2007) demonstrated that the interaction between $\alpha 4 \beta 1$ and the adhesion molecule VCAM-1 enhances the migration of human melanoma cells across activated endothelial cells. The transmigration through the endothelium of high metastatic human melanoma cell lines also occurred through the unstimulated VCAM-1 negative endothelial cells. The authors of this work used human melanoma cells that were devoid of $\beta 2$ integrins, therefore did not bind to ICAM-1. The transmigration through the endothelial cells is mediated via interactions of other adhesion molecules than VCAM-1 or ICAM-1 (Klemke *et al.*, 2007). Rebhun *et al.* (2010) also studied the importance of the integrin 4α binding existing in B16-F1 melanoma cells in the adhesion to the lymphatic vasculature, *in vitro* and how this mechanism impact the lymphatic metastization of melanoma cells *in vivo*, in mice. Lymphatic vessels express the VCAM-

1 molecule in endothelial cells. The B16-F1 metastasized in 30% in lymph nodes, while the B164 α metastasized 80% in the lymph nodes; B164 α (deficient in 4 α) was non-tumorigenic. This study demonstrated that 4 α expressed in melanoma cells contributes to the metastization of the lymph nodes, due to a stable connection established between the melanoma cell and the lymphatic vasculature (Rebhun *et al.*, 2010). Therapeutic strategies must be developed, avoiding these specific binding molecules. Using drugs that provide the capacity to compete and bind first to 4 α , avoiding the VCAM-1 contact or a drug that works through the weakness forces stabilizing between these binding complexes. Immunotherapy development could be based in this approach.

Kolasińska *et al.* (2019) also mentioned that sialic acid (SA) monosaccharides are commonly found at the outermost end of the sugar chains of various glycoconjugates. SA external position on cell surface molecules strongly influence cell behavior because SA may interact with other cell surface molecules and ECM proteins. SA has been reported to have an important role in the progression of melanoma cells. The ECM integrins also play an important role in melanoma cell adhesion to fibronectin (FN) as well as melanoma cell migration rate on FN. The main receptor of FN ($\alpha 5\beta 1$) can be modified by the $\alpha 2$ -3 and $\alpha 2$ -6 linked SA residues and its expression level on melanoma cells was positively associated with the progression of melanoma (Kolasińska *et al.*, 2019). The authors suggest that AFM molecular recognition force spectroscopy could be used to assess the unbinding forces between SA and $\alpha 2$ -3 and $\alpha 2$ -6 integrins. These findings could contribute to the development of new therapies that could avoid the bindings between the SA and the integrins.

Metastization

The main target organs for melanoma metastases are the lungs, the liver and the brain (Ju *et al.*, 2018). Metastization occurs after a cell/group of cells detached from the primary tumor, passed through the basal membrane, migrated through the ECM, entered the circulation system (blood and lymph nodes), adhered to epithelial cells, leaving again the circulatory system and entering finally to another organ(s), where the secondary tumor(s) establishes (Hofschroer *et al.*, 2017). Contributing factors and

associated physical phenomena can be observed and quantified during metastization. Like in other cancers (Kim *et al.*, 2016), melanocyte(s) detached from the primary tumor must have high “strength” that make them different from those that stayed in the primary tumor. These characteristics are related with the nuclear size, architecture and cytoskeleton network. The physical processes associated are related to cell deformability, contractility, migration, traction forces and invasion. As previously referred, the malignant cells need to cross the basement membrane that is associated with structure integrity, being stiff and leakiness. Blood or lymph vessels contribute to the cell mobility because of their structure integrity, leakiness and osmolality changes. Associated physical processes like fluid shear stress, friction, and adhesion and cell deformation contribute to mobility of malignant melanocytes through the flow due to its nuclear architecture, nuclear size, cytoskeleton network and surface molecules. The ECM also contributes to the cell migration to a new organ(s) through the pore size and substrate properties. The physical phenomena associated are: matrix stiffness, adhesion and traction stresses and invasion (Kim *et al.*, 2016). Melanoma cell invasion is facilitated by the relation between the melanoma, ECM and stroma, in which the mechanical ECM has an important role (Tanner, 2018). The tumor-stroma tissue involves extracellular matrices, fibroblasts, adipocytes, pericytes, endothelial, immune, and inflammatory cells, such as macrophages and neutrophils (Pietras and Ostman, 2010). Transformed CAFs have shown to facilitate tumor invasion through the integrin $\alpha V\beta 3$ -dependent fibronectin secretion, which promote the mechanical changes in the ECM through the contraction of collagen fibers (Gao *et al.*, 2013; Kim *et al.*, 2016). Ju *et al.* (2018) described the dynamic of 3D migration in metastatic melanoma. This process involves the mesenchymal and amoeboid migration (**Figure 7**). Mesenchymal process is based in a front-rear 3D polarity to generate a predominant pseudopodium, showing the spindle like shape and depending on the ECM degradation by the MMPs and integrin-dependent cell-matrix attachment protein complexes known as focal adhesions (FAs). These FAs work like exocytic structures for MMPs, mediated by the cortical microtubule stabilization complexes containing microtubule associated proteins. The amoeboid migration is based from blebbing, chimneying, to actin gliding modes. This last migration process requires little to no integrin activity and or MMP-mediated matrix degradation. Transformed cancer-associated fibroblasts (CAFs) are known to facilitate

ECM changes through deposition of fibronectin that crosslinks collagen I fibers. CAFs dependent contractility further in reorganization of ECM influencing melanoma migration and survival mechanosensory proteins (Ju *et al.*, 2018).

According to Huang *et al.* (2018), the fluid shear stress (FSS) is important for migration and metastization. The shear stress flow is a variable in tumor metastasis-related fluid microenvironment. Blood shear stress levels ($1\text{--}3000\text{ dyn/cm}^2$) is higher than interstitial flow (0.1 dyn/cm^2) and lymph flow (0.64 dyn/cm^2). In blood, only 0.02% of CTCs survive. In lymphatic fluid, the FSS generated at sentinel lymph nodes can significantly upregulate ICAM-1 in lymphoid endothelial cells, facilitating lymph node metastasis. Low FSS facilitate cell melanoma adhesion facilitating the extravasation and metastization (Huang *et al.*, 2018). The MelCTCs are promising cancer biomarkers but isolation from the blood is very challenging because of their huge heterogeneity, which makes difficult their biological and clinical study (Aya-Bonilla *et al.*, 2017). In melanoma, the metastization occurs in several organs being the lung, brain (Tawbi *et al.*, 2018) and liver, the most affected ones (Damsky *et al.*, 2010). “Why is melanoma so metastatic?” asked Braeuer *et al.* (2013). Melanoma cells are very efficient in invading the host immune system sharing several molecules with the vascular system, having a potentiality for angiogenesis, mesenchymal nature more than other tumors (Braeuer *et al.*, 2013).

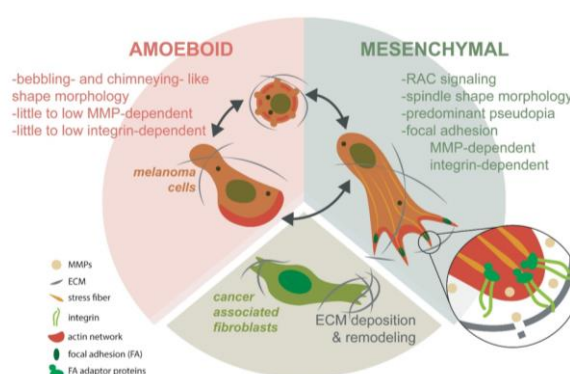


FIGURE 7 | Mesenchymal and amoeboidal 3D migration dynamic modes in metastatic melanoma. Mesenchymal migration depends on RAC signaling to establish front-rear 3D polarity. This polarity generates a predominant pseudopodia, producing the characteristic spindle like morphology. Mesenchymal migration depends on MMP-dependent ECM degradation and integrin-dependent cell-matrix attachment protein complexes known as focal adhesions (FAs). Most probably, FAs are related as being important for exocytic trafficking of MMPs. In contrast, amoeboid migration assumes different migration modes from blebbing, chimneying to actin gliding modes. Amoeboidal migration requires little to no integrin activity and or MMP-mediated matrix degradation. CAFs facilitate ECM changes through deposition of fibronectin that crosslinks collagen I fibers. CAF-dependent contractility further in reorganization of ECM influencing melanoma migration and survival mechanosensory proteins (based in Ju *et al.*, 2018).

The extravasation process, in melanoma, can be classified as paracellular and transcellular. The paracellular type is based on cell adhesion molecules (like CD44 and others). In transcellular extravasation, cancer cells can exit the circulatory system and penetrate through endothelial cells, not between endothelial cells (Lee *et al.*, 2019). Regarding the metastization to the brain, Central Nervous System (CNS) has no lymphatic system, being the blood stream and the Blood-Brain-Barrier (BBB) the pathway to melanoma reach the brain. Fazakas *et al.* (2011) investigated this process *in vitro* using cerebral endothelial cells (CECs), the A2058 metastatic lymph node and B16/F10 melanoma cell lines, respectively. Melanoma cells adhere to confluent brain endothelial cells. Melanoma cells migrate through the endothelial monolayer, continuing its movement beneath the endothelial cell layer. Melanoma cells, in contact with the brain endothelial cells, disrupted the TJ and AJ of CECs and used partially the paracellular transmigration pathway. During the experiment, melanoma cells produce and release large amount of secreted factors, which, if inhibited, decrease 44 to 55% of melanoma cell migration through the CECs. **Figure 8** presents a possible explanation for paracellular migration of melanoma cells through the endothelial monolayer (Fazakas *et al.*, 2011). In terms of forces involved in this process refer that this process is not totally understood and to our knowledge there is no literature related to this mechanism that could help to understand how the cells behave. In terms of biophysics, more work should be performed to understand the role of the forces and contractibility in the hypothesis proposed.

Melanoma cells may attach in clusters to the brain endothelial monolayer, through N-cadherin mediated junctions (Herman *et al.*, 2019).

Some studies about melanoma transmigration were performed using human lung microvascular endothelial cells, in which was found that melanoma cells used the transcellular migration pathway (Qi *et al.*, 2005). To our knowledge there are no studies regarding the migration (paracellular or transcellular), in liver metastization. It seems, based on literature, that both pathways can be used, being different if the metastasis achieve the brain or the lungs. In terms of driven forces in both processes, also to the best of the authors knowledge, there is no literature related to biophysical processes; more investigation should be performed in this field. Huang *et al.* (2018) referred that CD44 ligand-receptor interactions is also involved in cell migration and tumor cell

metastization. High levels of CD44 expression will be expressed in a poor diagnosis for melanoma patients when treated with decarbazine (DTIC).

AFM force spectroscopy was used to map CD44-coupled surface receptors of melanoma cells. The effect of DTIC treatment was assessed by the dynamic binding strength and ligand free energy landscape. The unbinding force between CD44 ligand and its receptor, even when the CD44 nano domains were reduced significantly, were not affected by the DTIC. Meanwhile, DTIC did affect the kinetic and thermodynamic interactions of the CD44 ligand–receptor, presenting greater dissociation rate, lower affinity, lower binding free energy, and a narrower energy valley for the free-energy landscape. The results demonstrated DTIC affects, but not completely inhibits, the binding of CD44 ligand to membrane receptors, suggesting the reason for the poor prognosis associated with DTIC treatment of melanoma. Therefore, AFM gives thermodynamic and kinetic insights into the effect of DTIC on the CD44 ligand-binding process (Hj *et al.*, 2017). Bobrowska *et al.* (2019) also demonstrated that vertical growth phase melanoma cells showed surface biochemical and biomechanical changes in parallel, which could be used as a fingerprint of melanoma progression (Bobrowska *et al.*, 2019).

Nikolovska *et al.* (2017) mentioned that when uronyl-2-O sulfotransferase (Ust) is knocked down in human melanoma cells, the amount of Ust protein is reduced, as well as adhesion and migration of melanoma cells (Nikolovska *et al.*, 2017). Adhesion, spreading and migration of some melanoma cells depend on the presence of collagen. Type IV collagen has a complex molecular basis which is partially dependent on RGD-related sequences (Chelberg *et al.*, 1989). Some integrins are also linked to the human melanoma progression such as the $\alpha v \beta 3$ integrin. The highly metastatic K1735M2 murine cells expressed $\alpha v \beta 3$ integrin, while the poorly murine metastatic K1735C23 cells did not present the $\alpha v \beta 3$ integrin (Li *et al.*, 2001).

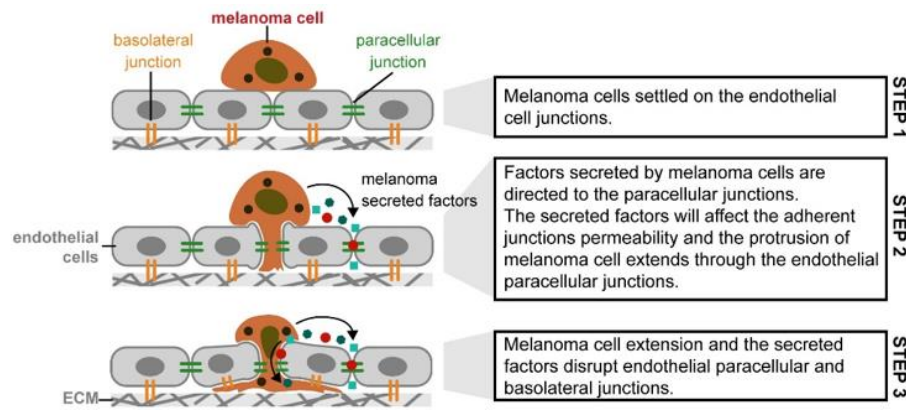


FIGURE 8 | Paracellular migration mechanism used by melanoma cells in metastization of brain tissue, through the cerebral endothelial cells. (Step 1) Melanoma cells settled on the endothelial cell junctions; (Step 2) Factors secreted by melanoma cells are directed to the paracellular junctions. The secreted factors will affect the adherent junctions permeability and the protrusion of melanoma cell extends through the endothelial paracellular junctions; (Step 3) Melanoma cell extension and the secreted factors disrupt endothelial paracellular and basolateral junctions. Based in Anchan *et al.* (2019) and Herman *et al.* (2019).

Sarna *et al.* (2014) also found that cell melanoma pigmentation interfere in cell transmigration *in vitro*. The transmigration efficiency decreased with the increase of the apparent Young's modulus, being proportional to the number of melanin granules present in the melanosomes inside the cell (Sarna *et al.*, 2014).

Targeting MelCTCs in personalized therapy using markers found in cells isolated from patients' blood stream, appears to be a promising way of preventing metastasis. The identification of MelCTCs in patient blood samples during the first year after surgical tumor removal, has an 83% correlation with the recurrence of disease after 8 months MelCTCs detection. The detection of MelCTCs in blood samples in the early stages (0-I) of melanoma development is indicative of patients with a high risk of developing metastasis (Mocellin *et al.*, 2006). The endothelium has a potential molecular target that can be used in order to block its interactions with melanoma cell molecules; this way invasion could be prevented. Targeting MelCTCs for personalized therapy using markers found in cells isolated from patients' blood can be a promising way of metastasis prevention (Mocellin *et al.*, 2006).

MELANOMA: FUTURE PERSPECTIVES

Several biophysical mechanisms, which are responsible for cell motility, invasion, adhesion, metastization, and progression in melanoma, were described in this review. It was shown that keratinocytes are an important factor in melanocyte's

control. If this control is hampered, it will lead to retraction of melanocyte's dendrites and promotion of the movement of melanocytes through the basement membrane. Other consequences of dendrite retraction are a hindered transfer of melanosomes and melanin, not being performed by the keratinocytes anymore. Melanoma cells are characterized by the disruption of cell-cell adhesion molecules between melanocytes and keratinocytes. The internal forces generated by keratin filaments drive melanoma cell motility, like in other cancer cells. For cell motility, progression and migration, shear stress forces influence the circulation of the cancer cells contributing to the metastization in other organs. In terms of therapeutic strategies, we must invest in the development of therapies targeting disruptions between keratinocytes-keratinocytes and/or between melanocytes-keratinocytes, and the retraction of melanocyte's dendrites. Force spectroscopy studies of adhesion molecules in adherent cells will contribute to define therapeutic strategies based on the quantification of binding forces, the energy landscape and dissociation constants of those adhesion molecules. Hampering these adhesion processes is intensively linked with the relation established by the ECM, cytoskeleton and cell nucleus. The forces produced by the tumor mass causes an increased pressure on healthy cells, which will activate several signaling pathways (mechanosensing vs. mechanotransduction), and thus transmitting these forces through the ECM, the cytoskeleton and the nucleus, causing several abnormalities in these structures, which will eventually lead to melanoma cancer progression. Keratins can be used as diagnostic biomarkers.

It was also highlighted that melanoma has a great potential of progression and metastization, and a special note was given to cancer-derived fibroblasts, since the identification of specific molecules is difficult, due to the existence of several fibroblast subpopulations. It is very important to identify the molecules participating in these pathways and quantify the forces involved, in order to understand how metastasis are formed and maintained. This also leads to the resistance of cancer to conventional treatments like radiotherapy and chemotherapy, once the specific genes expressed in this cancer, are highly mutated. These two conventional treatments affect not only tumor cells but also healthy cells. They showed fast results but harmful side effects for healthy cells. Although immunotherapy is not directed to the tumor cells themselves, reinforces the immunological system based in antibodies, vaccines and adoptive

therapies. This treatment can be personalized and combined with the chemotherapy (biochemotherapy), depending on the stage of disease and the mutations on specific genes. Researchers are trying to develop biological treatments to target specific oncogenes in cancerous cells. Occasionally, some patients do not respond to immunotherapy due to the instability of cancer cells. A possible way to overcome this problem is to target some stable and not mutated cells existing on the stroma like fibroblasts, endothelial cells, immune cells and activated fibroblasts. Another strategy could be derived from a detailed knowledge of the different subtypes of fibroblasts in order to target specific molecules. The development of transfection molecules, which could reach the cell nucleus, avoiding the expression of genes, causing mutations or changes in lamins, could be another strategy. Gene therapy approaches, that inhibit the BRAF gene, could also be a possible option. As shown in this review the investigation of melanoma using different approaches and techniques, bringing together biologists, physicists, biochemists and medical specialists, is very important to deepen our knowledge and to develop novel drugs and treatments for melanoma cancer.

AUTHOR CONTRIBUTIONS

MB conceived, designed, and wrote the manuscript. MR revised the manuscript, making substantial, direct and intellectual contribution to the work, and approved it for publication. SS helped MB to define the subject, gave intellectual contributions, revised the manuscript and approved it for publication. PG approved the manuscript for publication.

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4.2 Microrheological comparison of melanoma cells by atomic force microscopy

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Abstract

Melanoma is one of the most severe cancers due to its great potential for metastization. Recent studies showed the importance of mechanical properties assessment in metastization process, which depends on the cytoskeleton dynamics and cell migration. Although cells are considered purely elastic, they are viscoelastic entities. Microrheology atomic force microscopy (AFM) enables the assessment of elasticity and viscosity, which are relevant to cell behavior regulation. This work compares the mechanical properties of human neonatal primary melanocytes (HNPMs) with two melanoma cell lines (WM793B and 1205LU cells), using microrheology AFM. Immunocytochemistry of F-actin filaments phosphorylated focal adhesions (p-FAK) and migration cell assays were performed to understand the differences found in microrheology AFM regarding WM793B and 1205LU cell lines. AFM reveals that HNPMs and tumor cell lines had distinct mechanical properties. HNPMs were softer, less viscous, presenting a higher power law than the melanoma cells. The immunostaining of p-FAK showed that metastatic 1205LU cells expressed more p-FAK than WM793B cells. Melanoma cell migration showed that WM73B did not close the gap, in contrast to 1205LU cells, which closed the gap at the end of 23 h. These data seem to corroborate the high migratory behavior of 1205LU cells. The microrheology atomic force microscopy applied to HNPMs and melanoma cells allowed the quantification of elasticity, viscosity, glassy-phase, and power law properties, which have an impact in cell migration and metastization. The AFM study is important since it can be used as a biomarker of the different stages of the disease in melanoma.

Keywords: Melanocytes, melanoma, microrheology atomic force microscopy, viscoelasticity, frequency sweep, power law exponent.

Introduction

Melanoma is one of the most severe skin cancers in humans due to its high metastatic potential, with a mortality rate of 80% [1-4]. This type of cancer usually metastasizes, via blood or the lymphatic system, to several organs, such as the skin, lung, brain, liver,

bone, and intestine [5]. Melanoma is originated from the malignant transformation of melanocytes, which are the normal pigment-producing cells located at the stratum basal of the epidermis [6]. Malignant melanocytes are characterized by changes in biophysical properties, like shape, elasticity, and stiffness, which contribute to cell motility, invasion, and metastization [7, 8]. In this case, the plasticity of melanocytes is very important since, for that purpose, they need to be highly deformable [9].

In order to metastasize, tumor cells may detach from the primary tumor, invade blood or lymphatic vessels, extravasate from them, and establish new tumors at different sites of the primary one. To a large extent, the actin cytoskeleton is responsible for the elasticity of cells since it experiences assembly and disassembly during cell motion, regulating protrusion formation, focal adhesion behavior, and contractile filament organization. During migration of melanoma cells, integrins and focal adhesions play an important role since they establish the connection between the cell cytoskeleton with the extracellular matrix (ECM) [10, 11]. Focal adhesion kinase (FAK) has a crucial role in migration and invasion, mainly through the secretion of matrix metalloproteinases (MMP) that contribute to ECM degradation [12]. A high amount of FAK expression in human cancer was correlated with high malignancy and invasiveness [13].

The ECM, in particular its mechanics, is also known to contribute to cell migration since the ability of cells to migrate is influenced by stiffness gradients (from less stiff to more stiff substrates), a process called durotaxis [14, 15]. According to Huang *et al.*, for migration, and metastization, fluid shear stress (FSS) is also important and variable in the microenvironment of tumor metastasis-related fluid [16]. Accordingly, physical processes like FSS, friction, adhesion, and cell deformation contribute to the mobility of malignant melanocytes [16].

The assessment of the mechanical properties of cells has an increasingly prominent role in the understanding of various biological and biochemical mechanisms related to several cellular processes, such as migration and metastization. This is particularly relevant for cancer cells that adapt their elasticity for an efficient migration [17]. In most published works, the quantification of cell mechanical properties is performed using static methodologies, recurring to Hertz's model or its variants [18-20]. This model assumes that the cells are isotropic, homogenous, purely elastic and a semi-infinite material, resulting in the determination of only an apparent Young's modulus [21].

However, since cells are dynamic entities also exhibiting viscous properties, a more elaborated analysis is needed that includes the contribution of the viscoelastic nature of the cells neglected by the current methods of analysis.

In rheology, cells can be described as soft glassy materials, with properties varying according to time and frequency [22]. Some authors explain the cell mechanical behavior through the application of a power law exponent to the cells, which could be correlated to the cell “fluidity” of the cytosol [23-25].

The main goal of the present work was to quantify the cells’ elastic and viscous mechanical properties and discuss their contribution to the migratory behavior of melanoma cells, which is relevant to the metastization process. For that purpose, the same methodology used by Brás *et al.*, consisting in the dynamic atomic force microscopy frequency sweep was used, allowing the assessment of the storage and loss moduli, the power law exponent applied to the storage modulus, and the loss tangent, which has never been performed in melanoma cells [26]. The same methodology was applied to confirm that it can be applied to different cancer cell types. Additionally, this technique allowed the detection of the existence cells’ stress relaxation and creep, as a function of time [27]. Mechanical properties of human neonatal primary melanocytes (HNPMs) and two melanoma cell lines, WM793B (human cells established from the vertical growth phase of a primary skin melanoma lesion) and 1205LU cell lines (lung metastasis of WM793B) were also assessed.

Materials and methods

Culture of human neonatal primary melanocytes (HNPMs)

T25 cell culture flasks (Sigma, Taufkirchen, Germany) and Petri dishes (55 mm in diameter; Sigma, Taufkirchen, Germany) were coated with human collagen type IV (0.67 $\mu\text{g}/\text{cm}^2$; Sigma, Taufkirchen, Germany). The coated plastic materials were left inside a CO_2 incubator for a minimum of 16 h. After this period, the excess collagen was removed, and the coated Petri dishes were left to dry inside a laminar flow chamber for 2 h. All coated consumables were kept at 5°C if not used immediately. Before melanocyte thawing, the collagen-coated T25 flasks were washed with Dulbecco’s-buffered saline (D-PBS) (Invitrogen, Waltham, MA, USA). Thawing of HNPMs, from the foreskin, was performed according to the protocol provided by the HNPMs supplier (Coriell Institute,

Camden, NJ, USA). Medium 254 (Alfagene, San Francisco, CA, USA) supplemented with human melanocyte growth supplement (HMGS; Alfagene, San Francisco, CA, USA) at 37°C was placed in the collagen-coated T25 flasks and left in the CO₂ incubator for 16 h. After this period, the medium was replaced by 5 mL of fresh medium. When reaching 80% confluence, cells were washed with 3 mL of D-PBS and rinsed for a maximum of 5 min with 3 mL of versene (Invitrogen, Waltham, MA, USA). The previous solution was removed and 1 mL of a mixture of 0.05% trypsin (v/v) and 0.53 mM ethylenediaminetetraacetic acid (EDTA) (Gibco, Waltham, MA, USA) was added. The previous solution was neutralized with 5 mL of soybean trypsin inhibitor (Invitrogen, Waltham, MA, USA) and cells were centrifuged at 1200 rpm for 5 min. Cells (8×10^4) were seeded in collagen-coated Petri dishes and kept inside the CO₂ incubator at 37°C overnight before mechanical properties determination by AFM.

Culture of human melanoma cell lines

The WM793B and 1205LU cells were seeded in coated Petri dishes (IBIDI, Grafelfing, Germany). Cells were kindly provided by the Biophysics Institute (University of Bremen, Bremen, Germany). Both cell types were cultured in DMEM (Merck, Darmstadt, Germany), supplemented with 10% FBS and 1% penicillin/streptomycin (pen/strep). Cell subculturing was performed after trypsinization with 0.05% (v/v) trypsin/0.53 mM EDTA solution (Merck), followed by washing steps with PBS without Ca²⁺ or Mg²⁺. Cells (8×10^4) were then seeded in treated Petri dish and incubated for 24 h in the CO₂ incubator at 37°C before measurements.

Assessment of cell mechanics using AFM (static and dynamic methodologies)

Assessment of mechanical properties by AFM was performed as described in Brás *et al.* [26]. Briefly, force maps of single force-distance curve maps (6 × 6 pixels), followed by force maps of frequency sweep force-distance curves maps (4 × 4 pixels), were obtained on a cell area 5 × 5 μm², pointed nuclei of HNPMs and melanoma cell lines. The trigger point (maximum cantilever deflection) used was 3 nm. A scan rate of 2 Hz and velocity of 14.0 μm/s were used. In frequency sweep, the cantilever experienced in-phase and out-of-phase deflection signals, which were used to determine the complex shear modulus, creep compliance, and stress relaxation, as described by De Sousa *et al.* [28].

At the end of a conventional force-distance curve, a frequency sweep between 1 and 1000 Hz, with a dwell time of 8.7 s, was applied with a trigger point of 3 nm and an amplitude of 30 nm [28]. At least 40-70 cells were measured. Frequency sweep method allowed the determination of the power law exponent of the storage modulus as a function of frequency [26]. AFM measurements were always performed after the 2nd passage of the cells. The parameters used in the Hertz's fit as well as in the dwell window were used according to Brás *et al.* [26]. A b-hydrodynamic factor of cantilever was calculated [29]. In short, the response of the cantilever was recorded at several distances from the sample (0, 200, 300, 600, 1100, 2100, 3600 and 5100 nm), while the z-height of the sample was modulated at several amplitudes (50, 100, 200 and 500 nm) in 254 or DMEM culture media, as their viscosity could be different, as well as the viscous component of the cantilever. Following the procedure of Alcaraz *et al.*, the hydrodynamic correction factor b to a distance of 0 nm from the sample was extrapolated. This correction was used when fitting frequency data of cells was performed [29]. In the dwell window, the b-hydrodynamic factor introduced was 7.43×10^{-7} . Before cell measurements, the calibration of the cantilever (model PFQNM-LC-A-CAL; Bruker, Billerica, MA, USA) was performed according Brás *et al.* [26]. Example of a conventional force-distance curve, both fitted with the Hertz's model and a frequency sweep deflection as a function of time, using 1205LU cells, are presented in Supplementary Material, Figure S1.

The loss and storage moduli was calculated for each frequency (see Figure S2 in supplementary Material).

The apparent Young's, storage, and loss moduli, loss tangent, and the exponent of storage modulus of cells were determined. The latter one was obtained in the frequency sweep, through the calculation of the power law of the storage modulus, to enlighten the dynamic behavior of cells. Some previous works correlated the power law exponent applied to the storage modulus to the "fluidity" of the cell cytosol [23-25].

The quantification of the viscoelastic properties from the frequency sweep of the curve corresponds to the slow response behavior of cells, in the frequency range 1–100 Hz. In the present work, only one power law exponent was measured, obtained from the frequency sweep. The power law exponent showed how much the storage modulus changed as a function of frequency (being frequency-dependent).

Immunocytochemistry melanoma cells

Human melanoma cells were seeded on coverslips at a cell density of 3×10^4 cells/mL and incubated for 24 h at 37°C and 5% CO₂. After being washed with PBS, cells were fixed with 4% (v/v) paraformaldehyde (PFA; Electronic Sciences microscopy, Hatfield, PA, USA) in PBS for 20 min at room temperature (RT). Then, cells were washed 3 times with PBS for 5 min and maintained on the orbital shaker at 70 rpm. Next, cells were incubated with 0.25% (v/v) triton X-100 (ITW, Glenview, IL, USA) in PBS for 5 min at RT. Human melanoma cell structures stained were nuclei, F-actin filaments and phosphorylated focal adhesion kinase (p-FAK). For this purpose, samples were washed 3x with PBS for 5 min and incubated with 1% (w/v) bovine serum albumin (BSA; VWR International, Radnor, PA, USA) for 1 h at RT, involved in aluminum foil for protection from light. Subsequently, samples were incubated in 1% (w/v) BSA solution in PBS (1x) together with the primary antibodies anti-FAK (1:100, c-terminal rabbit; Invitrogen, Darmstadt, Germany) at 4°C during 3 h, under shaking at 70 rpm. The conjugated probe phalloidin/alexa fluor 488 (Molecular Probes-Invitrogen, Waltham, MA, USA) at 1:100 dilution for F-actin staining was added with the primary antibodies. Samples were then washed 3x with PBS for 5 min and kept overnight at 4°C for 18 h. The secondary antibody goat anti-rabbit alexa fluor 594 F(ab')₂ fragment was added for 3 h at RT (1:1000). Samples were then washed 3x with PBS for 5 min under shaking of 70 rpm. The nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; H-1200, Vector, Stuttgart, Germany) before being observed in a confocal scanning laser microscope (model SP3, Leica Microsystems, Wetzlar, Germany). Cells were imaged with an HCX PL APO CS 40x NA 1.3 oil objective (Leica Microsystems, Wetzlar, Germany). Fluorescence data were visualized with Image J software (version 1.53C). Three immunohistochemistry experiments were performed for each cell type.

Cell migration assays

Melanoma cell migration assays was carried out by measuring the surface area that cells occupy over time after creating a cell-free area through the scratch assay. Cells were seeded on Petri dishes and were kept in the CO₂ incubator until confluency was reached. A 200 µL tip was then used to create a scratch with a width of approximately 2500 µm. Five replicates of each melanoma cell line were prepared, each one in one Petri dish. Cells were placed in an inverted microscope (Zeiss, Oberkochen, Germany) and images

were taken every 5 min, for 23 h to follow their migration. The images were analyzed using ImageJ 1.53u software version, using the wound healing tool script.

Statistical analysis

The median and the 25th and 75th percentiles were used to visualize differences between cells. The Wilcoxon Rank's test was used to analyze the significance of differences. All statistical analysis were performed using the software package IGOR Pro 7 (Wavemetrics, Portland, OR, USA). Statistically significant differences were indicated with (**), i.e. the difference between groups of distinct cells was statistically significant at $p < 0.001$.

Results

Analysis of the apparent Young's modulus and viscoelastic properties

Aiming to analyze the mechanical properties of HNPMs and melanoma cancer cells, the tip was placed in the middle of the nucleus of each cell type (Figure 1), and a force map was obtained from conventional force-distance curves acquisition, followed by a frequency sweep map with a dwell time of 8.7 s.

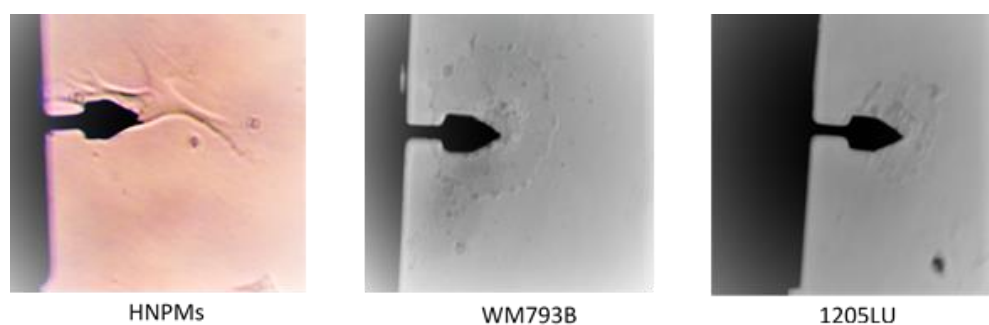


Fig. 1 Representative examples of indentation using AFM cantilever PFQNM-LC-A-CAL to quantify the mechanical properties of HNPMs, WM793B, and 1205LU cells. The tip was pointing at the cell nucleus.

Then, histograms (Figure 2) were obtained, and statistical data treatment was performed. Data is presented as a boxplot with median and min-to-max whiskers for all cells (40 to 70 cells measured).

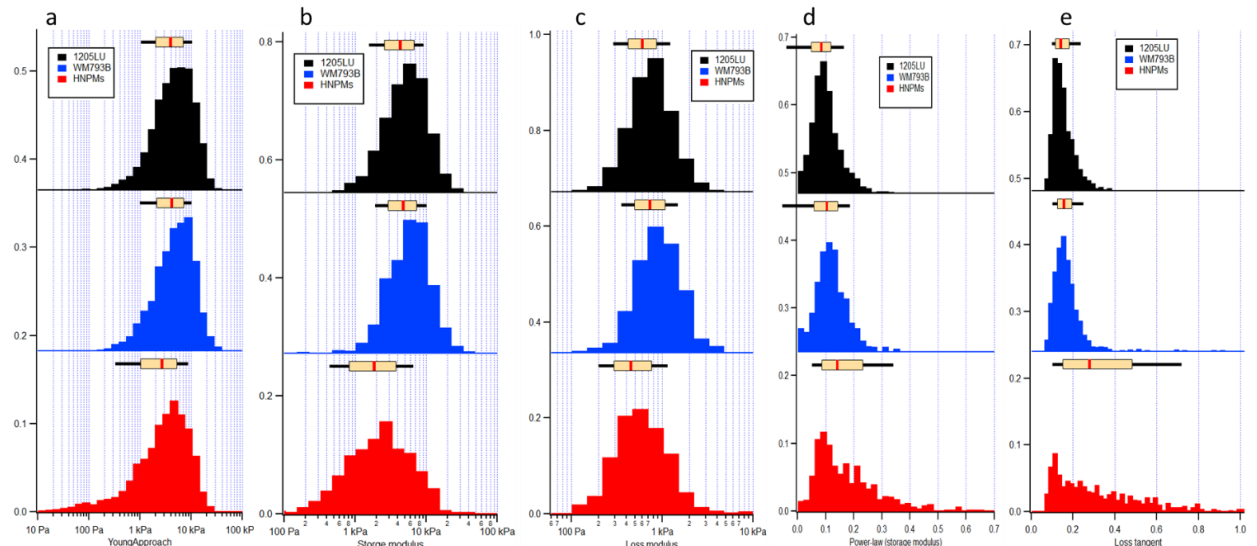


Fig. 2 Histograms of mechanical properties quantification of HNPMs (red), WM793B (blue), and 1205LU (black) cells: **a)** Apparent Young's modulus. **b)** Storage modulus. **c)** Loss modulus. **d)** Power-law exponent of the storage modulus. **e)** Loss tangent. The box plot above the histograms present the median (red line) and the 25th and 75th percentiles (extent of rectangle in cream color), and the 10th and 90th percentiles (black lines extending beyond the box).

The quantification of apparent Young's modulus and parameters obtained with frequency sweep for HNPMs, WM973B, and 1205LU cells are presented in Table 1.

Table 1 shows that the apparent Young's modulus of HNPMs was lower (2.94 kPa), when compared with the melanoma cells (4.58 kPa for WM793B and 3.89 kPa for 1205LU). The storage (1.94 kPa, 4.69 kPa and 4.27 kPa, respectively) and the loss (0.52 kPa, 0.73 kPa and 0.61 kPa, respectively) moduli showed the same trend as the apparent Young's modulus. For HNPMs, the loss modulus was lower by a factor of 3.7, when compared with the storage modulus; however, for WM793B and for 1205LU the loss modulus was lower by factors of 6.4 and 7, respectively, when compared with the storage modulus. As can be seen in Table 2, the apparent Young's and the storage moduli were significantly different when comparing HNPMs cells with WM793B and 1205LU cells. However, there were no significant differences between WM793B and 1205LU cells.

Table 1 Values of median and percentiles of Young's, storage, loss moduli, and power-law exponent applied to the storage modulus and loss tangent of HNPMs, WM973B, and 1205LU cells, quantified by AFM.

Cell type	HNPMs			WM793B			1205LU		
Mechanical properties	25th	Median	75th	25th	Median	75th	25th	Median	75th
Apparent Young's modulus (kPa)	1.68	2.94	4.34	2.79	4.58	6.28	2.47	3.89	6.38
Storage modulus (kPa)	1.32	1.94	2.81	3.55	4.69	6.39	2.92	4.27	6.09
Loss structure (kPa)	0.32	0.52	0.64	0.61	0.73	0.91	0.47	0.61	0.75
Power-law exponent of storage modulus	0.11	0.13	0.19	0.08	0.11	0.12	0.07	0.08	0.10
Loss tangent	0.18	0.25	0.44	0.14	0.16	0.18	0.12	0.13	0.17

In terms of loss modulus, there was no significant difference between the HNPMs and 1205LU cells, despite the former ones being healthy cells and the latter ones being a tumor metastatic cell line. This could be explained by the fact of HNPMs being neonatal cells, thus softer. This means that viscosity of these cells is similar, i.e. when cells were probed by the AFM tip, the movements of the cytosol and their components behave in the same way. Regarding the power law exponent of the storage modulus, values obtained presented a decreasing order (0.13, 0.11 and 0.08) regarding HNPMs, WM793B, and 1205LU cells, respectively. Table 2 has showed statistical differences in the power law exponent of the three cell types which may also be related to the loss tangent. The loss tangent of HNPMs was significantly different from that of WM793B and 1205LU cells. However, the loss tangent did not show significant differences between WM793B and 1205LU cells. This corroborates the inexistence of significant differences in the storage modulus between WM793B and 1205LU cells since the power law was calculated

through the storage modulus. HNPMs are statistically different from the WM793B and 1205LU cells regarding all mechanical parameters, except in viscosity, which was similar for HNPMs and 1205LU cells.

Table 2 Statistical data treatment of HNPMs and melanoma cell lines. The Wilcoxon Rank's test was used. (***) Corresponds to statistically significant differences between HNPMs and melanoma cell lines ($p < 0.001$).

Mechanical properties	HNPMs/WM793B	HNPMs/1205LU	WM793B/1205LU
Apparent Young's modulus (kPa)	7.21×10^{-9} ***	2.71×10^{-7} ***	0.11
Storage modulus (kPa)	6.20×10^{-12} ***	1.20×10^{-11} ***	0.14
Loss modulus (kPa)	6.7×10^{-7} ***	0.007	5.00×10^{-4} ***
Power-law exponent of storage modulus(***)	1.28×10^{-5}	2.22×10^{-4}	3.61×10^{-10}
Loss tangent	1.61×10^{-6} ***	3.61×10^{-10} ***	0.003

Cytoskeleton protein expression and localization and cell migration

The assessment of the mechanical properties by AFM showed interesting differences between both melanoma cell lines, despite being both cancer cells. To better understand the relation between the mechanical properties and the morphodynamic properties of the melanoma cell lines, WM793B and 1205LU, we used immunofluorescence to evaluate cytoskeletal organization with F-actin labelling and focal adhesion localization with p-FAK staining and a migration assay to assess the migratory cell behavior. As can be observed in Figure 3, F-actin is clearly present, forming a cortical ring in WM793B cells, while in 1205LU cells F-actin is organized in stress fibers throughout the cytoplasm and in lamellipodia, not showing a cortical distribution. Using p-FAK staining as a proxy of activated focal adhesions, one of the important structures for cell motility, it could be observed in the metastasis-derived 1205LU cells, that the protein was localized at the tip of several F-actin filaments (stress fibers) and lamellipodia, typical of the focal adhesion

complexes (Figure 5, bottom Z-stack). In WM793B, p-FAK could not be detected. Given this different organization of the F-actin in the melanoma cell lines, and taking into account that the presence of stress fibers and activated focal adhesions are important for cell contractility to provide the force for cell migration, both cell lines were assayed for cell migration.

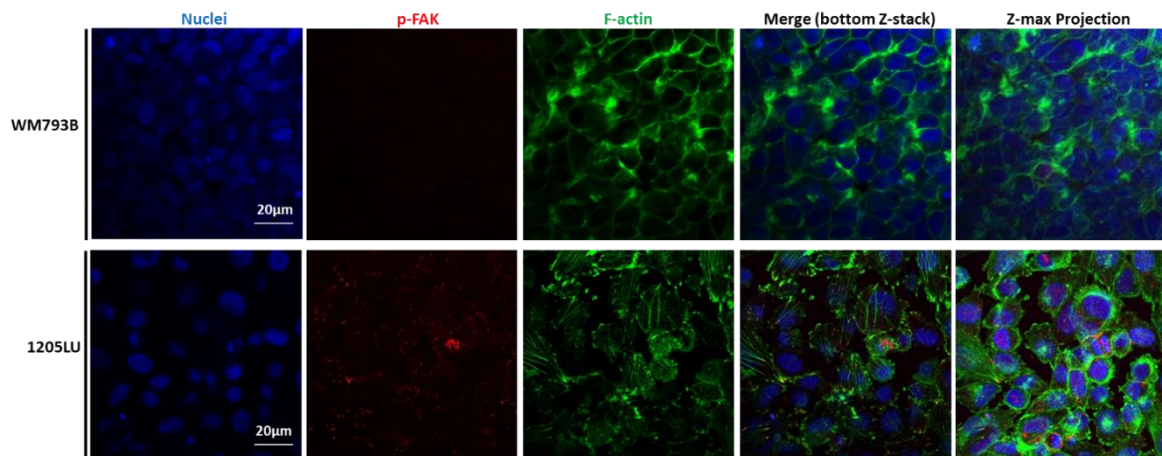


Fig. 3 F-actin distribution in melanoma cell lines (WM793B and 1205LU). Staining of cells for DAPI to identify the nuclei (blue), p-FAK (red) and phalloidin from F-actin filaments (green). The overlap of the three channels is presented in the bottom Z-stack and the Z-maximum projection of the imaged area. Scale bars: 20 μm

As shown in Figure 4, at the end of the assay the 1205LU metastatic cell line completely closed the gap, while WM793B only closed 30% of the gap, thus indicating that 1205LU migrated more than WM793B cells. Table 3 summarizes all the results obtained, namely the mechanical measurements, cytoskeleton structure and p-FAK, as well as cell migration assays.

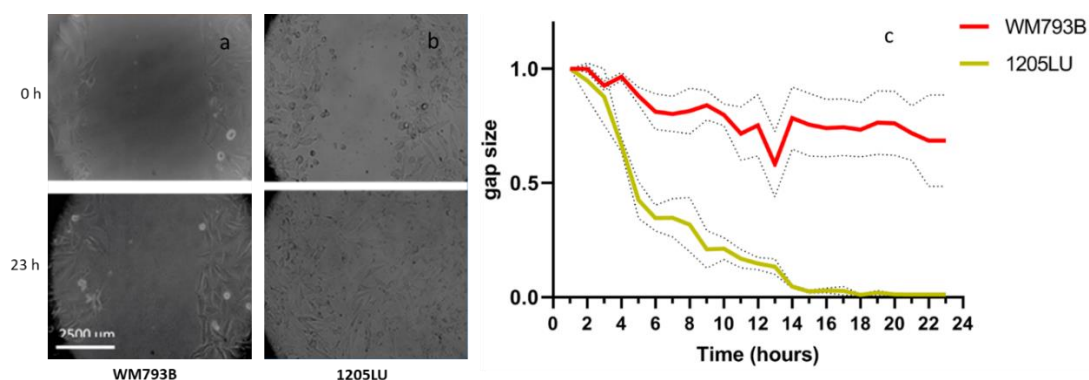


Fig. 4 Cell migration assay of WM793B and 1205LU cell lines between 0 and 23 h. **a)** WM793B cell line. **b)** 1205LU cell line. **c)** Gap size (i.e. state of closure) after 23 h, for both cell lines.

Table 3 Summary of the results related to mechanical properties, cytoskeleton organization structure and p-FAK.

Analyzed parameters	HNPMs	WM793B	1205LU
Apparent Young's modulus (kPa)	2.94	4.58	3.89
Storage modulus (kPa)	1.94	4.69	4.27
Loss modulus (kPa)	0.52	0.73	0.61
Power-law exponent of the storage modulus	0.13	0.11	0.08
Loss tangent	0.25	0.16	0.12
F-actin	NP	Abundant cortical actin	Distributed through the whole cell
p-FAK	NP	(-)	(++)
Wound healing (23 h)	NP	Did not close	Closed

Legend: (-) Absence; (+) low; (++) intermediate; (+++) high; (NP) not performed

Discussion

The main goal of this work was to compare the mechanical properties of human neonatal primary melanocytes (HNPMs) with that of two melanoma cell lines (WM793B and 1205LU cells), using a new AFM dynamic approach, which allows the quantification of not only the viscoelastic properties of the cells but also, the power law exponent of the storage modulus. The last one gives a measurement value how the storage modulus are frequency-dependent.

The vast majority of the published literature aiming to measure the mechanical properties of cells assume that cells are purely elastic, semi-infinite, homogeneous, and isotropic models, which is far from reality [18-20]. In fact, cells are dynamic entities that can be characterized by elasticity, viscosity, glassy-state, and by a power law exponent [25, 27]. As it was shown during this study, dynamic AFM frequency sweep, confirmed the existence of creep and stress relaxation of cells (se Figure S1, black arrow, in

Supplementary Material), which corroborated the complexity of cells, by not behaving elastically. The characterization of mechanical properties was performed using the determination of the apparent Young's modulus and the viscoelasticity, through the acquisition of conventional force-distance curves and frequency sweep, respectively. The frequency sweep allowed the determination of the storage and loss moduli, the loss tangent and the power law exponent applied to the storage modulus. The glassy-phase indicates if the cells are more solid-like (if the value are close to 0) or liquid-like (if the value are more close to 1). The higher the power law exponent of the cells, the higher is their motility, thus increasing their potential for metastization [30]. The quantification of the viscoelastic properties is very important regarding their proliferation, motility and regulation of disease state [31]. According to de Sousa *et al.*, there are studies mentioning that cells present two power law shear moduli, namely: high and low exponents corresponding to the fast and slow cell response, respectively [27]. In this work, a power law applied to the storage modulus of the frequency sweep was calculated, corresponding to the slow response of the cell [27]. The slow response is assigned to the glassy-like regimen of the cytoskeleton dynamics in cell mechanics [27].

Considering the obtained data shown in Table 3, the results in terms of mechanical properties can be summarized as follows:

- Apparent Young's modulus: WM793B > 1205LU > HNPMs
- Storage modulus: WM793B > 1205LU > HNPMs
- Loss modulus: WM793B > 1205LU > HNPMs
- Power law exponent of the storage modulus: 1205LU < WM793B < HNPMs
- Loss tangent: 1205LU < WM793B < HNPMs

Bobrowska *et al.* compared the apparent Young's modulus of melanocytes from adult skin with several melanoma cells, including WM793B and 1205LU [18]. The apparent Young's modulus of adult melanocytes was approximately 5 times higher than that obtained in our work. This can be explained due to the different sources of melanocytes. While the ones used in this work were from foreskin of a 1-year-old child, those from Bobrowska and co-workers were from adult skin [18].

Cells with low Young's modulus are claimed to exhibit the great potential to invade tissues, supporting the relevance of studying the biophysical properties, such as cell

elasticity of the different malignant cells [8]. Weder *et al.* also demonstrated by AFM that the transformation of the radial growth phase to the vertical growth phase contributed to melanoma progression that was associated with a decrease in cell stiffness, which promoted cell deformation and invasion into the surrounding stroma, as well as increased intravasation [32]. The storage modulus was correlated with the amount, distribution and crosslinking of F-actin filaments since these filaments were claimed to be, to a large extent, responsible for the resistance of cells to deformation/ indentation, i.e., it was claimed to corresponded to a measure of cell elasticity [33]. As previously discussed, melanoma cells presented higher storage modulus (4.69 kPa of WM793 B and 4.27 kPa of 1205LU, compared with 1.94 kPa of HNPMs). The difference between these values could be explained due to use of colloidal probes, which have a large hydrodynamic drag, making it more difficult to measure the viscous properties of soft materials. In addition, the experimental scheme (taking regular force curves and analyzing the response of the sample during loading) was also very sensitive due to misjudging the exact time of contact.

Regarding the exponent of the storage modulus, HNPMs showed the highest value (0.13 kPa) when compared with WM793B (0.11 kPa) and 1205LU (0.08 kPa) cells. According to Zhang *et al.*, this parameter can be correlated with the cell cytosol “fluidity” [34]. These results agree with the fact that WM793B and 1205LU were found to have a lower power law exponent, corresponding to the fact that these cells were stiffer (storage modulus values of 4.69 and 4.27 kPa, respectively) when compared with HNPMs. Sanchez *et al.* confirmed that the storage modulus varied inversely with the power law exponent [35]. The loss tangent, which is based on the ratio between the loss and the storage moduli (corresponding to the glassy-phase) is correlated with the solid-like or liquid-like state of the cell [28].

According to Table 2, the apparent Young’s modulus of HNPMs and melanoma cells were significantly different, except in the viscosity between HNPMs and 1205LU, where similar values were obtained. There were no significant differences between WM793B and 1205LU cell lines, either in the apparent Young’s modulus or storage modulus, which can likely be explained by the fact that these cells are cancer cells and potentially have a similar origin, since 1205LU are lung metastasis from WM793B cell line. Importantly, significant differences between the power law exponent of the storage

modulus and the loss tangent were observed, except between WM793B and 1205LU cell lines. These cells presented values of loss tangent of 0.16 and 0.12, respectively. They are more liquid-like, which is in accordance with their elasticity, translated by their storage modulus. This fact could mean that both melanoma cells are more liquid-like (when compared with HNPMs), which may explain the same trend followed in terms of storage modulus (4.69 and 4.27 kPa, respectively). The similarity of power law and loss tangent could be explained by an eventually similar state of the cytoskeleton and distribution of the organelles inside these cells, and the way the cytosol diffusion occurs through the various components. Wang *et al.* concluded that similar tumor cells present significant variations of cytoplasmatic viscosity within the same cell type, using micropipette aspiration [36]. Kwapiszewska *et al.* highlighted that the viscosity of the cytoplasm is not constant since it depends on the size of the components inside the cells, mentioning that an increase in viscosity is correlated with the increase in organelles size. The various organelles constitute an obstacle to probe indentation [37]. The cytosol must flow through the organelles, which is related to cytosol fluidity. Since interesting differences were found in AFM mechanical properties regarding WM793B and 1205LU cell lines, qualitative immunocytochemistry and cell migration assays of melanoma cells were performed in an attempt to correlate them. The mechanical properties were thus correlated with the cytoskeleton structure, focal adhesion and migratory behavior. Regarding the cytoskeleton structures, WM793B cells presented cortical F-actin filaments (Figure 3), while 1205LU cells presented F-actin distributed along the cell body, organized in stress fibers throughout the cytoplasm and lamellipodia structures, without cortical distribution. Regarding p-FAK, it was absent in WM793B, while it was present in 1205LU cells (Figure 3). Cells adhere to ECM or substrates using molecular adhesions, such as focal adhesions (FAs), FA complexes, or podosomes [38]. The presence of p-FAK in 1205LU cells (Figure 3), associated with their gap closure ability after 23 h, correlates well with their migratory behavior (Figure 4). Kahana *et al.* pinpointed that FAK might have a possible role in melanoma progression [39]. Interestingly, the presence of p-FAK correlated with the highest migratory behavior of 1205LU (where the gap is 100% closed after 23 h) compared with WM793B cells (where the gap was only ca. 30% closed after the same time period). This higher capability to migrate is not in accordance with the observed power law exponent. Unexpectedly, 1205LU cells presented the lower power

law exponent than WM793B cells. This fact indicates that other mechanisms probably also influence the mechanical properties of melanoma cells. In terms of WM793B cell line, they lacked p-FAK and it was observed that they only closed 30% of the gap after 23 h, which is in agreement with the result obtained (37%) for the same cells found by Wasinger *et al.* [40].

In terms of power law exponent, HNPMs showed a higher value, being more liquid-like, than the other cells studied. WM793B and 1205LU cells showed the same trend in terms of apparent Young's, storage and loss moduli, but not regarding the power-law exponent of the storage modulus (Tables 1 and 2).

Conclusions

HPNMs cells presented statistically significant differences in mechanical properties when compared with WM793B and 1205LU in all parameters, except in terms of viscosity that is comparable to 1205LU. WM793B and 1205LU are similar in Young's modulus, and storage modulus, but differ in viscosity and power law exponent. This could be understandable with the eventual differences in terms of organelles size and how they move rearrange themselves inside of the cell cytosol, and how the last one flow through, responding to the tip stress during the indentation.

Interestingly, although the power law exponent value of 1205LU was lower than that of WM793B cells, they presented a higher migratory behavior, which can be related to the presence of p-FAK and high levels of stress fibers. Eventually, the internal forces generated by this cytoskeleton rearrangement of 1205LU cells compensated the low power law exponent. Nevertheless, the cells originating from the primary tumor are more elastic and viscous and thus less deformable than the corresponding metastasis.

The innovative dynamic AFM methodology applied to HNPMs and melanoma cells allowed the quantification of elasticity, viscosity, glassy-phase, and power law properties (the latter according to some authors, are translated into "fluidity" of the cell cytosol), which have an impact in tumor cell properties such as migration and metastization. The study of dynamic mechanical properties of melanoma cells is important since they can be used as a biomarker of the different stages of the disease as this cancer type is one of the most aggressive. This work showed that the viscosity seems to contribute less to mechanical properties when compared to elasticity and the power

law exponent. Those last two parameters may be used as a biomarker of pathological cell state and in the metastization process. This work also have showed that microrheology AFM methodology can be applied to other cancer cell types as it was also observed by Brás *et al.* [26].

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Competing interests: The authors declare that they have no competing interests.

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Supplementary Materials

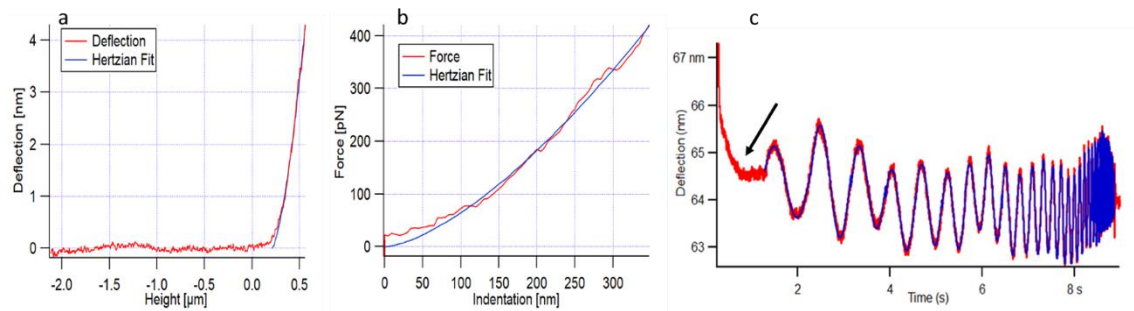


Fig. S1. An example of a conventional force-distance curve on a 1205LU cell, both fitted with Hertz's model and a frequency sweep deflection as a function of time: a) Deflection *vs.* height approach curve; b) Force *vs.* indentation; c) Representative curve of the deflection signal *vs.* time during dwell (tip contact for 8.7 s), in frequency sweep modulation. The first region of red curve (black arrow) corresponds to cells' stress relaxation and creep of the cell when the tip touches the surface during the tip approach. The analysis of the curve started with the curve fitting (blue color).

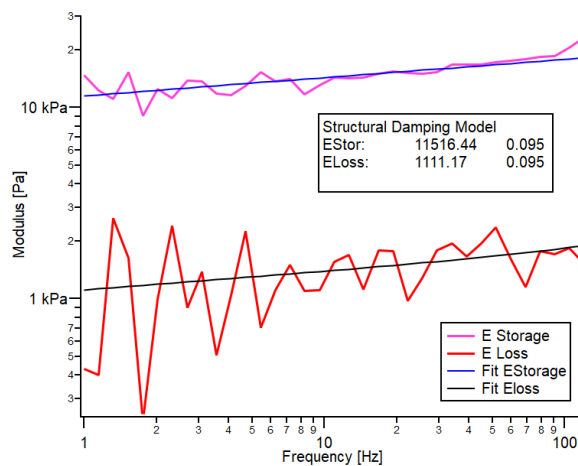


Fig S2. Representative example of loss and storage moduli *vs.* frequency curve, obtained by frequency sweep in a 1205LU cell. A power-law fit was applied to the storage modulus, whereas the structural damping model fit was applied to the loss modulus. Both fitting procedures resulted in a storage and loss moduli values for the lowest frequency (1 Hz) around 11.5 kPa and 1.1 kPa, respectively, and a power-law exponent of 0.095. Despite applying a correction for the hydrodynamic drag of the cantilever, the apparent loss modulus showed at higher frequency additional viscous damping, which was attributed to the viscosity of the medium. Thus, a double power-law fit was applied (lower exponent as determined from the elastic modulus plus a second one corresponding to hydrodynamic damping with an exponent of 1).

CHAPTER 5

CONCLUSION AND FUTURE PERSPECTIVES

5.1 Concluding remarks

In this work, AFM was used as a quantification technique, allowing the characterization of the mechanical properties in two different cancer types: CRC and melanoma, allowing the characterization of cells as tumoral and healthy cells. Two different cancer types were used to prove that mechanical properties can be used as a cancer biomarker. Colorectal cancer is included in the five cancer types, in which, the incidence and lethality are significant, in both genders being a tissue from an inner organ. In spite, of melanoma being not a cancer with high incidence, it has a great potential for metastization (having 80% of mortality). Skin tissue is the largest organ of the human body, establishing an interface between the interior and the exterior body. In this work, microrheology AFM allowed the quantification of several mechanical parameters such as the elasticity, viscosity, power law, and the glassy-phase state. Regarding results of CRC cell lines (HCT115, HCT116, and SW620), in terms of mechanical properties quantification, it was observed that apparent Young's modulus, elasticity, and viscosity of the SW620 cell line were higher (corresponding to the higher amount of F-actin filaments layers) than on HCT15 and HCT116. On the opposite, the "fluidity" of the cell cytosol (correlated with the quantification of the power law, according to some authors) was lower in the SW620 than in HCT15 and HCT116. These results were expected since cells need to deform more (having less elasticity and viscosity) to promote the metastization. This was corroborated by immunocytochemistry and migration assays. HCT15 and HCT116 cells presented abundant filopodia formation and closed the gap in migration assays faster (during the observation time of 144 h) compared with the SW620 cell line. The presence of total FAK was observed in the three cell types, being more predominant in HCT15 cells, which additionally justifies their migratory capacity. Tubulin associated with several cellular functions, such as force generation in cell division, showed to be higher in HCT15 than in HCT116 and SW620. Vinculin was present in HCT15 and HCT116, being abundant and well distributed through the whole cytoplasm, in the latter, while SW620 did not express vinculin. This fact was expected since the loss of vinculin is described as a promotor of cell metastasis predicting a poor prognosis in CRC.

For melanoma cells, it was observed that the apparent Young's modulus, elasticity, and viscosity of HNPMs were lower when compared with both melanoma cells type (WM793B and 1205LU). On the opposite, the "fluidity" of HNPMs (correlated with the determination of the power law) is higher as in melanoma cell lines. These results were as expected. WM793B and 1205LU cell lines, although both cancer cells, showed some differences between them. WM793B was stiffer and more viscous than 1205LU. This was also observed in elasticity and viscosity. These results were corroborated by immunocytochemistry, with WM793B presenting abundant cortical actin, in opposite to the 1205LU that presented actin distributed through the whole cell. In terms of the p-FAK presence, the 1205LU cells presented p-FAK, in contrast to WM793B cells where p-FAK was absent. In terms of migration, 1205LU cells showed a larger capability to migrate: the scratch was closed after 23 h, while WM793B cells had not closed the wound at the same time. The existence of p-FAK in 1205LU cells could explain its high potential to migrate. The migration and presence of p-FAK in 1205LU cells did not follow the tendency of the power-law exponent and the loss tangent, since these were lower when compared with WM793B cells. This fact contributes to the hypothesis that there may be other components in the 1205LU cells that must be explored to explain the contradiction observed since they were more viscous, having a lower power law exponent, which means that the viscoelastic properties do not depend weaker on frequency.

With this work, it has been proven that frequency sweep can be applied to different cancer types: melanoma cells were chosen here to corroborate the results obtained in CRC cells. A power law behavior (describing the frequency dependency of the storage modulus) was observed in these cells. This physical concept can be translated into the "fluidity" of the cytosol of the cell. The quantification of mechanical properties gave new insights in terms of cell deformability and metastization. In terms of clinical application, the measurement of the stiffness in tumor and healthy tissues, in real time in the surgery room, could be extremely valuable, in cases of carcinoma *in situ*.

2. Future perspectives

AFM is a promising tool for the diagnosis of diseases such as cancer, through the measurement of morphometric features of cells and tissues. Morphometry can provide

information about the level of pathologic or metastatic state of the cell through the topography and cytoskeleton (dis)organization. The stiffness of tissues may indicate the presence of cancer disease since diseased tissues usually present higher stiffness when comparing healthy ones. The translation of AFM to the clinics could be a great advantage in the diagnosis of cancer for the distinction of healthy and tumor tissues directly on the patient, in the surgery room, and during the procedure. This process would help to quantify the mechanical properties of the tumor mass from the center to the periphery, identifying the decreasing stiffness in the tissue, and allowing the identification of the interface borders by higher and lower stiffness (corresponding to the tumor and healthy tissue, respectively). Identifying the interface between the tumor and healthy tissue, the surgeon could do the incision adding only a marginal safety distance (of possible millimeters), assuring cleanness of them. This procedure, being complementary to anatomical pathology examination, could avoid additional surgeries for the same tumor in the same patient, thus reducing physical and psychological pain for the patients, and reducing overall health system costs.

A simple AFM instrument dedicated to this function should be developed, involving a research team integrating biophysicists, engineers, surgeons, and an AFM company to produce the instrument.