Extracellular vesicles and microRNAs emerge as biomarkers of breast cancer brain metastasis

iMed. ULisboa Research Institute for Medicines

Neurovascular Laboratory

U LISBOA | UNIVERSIDADE DE LISBOA

Godinho-Pereira J.[1,2], Figueira I.[1,3], Galego S.[1], Maia J.[4,5], Haskó J.[6], Molnár K.[6], Malhó R.[7], Costa-Silva B.[4], Wilhelm I.[6,8], Krizbai I.A.[6,8] and Brito M.A[1,2]

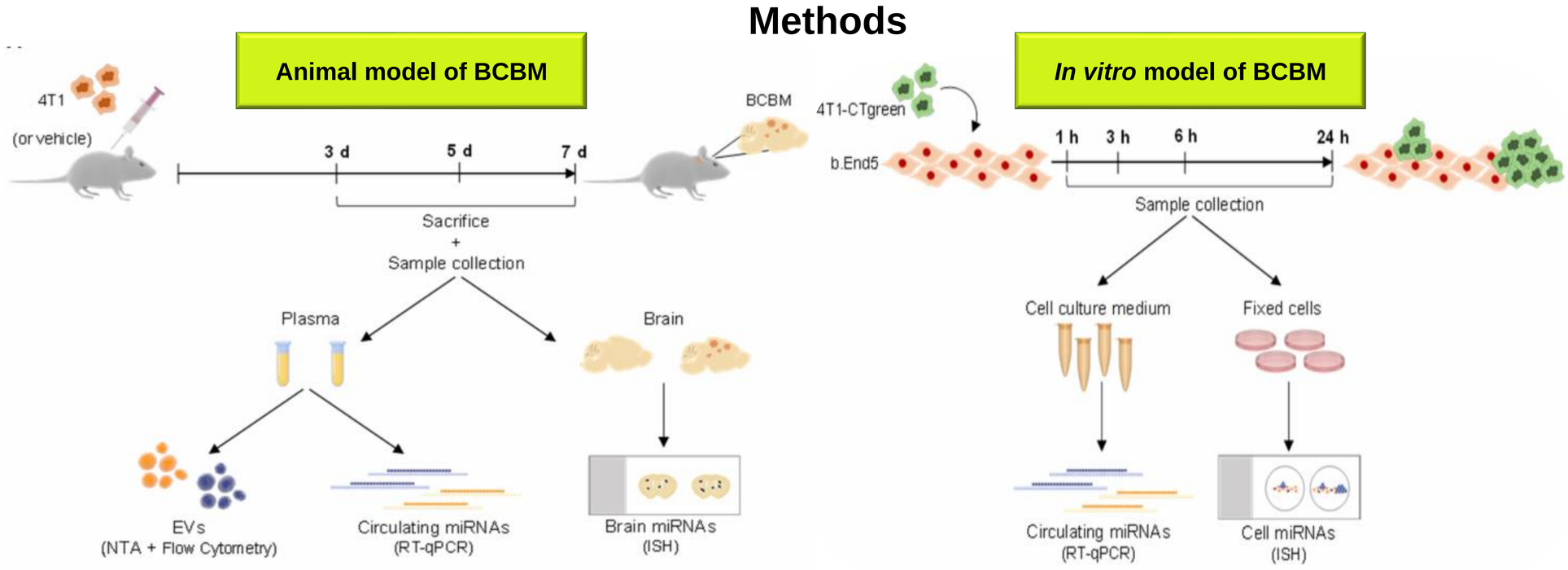
[1]Research Institute for Medicines (imed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal; [2] Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal; [3]Farm-ID—Associação da Faculdade de Farmácia para a Investigação e Desenvolvimento, Lisbon, Portugal; [4] Champalimaud Centre for the Unknown, Champalimaud Foundation, Lisbon, Portugal; [5] Graduate Program in Areas of Basic and Applied Biology, Universidade do Porto, Porto, Portugal; [6] Biological Research Centre, Eötvös Loránd Research Network (ELKH), Institute of Biophysics, Szeged, Hungary; [7] BioISI, BioSystems and Integrative Sciences Institute, Faculty of Sciences, Universidade de Lisboa, Lisbon, Portugal; [8] Institute of Life Sciences, Vasile Goldis Western University of Arad, Arad, Romania.

Joanagpereira@ff.ulisboa.pt

Background

Triple negative breast cancer (TNBC) presents higher mortality and poorer survival rates than other BC types, owing to the proneness to brain metastases formation and to the lack of biomarkers that precludes a timely diagnosis. Hence, the discovery of BC brain metastases (BCBM) biomarkers emerges crucial for an expedient intervention.

Methods



Objective Disclose microRNAs (miRNAs) and extracellular vesicles (EVs) in peripheral circulation as biomarkers of BCBM formation

Results

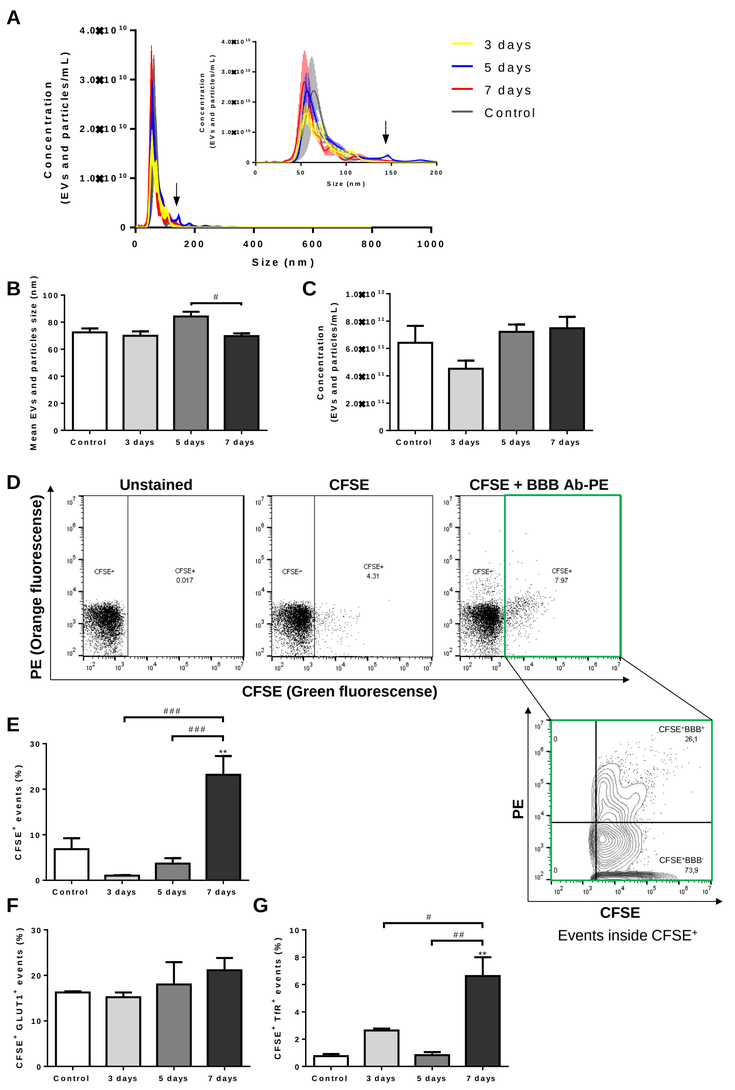
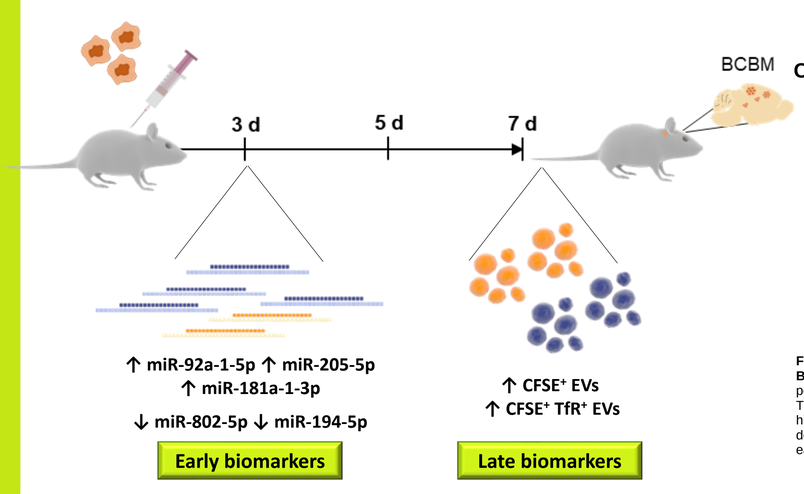


Fig 1 | Extracellular vesicles (EVs) are released by brain microvascular endothelial cells in advanced stages of breast cancer brain metastasis formation. 4T1 cells or vehicle (control) were inoculated in the carotid artery of female Balb/c mice, whole blood was collected upon sacrifice after 3, 5 and 7 days, and plasma was processed for EVs and particle analysis. Nanoparticle tracking analysis (NTA) was performed to assess EVs size distribution (**A**), mean particle size (**B**) and concentration (**C**), which revealed a sustained presence of exosome-like EVs along BCBM formation, with an additional tiny peak observed at 5 days (arrow). EVs brain endothelial origin was determined by flow cytometry using carboxyfluorescein diacetate succinimidyl ester (CFSE) to detect vesicular particles (CFSE⁺) and phycoerythin (PE)-conjugated antibodies against the blood-brain barrier (BBB) markers glucose transporter 1 (GLUT1-PE) and transferrin receptor (TfR-PE), collectively referred as BBB Ab-PE. Representative plots of unstained, only CFSE-labelled and CFSE+BBB Ab-PE-labelled plasma samples are presented, which allowed the quantification of vesicular particles (CFSE⁺) of brain endothelial origin (BBB⁺) inside the CFSE⁺ population (CFSE⁺ BBB⁺). (**D**). Percentage of vesicular particles (CFSE⁺) events increased at 7 days of 4T1 cells injection (**E**). No statistically significant differences were observed in CFSE⁺ GLUT1⁺ events within CFSE⁺ population along BCBM (**F**), while CFSE⁺ TfR⁺ events within CFSE⁺ population increased at 7 days (**G**). Statistical differences are denoted as **p<0.01 vs. control, and as #p<0.05, ##p<0.01 and ###p<0.001 between indicated conditions by One-way ANOVA. Data represented are means ± SEM, n=3.

Established metastases were associated with increased content of circulating **exosome-like EVs**, particularly of **Blood-brain barrier origin**



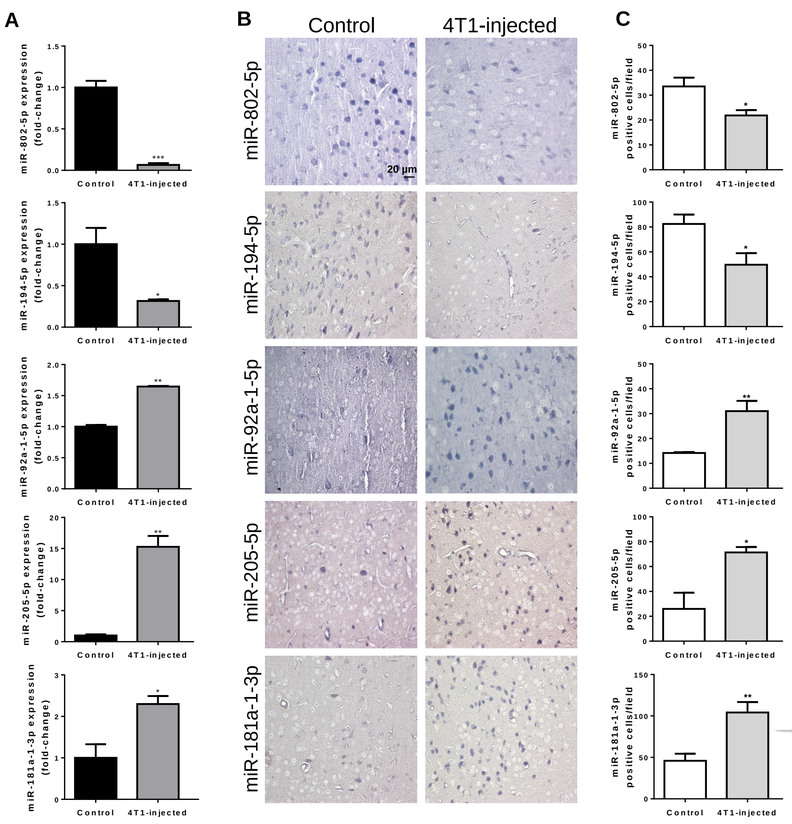
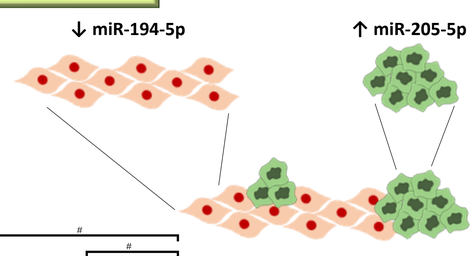


Fig. 2 | Circulating miRNAs alterations are related with their brain parenchyma deregulation in early stages of breast cancer brain metastasis formation. 4T1 cells or vehicle (control) were inoculated in the carotid artery of female Balb/c mice and after 3 days, whole blood was collected, and brains harvested after sacrifice. Plasma was processed for real time quantitative PCR (RT-qPCR) and sections of cranial hippocampus were processed for in situ hybridization (ISH) analysis of the different miRNAs. RT-qPCR analysis highlighted the downregulation of miR-802-5p and miR-194-5p and up-regulation of miR-92a-1-5p, miR-205-5p and miR-181a-1-3p in 4T1-injected mice plasma, with the results presented as fold-change vs. control (**A**). ISH analysis validated the same miRNAs downregulation and upregulation, as shown by the expression of these miRNAs in brain cells (blueish coloration, **B**), and ascertained by semi-quantitative analysis of the number of each miRNA positive cells per field (**C**). Statistical differences are denoted as *p<0.05, **p<0.01, ***p<0.001 vs. control by two-tailed unpaired Student's *t*-test. Data represented are means ± SEM, n=3.

Downregulation of miR-802-5p and miR-194-5p, and upregulation of miR-92a-1-5p, miR-205-5p and miR-181a-1-3p in circulation were observed **prior to BCBM detection**

miRNAs **brain origin** was established by parenchymal analysis, mirroring the alterations found in circulation



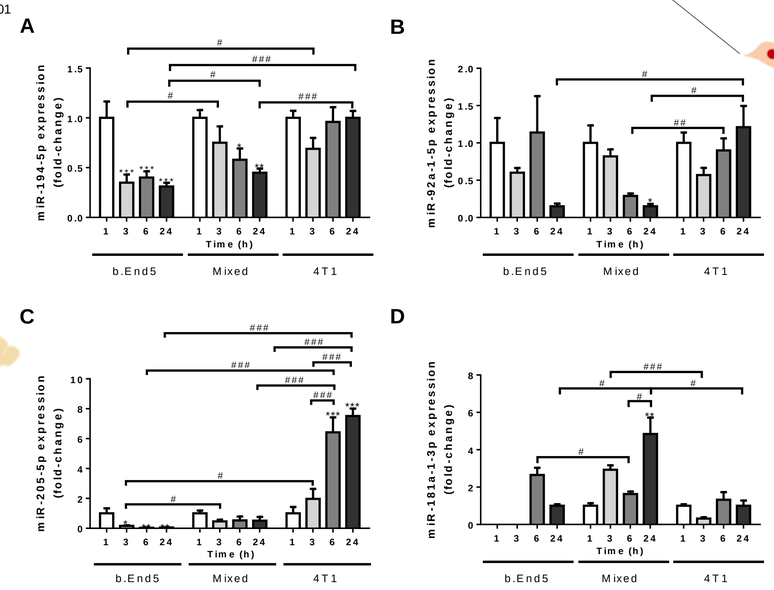


Fig. 3 | Differential release of miRNAs by single cultures of brain microvascular endothelial cells (BMECs) or breast cancer cells (BCCs), and mixed cultures of BMEC-BCC. Single cultures of 4T1 cells (BCCs), b.End5 cells (BMECs) and mixed cultures of b.End5 and 4T1 (previously labelled with CellTracker™ Green) cells, were performed under physiological shear stress for 1, 3, 6 and 24 h, after which the media were collected and processed for real time quantitative PCR of the indicated miRNAs. The results are presented as fold-change vs. 1 h of each culture system (with the exception of miR-181a-1-3p in b.End5 single cultures which were normalized for 24 h), and highlight significant alterations along time and between cultures for miR-194-5p (**A**), miR-92a-1-5p (**B**), miR-205-5p (**C**) and miR-181a-1-3p (**D**). Statistical differences are denoted as *p<0.05, **p<0.01, ***p<0.001 vs. 1 h of each culture, and by #p<0.05, ##p<0.01, ###p<0.001 between indicated conditions, determined by one-way ANOVA within each culture along time, and by two-tailed unpaired Student's *t*-test for each timepoint between cultures. Data represented are means ± SEM, n=3.

Endothelial cells from the BBB seem to account to the **decreased levels of miR-194-5p**

TNBC cells appear to significantly contribute to the **increased content of miR-205-5p**

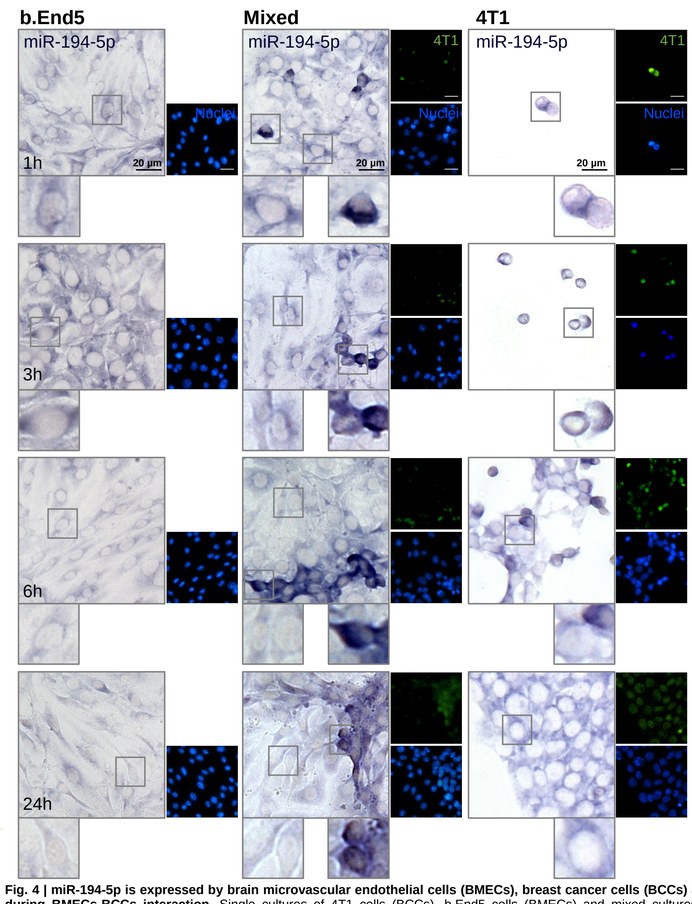


Fig. 4 | miR-194-5p is expressed by brain microvascular endothelial cells (BMECs), breast cancer cells (BCCs) and during BMECs-BCCs interaction. Single cultures of 4T1 cells (BCCs), b.End5 cells (BMECs) and mixed cultures of b.End5 and 4T1 cells (previously labelled with CellTracker™ Green) were performed under physiological shear stress for 1, 3, 6 and 24 h, after which cells were fixed and processed for in situ hybridization (ISH) for miR-194-5p and nuclei (blue) were counterstained with Hoechst 33342. Insets highlight major cellular alterations of miRNA expression in both cell populations (BMECs: left panels; BCCs: right panels) along time. ISH for miR-194-5p revealed a blueish coloration in BMECs, BCCs and particularly in BCCs interacting with BMECs.

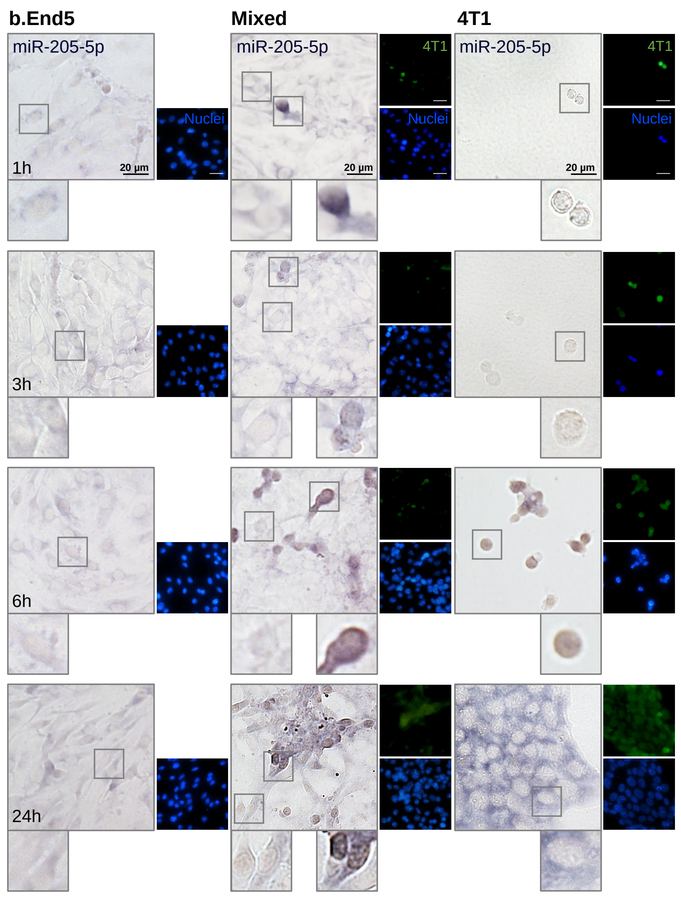


Fig. 5 | miR-205-5p is expressed by brain microvascular endothelial cells (BMECs), breast cancer cells (BCCs) and during BMECs-BCCs interaction. Single cultures of 4T1 cells (BCCs), b.End5 cells (BMECs) and mixed cultures of b.End5 and 4T1 cells (previously labelled with CellTracker™ Green) were performed under physiological shear stress for 1, 3, 6 and 24 h, after which cells were fixed and processed for in situ hybridization (ISH) for miR-205-5p and nuclei (blue) were counterstained with Hoechst 33342. Insets highlight major cellular alterations of miRNA expression in both cell populations (BMECs: left panels; BCCs: right panels) along time. ISH for miR-205-5p revealed a blueish coloration mainly in large BCCs clusters at later timepoints.

Take home message

TfR-positive EVs and specific miRNAs emerged as distinctive and novel biomarkers of BCBM in liquid biopsies, reflecting already established metastases and incipient ones, respectively.

Acknowledgments:

Portuguese Foundation for Science and Technology (PTDC/MED-ONC/29402/2017, UIDB/04138/2020 and UIDP/04138/2020; SFRH/BD/145522/2019, PD/BD/105866/2014); National Research, Development and Innovation Office (FK-124114, K-135425 and K135475); Executive Unit for Financing Higher Education, Research, Development and Innovation (PN-III-P1-1.1-TE-2019-1302 and PN-III-P4-ID-PCE-2020-1529); Champalimaud Foundation, H2020-MSCA-IF-2016 and La Caixa Foundation (grant 751547 from H2020-MSCAIF-2016 and grant LCF/PR/HR19/52160014); a node of the Portuguese Platform of BioImaging (PPBI-POCI-01-0145-FEDER-022122)