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Molecular profiling and function of exosomes derived from Non-Small Cell Lung Cancer. Diagnostic, predictive and prognostic biomarkers.

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LIST OF ABREVIATTIONS

ALK	anaplastic lymphoma kinase
AOBS	acousto-optical beam splitter
CAF	cancer associated fibroblast
DEX	dendritic cell-derived exosomes
ddPCR	digital droplet PCR
dsDNA	double-stranded DNA
EGFR	epithelial growth factor receptor
EML-4	echinoderm microtubule associated protein like-4
EMT	epithelial—mesenchymal transition
ER	estrogen receptor
ESCRT	endosomal sorting complex required for transport
EV	extracellular vesicle
FBS	fetal bovine serum
FFPE	formalin-fixed paraffin embedded
FISH	fluorescent in situ hybridization
GRB2	growth factor receptor-bound protein 2
HER2	receptor tyrosine-protein kinase erbB-2
MAGE	melanoma antigen gene
miRNA / miR-XX	micro-RNA
MHC	major histocompatibility complex
MVB	multivesicular body
NGS	next generation sequencing
NSCLC	non-small cell lung cancer
NYESO-1	new York esophageal squamous cell carcinoma
PDL	programmed death-ligand 1
PFS	progression free survival
PLAP	placental alkaline phosphatase
PLD	phospholipase D

PTEN	phosphatase and tensin homolog
SCC	squamous cell carcinoma
SEM	scanning electron microscopy
TKI	tyrosine kinase inhibitors
TLR3	toll-like receptor 3
TRK	tropomyosin receptor kinase
UTR	untranslated region
WT	wild-type

CHAPTER 1

Summary, aims and outline of the study

SUMMARY, AIMS AND OUTLINE OF THE STUDY

The main aim of this thesis is to increase the knowledge about exosomes in cancer. Specially, we would like to contribute to improve the imaging techniques for exosomes, and their role as predictive biomarkers and “shuttle” of crucial information between cells in the cancer process.

Every chapter of this thesis corresponds to a paper published, in process to be publish or submitted at the moment of thesis delivery.

After this initial summary, in the **second chapter** we provide an overview of the current landscape treatment in Non-Small Cell Lung Cancer (NSCLC) and the importance of liquid biopsy in this lethal disease IN the **third chapter**, we discus about the roles that exosomes might play in the NSCLC development through their pleiotropic roles. We also described the potential role of exosomes as diagnostic, predictive and prognostic biomarkers based on the study of miRNA, proteins, RNA and DNA contented in the exosomes.

Chapter four, is the first part of the thesis, describes our approach to improve cells and exosome imaging protocols. Starting for the minimum requirements for exosomes identification, scanning electron microscopy (SEM). Then, we propose our approach to improve a protocol for exosomes staining by the elimination of ultracentrifugations step, in order to simplify the technique and make it more accessible and reproducible. We apply this technique both in confocal microscopy and live cell/exosomes imaging through confocal microscopy. It is this last step where we elaborate a protocol in order to observe the internalization of exosomes in live imaging. Even if this technique requires further optimization, is settling the bases to understand one of the most important process for cross-talk, the exosomes internalization.

The **fifth chapter** is divided in two different sections, **5.1** and **5.2**, were we studied the exosomes as predictive biomarkers in a small subset of NSCLC patients. The first section of the chapter analysed the *ALK-EML4* translocation in exosomal RNA from patient’s samples and the concordance with tissue. The second section is focused on three *EGFR* (Epidermal Growth Factor Receptor) aberrations: deletion of the exon 19, L858R mutation and the acquired T790M mutation. Some of the test of this chapter are ongoing and we expect to have the results at the time of the defence of the thesis.

To conclude, the **sixth chapter** studies the functionality of exosomes derived from EGFR TKI (tyrosine kinase) lung cancer resistant cell lines when are internalized by a sensitive one. Firstly, we focused on the transference of genetic material and subsequently, we have studied whether the exosomes might induce partial or complete resistance in the recipient cells. In light of recent publications in other tumours, we also studied the effect of exosomes in cell survival and aggressiveness, that might be mediated through epithelial-mesenchymal transition. This might correlate with the changes of morphology observed on the cells after exosomes treatment.

CHAPTER 2

Non-Small Cell Lung Cancer

Extracted from:

"**Exosomes as a source of genetic material in non-small cell lung cancer: a truly Pandora's box"**

Pablo Reclusa, Rafael Sirera, Antonio Araujo, Marco Giallombardo, Anna Valentino, Laure Sorber, Ignacio Gil Bazo, Patrick Pauwels, Christian Rolfo

Published in "Translational Cancer Research"

NON-SMALL CELL LUNG CANCER

Lung cancer is the leading cause of cancer deaths worldwide, (1), being 85% of those Non-Small Cell Lung Cancer (NSCLC). The last data published by Cancer Research UK reported the 1-year overall survival rate of 32% for lung cancer patients while the 5-years survival rate is around 10%. Besides the development of new effective therapies, lung cancer is still today a disease difficult to control.

The advent of targeted agents represents one of the most important innovation, together with immunotherapy, in the treatment of lung cancer over the last years. The discovery of *epidermal growth factor receptor* (*EGFR*) activating mutations in 2004 as oncogene driver in a subgroup of patients with NSCLC led to the development of a new family of biological agents, called EGFR-TKIs, which were able to selectively bind and inhibit the EGFR molecular pathway. About eight phase III randomized clinical trials compared EGFR-TKI Gefitinib, Erlotinib, or Afatinib vs platinum-based chemotherapy as first-line treatment for *EGFR*-mutated NSCLC patients, all showing a significant survival benefit in favour of EGFR-TKIs. These drugs have revolutionized the clinical management of about 40% Asian and 12% -16% Caucasian NSCLC patients harbouring EGFR-mutations, whose survival outcomes nearly doubled compared to standard chemotherapy. Later the discovery of the *EML4-ALK* fusion gene in about 3-8% of patients with NSCLC and the subsequent clinical development of crizotinib represented an amazing success story leading to the recent approval of this compound as new standard first-line treatment in this subgroup of patients (2). Nevertheless, in both cases, despite an initial impressive benefit, patients inevitably experience tumour progression, because the tumour can generate resistance to these treatments through genetic modifications like mutations or amplifications. To avoid this problem, new drugs able to overcome resistance mechanisms are approved. New generations *EGFR* and *ALK* inhibitors have been recently investigated in randomized clinical studies, showing an impressive efficacy and tolerability in patients who failed prior TKIs. Particularly osimertinib, a third-generation EGFR-TKI in most advanced stage of clinical development which is active against both EGFR-sensitizing and resistant T790M mutation. The phase III AURA 3 study has recently shown a significant survival benefit in favour of osimertinib over platinum-chemotherapy in NSCLC patients who progressed to prior EGFR-TKI and were T790M positive (3). Recently, FDA (Food and Drug Administration) approved this compound in first line due to the FLAURA study. Similarly, the new generation ALK-inhibitors alectinib, ceritinib and brigatinib also demonstrated a significant improvement of PFS and OS in ALK-rearranged patients who failed

prior therapy with crizotinib (4). However, there are already some data showing that resistance mechanisms can occur also for these new generation drugs (5,6). In this scenario biomarker investigations have become one of the most interesting and studied fields of translational lung cancer research with the aim to estimate patients' prognosis, to monitor treatment response and to eventually predict both treatment efficacy and tumour recurrence (7,8).

The genetic analysis of both *EGFR* mutations and *EML4-ALK* translocation is a crucial step at the time of diagnosis, in order to plan the optimal treatment strategy for each patient. Furthermore, the analysis of *EGFR* mutations has acquired a growing importance also in the follow-up of TKI-treated patients. In fact, almost in nearly 60% of TKI-treated patients the treatment efficacy fails due to resistance mechanisms. The most common cause of TKI failure depends on the onset of secondary mutations; the exon 20 T790M is the most characterized resistance mutation in *EGFR* (9).

Therefore, *EGFR* mutational status should be monitored during treatment and mostly at relapse to choose the proper subsequent therapy. Tissue biopsy is a gold standard for the molecular analysis of a patient affected by NSCLC.

Even if there is a big consensus about the use of tissue biopsy as a primary source of genetic information, we still have to face the situation when "the tissue becomes the issue". This may happen when a strict "molecular follow-up" is mandatory to evaluate patient's disease evolution. To solve this problem, liquid biopsy has raised as the "new ambrosia of researchers" as it could help clinicians to identify both prognostic and predictive biomarkers in a more accessible way (10,11).

THE IMPORTANCE OF LIQUID BIOPSY IN NSCLC

Tissue biopsy is a procedure often limited by several features, including its invasiveness, the not easy access to different tumour sites, the high intra-tumour heterogeneity, and not ultimately the low patients' compliance (12). Moreover, a significant number of patients are not able to undergo rebiopsy due to their physical condition o tumour location, such as brain or bone marrow, thus, in the last decade many new non-invasive approaches have been studied

to overcome the aforementioned issues. Among these, liquid biopsy represents a valuable alternative for the detection of *the* mutational status once it cannot be performed on tissue samples according to international guidelines. Furthermore, a liquid biopsy can be easily repeated at different time-points allowing to follow the tumour molecular status during the treatment course (13). This could help clinicians to predict disease progression over time, to identify newly acquired molecular alterations and to observe how all these characteristics correspond to patient's status.

Nowadays important efforts in molecular profiling of the tumors are allowing to develop new technology that permits to analyze the tumor characteristics in peripheral blood. Accordingly, the liquid biopsy refers to the analysis of components that can be isolated and analyzed from a blood sample as, circulating tumor cells (CTCs), cell free circulating DNA (circulating tumor DNA, ctDNA) and exosomes (a part of the secreted micro-vesicles) (14-16). In this regard, CTCs have been demonstrated to be good predictors for risk of metastatic progression, to monitor the response of an undergoing treatment, or to identify new targets and resistance mechanisms (17). On the other hand, ctDNA the most used component of the liquid biopsies, is now being studied as possible biomarker and prognostic factor in cancer, although it is still difficult to standardize (18-21).

Nevertheless, the ultimate techniques developed in the field of sequencing and DNA amplification such as Next Generation Sequencing (NGS) and digital droplet PCR (ddPCR) are transforming the molecular profiling of the tumor in a fast, easy, reliable and affordable manner (22-25).

The field of study of this thesis is the least known member of liquid biopsies. Exosomes are one of the most promising tools of liquid biopsies and they will be described and discussed in the chapter three.

BIBLIOGRAPHY

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin [Internet]. 2016;66(1):7–30. Available from: http://dx.doi.org/10.3322/caac.21332

2. Sturgeon CM, Duffy MJ, Stenman U, Lilja H, Bru N, Chan DW, et al. Biochemistry Laboratory Medicine Practice Guidelines for Use of Tumor Markers in Testicular, Prostate, Colorectal, Breast, and Ovarian Cancers METHODS: RESULTS: 2008;79.

3. Group BDW. Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. Clin Pharmacol Ther [Internet]. 2001;69(3):89–95. Available from: http://dx.doi.org/10.1067/mcp.2001.113989

4. Yap TA, Lorente D, Omlin A, Olmos D, de Bono JS. Circulating tumor cells: a multifunctional biomarker. Clin Cancer Res. United States; 2014 May;20(10):2553–68.

5. Mabert K, Cojoc M, Peitzsch C, Kurth I, Souchelnytskyi S, Dubrovska A. Cancer biomarker discovery: current status and future perspectives. Int J Radiat Biol. England; 2014 Aug;90(8):659–77.

6. Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. Cancer Sci. England; 2010 Oct;101(10):2087–92.

7. Alix-Panabières C, Pantel K. Circulating tumor cells: liquid biopsy of cancer. Clin Chem. Am Assoc Clin Chem; 2013;59(1):110–8.

8. Yanagita M, Redig AJ, Paweletz CP, Dahlberg SE, O'Connell A, Feeney N, et al. A prospective evaluation of circulating tumor cells and cell-free DNA in EGFR mutant non-small cell lung cancer patients treated with erlotinib on a phase II trial. Clin Cancer Res. 2016 Jun;

9. Jansen MP, Martens JW, Helmijr JC, Beaufort CM, van Marion R, Krol NM, et al. Cell-free DNA mutations as biomarkers in breast cancer patients receiving tamoxifen. Oncotarget. 2016 May;

10. Hadano N, Murakami Y, Uemura K, Hashimoto Y, Kondo N, Nakagawa N, et al. Prognostic value of circulating tumour DNA in patients undergoing curative resection for pancreatic cancer. Br J Cancer. 2016 Jun;

11. Rolfo C, Castiglia M, Hong D, et al. Liquid biopsies in lung cancer: The new ambrosia of researchers. Biochim Biophys Acta. 2014 Oct;1846(2):539–46.

12. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol. United States; 2006 Apr; Chapter 3: Unit 3.22.

13. Thakur BK, Zhang H, Becker A, Matei I, Huang Y, Costa-Silva B, et al. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. Cell Res. 2014;24(6):766–9.

14. Yap TA, Lorente D, Omlin A, Olmos D, de Bono JS. Circulating tumour cells: a multifunctional biomarker. Clin Cancer Res. United States; 2014 May;20(10):2553–68.

15. Mabert K, Cojoc M, Peitzsch C, Kurth I, Souchelnytskyi S, Dubrovska A. Cancer biomarker discovery: current status and future perspectives. Int J Radiat Biol. England; 2014 Aug;90(8):659–77.

16. Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. Cancer Sci. England; 2010 Oct;101(10):2087–92.

17. Alix-Panabières C, Pantel K. Circulating tumor cells: liquid biopsy of cancer. Clin Chem. Am Assoc Clin Chem; 2013;59(1):110–8.

18. Yanagita M, Redig AJ, Paweletz CP, Dahlberg SE, O'Connell A, Feeney N, et al. A prospective evaluation of circulating tumor cells and cell-free DNA in EGFR mutant non-small cell lung cancer patients treated with erlotinib on a phase II trial. Clin Cancer Res. 2016 Jun;

19. Jansen MP, Martens JW, Helmijr JC, Beaufort CM, van Marion R, Krol NM, et al. Cell-free DNA mutations as biomarkers in breast cancer patients receiving tamoxifen. Oncotarget. 2016 May;

20. Hadano N, Murakami Y, Uemura K, Hashimoto Y, Kondo N, Nakagawa N, et al. Prognostic value of circulating tumour DNA in patients undergoing curative resection for pancreatic cancer. Br J Cancer. 2016 Jun;

21. Camps C, Jantus-Lewintre E, Cabrera A, Blasco A, Sanmartín E, Gallach S, et al. The identification of KRAS mutations at codon 12 in plasma DNA is not a prognostic factor in advanced non-small cell lung cancer patients. Lung Cancer. Elsevier Ireland Ltd; 2011 Jun;72(3):365–9.

22. Castera L, Krieger S, Rousselin A, Legros A, Baumann J-J, Bruet O, et al. Next-generation sequencing for the diagnosis of hereditary breast and ovarian cancer using genomic capture targeting multiple candidate genes. Eur J Hum Genet [Internet]. Macmillan Publishers Limited; 2014 Nov;22(11):1305–13. Available from: http://dx.doi.org/10.1038/ejhg.2014.16

23. Hortobagyi GN, Piccart-Gebhart MJ, Rugo HS, Burris HA, Campone M, Noguchi S, et al. Correlation of molecular alterations with efficacy of everolimus in hormone receptor-positive, HER2-negative advanced breast cancer: Results from BOLERO-2. In: ASCO Annual Meeting Proceedings. 2013. p. LBA509.

24. Chen WW, Balaj L, Liau LM, Samuels ML, Kotsopoulos SK, Maguire CA, et al. BEAMing and Droplet Digital PCR Analysis of Mutant IDH1 mRNA in Glioma Patient Serum and Cerebrospinal Fluid Extracellular Vesicles. Mol Ther Nucleic Acids [Internet]. American Society of Gene & Cell Therapy; 2013 Jul 23;2: e109. Available from: http://dx.doi.org/10.1038/mtna.2013.28

25. Oxnard GR, Paweletz CP, Kuang Y, Mach SL, O'Connell A, Messineo MM, et al. Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. Vol. 20, Clinical cancer research: an official journal of the American Association for Cancer Research. 2014. p. 1698–705.

CHAPTER 3

Exosomes

Partly adapted from:
"Exosomes as diagnostic and predictive biomarkers in lung cancer"

Pablo Reclusa*, Simona Taverna*, Marzia Pucci, Elena Durendez, Silvia Calabuig, Paolo Manca, Maria Jose Serrano, Laure Sober, Patrick Pauwels, Antonio Russo, Christian Rolfo

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"Exosomes as a source of genetic material in non-small cell lung cancer: a truly Pandora's box"

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Published in "Translational Cancer Research"

EXOSOMES

Exosomes are small nanovesicles, in a range between 30 to 150 nm. Were first describe in 1983 in two paper published simultaneously (1,2) . Exosomes are exocytosed by every cell in a constitutive manner, however, it has been demonstrated that tumour cells release higher amounts of exosomes than healthy ones and they can be found in different body fluids such as blood, semen, or ascites.

When discovered, exosomes were considered the garbage bags where the cells ousted the undesired components from themselves, however it has been demonstrated the implications of the exosomes in different functionalities of the tumour such as, immunomodulation (3) , pre-metastatic niche formation (4) , tumour growth (5) , treatment resistance mediation (6) and more recently, exosomes mediated drug expulsion (7) . Moreover, exosomes have been described to content messenger RNA (mRNA), microRNAS (miRNAs), double-stranded DNA (dsDNA) and proteins that could serve as diagnostic, predictive and prognostic biomarkers for the different tumours. More importantly, if exosomes are understood as horizontal cell communicators between cells, their content could mirror the one from the cell of origin; giving rise to a new and accessible source for tumour profiling analysis.

Extracellular vesicles such as exosomes accomplish a wide number of vesicles differing in size, origin and composition. Apoptotic bodies are big size vesicles ranging from 1000 to 5000 nm, smaller, macrovesicles can be found ranging from 200 until 1000 nm, and in the small spectrum exosomes can be found ranging between 50 and 200 nm (8) . However, due to their common multivesicular body origin, all exosomes have a common profiling that allows their identification, based on different membrane proteins such as ALIX, TSG101 and CD63.

Different methods for exosomes isolation have been successfully used. The most common methods are ultracentrifugation and sucrose density-gradient ultracentrifugation or exosomes immunoprecipitation. However, recently new isolation methods have been used such as extracellular vesicle array or immunobeads precipitation (9,10) . Both methods are based on antibodies recognition. Many other exosomes isolation kits have been developed recently based on filtration columns (11) .

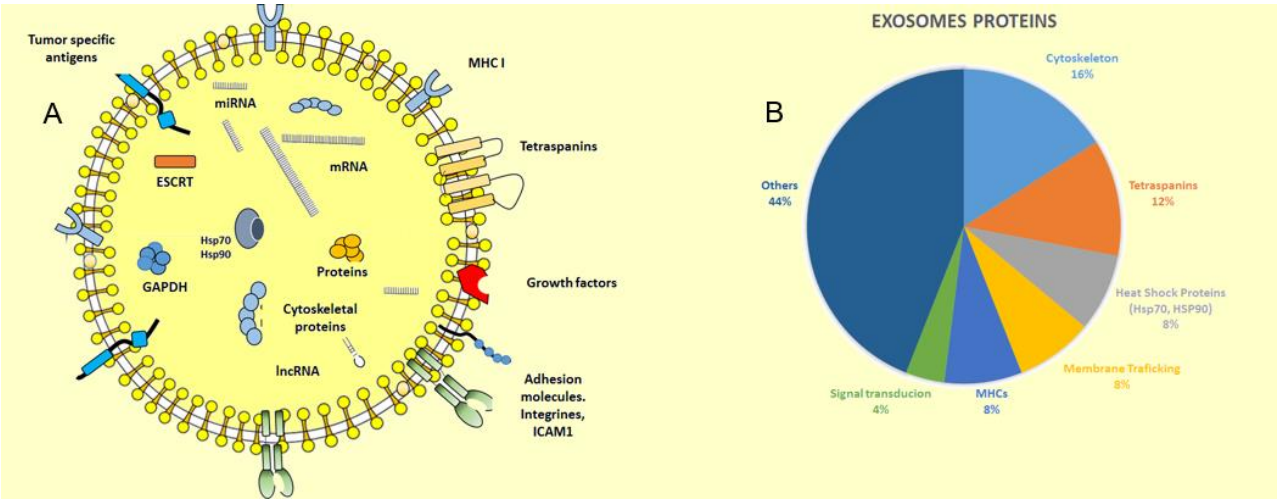

Figure 1. A) Schematic picture of the content of an exosome. B) Diagram showing the principal proteins of the exosomes. Adapted from “Tumor cell-derived exosomes: a message in a bottle.”. Kharaziha P. et al.

EXOSOMES BIOGENESIS AND TRAFFICKING

Originally, exosomes were thought to be released directly through fusion with the plasmatic membrane. However, was not until the 80s when it was described the existence of an intracellular endosome, that leaded to the formation of a multivesicular body (MVB), that later, could release exosomes through fusion with the plasma membrane (2) .

The Endosomal Sorting Complex Required for Transport (ESCRT) was firstly described to control the exosomes formation inside the MVB. It consists in four main complexes with other auxiliary proteins. The ESCRT-0 controls the cargo clustering in a ubiquitin dependent manner. The ESCRT-I and II induce the bud formation in the MVB and the ESCRT-III induce the vesicle scission from the MVB membrane. The auxiliary VPS4 protein is in charge of recycling the ESCRT machinery (12) (Fig. 2). Until recently, the ESCRT mechanism was the only known to induce the exosomes formation, however, in the last years; many articles have been published demonstrating alternative pathways for the exosomes formation ESCRT-independent. These mechanisms include proteins known to be present in the exosomes. The cholesterol has been described to induce the vesicles secretion in Flotillin-2 dependent

manner (13) . The phospholipase D2 (PLD2), increase the production of the intraluminal vesicles in the MVB through the increase of the inward curvature of the MVB membrane (14) . Moreover, different tetraspanins have been demonstrated to have any active role in the mRNA and protein cargo sorting in the exosomes (15,16) .

Once the exosomes are in the luminal space of the MVBs, SNARE family, and concretely, VAMP-7 could be enhancing the membrane fusion between the MVBs and the plasma membrane. However, the inhibition of the proteins from the SNARE family does not result in the complete depletion of exosomes secretion, suggesting other proteins could play this role (17,18) . Finally, the exosomes secretion is mostly inducing by proteins from the RAB family such as RAB27a and RAB27b (19) .

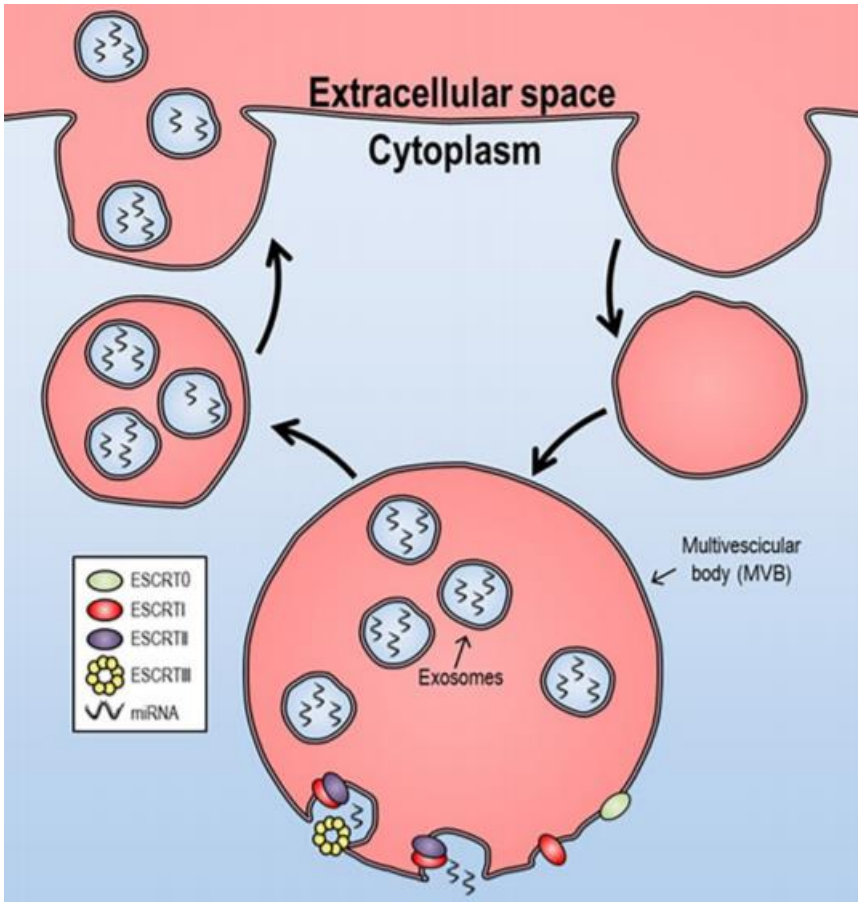

Figure 2. Detailed process of exosomes formation from the early endosome to the exosome's recreation. ESCRT, endosomal sorting complex required for transport.

PLEIOTROPIC ROLES OF EXOSOMES

In recent years, more and more studies are ongoing with regard to the function of exosomes and their application for tumor detection and treatment and several reports have demonstrated that exosomes can play pleiotropic roles affecting pivotal aspects of tumor development and growth (Fig. 3). For instance, exosomes derived from prostate tumor cells act in a paracrine fashion inducing a reprogramming of cell metabolism and enhancing of cell proliferation (20) . The fusion gene *TMPRSS2: ERG* and *EGFR*, both related with advanced prostate have been described inside the exosomes (21,22) . Cells from renal carcinoma are able to release exosomes containing some miRNAs and other RNAs that transform normal endothelial cells into an activated angiogenic phenotype and directing towards lung cancer metastasis (23). In other studies, it has been demonstrated that exosomes derived from highly metastatic melanoma cells promote the pre-metastatic niche formation through the education of the bone marrow and by reprogramming its progenitor cells to a pro-vasculogenic phenotype (24). On the other hand, another described role of exosomes in breast cancer and multiple myeloma is the ability to promote drug resistance through horizontal transfer (25,26). Exosomes are also involved in the creation of a pro-inflammatory microenvironment that promotes tumor growth by means of immunological proteins such as MHC-II, CD40 and CD40L. In breast cancer exosomes containing 27-Hydroxycholesterol (27-OHC), a lipid associated with proliferation and metastasis in estrogen receptor positive (ER+) tumor cells, open a new window to understand how exosomes genesis is achieved and its correlation with the content of the cells of origin (27). Other studies performed in exosomes derived from ovarian cancer cell lines show that exosomes can carry functional proteins that reprogram cell metabolism of the cell enhancing the pentose phosphate pathway that is crucial for resisting oxidant injury and favours tumor survival (28,29).

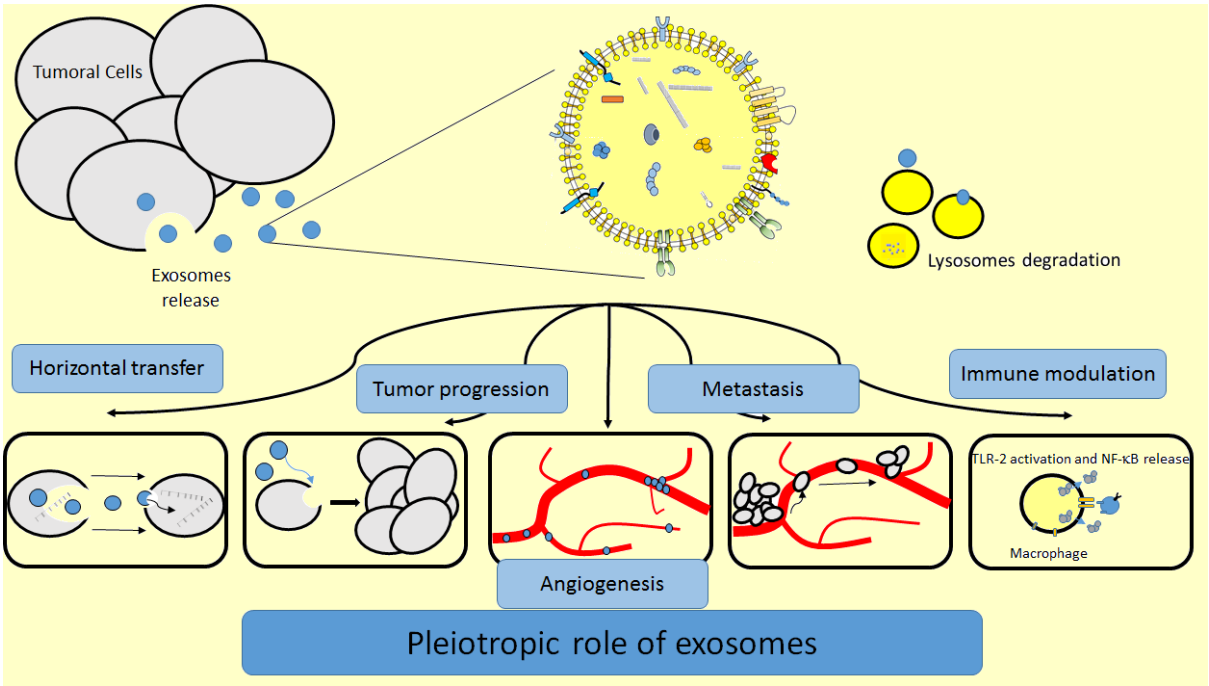

Figure 3. Pleiotropic roles of exosomes

EXOSOMES AS MARKERS FOR NON-SMALL CELL LUNG CANCER

One of the most attractive aspects of exosomes research is the discovery of novel biomarkers for the early and improved detection of lung cancer (30). The content of the exosomes has been widely described in different tumours. Micro RNAs (miRNAs) are probably the most studied molecules inside the exosomes. Among miRNAs, miRNA-21 have been widely described in NSCLC exosomes, and its impact in other malignancies has been described in several reports related with tumour growth in both haematological and solid malignancies, for example, in diffuse large B-cell lymphoma the AKT signalling pathway is constitutively activated due to the over expression of miR-21 that leads to the suppression of forkhead box protein O1 expression and the downregulation of *PTEN* expression, leading to a poor prognosis of the disease (31,32). Regarding the solid tumours, breast cancer and colorectal cancer have been demonstrated to upregulate miR-21 in advanced stages leading to a poor prognosis of the disease and high risk of metastasis (33,34). Also, messenger RNA has been described in the exosomes also as a possible predictive biomarker that could help to improve, in a non-invasive way, the treatment choice and to predict the resistance before clinical significances appear (35). Many proteomic studies have been performed in different tumours inside the exosomes with successfully results, being able to create protein profiles with

diagnostic and prognostic functions, and also to identify exosomal proteins that can play key roles in the receptor cells (35,36). In the last years and since the discovery of the exosomal dsDNA in 2014 (37) , the studies on the field has increased exponentially.

Many correlations have been found between tissue biomarkers and exosomal biomarkers in different tumours opening the door to improve our detection of diagnostic, predictive and prognostic biomarkers. Although none has been yet validated for NSCLC analysis, in the following paragraphs the main advances and most promising markers will be described. A summary of all the markers described can be found in Table 1.

microRNA

MicroRNAs (miRNAs) are small non-coding RNAs. They are recognized to regulate a number of genes by binding to the 3'UTR of their target mRNA, resulting in the alteration of the targeted gene expression. A single miRNA can influence multiple genes in a single cell. The utility of miRNAs for cancer study has been largely described. Unlike circulating miRNAs, exosomal miRNAs are protected from the degradation by RNAse in the blood torrent (38), thus miRNAs could serve as a useful tool in three fields of study; diagnostic of NSCLC, prognosis against a treatment and risk of resistance after a therapy.

The first group of miRNA was described to be useful to predict NSCLC over healthy patients by Rabinowits et al. in 2009, it consists of 12 miRNAs, miR-17-3p, miR-21, miR-106a, miR-146, miR-155, miR-199, miR-192, miR-203, miR-205, miR-210, miR-212 and miR-214, which were present in NSCLC both in tissue and in circulating exosomes being their levels non-detectable in healthy donors . In the same paper, they described that the miRNA levels inside the exosomes derived from NSCLC were higher compared to those from healthy donors (39). Cazzoli et al. were the first groups to develop 2 miRNA profiling, one for screening based on 4 miRNAs, miR-378a, miR-379, miR-139-5p and miR-200-5p, and one for diagnosis based on 6, miR-151a-5p, miR-30a-3p, miR-200b-5p, miR-629, miR-100 and miR-154-3p, both extracted from a wide analysis of 746 miRNAs analysed on a training set of 3 groups; Lung Adenocarcinomas; Lung Granulomas; healthy former smokers. These profiles were confirmed in a validation set of 50 patients. In the case of the screening test the sensitivity was 97.5% for 72% specificity and an AUC value of 0.908 while the diagnostic test exhibited a sensitivity of 96% for 60% of specificity with an AUC value of 0.76 (40). More recently, Jin et al. has developed a miRNA profile based on 4 miRNAs, let-7b-5p, let-7e-5p, miR-23a-3p, and miR-486-5p that exhibited sensitivity of 80.25% and a specificity of 92.31% with an AUC value of

0.899 being able to diagnose 43 NSCLC over 60 samples. In the same paper, levels of miR-181b-5p and miR-361b-5p, as well as miR-10b-5p and miR-320b were used to identify among the 43 patients 31 adenocarcinoma patients and 11 squamous cell carcinoma (SCC) patients respectively. The miRNA for adenocarcinoma and SCC diagnosis showed an AUC value of 0.936 and 0.911 respectively with a sensitivity of 80.65% and a specificity of 91.67% for adenocarcinoma and a sensitivity of 83.33% and a specificity of 90.32% for SCC (41).

One of the big problems of NSCLC is the high rates of progression and recurrence of the patients; thus, tools to predict this behaviour are needed to improve the medical treatment choices in the clinic. Many miRNAs have been reported to be differentially expressed in situations where the tumour is progressing before it is a detectable under Computerize Tomography Scan (CT).

The downregulation of the miRNA-146-5p is indicative of a poor progression free survival (PFS) compared to those patients with higher levels of the miRNA inside the exosomes. Moreover, its levels are correlated to the chemosensitivity of the tumour to Cisplatin mediated through the blockade expression of *Atg12*, an autophagy mediator gene (42). In another study with 10 lung cancer patients and 10 healthy controls, 83 cancer-related miRNAs were analysed. After informatics analysis, 9 miRNAs were differentially expressed and validated in a subset of 209 NSCLC patients. From those, miR-23b-3p, miR-10b-5p and miR-21-5p were validated as upregulated miRNAs in NSCLC compared to healthy controls. Separately, the 3 miRNAs showed significantly low overall survival in those patients with higher levels of the miRNA in their exosomes compared to those with low levels. Moreover, the 3 miRNAs model improves the AUC value from 0.88, from only the clinical variables, to 0.91 analysing both the miRNAs expression and the clinical variables such as sex, or tobacco consumption (43). Exosomal miRNA has been described also to play a role in the predictive response to a treatment as it has been explained before with the miRNA-146-5p (44). Two other independent studies show that upregulation of miR-1246 and miR-208-a is correlated with a high proliferation of the tumour and a resistant profile to radiotherapy by targeting the genes DR5 and p21 respectively. This could lead not only to a prognostic biomarker but to a new target against NSCLC (44,45). The miR-21 and miR-4257 were found among a subset of patients with recurrence after surgery, without recurrence and healthy donors. These results were validated in 201 cases with an upregulation of 2.5 and 2.9-fold of increase respectively between recurrence patients and healthy donors. Moreover, it was demonstrated that the disease-free survival- similar to PFS-, was lower in those patients with high expression of both miRNAs- considered high more than the mean of the expressions-. They described also the

correlation of the over expression of miR-21 with the tumour size and TNM stage, and the correlation between de levels of miR-4257 and lymphatic node invasion, histological type and TNM stage (46).

One of the problems of the research and analysis of exosomes is the amount of material available. In another study, exosomes isolated from H1299 (NSCLC cell line) and Beas-2b (bronchial-epithelial cell line), and isolated from mouse with H1299 after surgery, some being recurrent and some not. The authors faced a very low-positive amplification rate due to the low amount of extracted RNA, and high variability between replicates and groups. However, despite the low amplification rates, the authors showed that miR-21 and miR-155 may be upregulated in the group of mice recurrent after surgery in comparison with primary tumour mice (47).

One of the most significant papers regarding the utility of miRNA inside the exosomes has been recently published by Qin et al. They described that miR-100-5p is downregulated in exosomes derived from A549 resistant to Cisplatin (A549/DDP) in comparison with A549 wild-type. However, they saw that exosomes derived from A549/DDP were able to transfer Cisplatin resistance mediated by the binding of miR-100-5p to the 3' UTR of mTOR gene. Same experiments were performed in vivo with mice inoculated in different conditions of exosomes derived from A549, A549/DDP and inhibitors and mimics of miR-100-5p being the mice with exosomes of A549/DDP transfected with miR-100–5p control mimic they group with a higher tumour growth, lower levels of miR-100-5p and higher levels of mTOR (48).

Contrary to what it has been shown in this review, some papers have demonstrated a negative correlation between tissue and exosomal miRNA levels. Our group published an abstract with a follow up analysis of patients during Osimertinib treatment, a third-line tyrosine kinase inhibitor. The treatment lead to an upregulation of oncomiRs (hsa-miR-221-3p/222-3p) in exosomes isolated from patient's plasma respect to healthy control. However, the upregulation was correlated to a good clinical outcome suggesting that some these two miRNAs may be pump out of the cell due to the treatment (49).

All these studies suggest that exosomal miRNA varies their composition depending on their cellular origin being able to differentiate between NSCLC subtypes, and due to their stability and the stability of their content they may be perfect candidates for the diagnosis, or prognosis of NSCLC (40–42,47).

Exosomal protein

Exosomes can be considered as potential tools for tumour diagnosis and prognosis. These stable vesicles, contained in the blood stream, are tumour specific and protected from degradation by a lipid bilayer membrane. Recent studies identify the proteome of cancer exosomes; proteomic analyses show that several proteins are enriched in exosomes. These proteins are involved in exosome biogenesis, membrane transport and fusion and in various steps of tumour progression such as metastasis, angiogenesis and immunomodulation (50).

According to the database Exocarta (http://www.exocarta.org)], 9769 proteins have been identified in exosomes. Some exosomal proteins can mirror producer cells and pathological stage of disease (50). Moreover, some of these proteins play a key role in tumorigenesis. These features qualify exosomal protein as good diagnostic and prognostic potential biomarkers in lung cancer (51).

Lung cancer exosomes contain several tumours associated proteins, such as EGFR, KRAS, extracellular matrix metalloproteinase inducer (EMMPRIN), claudins and RAB-family proteins. CD91, CD317 and EGFR have been suggested as potential exosomal markers in NSCLC. Exosomal EGFR is one membrane-bound proteins that have been evaluated in NSCLC (52). Specifically, Huang et al. found that 80% of the exosomes isolated from NSCLC biopsies were EGFR positive (53).

Recently, it was tested exosomal proteins differential expressed in normal bronchial epithelial cells and NSCLC cells using a triple SILAC quantitative proteomic method. They found that NSCLC exosomes are enriched in proteins involved in cell signalling, cell adhesion extracellular matrix remodelling. They identify and quantify 721 exosomal proteins derived from three cell lines. Among the proteins associated with signal transduction, enriched in NSCLC exosomes, EGFR and SRC are upregulated as well as downstream effectors such as GRB2 and RALA. Moreover, it was demonstrated that MET receptor, RAC1, and KRAS proteins were increased in NSCLC exosomes (54).

Furthermore, Sandfeld-Paulsen demonstrated that in a cohort of 276 non-selected NSCLC patients of all stages, it was evaluated 49 exosomal membrane bound proteins and found that nine proteins have a potential as prognostic markers in NSCLC. In particular, they indicate that increasing concentration level of NYESO-1, EGFR and PLAP are prognostic markers of poor prognosis (55). Proteomic analysis of exosomes isolated from human malignant pleural effusions demonstrated that these vesicles contained MHC class I and II proteins, heat shock

and cytoskeletal proteins, and signal transduction–involved proteins. These proteins have already been reported as constituent of exosomes from other origin.

Exosomes from malignant pleural effusions contain peculiar proteins, such as Sorting-nexin (SNX) family, a group of hydrophilic proteins involved in the intracellular trafficking of proteins to different organelles. Acidic ribosomal phosphoproteins, from the 60S subunit of ribosomes, interact with elongation factors EF-1 and EF-2 and play an important role in the elongation step of protein synthesis. The presence in exosomes of proteins involved translation in may also be explained by their high concentration in the cytosol of cancer cells (56).

Nowadays, in the field of the biomarker discovery, the methodology that focuses only on a single exosome-protein has been overtaken that multiple protein markers panel (8). In particular, Jakobsen and colleagues used an extracellular vesicle array with 37 antibodies to capture exosomes directly from the plasma of NSCLC patients, the result indicated that the sensitivity, specificity and diagnostic accuracy of combined 30-marker model (57). A recent study used the extracellular vesicle array with 49 antibodies. Among the 49 exosomal proteins, CD151, CD171, and tetraspanin 8, have been considered the strongest markers to identify lung cancer of all histological subtypes from the control. It could important not only to evaluate protein profiling of exosomes from different lung cancer stage and histology, but also as potential diagnostic tool for lung cancer (58).

RNA & DNA

Due to the difficulties working with exosomes, it has not been until recent years with the improvement in the sensitivity of the techniques and the discovery of the high throughput technologies, such as NGS, that exosomal RNA and DNA may become a real alternative to the tissue analysis.

Our group has recently described for the first time the *ALK-EML4* translocation inside the exosomes with a sensitivity of 64% and a specificity of 100% over a subset of 17 patients being 14 positive and 3 negatives compared with tissue from the same patients (59). Just few months earlier, Krug, et al. detected for the first time RNA transcribed from the *EGFR* gene, in a study comparing the analysis of ctDNA for *EGFR* mutation with a combined analysis of ctDNA together with exosomal RNA presenting a better sensitivity for all the analysed mutations (60).

In 2014, Thakur et al. described for the first time the presence, inside chronic leukaemia, human colorectal carcinoma, and murine melanoma derived exosomes of dsDNA over other forms of DNA. However, the presence of exoDNA inside the lung cancer derived exosomes was lower than the in other tumours. To conclude the research, the authors performed an analysis of exosomal *EGFR* in 4 NSCLC cell lines with the objective of detecting mutations known to be present in those cell lines. The results showed that 100% of the exoDNA analysis were positive for the corresponding mutation (61).

ExoDNA may lead to improvements in the sensitivity and specificity of detecting tumour alterations that will prove useful in the clinical setting in the analysis of driver mutations (62). Moreover, their stability and abundance make them very good candidates over other liquid biopsy compartments exposed constantly to the conditions of the blood stream.

Molecule	**Utility**	**Sensitivity (%)/ Specificity (%)/ AUC Value**	**Reference**
miRNA			
miR-17-3p, miR-21, miR-106a, miR-146, miR-155, miR-199, miR-192, miR-203, miR-205, -miR 210, -miR -212, miR -214	Diagnosis NSCLC Vs Healthy Donors	NA*	(29)
miR-378a, miR-379, miR-139-5p, miR-200-5p	Screening Panel	97.5/72/0.908	(30)
miR-151a-5p, miR-30a-3p, miR-200b-5p, miR-629, miR-100, miR-154-3p	Diagnostic Panel	96/60/0.76	
let-7b-5p, let-7e-5p, miR-23a-3p, miR-486-5p	Diagnostic Panel NSCLC Vs Healthy Donors	80.25/92.31/0.899	(31)
miR-181b-5p, miR-361b-5p	ACC Vs	80.65/91.67/0.936	
miR-10b-5p, miR-320b		23.3/90.32/0.911	

	SQQ		
miR-146-5p	Poor PFS Chemo sensitivity	NA	(32)
miR-23b-3p, miR-10b-5p, miR-21-5p + Clinical Variables	Poor survival	NA/NA/0.91	(33)
miR-1246	Progression	NA	(34)
miR-208	Radiosensitivity	NA	(35)
miR-21, miR-4257	Poor PFS	NA	(36)
miR-21, miR-155	Recurrence	NA	(37)
miR-100-5p	Cisplatin resistance	NA	(38)
miR-221-3p, miR-222-3p	Osimertinib prognosis	NA	(39)
Proteins			
EGFR	Diagnosis	NA	(42,43)
EGFR, SRC, GRB2, RALA	NA	NA	(44)
NYESO-1, EGFR, PLAP	Poor prognosis	NA	(45)
CD151, CD171, Tetraspanin 8	Diagnosis	NA	(47)
30 protein panel		75/76/0.83	
RNA			
ALK-EML4	Diagnosis	64/100	(49)
EGFR	Diagnosis	NA	(50)
DNA			
EGFR	Diagnostic	NA	(51)

Table 1. Summary of the predictive exosomal components described until now, including their utility and their values represented by sensitivity, specificity and AUC if possible.

DISCUSSION AND FUTURE PERSPECTIVES

Exosomes is a relatively new field of study; thus, no established protocols are available for their isolation or analysis. However, and despite the lack of consensus about how to work with exosomes, many groups are publishing interesting researches on the field. These facts demonstrate that although there is still a long way to run in the understanding of exosomes and their functions, exosomes can play a role not only in the diagnosis and prognosis of cancer, but can help us to understand the mechanisms of the cancer, to elucidate new targets even to function as specific drug deliverers.

We have already written about the miR-100-5p described by Qin et al. In this case, miRNA does not play only a function of prognosis biomarker but induces a change in the receptor cell producing an increase in the resistance to Cisplatin (48). Similar results were described by Yanfank et al. They saw that the upregulation of the gene TLR3 increase the cytokine production leading to pre-metastatic niche formation and metastasis. However, they saw that the activation of TLR3 in the lung epithelial cells was produced by exosomes derived from the tumour, and concretely, by different U1 snRNA presented in very high concentrations, compared to non-tumoral derived exosomes (63). This means, that exosomes do actually play a function in the tumour progression and metastasis leading to new molecules that can be targeted in order to stop the tumour development.

Exosomes mirror the lipid bilayer present in the cells due to their exocytic origin, and, due to the same reason, they would be able to carry surface proteins and fuse with the membrane of the receptor cell to release their cargo, and due to their stability, they would be able to carry molecules of interest without risk of alteration or degradation (64). Based on this idea a Phase I immunotherapeutic study was carried on using Dendritic cell derived exosomes (DEX) loaded with Melanoma associated antigen (MAGE), the results showed a good NK reactivity with low side effects (65). In another study, it was analysed the effect of a second generation DEX coupled with chemotherapy showing a better activation of T cells compared to normal DEX resulting on a better control of the tumour (66). Very few research have been done in this field, but due to the autology on the use of DEX, it is easy to think that the side effects derived from an adjuvant therapy based on exosomes will not exceed the side effects of the principal therapy.

In conclusion, exosomes are still a new field of study, where a consensus is needed to be able to include protocols in the clinical practice as it is already established for Erlotinib and Osimertinib in *EGFR* mutation detected in cfDNA. Very few is still known about how to use exosomes as adjuvants in a therapy or as drug deliverers, however, the new technologies and investigations are bringing these advances closer in time. Also, there is a lack of information about how exosomes interact with other tissues in longer distances making difficult the understanding of their function in the tumour progression and metastasis. This current review has focused on how the exosomes can be the future of biomarkers identification for diagnostic and prognostic of NSCLC specially miRNA and proteins, the most studied. A deeper study of exosomes and its composition will lead to better biomarker identification and thus, an early detection of the tumour.

BIBLIOGRAPHY

1. Pan BT, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. Cell. 1983 Jul;33(3):967–78.

2. Harding C, Heuser J, Stahl P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. J Cell Biol. 1983 Aug;97(2):329–39.

3. Chow A, Zhou W, Liu L, Fong MY, Champer J, Van Haute D, et al. Macrophage immunomodulation by breast cancer-derived exosomes requires Toll-like receptor 2-mediated activation of NF-kappaB. Sci Rep. 2014 Jul;4:5750.

4. Costa-Silva B, Aiello NM, Ocean AJ, Singh S, Zhang H, Thakur BK, et al. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. Nat Cell Biol. 2015 Jun;17(6):816–26.

5. Raimondo S, Saieva L, Corrado C, Fontana S, Flugy A, Rizzo A, et al. Chronic myeloid leukemia-derived exosomes promote tumor growth through an autocrine mechanism. Cell Commun Signal [Internet]. 2015 Feb 3;13:8. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4320527/

6. Hupfeld T, Chapuy B, Schrader V, Beutler M, Veltkamp C, Koch R, et al. Tyrosinekinase inhibition facilitates cooperation of transcription factor SALL4 and ABC transporter A3 towards intrinsic CML cell drug resistance. Br J Haematol. 2013 Apr;161(2):204–13.

7. Safaei R, Larson BJ, Cheng TC, Gibson MA, Otani S, Naerdemann W, et al. Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells. Mol Cancer Ther. 2005 Oct;4(10):1595–604.

8. Whiteside TL. Tumor-Derived Exosomes and Their Role in Tumor-Induced Immune Suppression. Vaccines. 2016 Oct;4(4).

9. Jorgensen M, Baek R, Pedersen S, Sondergaard EKL, Kristensen SR, Varming K. Extracellular Vesicle (EV) Array: microarray capturing of exosomes and other extracellular vesicles for multiplexed phenotyping. J Extracell vesicles. 2013;2.

10. Zarovni N, Corrado A, Guazzi P, Zocco D, Lari E, Radano G, et al. Integrated isolation and quantitative analysis of exosome shuttled proteins and nucleic acids using immunocapture approaches. Methods. 2015 Oct;87:46–58.

11. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in Exosome Isolation Techniques. Theranostics. 2017;7(3):789–804.

12. Kowal J, Tkach M, Théry C. Biogenesis and secretion of exosomes. Curr Opin Cell Biol [Internet]. 2014;29:116–25. Available from: http://www.sciencedirect.com/science/article/pii/S095506741400057X

13. Strauss K, Goebel C, Runz H, Mobius W, Weiss S, Feussner I, et al. Exosome secretion ameliorates lysosomal storage of cholesterol in Niemann-Pick type C disease. J Biol Chem. 2010 Aug;285(34):26279–88.

14. Ghossoub R, Lembo F, Rubio A, Gaillard CB, Bouchet J, Vitale N, et al. Syntenin-ALIX exosome biogenesis and budding into multivesicular bodies are controlled by ARF6 and PLD2. Nat Commun. 2014 Mar;5:3477.

15. van Niel G, Charrin S, Simoes S, Romao M, Rochin L, Saftig P, et al. The tetraspanin CD63 regulates ESCRT-independent and -dependent endosomal sorting during melanogenesis. Dev Cell. 2011 Oct;21(4):708–21.

16. Nazarenko I, Rana S, Baumann A, McAlear J, Hellwig A, Trendelenburg M, et al. Cell Surface Tetraspanin Tspan8 Contributes to Molecular Pathways of Exosome-Induced Endothelial Cell Activation. Cancer Res [Internet]. 2010 Feb 14;70(4):1668 LP-1678. Available from: http://cancerres.aacrjournals.org/content/70/4/1668.abstract

17. Fader CM, Sanchez DG, Mestre MB, Colombo MI. TI-VAMP/VAMP7 and VAMP3/cellubrevin: two v-SNARE proteins involved in specific steps of the autophagy/multivesicular body pathways. Biochim Biophys Acta. 2009 Dec;1793(12):1901–16.

18. Rao SK, Huynh C, Proux-Gillardeaux V, Galli T, Andrews NW. Identification of SNAREs involved in synaptotagmin VII-regulated lysosomal exocytosis. J Biol Chem. 2004 May;279(19):20471–9.

19. Ostrowski M, Carmo NB, Krumeich S, Fanget I, Raposo G, Savina A, et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. Nat Cell Biol. 2010 Jan;12(1):13–9.

20. Zhao H, Yang L, Baddour J, Achreja A, Bernard V, Moss T, et al. Tumor microenvironment derived exosomes pleiotropically modulate cancer cell metabolism. Dang C Van, editor. Elife [Internet]. 2016 Feb;5:e10250. Available from: https://dx.doi.org/10.7554/eLife.10250

21. Kharmate G, Hosseini-Beheshti E, Caradec J, Chin MY, Tomlinson Guns ES. Epidermal Growth Factor Receptor in Prostate Cancer Derived Exosomes. Nie D, editor. PLoS One [Internet]. 2016 May 6;11(5):e0154967. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4859494/

22. Nilsson J, Skog J, Nordstrand A, Baranov V, Mincheva-Nilsson L, Breakefield XO, et al. Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer. Br J Cancer [Internet]. 2009 May 19;100(10):1603–7. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2696767/

23. Grange C, Tapparo M, Collino F, Vitillo L, Damasco C, Deregibus MC, et al. Microvesicles released from human renal cancer stem cells stimulate angiogenesis and formation of lung premetastatic niche. Cancer Res. 2011 Aug;71(15):5346–56.

24. Peinado H, Alečković M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med [Internet]. 2012 Jun;18(6):883–91. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3645291/

25. Ambudkar S V, Sauna ZE, Gottesman MM, Szakacs G. A novel way to spread drug resistance in tumor cells: functional intercellular transfer of P-glycoprotein (ABCB1). Trends Pharmacol Sci [Internet]. 2005;26(8):385–7. Available from: http://www.sciencedirect.com/science/article/pii/S0165614705001318

26. Wang J, Hendrix A, Hernot S, Lemaire M, De Bruyne E, Van Valckenborgh E, et al. Bone marrow stromal cell{\textendash}derived exosomes as communicators in drug resistance in multiple

myeloma cells. Blood [Internet]. 2014;124(4):555–66. Available from: http://www.bloodjournal.org/content/124/4/555

27. Roberg-Larsen H, Lund K, Seterdal KE, Solheim S, Vehus T, Solberg N, et al. Mass spectrometric detection of 27-hydroxycholesterol in breast cancer exosomes. J Steroid Biochem Mol Biol. 2016 Feb;

28. Yi H, Zheng X, Song J, Shen R, Su Y, Lin D. Exosomes mediated pentose phosphate pathway in ovarian cancer metastasis: a proteomics analysis. Int J Clin Exp Pathol. 2015;8(12):15719–28.

29. D'Alessandro A, Amelio I, Berkers CR, Antonov A, Vousden KH, Melino G, et al. Metabolic effect of TAp63alpha: enhanced glycolysis and pentose phosphate pathway, resulting in increased antioxidant defense. Oncotarget. 2014 Sep;5(17):7722–33.

30. Alipoor SD, Mortaz E, Garssen J, Movassaghi M, Mirsaeidi M, Adcock IM. Exosomes and Exosomal miRNA in Respiratory Diseases. Mediators Inflamm. 2016;2016:5628404.

31. Go H, Jang J-Y, Kim P-J, Kim Y-G, Nam SJ, Paik JH, et al. MicroRNA-21 plays an oncogenic role by targeting FOXO1 and activating the PI3K/AKT pathway in diffuse large B-cell lymphoma. Oncotarget. 2015 Jun;6(17):15035–49.

32. Hong JY, Hong ME, Choi MK, Kim YS, Chang W, Maeng CH, et al. The impact of activated p-AKT expression on clinical outcomes in diffuse large B-cell lymphoma: a clinicopathological study of 262 cases. Ann Oncol Off J Eur Soc Med Oncol. 2014 Jan;25(1):182–8.

33. Yan L-X, Huang X-F, Shao Q, Huang M-Y, Deng L, Wu Q-L, et al. MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. RNA. 2008 Nov;14(11):2348–60.

34. Nielsen BS, Jorgensen S, Fog JU, Sokilde R, Christensen IJ, Hansen U, et al. High levels of microRNA-21 in the stroma of colorectal cancers predict short disease-free survival in stage II colon cancer patients. Clin Exp Metastasis. 2011 Jan;28(1):27–38.

35. Jenjaroenpun P, Kremenska Y, Nair VM, Kremenskoy M, Joseph B, Kurochkin I V. Characterization of RNA in exosomes secreted by human breast cancer cell lines using next-generation sequencing. PeerJ. 2013;1:e201.

36. Silva J, Garcia V, Rodriguez M, Compte M, Cisneros E, Veguillas P, et al. Analysis of exosome release and its prognostic value in human colorectal cancer. Genes Chromosomes Cancer. 2012 Apr;51(4):409–18.

37. Kahlert C, Melo SA, Protopopov A, Tang J, Seth S, Koch M, et al. Identification of double-stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer. J Biol Chem. 2014 Feb;289(7):3869–75.

38. Gallo A, Tandon M, Alevizos I, Illei GG. The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. PLoS One. 2012;7(3):e30679.

39. Rabinowits G, Gerçel-Taylor C, Day JM, Taylor DD, Kloecker GH. Exosomal MicroRNA: A Diagnostic Marker for Lung Cancer. Clin Lung Cancer [Internet]. 2016 Jun 12;10(1):42–6. Available from: http://dx.doi.org/10.3816/CLC.2009.n.006

40. Cazzoli R, Buttitta F, Di Nicola M, Malatesta S, Marchetti A, Rom WN, et al. microRNAs derived from circulating exosomes as noninvasive biomarkers for screening and diagnosing lung cancer. J Thorac Oncol. 2013 Sep;8(9):1156–62.

41. Jin X, Chen Y, Chen H, Fei S, Chen D, Cai X, et al. Evaluation of Tumor-Derived Exosomal miRNA as Potential Diagnostic Biomarkers for Early-Stage Non-Small Cell Lung Cancer Using Next-Generation Sequencing. Clin Cancer Res. 2017 Sep;23(17):5311–9.

42. Yuwen D-L, Sheng B-B, Liu J, Wenyu W, Shu Y-Q. MiR-146a-5p level in serum exosomes predicts therapeutic effect of cisplatin in non-small cell lung cancer. Eur Rev Med Pharmacol Sci. 2017 Jun;21(11):2650–8.

43. Liu Q, Yu Z, Yuan S, Xie W, Li C, Hu Z, et al. Circulating exosomal microRNAs as prognostic biomarkers for non-small-cell lung cancer. Oncotarget. 2017 Feb;8(8):13048–58.

44. Yuan D, Xu J, Wang J, Pan Y, Fu J, Bai Y, et al. Extracellular miR-1246 promotes lung cancer cell proliferation and enhances radioresistance by directly targeting DR5. Oncotarget. 2016;

45. Tang Y, Cui Y, Li Z, Jiao Z, Zhang Y, He Y, et al. Radiation-induced miR-208a increases the proliferation and radioresistance by targeting p21 in human lung cancer cells. J Exp Clin Cancer Res. 2016;35:7.

46. Dejima H, Iinuma H, Kanaoka R, Matsutani N, Kawamura M. Exosomal microRNA in plasma as a non-invasive biomarker for the recurrence of non-small cell lung cancer. Oncol Lett. 2017 Mar;13(3):1256–63.

47. Munagala R, Aqil F, Gupta RC. Exosomal miRNAs as biomarkers of recurrent lung cancer. Tumour Biol. 2016 Aug;37(8):10703–14.

48. Qin X, Yu S, Zhou L, Shi M, Hu Y, Xu X, et al. Cisplatin-resistant lung cancer cell-derived exosomes increase cisplatin resistance of recipient cells in exosomal miR-100-5p-dependent manner. Int J Nanomedicine. 2017;12:3721–33.

49. Giallombardo M, Jorge Chacartegui J, Reclusa P, Van Meerbeeck JP, Alessandro R, Peeters M, et al. Follow up analysis by exosomal miRNAs in EGFR mutated non-small cell lung cancer (NSCLC) patients during osimertinib (AZD9291) treatment: A potential prognostic biomarker tool. ASCO Meet Abstr [Internet]. 2016 May;34(15_suppl):e23035+. Available from: http://meeting.ascopubs.org/cgi/content/abstract/34/15_suppl/e23035

50. Zhou L, Lv T, Zhang Q, Zhu Q, Zhan P, Zhu S, et al. The biology, function and clinical implications of exosomes in lung cancer. Cancer Lett. 2017 Aug;407:84–92.

51. Mirzaei H, Masoudifar A, Sahebkar A, Zare N, Sadri Nahand J, Rashidi B, et al. MicroRNA: A novel target of curcumin in cancer therapy. J Cell Physiol. 2017 Jun;

52. Yamashita T, Kamada H, Kanasaki S, Maeda Y, Nagano K, Abe Y, et al. Epidermal growth factor receptor localized to exosome membranes as a possible biomarker for lung cancer diagnosis. Pharmazie. 2013 Dec;68(12):969–73.

53. Huang S-H, Li Y, Zhang J, Rong J, Ye S. Epidermal growth factor receptor-containing exosomes induce tumor-specific regulatory T cells. Cancer Invest. 2013 Jun;31(5):330–5.

54. Clark DJ, Fondrie WE, Yang A, Mao L. Triple SILAC quantitative proteomic analysis reveals differential abundance of cell signaling proteins between normal and lung cancer-derived exosomes. J Proteomics. 2016 Feb;133:161–9.

55. Sandfeld-Paulsen B, Aggerholm-Pedersen N, Baek R, Jakobsen KR, Meldgaard P, Folkersen BH, et al. Exosomal proteins as prognostic biomarkers in non-small cell lung cancer. Mol Oncol. 2016 Dec;10(10):1595–602.

56. Bard MP, Hegmans JP, Hemmes A, Luider TM, Willemsen R, Severijnen L-AA, et al. Proteomic analysis of exosomes isolated from human malignant pleural effusions. Am J Respir Cell Mol Biol. 2004 Jul;31(1):114–21.

57. Melton L. Protein arrays: proteomics in multiplex. Nature. 2004 May;429(6987):101–7.

58. Jakobsen KR, Paulsen BS, Baek R, Varming K, Sorensen BS, Jorgensen MM. Exosomal proteins as potential diagnostic markers in advanced non-small cell lung carcinoma. J Extracell vesicles. 2015;4:26659.

59. Rolfo C, Laes JF, Reclusa P, Valentino A, Lienard M, Gil-Bazo I, et al. P2.01-093 Exo-ALK Proof of Concept: Exosomal Analysis of ALK Alterations in Advanced NSCLC Patients. J Thorac Oncol [Internet]. 2017 Sep 14;12(1):S844–5. Available from: http://dx.doi.org/10.1016/j.jtho.2016.11.1145

60. Krug AK, Karlovich C, Koestler T, Brinkmann K, Spiel A, Emenegger J, et al. Abstract B136: Plasma EGFR mutation detection using a combined exosomal RNA and circulating tumor DNA approach in patients with acquired resistance to first-generation EGFR-TKIs. Am Assoc Cancer Res [Internet]. 2016;14(12 Supplement 2):B136--B136. Available from: http://mct.aacrjournals.org/content/14/12_Supplement_2/B136

61. Thakur BK, Zhang H, Becker A, Matei I, Huang Y, Costa-Silva B, et al. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. Cell Res. 2014;24(6):766–9.

62. Lyden DD. Tests for Double-Stranded DNA Fragments in Exosomes Could Lead to Earlier, Easier Cancer Diagnoses and Targeted Treatments [Internet]. Newsroom Weill Cornell Medicine. 2014 [cited 2017 Sep 14]. p. 1. Available from: http://news.weill.cornell.edu/news/2014/05/tests-for-double-stranded-dna-fragments-in-exosomes-could-lead-to-earlier-easier-cancer-diagnoses-an

63. Liu Y, Gu Y, Han Y, Zhang Q, Jiang Z, Zhang X, et al. Tumor Exosomal RNAs Promote Lung Pre-metastatic Niche Formation by Activating Alveolar Epithelial TLR3 to Recruit Neutrophils. Cancer Cell. 2016 Aug;30(2):243–56.

64. Johnsen KB, Gudbergsson JM, Skov MN, Pilgaard L, Moos T, Duroux M. A comprehensive overview of exosomes as drug delivery vehicles - endogenous nanocarriers for targeted cancer therapy. Biochim Biophys Acta. 2014 Aug;1846(1):75–87.

65. Morse MA, Garst J, Osada T, Khan S, Hobeika A, Clay TM, et al. A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. J Transl Med [Internet]. 2005;3(1):1–8. Available from: http://dx.doi.org/10.1186/1479-5876-3-9

66. Taieb J, Chaput N, Novault S, Terme M, Ghiringhelli F, Martin F, et al. Dendritic cell derived-exosomes boost effector CD8+ T cells in the absence of tumor induced-T regulatory cells: Synergistic antitumor effects of cyclophosphamide and exosome-based vaccines. Cancer Res [Internet]. 2014 Oct 23;65(9 Supplement):623 LP-624. Available from: http://cancerres.aacrjournals.org/content/65/9_Supplement/623.4.abstract

CHAPTER 4

Old methods in a hot topic field. Improving exosomes visualization.

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ABSTRACT

Extracellular vesicles have become a hot topic during the last years. The last discoveries regarding their composition and functionality has made them a major field of interest in cancer research. Extracellular vesicles, through the integrins expressed in the parental cells, are able to be internalize in a tissue specific manner being partially responsible of the expansion of the tumour and creation of the pre-metastatic niche formation among others. However, this specificity might lead also to new opportunities in the treatment of the tumour by using extracellular vesicles as containers for drug delivery. In order to further explore this topic, protocols and methods for extracellular vesicles tracking and observation are required. Our group has put efforts on developing a protocol for exosomes staining without ultracentrifugation, reducing the time required for the experiment and simplifying the technical issues of using an ultracentrifugation. In this paper, we also described for the first time a protocol to track exosomes internalization in vivo through confocal microscopy.

INTRODUCTION

Exosomes were first observed 50 years ago in plasma by Wolf, who mentioned them as “platelet dust” (1). Two main types of EVs have been described based on their mechanism of release and size: microvesicles and exosomes. Microvesicles are released directly from the plasma membrane of living cells. In the 1980s the Stahl’s and Johnstone’s pioneering study showed that nano-vesicles, called exosomes, were released from maturing reticulocytes carrying transferrin receptors. This process was considered a system to eliminate cellular waste (2,3). After several years the researchers change the image of exosomes as “cellular garbage collectors” to exosomes as information shuttle. Nowadays, exosomes are emerging as superbly armed vehicles for intercellular communication (4). In this work we focus on extracellular vesicles, group of nano-vesicles of less than 150 nm in diameter, secreted by all cells and circulate in the body fluids. These nano-size carriers are released in extracellular space after fusion of the multivesicular bodies (MVBs) with the plasma membrane (5,6). Exosome membrane is a lipid bilayer containing enriched sphingomyelin and decreased phosphatidylcholine (7). The exosomal content is heterogeneous and in a dynamic state, depending on the parental cells and its physiological and pathological state (8). Proteomic analyses reveal that exosomes includes endosomal, plasma, cytosolic, and nuclear proteins. Exosomal cargoes are proteins associated with membrane transport and fusion, including Rab GTPases and annexins, as well as proteins involved in exosome biogenesis (ESCRT complex, ALIX, TSG101), tetraspanins (CD63, CD9, and CD81) and other enzymes (9,10). Exosomes also contain genetic material such as mRNA, long noncoding RNA, microRNA (miRNA) and double-stranded DNA. A growing number of the papers report that tumour derived-exosomes have a key role in tumorigenesis, tumour growth, angiogenesis, metastasis, tumour immune escape and drug resistance. These vesicles are also involved in the intercellular communication between cancer cells, endothelial, stromal cells and cancer-associated fibroblasts (8). Cell-to-cell communication has a crucial role in different steps of tumour progression; the communication network that exosomes create is tumour-driven and promote tumour progression, metastasis by silencing anti-tumour immune responses or by altering stromal cell responses, supporting new vessel growth or promoting survival of tumour cells (11,12). Both direct and indirect evidence suggest that exosomes are internalized into recipient cells; exosomes transfer functional molecules from donor to target cells. Although some models of exosomes internalization by target cells have been proposed, there is not a consensus about the mechanisms. The first papers on EV internalization propose interaction with recipient cells through receptor-ligand binding (13), direct fusion with the plasma

membrane (14,15) or phagocytosis (16). Other models reported the mechanism of exosomal uptake via energy-dependent, receptor-mediated endocytosis (17,18) or micropinocytosis (19,20). Heusermann et al (2016) reported that exosomes enter into the cells as intact vesicles surfing on filopodia to sort into endocytic hot spots, traffic within endosomes, and are targeted to the endoplasmatic reticulum (21). It was also demonstrated that cellular uptake of extracellular vesicles is mediated by clathrin-independent endocytosis and micropinocytosis (22). Recently, Schneider and colleagues showed that alveolar macrophage-derived EVs were internalized by alveolar epithelial cells in a time-dose-and temperature-dependent manner. The uptake was dependent on dynamin and actin polymerization. It was neither saturable nor dependent on clathrin or receptor binding. Internalization was improved by extracellular proteins, but was inhibited by cigarette smoke extract via oxidative disruption of actin polymerization (23). Taken together these data indicate that exosomes internalization is not a passive process (24) and different kind of cells can internalize exosomes with a specific mechanism.

Actually, exosomal subcellular fate within recipient cells and their mechanisms of cargo release remains mysterious. It is unclear how cargo delivery arises inside cells, but it is possible that either a transient ‘kiss-and-run’ fusion event or full fusion between the EV and endosomal membranes might deliver cargo into the cytoplasm before lysosome fusion with subsequent degradation of cargo (25).

The last years many studies have described exosomes internalization in confocal microscopy, however, still nowadays there is no consensus on how to perform these experiments, leading to difficulties and different results. In this work, we aim to standardize a protocol for exosomes visualization through different techniques, and to improve the staining of the EVs through the optimisation of a known protocol in order to make it less time consuming, showing the results in different microscopes. Moreover, we describe for the first time a protocol to observe exosomes trafficking and internalization in living cells.

Exosomes preparation

The exosomes used in the experiments were isolated from cell culture media from the CRL-5908 cell line, a lung cancer cell line with a mutation in EGFR that confer resistance to Erlotinib and Gefitinib treatments. Cells were plated at a concentration of 25000 cells/cm^2 in RPMI-1640 L-Glutamine (Gibco. Ref 11875-093) supplemented with 10% FBS, 1% Pen-Strep, 1% L-Glutamine. After 24h of incubation, when the cells attached to the bottom, the medium was removed, and the cells were cleaned with PBS. 40 mL of exosomes depleted medium were added and incubated for 24h. This conditional medium was collected and centrifugated at 500g x 5', 3000g x 15' at 4 ºC, followed by a slow ultracentrifugation of 10000g x 30' in Quick-Seal® Polypropylene tubes (Beckman Coulter, Ref. 342414) where the medium was collected and ultracentrifugated at 100000g x 1h 45' in Quick-Seal Ultra-Clear tubes (Beckman Coulter, Ref. 344326) both in a 70ti Rotor. The pellet of each tube was resuspended in 40 μL of PBS and its concentration was measured by Pierce™ BCA Protein Assay Kit (Thermo Fisher. Ref 23225) and exosomes were stored at -20 ºC until being used.

Scanning electron microscopy

One of the problems in the workflow with exosomes is the isolation protocols. Although new methods are being explored based on membrane protein markers, the gold standard for its isolation is still the ultracentrifugation. Membrane affinity-based isolation has been implemented in standardize kits such as ExoEasy and could represent a fast and reproducible technique for the implementation of future protocols in the clinic. For this reason, we analysed 3 different conditions in the electron microscopy, single ultracentrifugation protocol, double ultracentrifugation protocol, coupling the protocol described above with a second ultracentrifugation where the tubes were filled with PBS, and a third isolation protocol with ExoEasy Maxi Kit (Qiagen. Ref 76064) coupling the 10000g x 30' centrifugation with the protocol of the kit.

After isolation, exosomes were fixed with 2.5% glutaraldehyde in phosphate buffered saline (PBS) for 10 minutes. [1] Then the solution was attached and dried on stubs. In order to inhibit charging and to improve the secondary electron signal, a 15 nm conductive gold layer was deposited by sputter coating. This procedure allowed the visualization of exosomes.

Secondary electron SEM images of the exosomes were acquired using a FEI Quanta 250 FEG environmental scanning electron microscope. The microscope was operated at 30 kV.

The double ultracentrifugation protocol produces a very pure exosomes precipitation. Vesicles with a diameter between 70 nm and 193 nm are observed in the images, with a very low quantity of protein aggregates, crystals or residues derived from the cell culture media (Fig 1a), based on the SEM images the average diameter could be determined (Fig 1b). This image is completely opposite to the one observed in the single protocol ultracentrifugation where impurities derived from the cell culture, protein aggregates and crystal precipitates were observed making it impossible to detect exosomes in the image (Fig 1c). Some impurities were found on the samples isolated through exoEasy kit, mainly homogenous salt precipitates along the sample (Fig 1d). However, spherical particles with a size correlated with the exosomes and with a good purity were clearly observed.

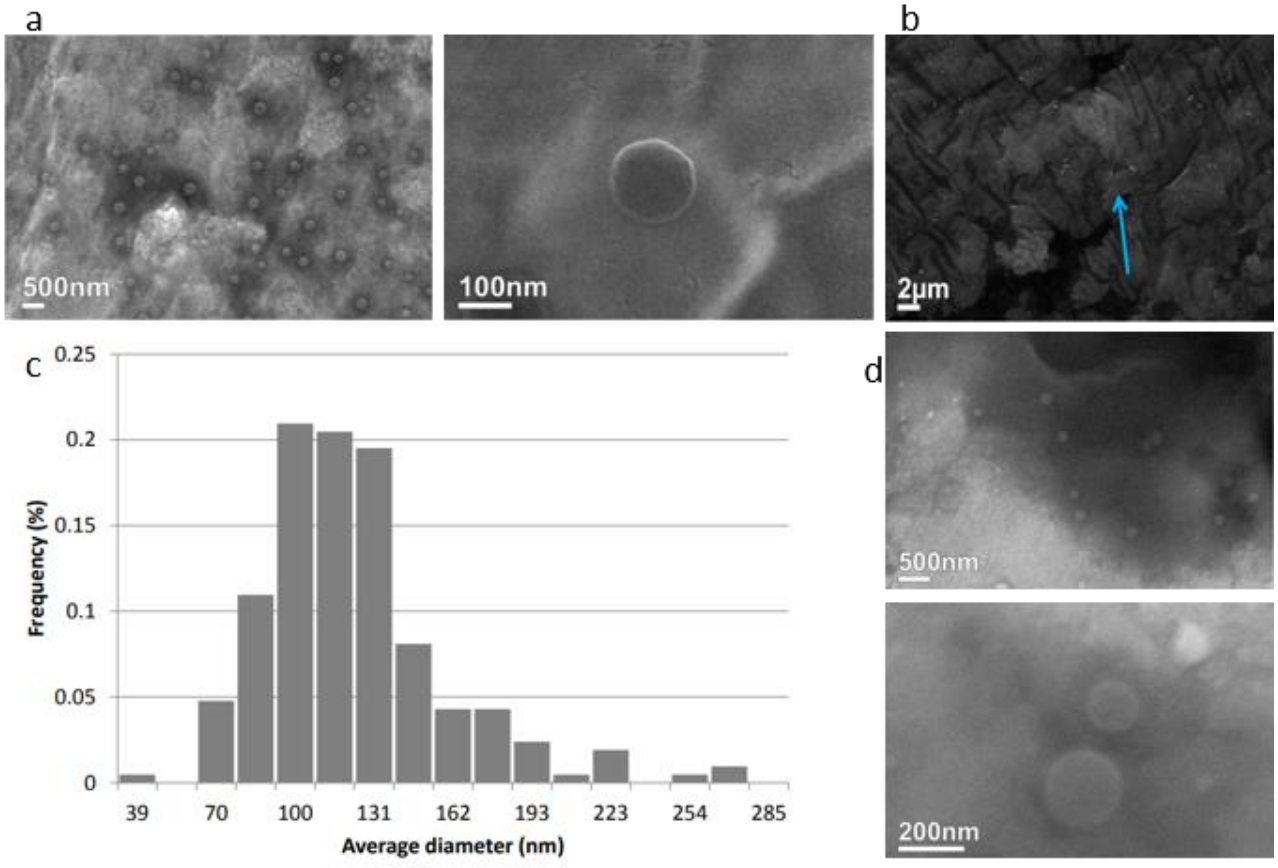

Figure 1. Scanning electron microscopy. (a) Exosomes isolated through double ultracentrifugation are observed. (b) Results of single ultracentrifugation at electron microscopy levels. The blue arrow points the protein or salt aggregates(c) Diagram showing the diameter of exosomes (nm) separated in its frequency with a peak on 100 to 140 nm. (d) Electron microscopy analysis of exosomes isolated with ExoEasy Kit.

Microscopic analysis

Nowadays exosomes are becoming a hot topic in cancer research. Its stability allows them to contain protein and genetic molecules such as double stranded DNA, messenger RNA and miRNA among others. However, in the last years, exosomes have started to be studied as functional elements in the progression of the cancer. Exosomes have been described to play pleiotropical roles in the cells where they are internalized (26). Regarding the internalization, there is still not a consensus on the best method to stain exosomes successfully and how to visualize internalization.

With this purpose, sterile coverslips were placed in 24 well plate and treated with 0.1% gelatine diluted in sterile water for 45 minutes at 37 ºC. In each well, 50000 CRL-2868 cells were plated for 24 hours. Both 20 and 50 μg/ml of exosomes were incubated with PKH26 (λexc 551 nm λem 567 nm) (Sigma-Aldrich. Ref PKH26GL-1KT) and PKH67 (λexc 490 nm λem 502 nm) (Sigma-Aldrich Ref PKH67GL-1KT).

Both colorants used for the exosomes are lipophilic colorants, and optimization of the original protocol was required prior its use with cells to get a perfect exosome staining without uptake by the cells. We prepared a dilution 1:100 of the dye in the diluent and mixed with 70 μg of exosomes diluted in PBS at the same volume as the final volume of colorant to have a 1:1 ratio of exosomes/colorant. The suspension was incubated at room temperature for 10 minutes and centrifugated at 14000g for 10 minutes at 4 ºC. The pellet is washed in PBS to avoid free colorant in the suspension and to eliminate the excess of colorant in the exosomes membrane. This step must be repeated at least twice, or until the supernatant is transparent after the centrifugation. The staining of the exosomes must be done maximal 3 hours prior to the treatment.

Cells were fixed with 4% paraformaldehyde for 1 hour. After permeabilization with Triton X-100 for 3 minutes, cells were stained with SiR-actin (100 nM, SiR-actin; λexc 640 nm λem 705 nm; Cytoskeleton Inc CY-SC001). Nuclei were visualized using VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories. Ref H-1200). To obtain detailed images of intracellular exosomes labelled with PKH26, confocal recordings were made using a Leica TSC SP8 scanning laser confocal system (Leica Microystems, Wetzlar, Germany), attached to a DMI8 inverted microscope and equipped with a 405-diode laser and a white light laser. On the white light laser, excitation wavelengths were set at 551 nm and 645 nm for the excitation of exosomes and SiR-actin respectively. DAPI staining was visualized by the 405-diode laser. An AOBS was used to select the corresponding emission spectra, i.e. 410 nm – 460 nm for

DAPI 560 nm – 640 nm for exosomes - PKH26, 658 nm – 783 nm for SiR-actin. The signal of the exosomes - PKH26 was detected using a classic PMT detector, while the DAPI and SiR-actin were visualized by a Hybrid or HyD detector in standard mode. Recordings were performed using a 63x water immersion objective (N.A. 1.2) and a pinhole of 1 AU (Airy Unit). Images were taken with a pixel size of 1024 x1024. Pixel saturation was prevented by adjusting the laser intensities and amplifier gains of the detectors. To avoid cross-talk between the three fluorophores image acquisition was performed sequentially. For each recording a z-stack was taken. Maximum intensity projections were obtained via the Leica TSC SP8 software. All detectors use grey levels, and thus the colours observed are pseudo-colours. High magnification images of exosomes labelled with PKH67 were recorded as described in the next paragraph for live cell imaging.

Contradictory, cells incubated with 20 μg/ml of exosomes stained with both colorants (Fig. 2.a.1 and 2.b.1 & .2) showed a much higher proportion of exosomes in the cells compared with the cells incubated with 50 μg/ml (Fig. 2.a.2). Both exosomes colorants give a good signal under the confocal microscope, even after 12 hours of exosomes staining, however, it seems that exosomes stained with PKH67 provides a better intensity and contrast than those with PKH26.

Live cell imaging

It is well known that exosomes are internalized by different cell types, and that this internalization can depend on exosomal membrane proteins, on direct membrane fusion, or on endocytic processes. However, the relative contribution of these mechanisms in different cell types are still unclear. Their elucidation could enhance the study of exosomes as biological drug carriers (27), or the study of specific exosomes uptake blockade to avoid pathological process like angiogenesis or metastasis. To help to the clarification, our group has put effort on developing for the first time a protocol for imaging of exosomes internalization in living cells.

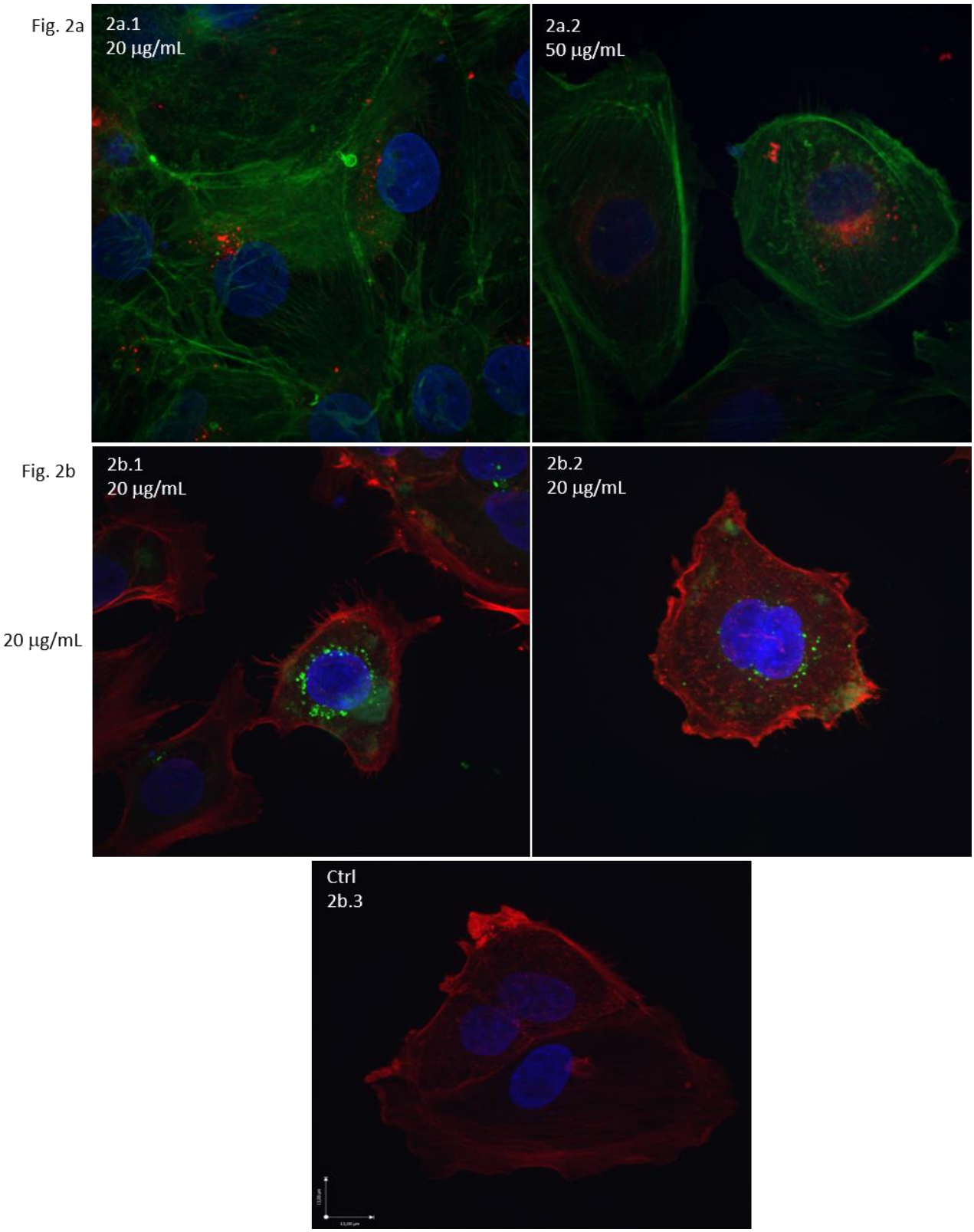

Figure 2. Confocal microscopy. (a) Confocal cell imaging of cells treated with exosomes stained with PKH26 at 20 and 50 ug/mL. **EV-PKH26 – SiR Actine - DAPI** (b) Confocal imaging of cells treated with PKH67 staining exosomes, at a concentration of 20 ug/mL and 50 ug/mL (not shown). **EV-PKH67 – SiR Actine –- DAPI**

For live imaging of exosome uptake and intracellular mobility, CRL-2868 cells were grown in 96-well plates with chimney wells (Greiner µClear 655090) at a density of 2000 cells per well in duplicates for 24 hours prior exosomes incubation. Exosomes were labelled with PKH67 as described above. Five hours prior to imaging, cells were incubated with 20 µg/ml of stained exosomes diluted in RPMI-1640. To allow robust identification of nuclei and cell contours, the cultures were incubated with the membrane-permeable dyes Hoechst 33342 (final concentration 10 µg/ml, ThermoFisher H3570) and SiR-actin (final concentration 100 nM, Cytoskeleton Inc CY-SC001) for 30 min at 37 °C one hour before starting the image acquisition. The acquisition of the images started 5 hours after the plating of the exosomes with the cells. The timing was based on previous experiments of internalization carried by our group (data not shown). However, as it is well known, the time of internalization may vary depending on the cells of origin and recipient cells.

Microscopic imaging was performed on a dual spinning disk confocal microscope (Ultra*VIEW* VoX system with Volocity software, PerkinElmer, Seer Green, United Kingdom) at 37°C and 5% CO2 in growth medium. A water immersion objective (60X, NA 1.2, Plan Apochromat) was used to acquire images at high resolution. The motorized stage was programmed to visit multiple locations in different wells with intervals of 5 minutes per location. Areas were selected based on the following criteria: isolated cell, edge of group of cells, and centre of big groups of cells. At each location and time point, the Z-focus was determined by means of image autofocus in the SiR-actin channel, after which 20 Z-planes were recorded with 1 µm spacing. For each channel (Hoechst λexc 405 λem 445, exosomes λexc 488 λem 525, SiR-actin λexc 640 λem 705 nm), acquisition parameters were set to limit photodamage while having sufficient signal-to-noise ratio during the course of an overnight experiment. Images were exported as tiff for further analysis in imageJ freeware (Schneider et al., 2012). Hyperstacks containing the time frames of each fluorescence channel were construed, after which channels were merged into RGB colour frames. The resulting image stack was exported as AVI with a frame rate of 5 frames/sec (Vid 1 & 2) and 3 frames/sec (Vid 3). Videos were edited afterwards in order to focus more on the field of interest.

Due to the exposition, cells under the laser start to present signs of death, with malformation of the nucleus, loose of the cellular shape, or movement stop of the cells. In the images captured on the isolated cell, movement of exosomes is observed in the perinuclear area which become aggregated after a short period inside the cell, moreover, a small membrane prolongation is observed during the first seconds on the bottom of the cells (Video 1). In the case of the composition of images captured on the edge of the growing cells, in Video 2, it is possible to

see high concentrations of exosomes in the perinuclear area of the peripheric cells. As described in the Video 1, exosomes seem to aggregate in the cytoplasm of the cells after long period of incubation. More interestingly, in the Video 3, it is possible to observe the internalization of the exosomes for the first time in live cell analysis. After 2 hours of imaging captures, on the left area of the image, it is possible to observe cytoskeleton filopodia, from a cell out of the area of observation, capturing exosomes that were free in the medium and mobbing them towards the body of the cell.

DISCUSSION

Isolation methods for exosomes are still a bump in the road for exosomes workflow. Currently exosomes isolation gold standard is the ultracentrifugation, but this method is time consuming and require high technical skills. Moreover, as observed in Fig. 1b when only one ultracentrifugation is performed the amount of impurities and protein aggregates is very high making it impossible to observe any exosomes. This technique is not very suitable for experiments where an accurate number of exosomes might be required. In these cases, a double ultracentrifugation will provide a purer exosome suspension, but doubling the time required for the isolation. Further studies must be performed regarding the reports of exosomes measurements to elucidate the amount of protein contamination after different isolation methods. Nonetheless, new standardize, simple and fast methods will be required if we expect exosomes to be part of the clinical practice as source of biomarkers or as drug carriers. For this reason, we performed a third isolation with exoEasy Kit from Qiagen based membrane affinity. The suspension is free of big protein contamination and crystal precipitates. Although effective, the kit presents some troubles regarding downstream research, due to its elution buffer and in its implementation in the clinical practice. The elution buffer of the kit is not replaceable apart of using an ultrafiltration column with the consequent loss of material, making the downstream applications such as DNA analysis or Western-Blot more difficult to perform. Moreover, the minimum elution volume for one flask of medium for a Maxi Kit column is 400 μL, making the sample too diluted for some downstream experiments.

Different methods have been used for the staining of exosomes, however, in our experiences, PKH colorants are the best performers for this task. Other methods described such as Calcein AM, SYTO RNA or ExoGlow, have different problems, the lack of possibilities to eliminate the excess of colorant is one of the most important together with the poor definition of the exosomes in the images (28–30). To avoid this, PKH26 and 67 binds to the lipidic membrane

of the exosomes, in short incubation time at room temperature, making the exosomes being able to pellet at lower speeds than normal ultracentrifugation, and thus, improving the time and the efforts spent in the staining and making possible to remove the excess of colorant from the supernatant, avoiding possible staining of the target cell.

Among both colorants, in our experience, the PKH67, provides a better intensity under the microscope, making the exosomes more visible without saturating the other DYEs in the sample as shown in other publications when using PK26 (31,32). The staining of exosomes with PKH67 requires more centrifugations and washings of the pellet, but this allows a very good removal of the exceeding colorant allowing clearer analysis of the samples.

Exosomes are nowadays of major interest due to its functionality. Many studies are trying to use exosomes as drug carriers due to its tissue specificity. However, in order to track the exosomes, visualization is a key step. For the first time, we have shown a live cell experiment for the internalization of exosomes, and we have developed a protocol to perform it. As it can be seen in the Video 3, the exosomes are internalized through filopodia extensions from the cell membrane. In the video, it is possible to see how the filopodia moves towards a specific area and afterwards. Specific studies are required to elucidate the exact mechanism for exosomes internalization, but the protocol described above can be of interest to the community to set-up such experiments. One of the weak spots of our protocol is the time of exposure of the cells to the laser, making them get necrotic as seen in the 3 videos, this can be avoided by less laser power (compensated by increased detector gain), less Z-stacks or less time resolution. Less time between images capture would be optimal since the movement of exosomes getting into the cell, and in the cell, itself are too fast to be captured every 5 minutes. This article tries to summarize some techniques for exosomes workflow such as an overview on its isolation, and how to proceed with a proper exosome staining and live cell imaging internalization. Our protocol, is a good first step to detect the internalization of exosomes inside the cells, however, further conformational studies must be done in order to confirm the suitability of our technique in other cells or with other exosomes, to improve the acquisition of images and reduce the toxicity for the cells, and more importantly, to elucidate what is the mechanisms of the internalization of the exosomes in the cell.

BIBLIOGRAPHY

1. Wolf P. The nature and significance of platelet products in human plasma. Br J Haematol. England; 1967 May;13(3):269–88.

2. Pan BT, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. Cell. United States; 1983 Jul;33(3):967–78.

3. Harding C, Heuser J, Stahl P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. J Cell Biol. United States; 1983 Aug;97(2):329–39.

4. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol. United States; 2013 Feb;200(4):373–83.

5. Kowal J, Tkach M, Théry C. Biogenesis and secretion of exosomes. Curr Opin Cell Biol [Internet]. 2014;29:116–25. Available from: http://www.sciencedirect.com/science/article/pii/S095506741400057X

6. Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu Rev Cell Dev Biol. United States; 2014;30:255–89.

7. Laulagnier K, Motta C, Hamdi S, Roy S, Fauvelle F, Pageaux J-F, et al. Mast cell- and dendritic cell-derived exosomes display a specific lipid composition and an unusual membrane organization. Biochem J. England; 2004 May;380(Pt 1):161–71.

8. Guo W, Gao Y, Li N, Shao F, Wang C, Wang P, et al. Exosomes: New players in cancer (Review). Oncol Rep. Greece; 2017 Aug;38(2):665–75.

9. Kalluri R. The biology and function of exosomes in cancer. J Clin Invest. United States; 2016 Apr;126(4):1208–15.

10. Kosaka N, Yoshioka Y, Fujita Y, Ochiya T. Versatile roles of extracellular vesicles in cancer. J Clin Invest. United States; 2016 Apr;126(4):1163–72.

11. Whiteside TL. Tumor-Derived Exosomes and Their Role in Cancer Progression. Adv Clin Chem. United States; 2016;74:103–41.

12. Lo Cicero A, Stahl PD, Raposo G. Extracellular vesicles shuffling intercellular messages: for good or for bad. Curr Opin Cell Biol. England; 2015 Aug;35:69–77.

13. Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding C V, Melief CJ, et al. B lymphocytes secrete antigen-presenting vesicles. J Exp Med. United States; 1996 Mar;183(3):1161–72.

14. Parolini I, Federici C, Raggi C, Lugini L, Palleschi S, De Milito A, et al. Microenvironmental pH is a key factor for exosome traffic in tumor cells. J Biol Chem. United States; 2009 Dec;284(49):34211–22.

15. Del Conde I, Shrimpton CN, Thiagarajan P, Lopez JA. Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. Blood. United States; 2005 Sep;106(5):1604–11.

16. Feng D, Zhao W-L, Ye Y-Y, Bai X-C, Liu R-Q, Chang L-F, et al. Cellular internalization of exosomes occurs through phagocytosis. Traffic. Denmark; 2010 May;11(5):675–87.

17. Svensson KJ, Christianson HC, Wittrup A, Bourseau-Guilmain E, Lindqvist E, Svensson LM, et al. Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1. J Biol Chem. United States; 2013 Jun;288(24):17713–24.

18. Tian T, Zhu Y-L, Hu F-H, Wang Y-Y, Huang N-P, Xiao Z-D. Dynamics of exosome internalization and trafficking. J Cell Physiol. United States; 2013 Jul;228(7):1487–95.

19. Tian T, Zhu Y-L, Zhou Y-Y, Liang G-F, Wang Y-Y, Hu F-H, et al. Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery. J Biol Chem. United States; 2014 Aug;289(32):22258–67.

20. Fitzner D, Schnaars M, van Rossum D, Krishnamoorthy G, Dibaj P, Bakhti M, et al. Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. J Cell Sci. England; 2011 Feb;124(Pt 3):447–58.

21. Heusermann W, Hean J, Trojer D, Steib E, von Bueren S, Graff-Meyer A, et al. Exosomes surf on filopodia to enter cells at endocytic hot spots, traffic within endosomes, and are targeted to the ER. J Cell Biol. United States; 2016 Apr;213(2):173–84.

22. Costa Verdera H, Gitz-Francois JJ, Schiffelers RM, Vader P. Cellular uptake of extracellular vesicles is mediated by clathrin-independent endocytosis and macropinocytosis. J Control Release. Netherlands; 2017 Sep;266:100–8.

23. Schneider DJ, Speth JM, Penke LR, Wettlaufer SH, Swanson JA, Peters-Golden M. Mechanisms and modulation of microvesicle uptake in a model of alveolar cell communication. J Biol Chem. United States; 2017 Nov;

24. Bastos N, Ruivo CF, da Silva S, Melo SA. Exosomes in cancer: Use them or target them? Semin Cell Dev Biol. England; 2017 Aug;

25. Maas SLN, Breakefield XO, Weaver AM. Extracellular Vesicles: Unique Intercellular Delivery Vehicles. Trends Cell Biol. England; 2017 Mar;27(3):172–88.

26. Reclusa P, Sirera R, Araujo A, Giallombardo M, Valentino A, Sorber L, et al. Exosomes genetic cargo in lung cancer: a truly Pandora’s box. Transl lung cancer Res. China; 2016 Oct;5(5):483–91.

27. Ha D, Yang N, Nadithe V. Exosomes as therapeutic drug carriers and delivery vehicles across biological membranes: current perspectives and future challenges. Acta Pharm Sin B [Internet]. Elsevier; 2016 Jul 8;6(4):287–96. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4951582/

28. Biosciences S. Exo-GlowTM Exosome Labeling Kits [Internet]. 2017 [cited 2018 Mar 7]. p. 12. Available from: https://www.systembio.com/wp-content/uploads/MANUAL_EXOG100A-1_EXOR200A-1_EXOC300A-1-1.pdf

29. ThermoFisher. SYTO ® RNASelect™ Green Fluorescent Cell Stain (S32703) [Internet]. 2004 [cited 2018 Mar 7]. p. 3. Available from: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/mp32703.pdf

30. Gray WD, Mitchell AJ, Searles CD. An accurate, precise method for general labeling of extracellular vesicles. MethodsX [Internet]. 2015;2:360–7. Available from: http://www.sciencedirect.com/science/article/pii/S2215016115000448

31. Muller L, Simms P, Hong C-S, Nishimura MI, Jackson EK, Watkins SC, et al. Human tumor-derived exosomes (TEX) regulate Treg functions via cell surface signaling rather than uptake mechanisms. Oncoimmunology. United States; 2017;6(8):e1261243.

32. Charrier A, Chen R, Chen L, Kemper S, Hattori T, Takigawa M, et al. Exosomes mediate intercellular transfer of pro-fibrogenic connective tissue growth factor (CCN2) between hepatic stellate cells, the principal fibrotic cells in the liver. Surgery. United States; 2014 Sep;156(3):548–55.

CHAPTER 5

Exosomes as predictive biomarkers

5.1. ExoALK: Exosomes as new predictive biomarkers in Non-Small Cell Lung Cancer

5.2. ExoEGFR. Exosomes as predictive biomarkers for EGFR detection

CHAPTER 5.1

ExoALK: Exosomes as new predictive biomarkers in Non-Small Cell Lung Cancer*

*Amplified chapter from “EML4-ALK translocation identification in RNA exosomal cargo (ExoALK) in NSCLC Patients: a novel role for liquid biopsy”

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ABSTRACT

Lung cancer treatment landscape has completely changed in the last decade. The introduction of druggable targets in the therapeutic scenario gave the opportunity to significantly improve the outcomes in the most lethal malignant disease. *EML4-ALK* translocation represents a story of success in drug development. The discovery of the aberration in tumours of patients with lung cancer in 2007 soon translated into a rapid approval of the first ALK tyrosine kinase inhibitor (TKI), crizotinib, and the subsequent early arrival of second and third generation TKIs, all happening in roughly a decade. The current standard for the identification of the target necessarily implies identification of rearrangement in the tumor tissue either by FISH or immunohistochemistry. Unfortunately, despite the efforts, a big number of non-small cell lung cancer (NSCLC) patients have no tissue available for determination. The isolation of ctDNA is a novel approach, but still not validated for *EML4-ALK* translocation. Exosomes, new members of the liquid biopsy family, carrying genetic material represent an important tool for biomarkers discovery. Our group has identified, for the first time, *EML4-ALK* translocation in exosomes (*ExoALK*), using a Next Generation Sequencing technique.

Exosomes and exosomal RNA analysis in other tumours.

RNA, proteins and, lipids can be shuttled from one cell to another via small extracellular vesicles called exosomes. Exosomes (40-100 nm sized) are constantly released from cells in both normal and pathological status, both in culture and *in vivo* (such as plasma, urine, cerebrospinal fluid, ascites, breast milk, fluid, bile, amniotic fluid, semen, saliva and sputum). These vesicles play an important role in cell-to-cell communication transferring their content through membrane receptors, suggesting a regulatory function in the recipient cells (1). Therefore, they may be a new promising class of potential non-invasive diagnostic and prognostic biomarkers in cancer.

Exosomes have different roles depending on the cells or tissue of origin as immune response, apoptosis, inflammation, angiogenesis, antigen presentation, and dissemination of oncogenes from cancer cells (2).

Regarding the exosomal cargo, until recently, the gold standard for cargo isolation was the ultracentrifugation coupled with RNA, DNA or protein extractions kit, however, in the last years some kits have been produced for direct isolation of the exosomal RNA including mRNA, miRNA and long non-coding RNA (lncRNA).

It has been demonstrated that microRNAs encapsulated in exosomes are very stable (3), this has led to studies showing that exosomal miRNAs might be great biomarkers for prognostic and predictive applications (4). These affirmations lead us to the idea that RNA might be also a good provider of information in predictive biomarkers through the identification of mutations inside the exosomes. Very few has been studied of the exosomal RNA inside the exosomes. Until know, predictive mutations in liquid biopsy has been focused on the detection of circulating tumour DNA. Nevertheless, big improvements have been done in the detection of *EGFR* mutations in liquid biopsy with the approval of the prescription of Erlotinib after the detection of EGFR in circulating tumour DNA (5). However, translocations are difficult to find in DNA, thus, the study of exosomal RNA might lead to new detection of alterations in liquid biopsy.

ALK-EML4 translocation in Non-Small Cell lung cancer.

The human Anaplastic lymphoma kinase, whose acronyms is *ALK*, is a tyrosine kinase protein with a single transmembrane domain consisting of 1,620 amino acid. (Figure 1). (Thai AA et al 2017) (Tsuyama N et al 2017) ALK extracellular domain contains 2 meprin, A5 protein, and protein phosphatase μ domains and a putative ligand-binding domain (6). From the phylogenetic point of view, ALK is relatively isolated in tyrosine kinase superfamily; its kinase domain show 50% identity with the proto-oncoprotein ROS1.

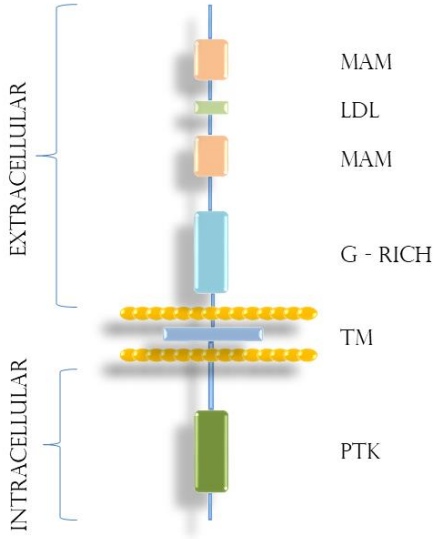

Figure 1. Schematic representation of human ALK structure. Starting from N – terminal region, two MAM, one LDLa and one G – Rich domains are showed. The only transmembrane (TM) domain connect the extracellular ALK structure with intracellular C – terminal protein tyrosine kinase domain (PTK).

In approximately 5% (ranging from 3% to 7%) of NSCLC patients occur an actionable driver *ALK* gene rearrangements, specifically adenocarcinomas and usually in younger and light or never smokers (7–10). From the morphological point of view, the tumour harbouring an ALK rearrangements are characterized by signet ring cells with a cribriform pattern and an abundant extracellular mucin component (7).

The oncogenic echinoderm microtubule-associated protein-like 4 gene *(EML4)-ALK* fusion transcript represent the most common *ALK* rearrangement and is caused by an intrachromosomal inversion within the short arm of chromosome 2 (7,8). The breakpoint of *ALK* is constant at exon 20, but the breakpoints variability in the amino terminal domain of *EML4* (from exon 2 to exon 20) lead to more than 21 different *EML4-ALK* gene fusions products (7,9,10). Other rare fusion *ALK* partners are represented by Kinesin family member 5B, the tropomyosin receptor kinase *(TRK)*-fused gene, and kinesin light chain 1 (11). The presence of an *ALK* rearrangements in NSCLC patients represent a positive predictive factor for the target treatment with a first (e.g. crizotinib) and second (e.g. ceritinib, brigantinib, alectinib) generation *ALK* inhibitors, characterized by approximately 60%-70% objective response rates and by a longer progression-free survival compared with standard chemotherapy (12).

Current detection methods for the translocation

In the crizotinib PROFILE 1014 clinical trials, the FISH assay carried out by using “breakapart” probes (ALK FISH Break-Apart Kit; Vysis, Abbott Molecular, Abbott Park, Illinois) was selected as the companion diagnostic test. This FDA approved test consists of 3’ end red and 5’ end green probes flanking the conserved ALK gene translocation breakpoint. While the normal cells are characterized by a yellow signal, neoplastic cells (with an ALK rearrangements) show a split red and green signals (Figure 2) (13,14). When 15% or more tumour cell nuclei exhibit a distinctly separated green and red signals (or isolated red signals) the analysed sample is defined as positive for an *ALK* translocation. The application of these analytical threshold on routine cito – histological samples represent one of the most discussed point to define a clinical validity of this approach. Another limitation of FISH based assays was represented by the use of targeted probes, leading to the non – identification of rarer, but clinically relevant, translocations. Also, to overcome these limitations, more recently, in the ceritinib and alectinib clinical trial programs the Ventana ALK (D5F3) immunohistochemistry (IHC) companion diagnostic assay (Medical Systems Inc, Tucson, Arizona), was selected as a FISH cost-effective surrogate and received the FDA approval (15).

Since both FISH that IHC approaches are validated for diagnostic use on Formalin Fixed Paraffin Embedded (FFPE) samples, currently, ALK testing guidelines recommend, also for cytological samples the FFPE preparation (cell block) and in cases of equivocal immunostaining a confirmatory FISH assay is suggested (16)

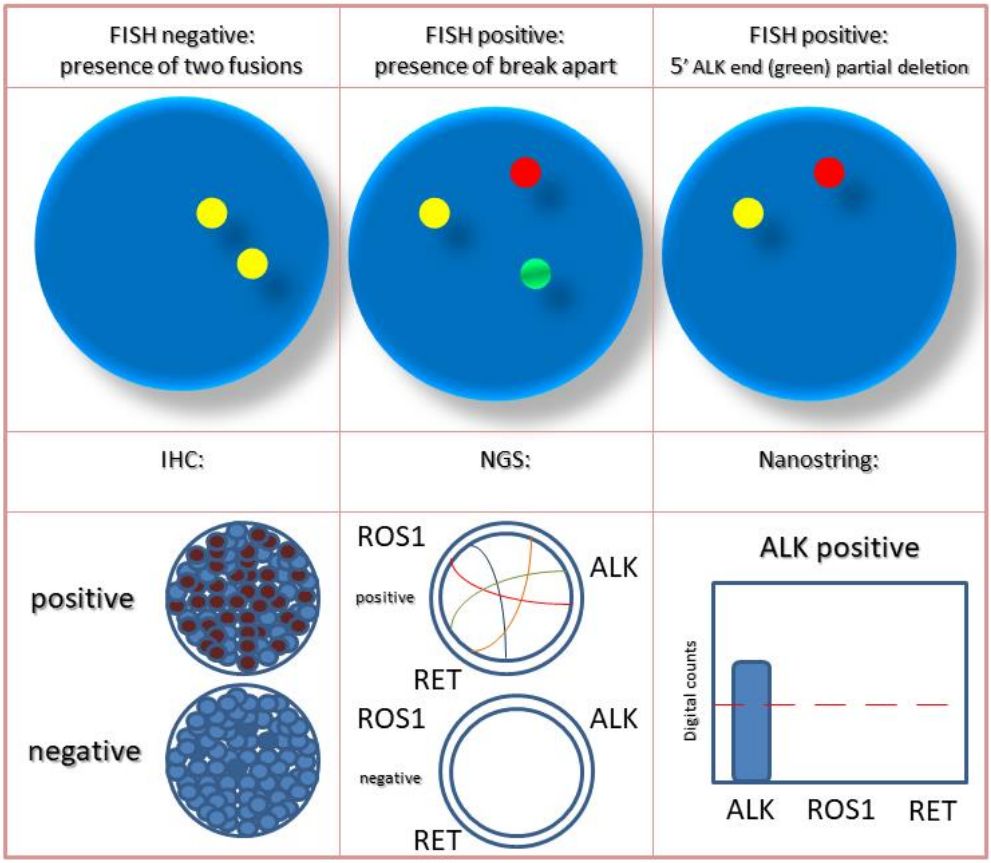

Figure 2. In the upper part of the figure are schematically represented a not – rearranged ALK gene (FISH negative – yellow), rearranged ALK gene (FISH positive, split signal red and green probes) and a partial deletion of 5' end of ALK gene (green probe – FISH positive). In the bottom part, a schematization of positive (brown) and negative (blue) IHC signals, positive (circle plot with interconnection) and negative (circle plot without interconnection) NGS results and nanostring positive ALK digital count of fusion mRNA are represented.

Issues for the detection of ALK-EML4 translocation

In more than 80% of NSCLC metastatic patients the samples are collected by using minimally invasive procedures (small biopsy or cytological samples) making difficult the recovery of an adequate quantity of biological material to exploit the complete characterization of NSCLC samples, not only from the morphological point of view, but also for *ALK* translocations, that within Epidermal Growth Factor Receptor mutations and PD – L1 expression assessment represent the fundamental elements to define the most appropriate therapeutic strategies

(17,18). In some cases that vary from low percentages up to 40% the tissue material collected results inadequate to define the ALK translocations and other clinical relevant predictive biomarkers status and in these landscape the implementation of new generation technologies approaches, based on high throughput, blood derived RNA procedures, such as, NGS and the nCounter methodology (NanoString Technologies), may represent robust alternatives to FISH and IHC testing enabling the simultaneous screening of large numbers of other actionable gene fusions and alterations (Figure 2) (7). The main limitation for the implementation of these new approaches in clinical setting is represented by the need to recover a good quality and quantity circulating tumour derived RNA, which nowadays is not feasible.

In this setting, and thanks to the new methods for exosomes workflow, exosomal RNA may represent an optimal source of Next Generation Technologies analysable RNA to define the *ALK* translocation status (7,18,19).

MATERIAL AND METHODS

Clinical data and patients selection

Exosomal RNA material is extracted from plasma from 19 patients with diagnosed NSCLC at different disease stages. The blood samples were collected from patients according to the following criteria: confirmed diagnosis of NSCLC with stage IIIa-b or stage IV at the time of blood collection, independent of the metastasis type, age 18 or older at the time of informed consent signature, smoker status independent and both naïve and under treatment at the time of diagnosis. Out of the 19 patients included in the study, 16 of the patients were positive for *ALK-EML4* translocation detected in tissue and 3 were negative.

Exosomal RNA extraction and yield measurement

The kit used is distributed by QIAGEN, ExoRNeasy Serum/Plasma Midi KIT (Cat No./ID: 77044) (Fig. 3). Once the sample is collected, the plasma is separated through centrifugation, this step may variate on dependence of the protocol of the biobank. The starting volume for the samples ranged between 800 μL until 1 mL per patient. The first step of the Kit was modified to follow our standards for exosomes workflow. Instead of performing a 0.8 μM filtration and to avoid the loss of material and volume, we performed 3 serial centrifugations at

4 ºC as described previously for cell culture media, of 500g x 5', 3000g x 15' and 10000g x 30', collecting in every step the supernatant and avoiding contamination by the pellet. After the last centrifugation the isolation is performed following the protocol provided by QIAGEN. The RNA yield is assessed in a Nanodrop ND-1000, Thermo Scientific, with 1 μL of sample.

NGS

The fusions are analysed using the Ion Ampliseq RNA Fusion Lung Cancer panel (Thermo Fisher Scientific, Waltham, USA). Briefly 10 ng of RNA are retrotranscribed into cDNA, the cDNA is amplified using the specific primer pool present in the kit. The primers used for amplification are then partially digested by the Pfu enzyme. The product of digestion is then ligated with corresponding barcoded adapters and purified using Ampure Beads (Agilent Genomics Inc). The product of purification is amplified for 5 more cycles and subsequently purified using Ampure Beads. The quality of the libraries was assessed using the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, Waltham, USA). 10 pM of each library was loaded into the IonChef system (Thermo Fisher Scientific, Waltham, USA) for the emulsion polymerase chain reaction (PCR) and then loaded in the chip. The libraries are then sequenced on either the PGM or the Ion S5 (Thermo Fisher Scientific, Waltham, USA).

The quality of the data is assessed using the Torrent suite software (Thermo Fisher Scientific, Waltham, USA) associated with the sequencing machine. The minimum read length for fusion detection is set to 50bp (base pair) under this value, the reads are too short to overlap targeted fusion breakpoint. In addition, to assess the detection quality, at least 20 000 reads are required per sample.

Analysis steps

Alignment of reads on a reference sequences set. Those sequences were built by first getting CDS sequence of targeted gene fusion, then every fusion was built taking into account the different combinations. An additional file indicating for each sequence the exact location of breakpoint was also added to allow the software to recognize those breakpoints during the alignment process.

The counting of fusions identified. For those particular reads, only the ones overlapping expected breakpoint are kept. A fusion is labelled as positive if at least 20 reads of 50bp length min are aligned over fusion breakpoint and at least 20% of reads are aligned over control sequences (wild types).

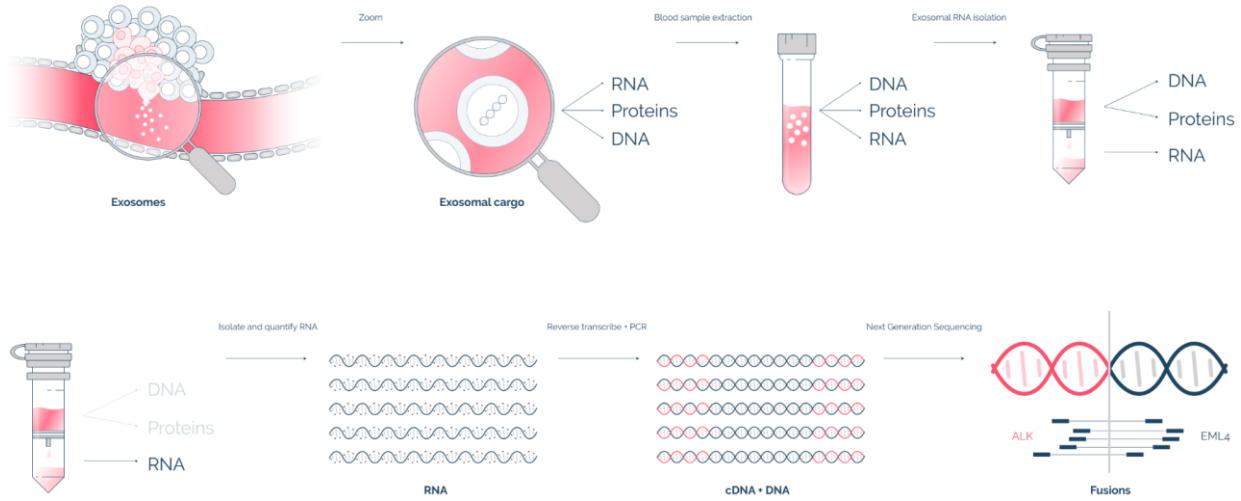

Figure 3. Schematic representation of *ExoALK* proof of concept study

RESULTS

RNA concentration

The RNA concentration ranged from 0.5 mg/ml until 121 mg/mL. Two samples provide no concentration in the nanodrop due to melting during the shipment, but new samples were provided to substitute them. No correlation was found between the RNA yield and the type of sample or its clinical variables. Table 1 shows the RNA concentration from every patient.

Exo ALK-EML4 identification

Out of the 19 patients included in the study, 16 patients were positive for *ALK-EML4* translocation, the 3 others had no presence of the fusion gene. After the analysis of the RNA samples, 2 out of the 19 samples were non-informative due to degradation being these positive for tissue. From the other 17 samples, 9 were detected as positive and 8 were detected as negative. When matching the results of the tissue and the liquid biopsy, we found that out of the 15 positive samples in tissue, 9 were positive too in liquid biopsy and 6 were detected negative reporting a sensitivity of 63%. Regarding the 3 patients negative for the fusion gene, the 3 of them were reported as negative in the liquid biopsy analysis giving a specificity of 100% (Table 1).

		ALK-EML4 FISH detection in Tissue	
		Positive (16)	**Negative (3)**
ALK-EML4 Exosomal RNA detection. OncoDNA/UZA	**Positive (9)***	9	0
	Negative (8)	5	3
		Sensitivity 64%	**Specificity 100%**

*2 RNA (positive) samples get degraded during the delivery

Table 1. Concordance between tissue and *ExoALK*

DISCUSSION

Exosomes may be the future source of mutational status for cancer patients, however, the work with exosomes is not fully standardized. When the yield of exosomes is measured and due to the presence of phenol in the Qiazol, the 260/280 ratio in the Nanodrop is not accurate, and thus is difficult to assess the purity of your RNA. Moreover, the fusion gene *ALK-EML4* presents over 20 variants, in which different exons are present from *ALK* and *EML4* thus, making more difficult a genetic analysis for all the isoforms.

Thanks to the effort of different companies, new kits are available for the exosomes workflow, allowing the standardization of protocols such as the exosomal RNA extraction, opening a new window for the identification of exosomal RNA in the clinic routine as a minimum invasive procedure for the substitution of the current tissue biopsy, and in this study we demonstrated that, and without any special formation and due to its simplicity the exosomes analysis could be implemented in the clinic. Moreover, we have demonstrated for the first time the presence of the fusion gene *ALK-EML4* through NGS inside the exosomes. This discovery opens the windows to the possibility of making a full gene analysis only based on the exosomes content, that due to their composition and concentration, provides a very stable source of genetic material.

BIBLIOGRAPHY

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1. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9(6):654–9.

2. Li M, Rai AJ, DeCastro GJ, Zeringer E, Barta T, Magdaleno S, et al. An optimized procedure for exosome isolation and analysis using serum samples: Application to cancer biomarker discovery. Methods. 2015 Oct;87:26–30.

3. Cazzoli R, Buttitta F, Di Nicola M, Malatesta S, Marchetti A, Rom WN, et al. microRNAs derived from circulating exosomes as noninvasive biomarkers for screening and diagnosing lung cancer. J Thorac Oncol. 2013 Sep;8(9):1156–62.

4. Cheng L, Sharples RA, Scicluna BJ, Hill AF. Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood. J Extracell vesicles. 2014;3.

5. cobas EGFR Mutation Test v2 [Internet]. 2016 [cited 2018 Nov 5]. p. 1. Available from: https://www.fda.gov/drugs/informationondrugs/approveddrugs/ucm504540.htm

6. Loren CE, Englund C, Grabbe C, Hallberg B, Hunter T, Palmer RH. A crucial role for the Anaplastic lymphoma kinase receptor tyrosine kinase in gut development in Drosophila melanogaster. EMBO Rep. 2003 Aug;4(8):781–6.

7. Pisapia P, Lozano MD, Vigliar E, Bellevicine C, Pepe F, Malapelle U, et al. ALK and ROS1 testing on lung cancer cytologic samples: Perspectives. Cancer Cytopathol. 2017 Nov;125(11):817–30.

8. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature. 2007 Aug;448(7153):561–6.

9. Wong DW-S, Leung EL-H, So KK-T, Tam IY-S, Sihoe AD-L, Cheng L-C, et al. The EML4-ALK fusion gene is involved in various histologic types of lung cancers from nonsmokers with wild-type EGFR and KRAS. Cancer. 2009 Apr;115(8):1723–33.

10. Kwak EL, Bang Y-J, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N Engl J Med. 2010 Oct;363(18):1693–703.

11. Pan Y, Zhang Y, Li Y, Hu H, Wang L, Li H, et al. ALK, ROS1 and RET fusions in 1139 lung adenocarcinomas: a comprehensive study of common and fusion pattern-specific clinicopathologic, histologic and cytologic features. Lung Cancer. 2014 May;84(2):121–6.

12. Tsuyama N, Sakamoto K, Sakata S, Dobashi A, Takeuchi K. Anaplastic large cell lymphoma: pathology, genetics, and clinical aspects. J Clin Exp Hematop. 2017;57(3):120–42.

13. Shaw AT, Yeap BY, Mino-Kenudson M, Digumarthy SR, Costa DB, Heist RS, et al. Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. J Clin Oncol. 2009 Sep;27(26):4247–53.

14. Savic S, Bode B, Diebold J, Tosoni I, Barascud A, Baschiera B, et al. Detection of ALK-positive non-small-cell lung cancers on cytological specimens: high accuracy of immunocytochemistry with the 5A4 clone. J Thorac Oncol. 2013 Aug;8(8):1004–11.

15. Thorne-Nuzzo T, Williams C, Catallini A, Clements J, Singh S, Amberson J, et al. A Sensitive ALK Immunohistochemistry Companion Diagnostic Test Identifies Patients Eligible for Treatment with Crizotinib. J Thorac Oncol. 2017 May;12(5):804–13.

16. Lindeman NI, Cagle PT, Beasley MB, Chitale DA, Dacic S, Giaccone G, et al. Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. Arch Pathol Lab Med. 2013 Jun;137(6):828–60.

17. Sgariglia R, Pisapia P, Nacchio M, De Luca C, Pepe F, Russo M, et al. Multiplex digital colour-coded barcode technology on RNA extracted from routine cytological samples of patients with non-small cell lung cancer: pilot study. J Clin Pathol. 2017 Sep;70(9):803–6.

18. Malapelle U, Sirera R, Jantus-Lewintre E, Reclusa P, Calabuig-Farinas S, Blasco A, et al. Profile of the Roche cobas(R) EGFR mutation test v2 for non-small cell lung cancer. Expert Rev Mol Diagn. 2017 Mar;17(3):209–15.

19. Taverna S, Giallombardo M, Gil-Bazo I, Carreca AP, Castiglia M, Chacartegui J, et al. Exosomes isolation and characterization in serum is feasible in non-small cell lung cancer patients: critical analysis of evidence and potential role in clinical practice. Oncotarget. 2016 Feb.

CHAPTER 5.2

ExoEGFR. Exosomes as predictive biomarkers for EGFR detection in NSCLC

Research ongoing

Pablo Reclusa, Karen Zwaenepoel, Marzia Pucci, Laure Sorber, Simona Taverna, Elena Durendez, Christian Rolfo

ABSTRACT

Lung cancer treatments has changed dramatically during the last years. Targeted mutations has significantly improved the outcome of NSCLC.

EGFR represents the must common targetable mutation in NSCLC with a percentage of mutation from 50% in Asian population to 15% in Caucasians. The current standard of detection is by genetic analysis of tissue biopsy or genetic analysis of circulating tumour DNA. However, the first method is commonly difficult because of the impossibility of obtaining biopsy due to the location of the tumour or the condition of the patient. Moreover, in recurrent patients developing resistance to the treatment, it becomes even more challenging.

The analysis of EGFR in liquid biopsy has been approved by the FDA, has meant an improvement in the detection of mutations. However, ctDNA represents a small percentage of the total cell-free DNA and this introduce a big variability in the assay.

The study of exosomes as predictive biomarkers might be able to solve these problems. Exosomes are released actively by the tumoral cells into the body fluids, and its composition protects the content from degradation.

Our objective is to demonstrate the presence of exosomal DNA, standardize its isolation through the design of protocols. To finish, evaluate the mutational status of three EGFR mutations in exosomal DNA, and correlate the results with the analysis performed in tissue.

INTRODUCTION

As explained in Chapter 2, lung cancer is the leading cause of cancer-related mortality in western countries with more than a million deaths every year (1). Identification of druggable targets such as *Epidermal Growth Factor Receptor* mutated non-small cell lung cancer (NSCLC), with a prevalence of 15% in European countries, and up to 50% in Asian countries, *EML4* translocation with a prevalence around 6%, *cMET* amplification or ROS1 translocation with 5% and 2% respectively, has led to an improvement on the treatment of NSCLC. (1–3).

Epidermal Growth Factor Receptor in cancer

Epidermal Growth Factor receptor (*EGFR*) also seen as *ErbB1* and *HER1* is included in a big family of tyrosine kinases and is able to activate a wide number of signalling pathways with roles in cell proliferation. (4). It is composed of 4 extracellular domains, 2 L domains and 2 CR domains, that are the EGF binding sites. The transmembrane domain links the extracellular part of the protein with the intracellular tyrosine-kinase domain and the C-terminal coil (Figure 1)

There are different mechanisms that alter the function of EGFR, and thus, its downstream pathways resulting in abnormal cell growth, and in some cases to the development of cancer. The most common *EGFR* aberrations are in-frame deletions in exon 19 (del19) and the L858R point mutation in exon 21, which lead to a constitutive activation of the kinase activity and thus, activation of its downstream pathways. However, was not until 2004 that two groups simultaneously discovered correlation of somatic mutations in patients with the response to Gefitinib therapy (5,6). After these studies, many others provide evidences of the better prognostic in those patients with *EGFR* mutation undergoing Gefitinib or Erlotinib treatment, both first generation Tyrosine Kinase Inhibitors (TKIs) compared to chemotherapy (6–8). Both treatments block the ATP binding site of *EGFR*, resulting on the blockade of the downstream cascade.

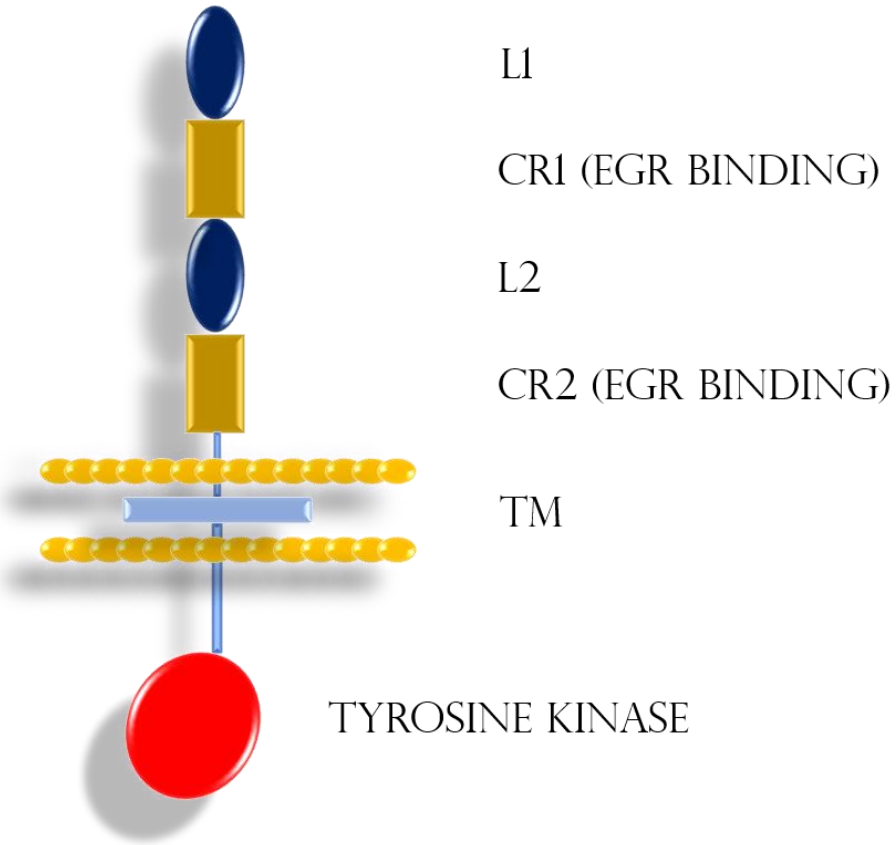


Figure 1. Schematic representation of human *EGFR* structure. Starting from L1 domain, one CR1, followed by another L2 and CR2. Both CR domains are EGR binding domains. The only transmembrane (TM) domain connect the extracellular *EGFR* structure with intracellular C – terminal protein tyrosine kinase domain.

Unfortunately, most of the patients undergoing TKI treatment progress due to the acquisition of resistance mechanisms. The T790M point mutation in exon 20 is the most common resistance mechanism, responsible for approximately 50 to 60% of cases (9–11). Second generation TKIs, as Afatanib were studied to overcome these alterations, however, high concentrations of the drug were required resulting on high toxicities (12). Third generation TKIs such as Osimertinib have been already approved for the treatment of these mutations due to their good performance mostly based on the Phase-II AURA2 clinical trial to treat patients with T790M resistance mutations (13,14). Moreover, Osimertinib has been recently approved for first line treatment in NSCLC patients with del19 or L858R mutations proving a better Overall-Survival (OS) than Erlotinib or Gefitinib treated patients in first line (15).

Current methods for EGFR detection

The clinical indications for *EGFR* TKI treatments are based on the detection of *EGFR* mutations. In 2009, the European EGFR Workshop group recommended, if possible, that clinicians should obtain tissue biopsies at time of diagnosis (16). However, as reported by Malapelle et al. a subset of this samples, 12,2% in this study, were not suitable for DNA test (17). This is because these patients are not undergoing surgery, and thus, the sample is based on minimal invasive biopsies or small cytologies, deriving on bad quality of the samples (17). Moreover, when follow up is necessary in order to understand the mutational evolution of the patient, biopsies are often dangerous for the patient due to the location of the tumour (18).

Hence, liquid biopsies have become an important complementary tool in several aspects of medicine, including oncology. In 2016, the first ctDNA based test for *EGFR* mutations was approved by the FDA (19). ctDNA is passively released by tumour cells after apoptosis or necrosis. However, and since both are natural processes in the body, the presence of ctDNA in the blood is 0,5% compared to the total circulating free DNA However, ctDNA levels are known to be highly variable and can even comprise less than 1% of the total cfDNA (20). Hence, highly sensitive techniques are necessary to detect these low abundant ctDNA fractions. Despite these data, and although it can be improved, ctDNA is nowadays a feasible alternative in those patients where biopsy is not possible, moreover, ctDNA analysis represents a good alternative for follow up by anticipating the response to a treatment.

However, and in order to complement and improve ctDNA analysis, exosomes could play an important role due to the characteristics of their biogenesis that increases the proportion of tumoral DNA compared to non-tumoral DNA. Exosomes from tumour cells are released in much higher amount compare to the normal cells. As indicated previously in chapter 3, the composition of exosomes enables too, the protection of the internal material from degradation due to DNases and RNAses present in the blood stream. Altogether, might make the identification of *EGFR* theoretically more stable and easier.

Exosomal DNA

Exosomal DNA is the last component discovered inside the exosomes in 2011 (21). However, was not until 2014 were the first mutations, *p53* and *KRAS* were described in exosomal DNA from cell lines and mice, from different tumours like melanoma, pancreatic cancer, breast cancer or lung cancer among others (22,23)**.** Moreover, this exosomal DNA seems to contain

information derived from all the chromosomes that could lead to a better understanding of the tumour prognosis.

However, based on these studies it is unclear whether the DNA is completely derived from exosomes or if it also includes DNA bound to proteins or other components in the plasma.

However, the protocols used for these experiments lack samples where the exosomes are destroyed prior to a DNase treatment, in order to ensure the complete negativity of the sample and that the DNA detected is indeed coming from the exosomes and not DNA bonded to proteins or protected by other components of the plasma, in case of in vivo samples.

In order to verify the existence of mutated exosomal DNA our group has first analysed exosomes derived from a cell line containing the T790M mutation. Moreover, two exosomal DNA isolation protocols have been compared to isolate exosomal DNA including a detergent based control in which exosomes are permeabilize prior to the isolation of DNA. We will compare the *EGFR* mutational status of matched tissue and exosomes derived from plasma samples of 19 patients.

MATERIAL AND METHODS

Cell lines

The NSCLC cell line CRL-5908 was purchased from ATCC and the cells were grown in RPMI-1640 (Thermo Fisher, US) supplemented with 10% Fetal Bovine Serum (FBS) (Thermo Fisher, US), 1% Penicillin/Streptomycin (P/S) (Thermo Fisher, US) and 1% L-Glutamine (Thermo Fisher, US) at 37 ºC with 5% of CO2. This cell line is resistant to Erlotinib through the single T790M mutation in the exon 20.

When the cell lines were cultured for exosomes isolation, the FBS used to complete the medium was ultracentrifugated at 100.000g for 90 minutes and filtered in 0,20 mM nylon filters in order to deplete the exosomes contained in the FBS.

Exosomes isolation

Cells were seeded at a concentration of 20000 cells/cm2 with standard medium. After 24 hours, the medium was removed, and the cells were washed with PBS. Exosomes depleted medium (RPMI-1640 with exosomes depleted FBS) was added to the cells. Upon reaching a confluence of 80% (24 hours), the medium was collected. Three centrifugation steps were performed at 4°C; 500 g x 5', 3000 g x 15', and 10000g x 30' at 4 ºC, to remove dead cells, apoptotic bodies and cell debris, and macrovesicles respectively. The Eppendorf centrifuge 5810R was used for the first two centrifugation steps, while the last step was performed in a Beckman Coulter OptimaTM XPN – 80 -IDV in Ultraclear tubes in a 70 rotor from Beckman Coulter. The medium was collected after every centrifugation step. In order to isolate exosomes, the resulting supernatant was ultracentrifuged at 100,000 g x 90'. The pellet was resuspended in PBS and pooled. This was repeated to eliminate protein precipitates.

Western Blot

The presence of exosomes was verified by Western blot according to the protocol previously described by Pauwels B et al. (24). The pellet was resuspended in lysis buffer, after which blocking and primary and secondary antibody incubation were performed using the SNAP id® 2.0 protein detection system (Merck Millipore, Burlington, MA, USA) according to the manufacturer's instructions. Mouse anti-ALIX and CD63 (Abcam, UK) were used as primary antibodies and Anti-mouse IgG HRP-linked (Cell signalling, US) as secondary antibody at recommended concentrations for Western-Blot. Chemiluminescent detection was performed using the LuminataTM Forte Western HRP Substrate (Merck Millipore).

Scanning electron microscopy (SEM)

Scanning electron microscopy was performed from a pellet of exosomes after double ultracentrifugation as described in Chapter 4.

Exosomal DNA isolation from cell line

Exosomal DNA was isolated using the QIAmp DNA FFPE Tissue kit (Qiagen, Germany). The pellet of exosomes was resuspended in Lysis Buffer mixed with proteinase K as per manufacturers' instruction

Patients selection

The blood samples were collected at Ospedale Universitario di Palermo, Italia and at the Antwerp University Hospital, Belgium in 10 mL EDTA tubes from patients according to the

following criteria: confirmed diagnosis of NSCLC independently of the stage, age 18 or older at the time of informed consent signature and both naïve and under treatment at the time of diagnosis. The positive patients must contain at least one of the following EGFR mutation; del19, L858R or T790M. The negative patients were NSCLC patients that match all the previous criteria but without targetable mutations. Informed consent was obtained according to the Declaration of Helsinki and with hospital ethics committee approval (Antwerp University Ethics committee n. 14/17/206). Plasma was generated within 2 h of blood collection, pooled and transported on dry ice. Sample collection, processing and storage was performed by Biobank@UZA (Antwerp, Belgium; ID: BE71030031000; Belgian Virtual Tumourbank funded by the National Cancer Plan, BBMRI-ERIC; No. Access: 1, Last: 25 September 2017) (25).

Exosomal DNA isolation from patients

In the second part of this study, a novel isolation workflow was performed to verify the presence of exosomal DNA. Isolation was performed using two EGFR WT and one EGFR mutated plasma sample. In order to permeabilize the exosomes, one mL of plasma was treated with 1% Triton X-100 solution for 30 minutes as described by Palanisamy V et al. (26).

Next, DNAse-free RNAse (Qiagen, Germany) was added to eliminate ctDNA as well as exosomal DNA. Exosomes were isolated using ExoEasy Maxi Kit (Qiagen, Germany) as per manufacturers' instructions. The exosomes were eluted in 400 mL of Xe elution buffer. DNA was isolated from the exosomes with the QIAmp DNA FFPE tissue protocol (Qiagen, Germany), as described previously.

In the last part of this study, exosomes were isolated from patient samples. Plasma samples were processed in the same manner as cell-derived exosomes. Plasma samples were centrifuged three times and exosomes were isolated by two step ultracentrifugation. The QIAmp DNA FFPE Tissue was also used for exosomal DNA isolation.

Exosomes isolation was performed through Isolated exosomal DNA from all study phases were stored at 4 ºC or -20 ºC until ddPCR analysis.

Mutational analysis of the samples

Digital droplet PCR (ddPCR) analysis was performed by the department of pathology (UZA) to assess the EGFR mutation status of the exosomal DNA. Samples derived from cell lines and patient samples processed by the novel isolation workflow were screened using the T790M

primer/probe assay from BioRad, as described by Sorber et al (27). To correct for sampling error and to increase detection rate, samples were analysed in triplicate. Exosomal DNA isolated from patients in the third phase, were screened by the T790M assay as well as one of two multiplex ddPCR assays to detect both the EGFR T790M and activating mutation, respectively.

RESULTS

Exosomes characterization

CRL-5908 derived exosomes were characterized by Western Blotting and SEM. ALIX and CD63 proteins are known as exosome markers and were detected in the pellet of exosomes after double ultracentrifugation from the conditioning medium (Figure 2a). Moreover, it is clearly visible in the images obtained through SEM, rounded vesicles ranging from 70 to 180 nm (Figure 2b).

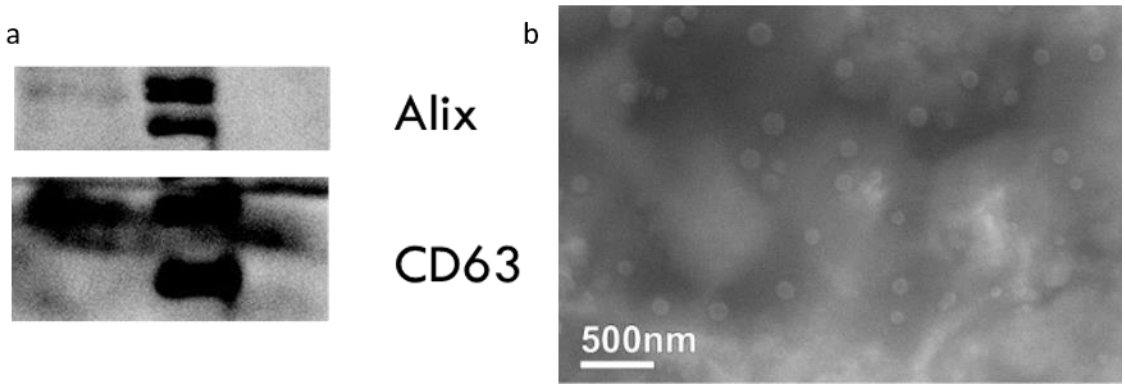


Figure 2. (a) Western-blot for the exosomes markers Alix and CD63. (b) Scanning electron microscopy showing rounded micro-vesicles with a size ranging from 70-180 nm.

Cell line analysis

DdPCR analysis of the exosomal DNA revealed that 81.6% of all DNA molecules carried the *EGFR* T790M mutation. The sensitivity of the triplicates was adequate with a value of 0,05% (EGFR mutations are detectable at a frequency of 0,05 in the total exosomal DNA) (Figure 3).

Patients analysis

In the second phase of this study, we tried to verify the exosomal DNA content. No signal of either T790M or T790 WT was detected by ddPCR analysis, indicating that no DNA was isolated (Figure 4)

The third phase of this study, consisting of exosome isolation via ultracentrifugation is currently ongoing. The results will be available at the time of the thesis presentation, or if possible, in a modified version of this thesis.

DISCUSSION

Exosomes contain a high number of molecules, such as proteins, miRNAs, RNA and DNA derived from their cell of origin, making them a suitable biomarker for diagnostic, predictive and prognostic purposes. Exosomal miRNA signatures have already been described as possible prognostic profiles with regards to response to therapy in different tumour types. Our group highlighted the potential of using exosomal RNA for the detection of *EML4-ALK* translocations (Chapter 5.1). Presently, tissue samples are the preferred matrix, due to the instability of RNA in the circulation and the technical difficulties associated with cfDNA analysis.

In 2011, exosomes were found to carry single-stranded DNA molecules (21). Several groups described the detection of long fragments of exosomal DNA. *KRAS* and *p52* mutations were detected in exosomes isolated from pancreatic cancer cell lines and patients. Furthermore, the exosomal DNA was found to originate from all chromosomes (28). Cancer associated mutations of several tumour types were detected in exosomes generated from cancer cell lines, mice, and patients (23). Similarly, our study demonstrates the presence of the *EGFR* T790M mutation in approximately 80% of DNA isolated from cell line derived exosomes, highlighting the potential of exosomes. In this study, as previously shown by other groups, we were able to detect the mutation T790M in exosomes derived from the CRL-5908 cell line in a frequency of 80,6%. This result correlates with similar results published by other groups

suggesting that exosomes carry important genetic material from the cell of origin, and thus, they can be useful as complementary biomarkers to tissue of ctDNA analysis.

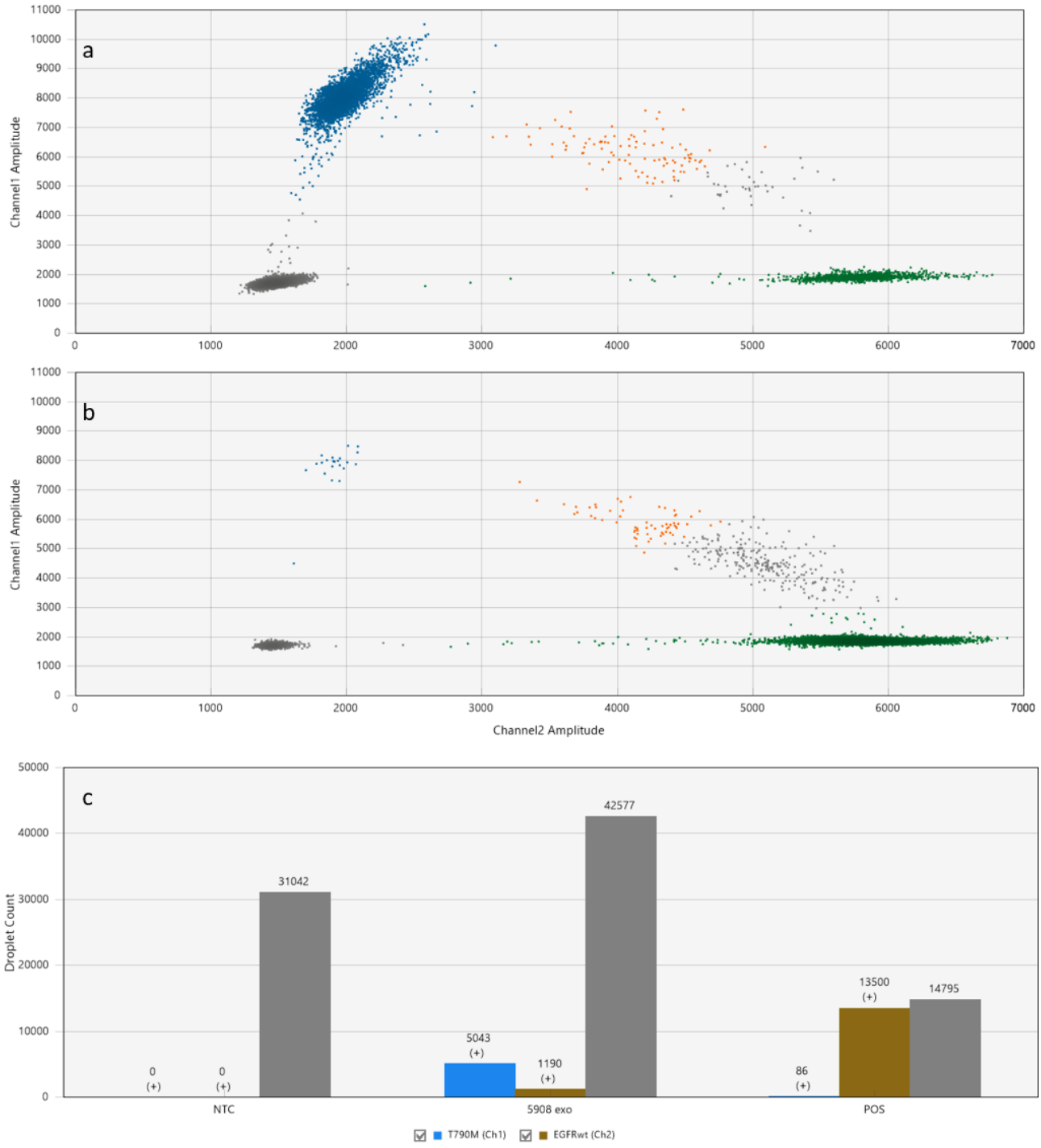


Figure 3. ddPCR of exosomal DNA isolated from the cell line CRL-5908. (a) 2D graphic from the exosomal DNA isolated from the cell line CRL-5908. **Undetermined droplets – T790M – WT – Empty droplets**. (b) 2D graphic from the positive template for the T790M mutation. **Undetermined droplets – T790M – WT – Empty droplets**. (c) Comparative graphic with

NTC- No template control; POS- Positive control and 5908exosomes. **WT – T790M –Total number of droplets.**

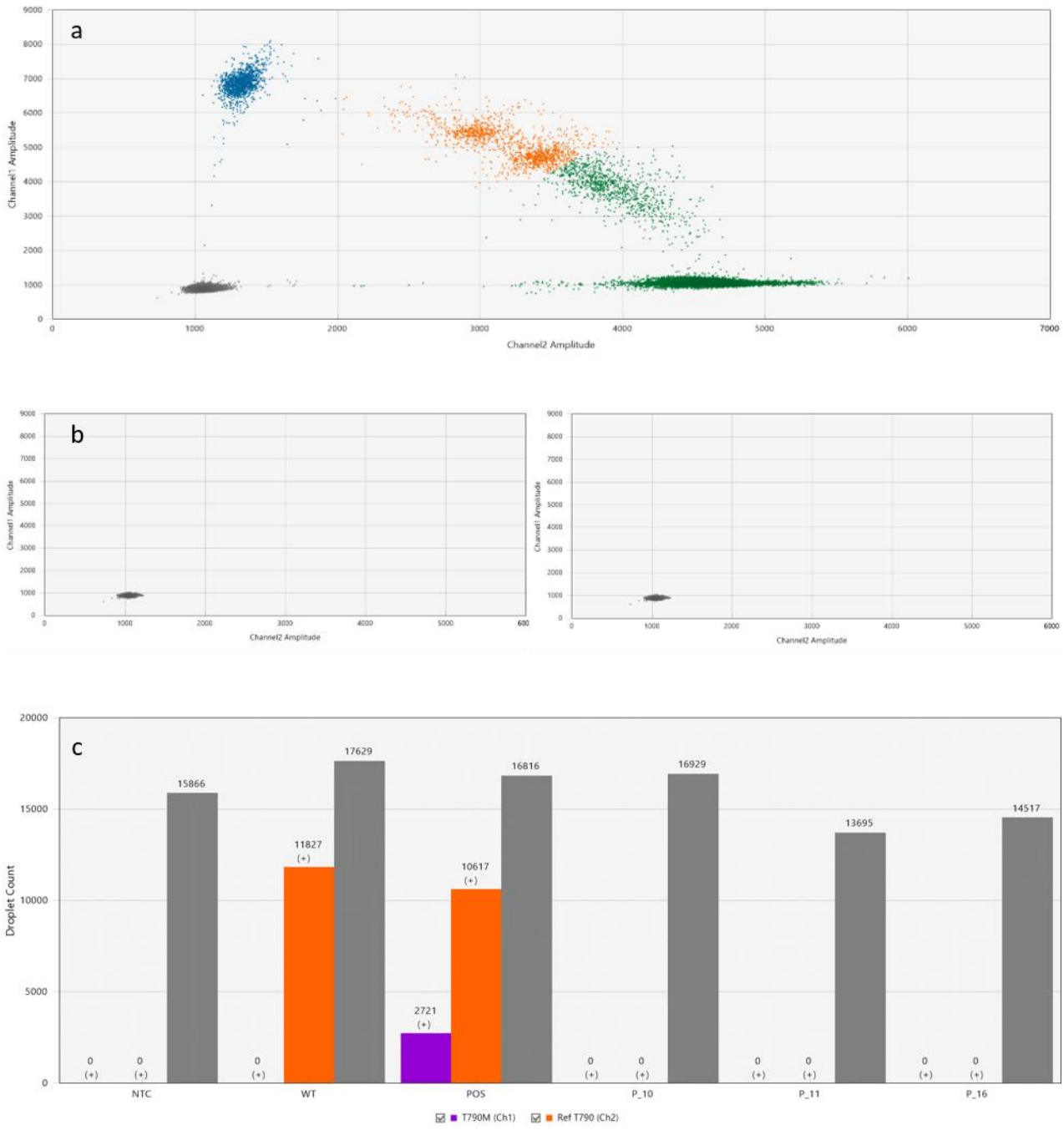


Figure 4. ddPCR analysis of exosomal DNA isolated from 3 patients through kits coupling. (a) Graphic from the positive template for the T790M mutation. **Undetermined droplets – T790M – WT – Empty droplets.** (b) Example from the P10 and P11 patients showing no DNA molecules in the sample. (c) Comparative graphic with all the samples included in the assay. NTC- No template control; WT- Wild-type; POS- Positive control; P10,11,12- Patients. **WT – T790M –Total Number of droplets.**

However, whether the DNA analysed in these studies is actually exosomal DNA, remains controversial. It is possible that a part of this DNA is bound to proteins or adhered to the membrane of microvesicles, thereby evading degradation by DNases. Furthermore, the complexity of the ultracentrifugation-based workflow, and lack of standardization hamper implementation of exosomes in the clinic. To address these issues, our group has put the effort on developing a protocol for exosomal DNA extraction based on using commercialized kits. However, and despite the previous experiments performed to set up the conditions (data not shown), no exosomal DNA could be generated by this workflow. It is possible that the ExoEasy and QIAmp FFPE DNA kit are not compatible. The ExoEasy kit uses a saline based buffer, which might, through dilution, or through some of its components, inhibit the proper function of the lysis buffer from the QIAmp FFPE DNA kit. Hence, there will be insufficient, or no lysis of the exosomes and the DNA fraction will remain inside the exosomes. This issue might be resolved by switching to a DNA extraction kit from blood as previously described or replacing the saline buffer by a lysis buffer through ultrafiltration. Further research into optimal and standardized workflows is crucial.

In the third phase of our study, which is still ongoing, we are examining exosomal DNA derived from patients. Despite the complexity, double ultracentrifugation remains the gold standard to isolate exosomes. These results will be discussed at the thesis defence.

In conclusion, the use of exosomes as predictive biomarkers is still in its early stages. Further research is necessary to investigate the potential of mutation analysis of exosomal DNA as a complementary technique for tissue and cfDNA analysis. Moreover, different studies have shown that exosomes reflect the content of their cells of origin, and thus, might content the complete mutational status of the tumour cells (29–31). As exosomes reflect the content of their cell of origin, analysis of their content could lead to a complete identification of the genetic landscape of the tumour. However, the optimization and standardization of the exosome workflow remains to be accomplished.

BIBLIOGRAPHY

1. Ridge CA, McErlean AM, Ginsberg MS. Epidemiology of lung cancer. Semin Intervent

Radiol [Internet]. 2013 Jun;30(2):93–8. Available from: https://www.ncbi.nlm.nih.gov/pubmed/24436524

2. Chan BA, Hughes BGM. Targeted therapy for non-small cell lung cancer: current standards and the promise of the future. Vol. 4, Translational Lung Cancer Research. 2015. p. 36–54.

3. Midha A, Dearden S, McCormack R. EGFR mutation incidence in non-small-cell lung cancer of adenocarcinoma histology: a systematic review and global map by ethnicity (mutMapII). Am J Cancer Res [Internet]. 2015 Aug 15;5(9):2892–911. Available from: https://www.ncbi.nlm.nih.gov/pubmed/26609494

4. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol. 2001 Feb;2(2):127–37.

5. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science. 2004 Jun;304(5676):1497–500.

6. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med [Internet]. 2004;350. Available from: http://dx.doi.org/10.1056/NEJMoa040938

7. Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, et al. EGF receptor gene mutations are common in lung cancers from “never smokers” and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A. 2004 Sep;101(36):13306–11.

8. Giaccone G. Epidermal growth factor receptor inhibitors in the treatment of non-small-cell lung cancer. J Clin Oncol. 2005 May;23(14):3235–42.

9. Pasquini G, Giaccone G. C-MET inhibitors for advanced non-small cell lung cancer. Expert Opin Investig Drugs. 2018 Apr;27(4):363–75.

10. Sequist L V, Waltman BA, Dias-Santagata D, Digumarthy S, Turke AB, Fidias P, et al. Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. Sci Transl Med. 2011 Mar;3(75):75ra26.

11. Yu HA, Arcila ME, Rekhtman N, Sima CS, Zakowski MF, Pao W, et al. Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers. Clin Cancer Res. 2013 Apr;19(8):2240–7.

12. Russo A, Franchina T, Ricciardi GRR, Picone A, Ferraro G, Zanghì M, et al. A decade of EGFR inhibition in EGFR-mutated non small cell lung cancer (NSCLC): Old successes and

future perspectives. Oncotarget [Internet]. 2015 Jun 12;6(29):26814–25. Available from: https://www.ncbi.nlm.nih.gov/pubmed/26308162

13. Goss G, Tsai C-M, Shepherd FA, Bazhenova L, Lee JS, Chang G-C, et al. Osimertinib for pretreated EGFR Thr790Met-positive advanced non-small-cell lung cancer (AURA2): a multicentre, open-label, single-arm, phase 2 study. Lancet Oncol. 2016 Dec;17(12):1643–52.

14. Kiura K, Yoh K, Katakami N, Nogami N, Kasahara K, Takahashi T, et al. Osimertinib in patients with epidermal growth factor receptor T790M advanced non-small cell lung cancer selected using cytology samples. Cancer Sci [Internet]. 2018/02/27. 2018 Apr;109(4):1177–84. Available from: https://www.ncbi.nlm.nih.gov/pubmed/29363250

15. Soria J-C, Ohe Y, Vansteenkiste J, Reungwetwattana T, Chewaskulyong B, Lee KH, et al. Osimertinib in Untreated EGFR-Mutated Advanced Non-Small-Cell Lung Cancer. N Engl J Med. 2018 Jan;378(2):113–25.

16. Pirker R, Herth FJF, Kerr KM, Filipits M, Taron M, Gandara D, et al. Consensus for EGFR mutation testing in non-small cell lung cancer: results from a European workshop. J Thorac Oncol. 2010 Oct;5(10):1706–13.

17. Malapelle U, Bellevicine C, De Luca C, Salatiello M, De Stefano A, Rocco D, et al. EGFR mutations detected on cytology samples by a centralized laboratory reliably predict response to gefitinib in non–small cell lung carcinoma patients. Cancer Cytopathol [Internet]. 2013;121(10):552–60. Available from: https://doi.org/10.1002/cncy.21322

18. Sundaresan TK, Sequist L V, Heymach J V, Riely GJ, Janne PA, Koch WH, et al. Detection of T790M, the Acquired Resistance EGFR Mutation, by Tumor Biopsy versus Noninvasive Blood-Based Analyses. Clin Cancer Res. 2016 Mar;22(5):1103–10.

19. cobas EGFR Mutation Test v2 [Internet]. 2016 [cited 2018 Nov 5]. p. 1. Available from: https://www.fda.gov/drugs/informationondrugs/approveddrugs/ucm504540.htm

20. Malapelle U, Pisapia P, Rocco D, Smeraglio R, di Spirito M, Bellevicine C, et al. Next generation sequencing techniques in liquid biopsy: focus on non-small cell lung cancer patients. Transl lung cancer Res. 2016 Oct;5(5):505–10.

21. Balaj L, Lessard R, Dai L, Cho Y-J, Pomeroy SL, Breakefield XO, et al. Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. Nat Commun. 2011 Feb;2:180.

22. Lyden DD. Tests for Double-Stranded DNA Fragments in Exosomes Could Lead to Earlier, Easier Cancer Diagnoses and Targeted Treatments [Internet]. Newsroom Weill Cornell Medicine. 2014 [cited 2017 Sep 14]. p. 1. Available from: http://news.weill.cornell.edu/news/2014/05/tests-for-double-stranded-dna-fragments-in-exosomes-could-lead-to-earlier-easier-cancer-diagnoses-an

23. Thakur BK, Zhang H, Becker A, Matei I, Huang Y, Costa-Silva B, et al. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. Cell Res. 2014;24(6):766–9.

24. Pauwels B, Korst AEC, Andriessen V, Baay MFD, Pattyn GGO, Lambrechts HAJ, et al. Unraveling the mechanism of radiosensitization by gemcitabine: the role of TP53. Radiat Res. 2005 Nov;164(5):642–50.

25. Sofie Goethals, Annemieke De Wilde, Katrien Lesage, Elke Smits, Patrick Pauwels MP. Tumorbank@uza: A Collection of Tissue, Fluid Samples and Associated Data of Oncology Patients for the Use in Translational Research. Open J Bioresour. 2018;

26. Palanisamy V, Sharma S, Deshpande A, Zhou H, Gimzewski J, Wong DT. Nanostructural and transcriptomic analyses of human saliva derived exosomes. PLoS One. 2010 Jan;5(1):e8577.

27. Sorber L, Zwaenepoel K, De Winne K, Van Casteren K, Augustus E, Jacobs J, et al. A Multicenter Study to Assess EGFR Mutational Status in Plasma: Focus on an Optimized Workflow for Liquid Biopsy in a Clinical Setting. Cancers (Basel). 2018 Aug;10(9).

28. Kahlert C, Melo SA, Protopopov A, Tang J, Seth S, Koch M, et al. Identification of double-stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer. J Biol Chem. 2014 Feb;289(7):3869–75.

29. Li M, Zeringer E, Barta T, Schageman J, Cheng A, Vlassov A V. Analysis of the RNA content of the exosomes derived from blood serum and urine and its potential as biomarkers. Philos Trans R Soc Lond B Biol Sci [Internet]. 2014 Sep 26;369(1652):20130502. Available from: https://www.ncbi.nlm.nih.gov/pubmed/25135963

30. Yáñez-Mó M, Siljander PR-M, Andreu Z, Zavec AB, Borràs FE, Buzas EI, et al. Biological properties of extracellular vesicles and their physiological functions. J Extracell vesicles [Internet]. 2015 May 14;4:27066. Available from: https://www.ncbi.nlm.nih.gov/pubmed/25979354

31. Revenfeld ALS, Bæk R, Nielsen MH, Stensballe A, Varming K, Jørgensen M. Diagnostic and Prognostic Potential of Extracellular Vesicles in Peripheral Blood. Clin Ther [Internet]. 2014;36(6):830–46. Available from: http://www.sciencedirect.com/science/article/pii/S0149291814002653

CHAPTER 6

Exosomes as mediators of phenotypical and genotypical changes in the recipient cells

ABSTRACT

Exosomes have been described to play pleiotropic roles that involves the communication between cells. These pleiotropic roles involve horizontal transfer of information, pre-metastatic niche formation, angiogenesis, tumour progression and immunomodulation.

These roles might lead to exchange of information between cells, and thus, on a change of phenotype in the recipient cells.

As our and other groups have described, this is not a passive process but an active process in which cells uptake the exosomes actively through membrane extensions.

This knowledge, lead us to think, that tumoral cells obtain advantages from the exosomes in order to promote their survival.

To prove that, we have performed a sequence of experiments in which we incubate sensitive cells with exosomes derived primary resistant cells and acquired resistant cells. Afterwards we evaluate the changes that exosomes have in different aspects such as, response to the treatment, survival, response under stress and aggressiveness.

This might help us to understand the exact role that exosomes play in tumour development, which at the same time, could lead in a future to block these effects.

Exosomes mediates communication between cells

As explained in Chapter 3, exosomes have an impact in the communication between cells, this function is named as, pleiotropic roles. These roles involve the preparation of the pre-metastatic niche, as Peinado H et a. explained how melanoma exosomes alters the bone marrow cells making it more accessible for cancer cells, same results were shown for lymph nodes (1,2), but also angiogenesis, tumour progression or immunomodulation (3–5) .

In the last months, many articles have been published concluding that exosomes might mediate, at least partially, the resistance to cancer treatments. However, different mechanisms have been proposed to mediate this resistance; horizontal transfer; drug export; drug blockade.

The most described mechanism involves exosomes carrying genetic material, generally miRNAs, and being internalized by sensitive cells developing a resistance to the treatment. This was published for breast, prostate and lung cancer cell lines (6–8) . Safaei et al. described that cisplatin internalized by the target cells, was afterwards exported inside the exosomes from ovarian cancer cells (9) . Other publication described that the breast cancer cell lines increased the production of exosomes HER-2, which at the same time, were able to block the Anti-Her2 drug, Trastuzumab suggesting that these extracellular vesicles are able to reduce the effect of Trastuzumab by reduce the free-drug concentration (10) . Despite these discoveries, there is lack of information in whether the exosomes are able through horizontal transfer to promote the resistance to targeted therapies.

Exosomes as inducers for aggressiveness

Exosomes are able to carry different factors derived from the cell of origin that might trigger different functions in the recipient cells. When this is applied to tumoral exosomes, they might be able to induce pro-tumoral changes in the surrounding cells making them more aggressive, inducing this way, the growth of the tumour.

Recently, Shimada Y. et al. Published an abstract describing that exosomes derived from lung mesenchymal NSCLC cell lines, transfer active epithelial-mesenchymal transition proteins and

transcriptomes in sufficient amounts to induce phenotypical changes in bronchial epithelial immortalized cells, promoting lung cancer invasion and metastasis (11). Similar results were published in ovarian cancer, where exosomes derived from two ovarian cancer cell lines were able to induce migration in parental mesenchymal stem cells in vivo (12).

Another article demonstrated, that exosomes derived from breast cancer cell lines with different metastatic potential, also lead to different levels of migration in breast cancer cell lines (13). Goulet C.R. showed that exosomes derived from ovarian cancer cells, were able to induce tumorigenic status to healthy fibroblast in the surroundings of the tumour converting them in cancer-associated fibroblasts (CAFs) (14) , this is of high interest because recently, Jacobs J. described that CAFs alone, without other stimulus, are able to induce migration in colorectal cancer cell lines (15) .

Our hypothesis is that, in vivo, lung cancer starts developing a resistant phenotype due to continue exposure of targeted therapies such as Erlotinib, and these cells through exosomes secretion, induce resistance and aggressiveness in the surrounding tumoral cells. To prove our hypothesis, different experiments were carried out with exosomes derived from two different cells lines resistant to Erlotinib, one with an acquired resistance and another one with primary resistance.

MATERIAL AND METHODS

Cell culture

The NSCLC cell lines CRL-5908 and CRL-2868 were purchased from ATCC and the cells were grown in RPMI-1640 (Thermo Fisher, US) supplemented with 10% Fetal Bovine Serum (FBS) (Thermo Fisher, US), 1% Penicillin/Streptomycin (P/S) (Thermo Fisher, US) and 1% L-Glutamine (Thermo Fisher, US) at 37 ºC with 5% of CO2. This CRL-5908 cell line is resistant to Erlotinib through the single T790M mutation in the exon 20 of the *EGFR* gene. The cell line CRL-2868 is an is sensitive to Erlotinib through the deletion of the exon 19 (del19) in the same gene. The cell line CRL-2868 GR5 c-met was obtained from Prof. Elisa Giovannetti from the VU University Medical Centre in Amsterdam. This an acquired resistant cell line isogenic from CRL-2868, produced through exposure to increasing concentrations of Erlotinib, with an amplification of *c-met* and it was cultured in the same conditions as the other two (16) .

When the cell lines were cultured for exosomes isolation, the FBS used to complete the medium was ultracentrifugated at 100.000g for 90 minutes and filtered in 0,20 mM nylon filters in order to deplete the exosomes contained in the FBS.

Sulforhodamine B assay

Sulforhodamine B assay (SRB assay) was performed in order to assess the IC50, described as the drug concentrations that inhibits 50% of the cell growth. The assay was performed 3 times for every cell line, and in triplicates (3 wells per condition) every time with a negative control, without treatment.

The cell lines CRL-2868 GR5 c-met and CRL-5908 were incubated at 2000 cells/well for 24 hours prior to treatment with Erlotinib. A total of eleven different concentrations were prepared for the assay ranging from 100 mM to 1 nM applying a dilution 1 /2 and 1 /5 alternatively from the more concentrated to the least.

The cell line CRL-2868 was incubated at 3000 cells/well for 24 hours prior to treatment with Erlotinib. As before, a total of 11 concentrations were used, but ranging from 50 mM to 0,5 nM with the same dilution system described above.

The protocols were performed as described before by Orellana et al. (17) .

The results were transformed into a percentage taking as 100% the untreated cells. And then carried to a relative number between 0 and 1, to be analysed in Phoenix WinNonlin software using a pharmacodynamic model to calculate IC50.

Exosomes isolation

Exosomes isolation was performed as described in chapter 4 for CRL-2868 GR5 c-met and CRL-5908.

Mutation transfer to recipient cells

CRL-2868 well seeded at a concentration of 2000 cells/well in a 6 well-plate. After 24 hours the cells were treated twice for 48 hours consecutively with 20 μg/mL of exosomes derived from the cell line CRL-5908 and incubated for 72 hours at 37 ºC 5% CO2.

The cells were washed 3 times in order to remove non-internalized exosomes, scrapped and resuspended in lysis buffer from QIAmp FFPE Tissue DNA isolation Kit and the DNA was isolated following the manufacturers' instruction.

EGFR analysis

The mutational status of *EGFR* in the cells from the previous assay was performed for the T790M mutation as described in the Chapter 5.2 through ddPCR. Thresholds for the T790M assay were settled as: >1% non-informative, 0.1–1% for restricted, and <0.1% for adequate. The DNA from the cell line CRL-5908 was used as positive sample.

Assay for the transfer of the resistance. Incucyte.

CRL-2868 cells were cultured in 96-well clear flat-bottom polystyrene tissue-culture plates at the same concentration as in the SRB assay in 100 mL of medium. The experiment was performed in triplicates. After 24 hours exosomes derived from CRL-5908 or CRL-2868 GR5 c-met were added to the cells at a concentration of 10 μg/mL and 20 μg/mL twice along 48 hours (2 treatments of exosomes) *. Erlotinib HCl (OSI-744) (Selleckchem, US) IC50 was added 24 hours after the last exosomes treatment together with IncuCyte® Cytotox Green Reagent (Essen Bioscience, UK) according to the manufacturer protocol. The plate was incubated at 37 ºC with 5% CO2 in the Incucyte.

Images were taken every 2 hours since the plating of the cells and the final time point was analysed with the Incucyte ZOOM software based on the confluence of the cells in the well taking as positive control the triplicates without treatment.

Each well was corrected based on the percentage of surface of the IncuCyte® Cytotox Green Reagent. 3 wells with CRL-5908 and CRL-2868 GR5 c-met (at a concentration of 2000 cells/well) were used as reference for resistant cells to Erlotinib.

*Other combinations of exosomes treatment were used; 2 exosomes treatment; 1 exosomes treatment followed by a 24 hours incubation prior to Erlotinib addition.

Starving assay

In order to evaluate the influence of exosomes on the status of the cell, CRL-2868 cells were seeded in in 96-well clear flat-bottom polystyrene tissue-culture plates at the same concentration stated before. After 24 hours of incubation exosomes at a concentration of 20

μg/mL were plated with the cells. The cells were left in the Incucyte for 7 days at 37 ºC 5% CO2 without further changes. Images were observed after 168 hours.

Scratch assay

CRL-2868 cell line was plated at a concentration of 40.000 cells/well in a 96 well IncuCyte® ImageLock Plate for 24 hours. Exosomes were later added to the cells at a concentration of 20 μg/mL at different times in triplicates per condition, being these (using the scratch time as reference): -24 hours, - 6 hours, - 4 hours, 0 hours (just after the scratch) and +4 hours.

The scratch was performed using the WoundMaker™ (Essence Bioscience, UK) and following the washing and 96-Well Scratch Wound Cell Migration & Invasion Assays provided by the manufacturers. Wound confluency was monitored every 2h with the Incucyte LiveCell Imaging System (Essen Bioscience) for a total of 24h as described by Jacobs et al (15) .

The IncuCyte scratch-wound analysis software allowed for quantification of the increasing cell

confluence inside the wound using the “Relative Wound Density” metric. Scratches were performed in triplicate and wells with inappropriate scratches were excluded from analysis.

RESULTS

SRB assay

The experiment was performed three times for each cell line in triplicates. CRL-2868 had an IC50 value of 0.052 mM, much lower than CRL-5908 and CRL-2868 GR5 c-met with an IC50 value of 7.49 and 6.23 mM respectively (Table 1) (Figure 1).

The absorbance values for the Plate 2 of the cell line CRL-5908 presented a high standard deviation between several wells so it was not considering for the measurements.

Cell Line	IC50 Plate 1	IC50 Plate 2	IC50 Plate 3	IC50 Value (mM)
CRL-2868	0,059	0,043	0,054	0,052
CRL-5908	7,49		7,49	7,49
CRL-2868 GR5 C-Met	6,62	6,81	5,26	6,23

Table 1. IC50 values per cell line and plate.

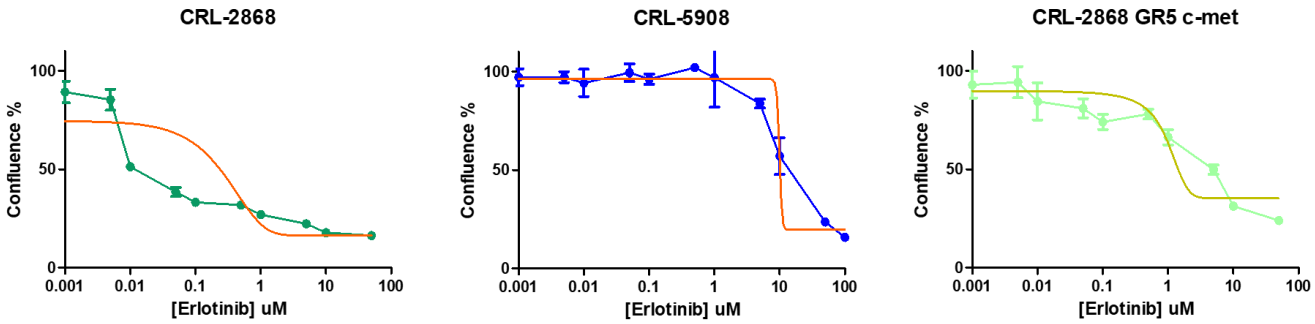


Figure 1. SRB results for SRB with standard deviation for every condition (n=3). CRL-5908 (n=2). The brown tendency line refers to the line designed by the software to calculate the IC50.

Detection of mutations in the recipient cells

DdPCR analysis of the DNA from the CRL-2868 after incubation with exosomes derived from the CRL-5908 was able to detect 3 copies of the *EGFR* T790M with a sensitivity of 0.02% meaning that it was an adequate analysis (Figure 2c). The cell line 5908 shows a clear positivity for the T790M mutation (Figure 2b).

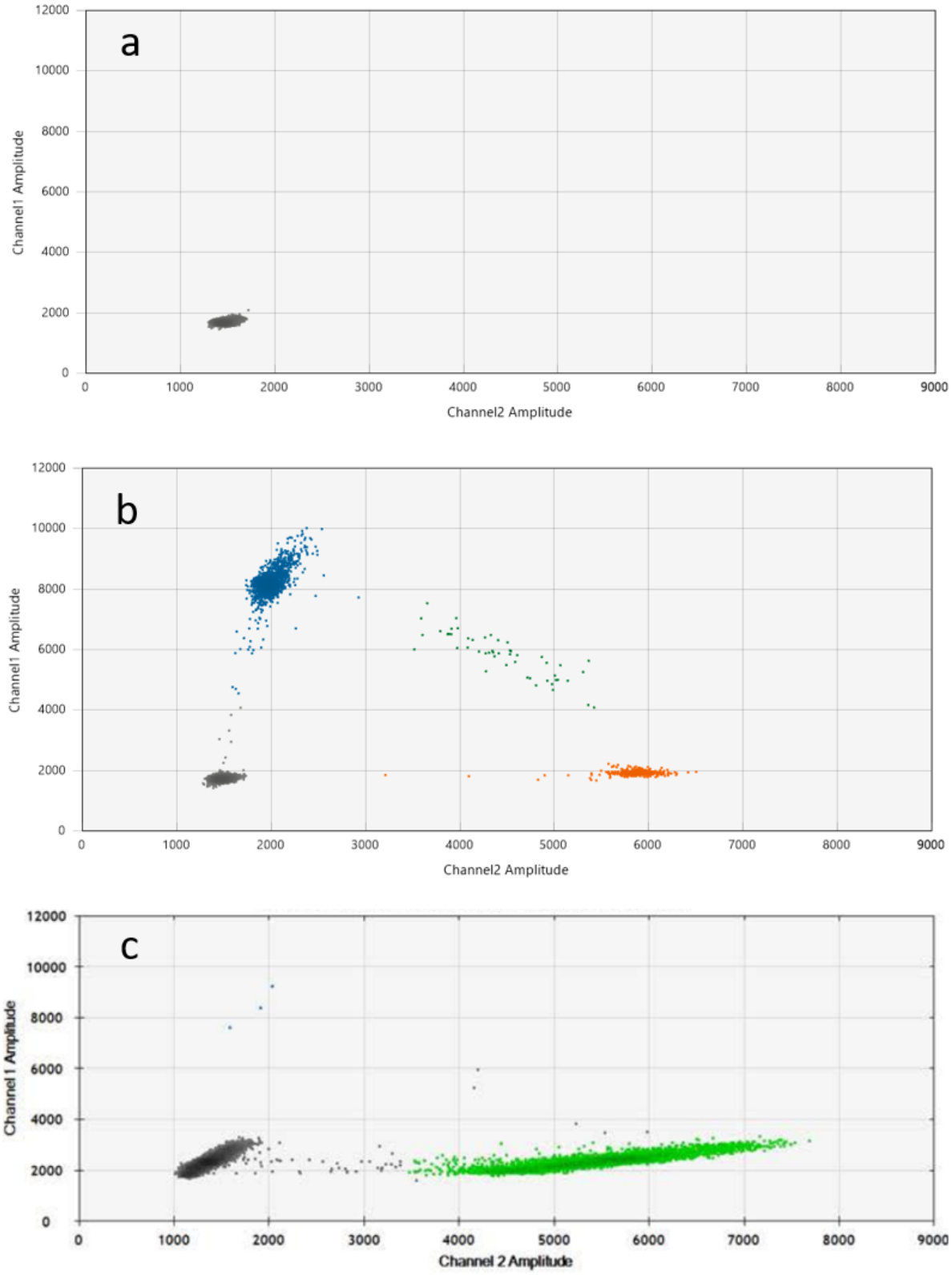
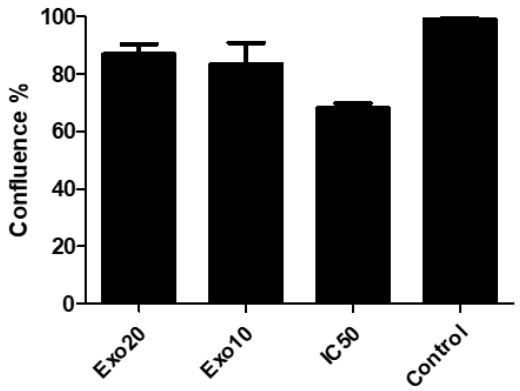


Figure 2. 2D diagram of the ddPCR. (a) No template control. (b) DNA from CRL-5908. (c) DNA from CRL-2868 incubated with exosomes derived from CRL-5908.

Effect of exosomes on the proliferation after treatment

The first experiment was performed with a single incubation of exosomes derived from the cell line CRL-5908 for 24 hours prior to Erlotinib treatment (data not shown) without appreciable differences between the different conditions. The next experiment consisted on a double treatment of exosomes from the same cell line for 48 hours prior to Erlotinib treatment. The experiment was performed in duplicates for the exosomes and triplicates for the controls. Clear differences are observable between the samples previously treated with exosomes and those that were treated with Erlotinib. While the cells treated with exosomes derived from were at an 86,96% and 83,42% for 20 μg/mL and 10 μg/mL of exosomes respectively. The wells treated only with Erlotinib had a confluence of 68,16% (Figure 3).

a



b

	Exo20	Exo10	IC50	Control
Confluence (%)	89,3539	78,177	67,0112	99,1216
	84,5856	88,6697	69,3195	98,7379
			*92,087	99,4156
X̄ Confluence (%)	86,96975	83,42335	68,16 (76,3369*)	99,0917

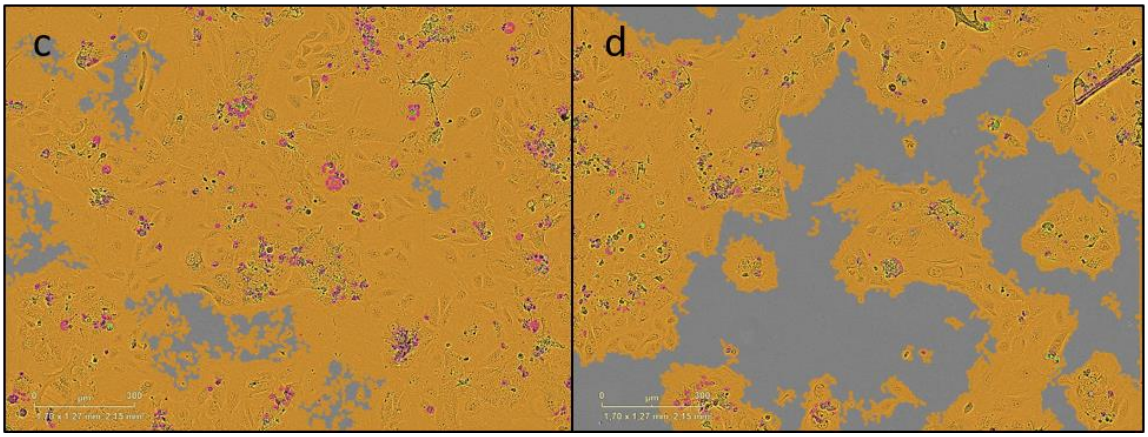


Figure 3. (a) Graphic comparing the confluence at end-point of CRL-2868 cells after treatment with exosomes and Erlotinib and cells with only Erlotinib treatment. (b) Percentages of the confluences per well based on the analysis of four images. *The well was considered as an outlier due to technical problems and was removed from the final calculations. (c & d) Images extracted from the experiment showing the confluence of cells treated with 20 μg/mL of exosomes and Erlotinib (c) and cells only treated with Erlotinib (d) at the end of the experiment.

Two other experiments with an interval of 24 hours after exosomes incubation and prior to Erlotinib treatment were performed without successful results (data not shown).

We performed two assays with exosomes derived from the cell line CRL-2868 GR5 c-met mirroring the conditions of the previous experiment. Unexpectedly, the results at the end of the experiment were not positive with a confluence of the wells treated with exosomes lower or similar to the ones treated only with Erlotinib (data not shown). However, after 24 hours of 20 μg/mL of exosomes incubation there was a clear change of morphology in the recipient cells compared to the untreated ones (Figure 4). The morphology of the CRL-2868 cells is rounded without prolongations, with a very diffuse membrane difficult to differentiate from the back ground (Figure 4a) while the same cells previously treated with exosomes presents an angular shape on their membrane, with prolongations of the cytoplasm, and a clearly defined shape when comparing with the background (Figure 4b).

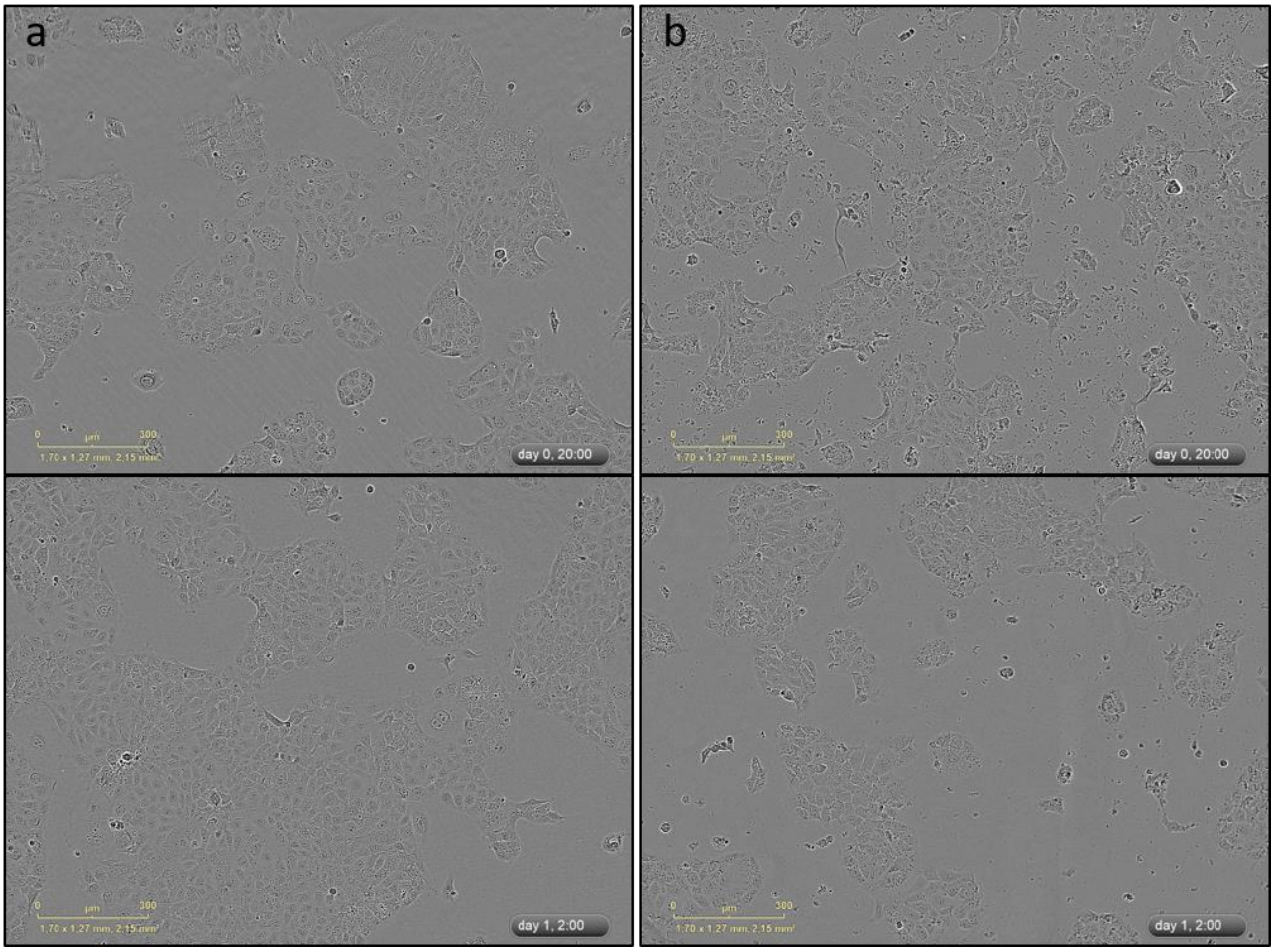


Figure 4. Example of 2 images of the CRL-2868 cells with (b) or without (a) exosomes at the same time point.

Role of exosomes in survival

After 7 days of incubation in the Incucyte, the cells that were treated with exosomes presented a well-defined body with long filopodia, characteristic from very aggressive cells (Figure 5a). In the same, the untreated cells presented a very diffuse shape, with multiple apoptotic bodies around them (Figure 5b).

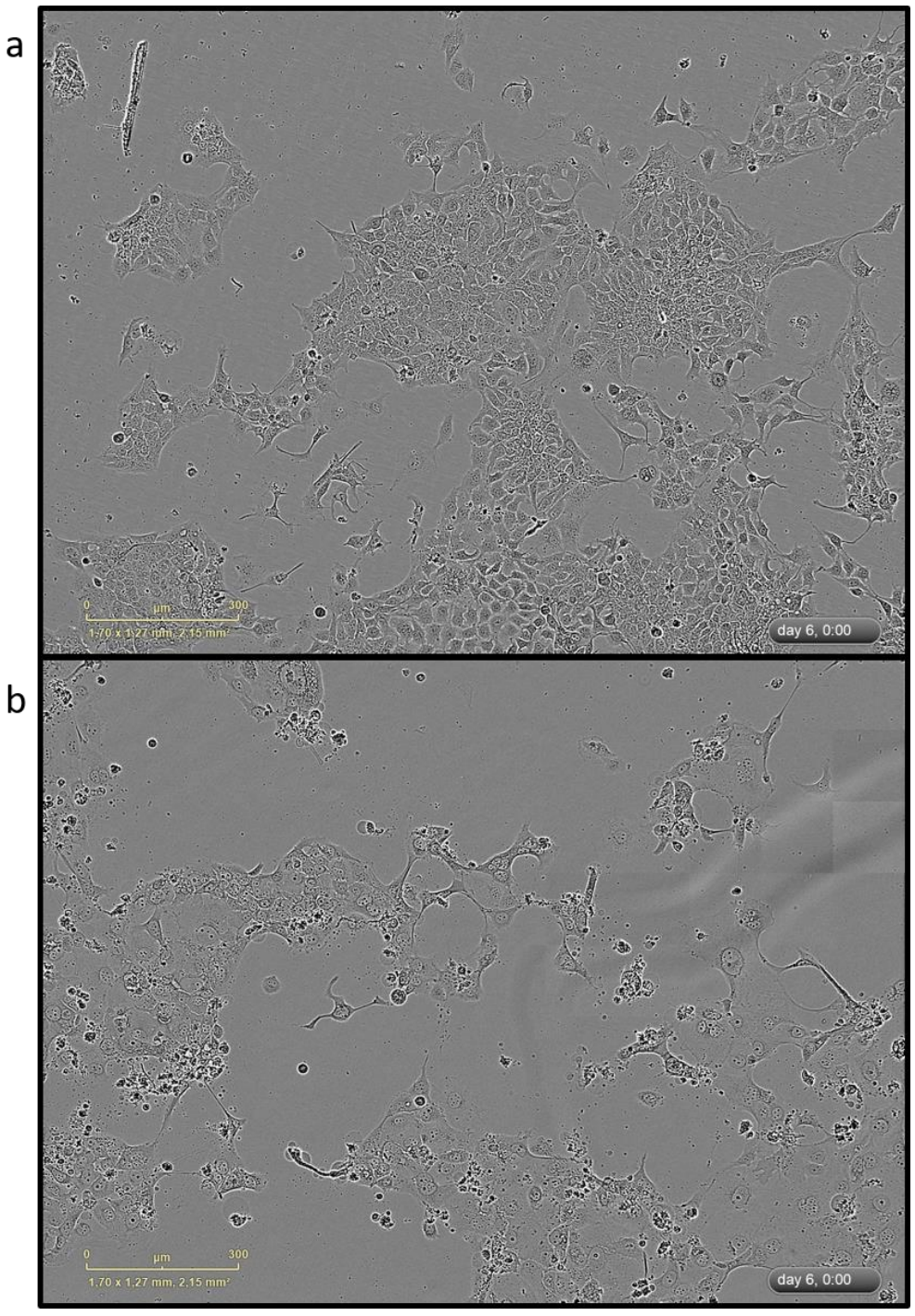


Figure 5. (a) Cells treated with exosomes 20 μg/mL (b) Negative control. Untreated cells.

Role of exosomes in migration and invasion

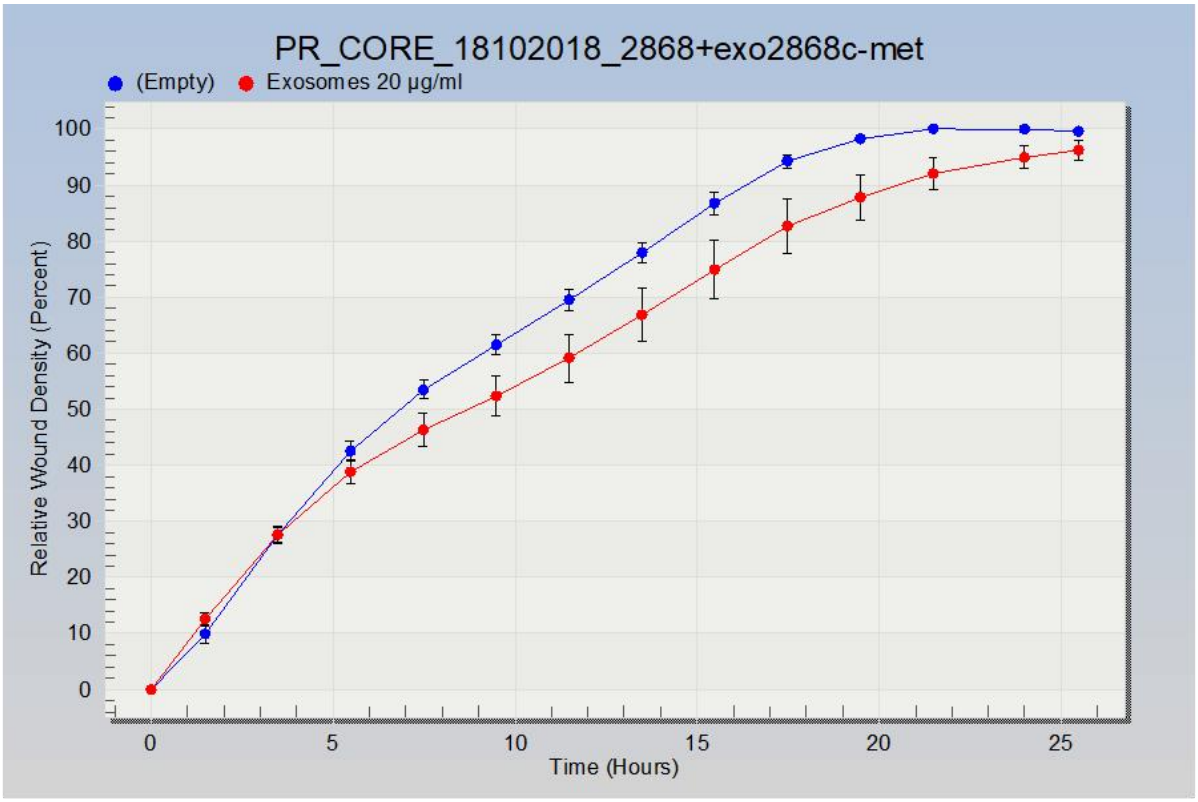
As explained before, exosomes might mediate phenotypical and genotypical changes in the cell that might lead to an increase in the aggressiveness of the cells, thus, we analysed the capacity of exosomes derived from CRL-2868 GR5 c-met cells of increase the migratory capacity of the CRL-2868 cells by real time analysis of wound closure.

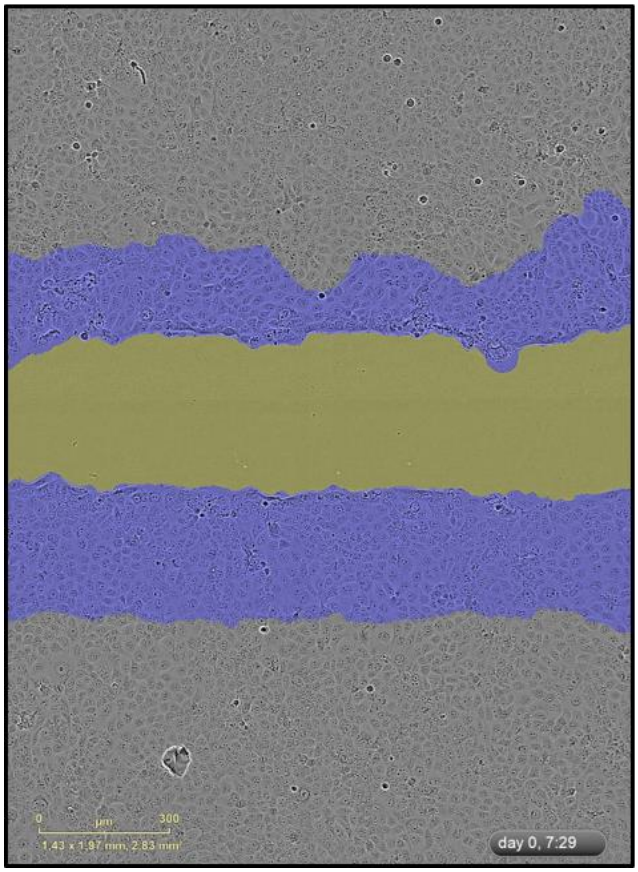
Due to technical problems the scratch was not regular in all the wells, thus, it was not possible to analyse the assay as it was designed. Moreover, regardless of our hypothesis, making a comparison of treated cells prior, or at the time of scratch or not treated cells, exosomes were not able to increase the capacity of invasion or migration in the cells at any time point compared to the cells alone. However, looking at the graph generated, it is clearly visible that there is a delay invading the scratch of those cells treated with exosomes (Figure 6a), something that is clearly visible in the images 7 hours after the scratch (Fig 6b & c).

DISCUSSION

The role of exosomes in different features of cancer progression has been described over the last years. However, these publications only investigate some tumoral types, and most of them research how exosomes derived from tumoral cell lines, induce phenotypical changes in surrounding healthy cells (11,12) . Therefore, the mechanisms and how these changes might affect in the real tumoral status are unknown.

Our results seem to not completely solve these questions. Despite the experiments where we clearly see that exosomes gets internalized by the sensitive cells (Chapter 4) and that these exosomes contain the T790M mutation releasing it inside the recipient cells, we were not able to show a robust effect of the exosomes inducing resistance to Erlotinib with CRL-5908, although we could see a tendency based on one experiment in duplicates. Moreover, using exosomes derived from CRL-2868 GR5 c-met, an isogenic cell line to CRL-2868, we obtained no difference compared to the cells only treated with exosomes in terms of confluence.





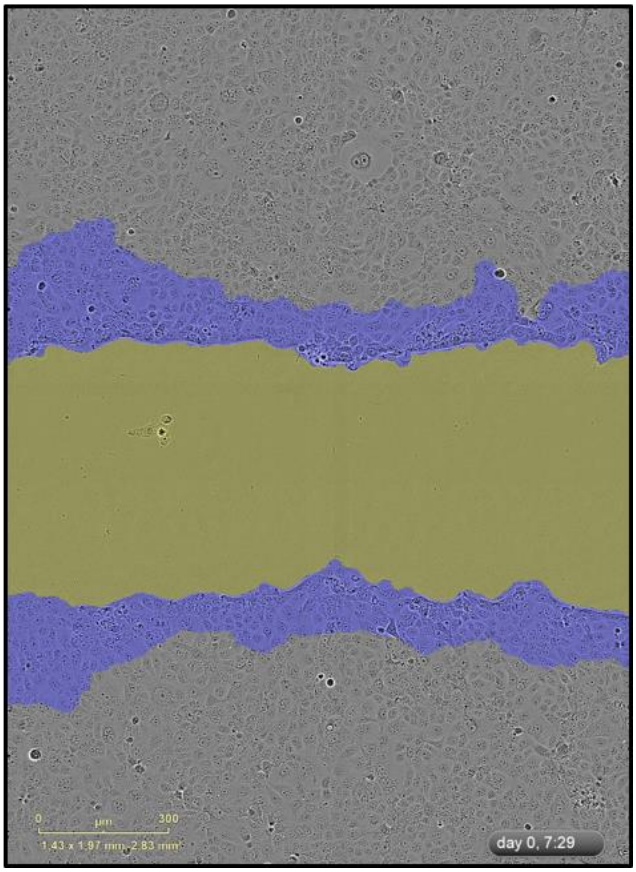


Figure 6. (a) Timeline of the relative wound density comparing untreated cells with cells treated with exosomes, prior and at the time of the scratch. (b) Image of untreated cells 7 hours after the scratch. (c) Image of treated cells with 20 µg/mL of exosomes 7 hours after the scratch.

On the other hand, as seen in Figure 4. there is a clear change of morphology on the CRL-2868 treated with exosomes compared to the untreated cells. This change of morphology, might demonstrate what we described with the starving assay. The cells treated with exosomes, seem to perform better on stressful environment, where, after 7 days, there is absence of nutrients in the medium and the untreated cells start to become apoptotic. These morphological changes shown in the different experiments, might be related to an Epithelial-mesenchymal transition induce by exosomes. This process has been already described in cells with acquired resistant to TKI treatments (18) . In order to confirm this hypothesis, a genetic analysis in the exosomes and in the cells after exosomes treatment should be perform for classical EMT markers such as Vimentin, E- and N-Cadherin among others.

To finish, we decided to perform a scratch assay to assess the changes that exosomes might produce in terms of aggressiveness. We saw that there was a delay in starting the wound closure on those cells treated with exosomes at different times compared to the untreated cells, however, this experiment just gives us a tendency, since many wells were not possible to be analysed in both, treated and untreated conditions, due to technical problems with the Wound Maker.

Looking at the results, it is clear that exosomes derived from resistant cells induce changes in the sensitive cell line, however, we require further research to extract robust conclusions out of them. Until now, our experiments were based on one or two incubations of exosomes for a period of 6 to 48 hours, nevertheless, it seems that exosomes might be inducing the phenotypical changes later in time, thus, the effect on the resistance to the treatment is not observable due to the quick addition of Erlotinib to the cells. In our opinion, this is happening overall in the scratch assay. Taking into account that the wound is closed in less than 24 hours, exosomes might not have enough time to induce the changes.

To solve these problems, new protocols must be designed to confirm our hypothesis. Growth of cells in presence of exosomes, might induce long term changes in the sensitive cells, increasing the doses required for treat them. This could also result on a more aggressive phenotype, increase survival, migration and invasion of the cells.

BIBLIOGRAPHY

1. Hood JL, San RS, Wickline SA. Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis. Cancer Res. 2011 Jun;71(11):3792–801.

2. Peinado H, Alečković M, Lavotshkin S, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med. 2013;18(6):883–91.

3. Zhao H, Yang L, Baddour J, et al. Tumor microenvironment derived exosomes pleiotropically modulate cancer cell metabolism. Dang C Van, editor. Elife [Internet]. 2016 Feb;5:e10250. Available from: https://dx.doi.org/10.7554/eLife.10250

4. Grange C, Tapparo M, Collino F, et al. Microvesicles released from human renal cancer stem cells stimulate angiogenesis and formation of lung premetastatic niche. Cancer Res. 2011 Aug;71(15):5346–56.

5. Roberg-Larsen H, Lund K, Seterdal KE, et al. Mass spectrometric detection of 27-hydroxycholesterol in breast cancer exosomes. J Steroid Biochem Mol Biol. 2016 Feb;

6. Qin X, Yu S, Zhou L, et al. Cisplatin-resistant lung cancer cell-derived exosomes increase cisplatin resistance of recipient cells in exosomal miR-100-5p-dependent manner. Int J Nanomedicine. 2017;12:3721–33.

7. Lv M, Zhu X, Chen W, et al. Exosomes mediate drug resistance transfer in MCF-7 breast cancer cells and a probable mechanism is delivery of P-glycoprotein. Tumour Biol. 2014 Nov;35(11):10773–9.

8. Corcoran C, Rani S, O'Brien K, et al. Docetaxel-Resistance in Prostate Cancer: Evaluating Associated Phenotypic Changes and Potential for Resistance Transfer via Exosomes. Kyprianou N, editor. PLoS One [Internet]. 2012 Dec 10 [cited 2018 Nov 17];7(12):e50999. Available from: https://dx.plos.org/10.1371/journal.pone.0050999

9. Safaei R, Larson BJ, Cheng TC, et al. Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells. Mol Cancer Ther. 2005 Oct;4(10):1595–604.

10. Ciravolo V, Huber V, Ghedini GC, et al. Potential role of HER2-overexpressing exosomes in countering trastuzumab-based therapy. J Cell Physiol. 2012 Feb;227(2):658–67.

11. Shimada Y, Okano T, Yenerall P, et al. Exosomes secreted from mesenchymal NSCLC cells to promote invasiveness and metastatic capability of recipient cells through UCHL1. J Clin Oncol [Internet]. 2018 May 20;36(15_suppl):e24008–e24008. Available from: https://doi.org/10.1200/JCO.2018.36.15_suppl.e24008

12. Sharma S, Alharbi M, Kobayashi M, et al. Proteomic analysis of exosomes reveals an association between cell invasiveness and exosomal bioactivity on endothelial and mesenchymal cell migration in vitro. Clin Sci (Lond). 2018 Sep;132(18):2029–44.

13. Harris DA, Patel SH, Gucek M, Hendrix A, Westbroek W, Taraska JW. Exosomes Released from Breast Cancer Carcinomas Stimulate Cell Movement. PLoS One [Internet]. 2015 Mar 23;10(3):e0117495. Available from: https://doi.org/10.1371/journal.pone.0117495

14. Ringuette Goulet C, Bernard G, Tremblay S, Chabaud S, Bolduc S, Pouliot F. Exosomes Induce Fibroblast Differentiation into Cancer-Associated Fibroblasts through TGFbeta Signaling. Mol Cancer Res. 2018 Jul;16(7):1196–204.

15. Jacobs J, Deschoolmeester V, Zwaenepoel K, et al. Unveiling a CD70-positive subset of cancer-associated fibroblasts marked by pro-migratory activity and thriving regulatory T cell accumulation. Oncoimmunology. 2018;7(7):e1440167.

16. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET Amplification Leads to Gefitinib Resistance in Lung Cancer by Activating ERBB3 Signaling. Science (80-) [Internet]. 2007 May 18;316(5827):1039 LP-1043. Available from: http://science.sciencemag.org/content/316/5827/1039.abstract

17. Orellana EA, Kasinski AL. Sulforhodamine B (SRB) Assay in Cell Culture to Investigate Cell Proliferation. Bio-protocol [Internet]. 2016 Nov 5;6(21):e1984. Available from: https://www.ncbi.nlm.nih.gov/pubmed/28573164

18. Fustaino V, Presutti D, Colombo T, et al. Characterization of epithelial-mesenchymal transition intermediate/hybrid phenotypes associated to resistance to EGFR inhibitors in non-small cell lung cancer cell lines. Oncotarget [Internet]. 2017 Sep 22;8(61):103340–63. Available from: https://www.ncbi.nlm.nih.gov/pubmed/29262566

CHAPTER 7

Discussion

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Exosomes are a novel field of research. When discovered, it was believed that they were merely trash bags used by the cells to eliminate non-desire contents. However, in the last years the research on exosomes has increased dramatically from 100 publications ten years ago, to 1800 publications in 2018.

This increasing interest is due to the promising results that exosomes has shown in different fields. Among them, cancer exosomes it's one of the most studied. Exosomes are composed by a lipid bilayer that provides them with a big stability in hostile environments protecting their content from the different components in the blood stream, urine and other biological fluids (1).

However, due to the novelty of the topic, there is no consensus on the methodological area regarding exosomes workflows. As published by Pathan M. et al. (2) . nowadays ultracentrifugation is the most common method for exosomes isolation, however, up to nine different methods are commonly used for isolation, some of them, combined. This paper, highlights the importance of standardization of the protocols used to work with exosomes. On the same path, Van Deun, J. and Hendrix A. created a knowledge database in order to evaluate the transparency and rigour of exosomes publications to improve reproducibility and standardization in extracellular vesicles research (3) .

In chapter 4, we have described three protocols for exosomes imaging. One of the common experiments required to demonstrate the correct isolation of exosomes, is the Electron Microscopy, both scanning and transmission are valid. Until now, many articles have published images in electron microscopy, however, these images usually present a poor quality, and few information can be extracted from them (4,5) . We have prepared an electron microscopy protocol setting the values for the Voltage, and the staining of the exosomes obtaining clear images, being possible to used together with the detection of surface components through Immunostaining. We have also obtained the first images of SEM after exosomes isolation through ExoEasy.

Exosomes are also being studied as transmitters of information between cells, especially in cancer cells (6–11) . Thus, the importance of imaging techniques where it is visible the

internalization of exosomes is required. Many protocols have been published for exosomes staining. In our articles, we have shown a comparison between colourants for exosomes, and we have included a protocol for exosomes staining removing the ultracentrifugation steps, in order to simplify the protocol.

The last protocol designed, have described the observation of live uptake of exosomes. In it, we have clearly seen how the exosomes are uptaken by the recipient cells, not just as a passive action, but how clearly the cell creates filopodia and how the exosomes move along the filaments of actin. The protocol is not completely set up, due to the cell dead that can be seen in the videos. However, is a good starting point to optimize settings and concentrations of colourants to find the best performance. This type of protocols might be extremely interesting for those projects that start using exosomes as drug delivery systems in order to analyse the performance of exosomes internalization and thus the concentrations of drug that might be active inside the cells (12,13) .

Non-Small Cell Lung Cancer (NSCLC) remains as the most lethal type of cancer. Its 5-year overall survival remains below 20% and it produce around 1.3 million per year (14) . Despite the increase of treatment options for different subsets of NSCLC, such as targeted therapies and immunotherapy, there has not been major improvements in terms of overall survival (OS) (15) .

Despite the statistics, both targeted therapies and immunotherapy meant a revolution on the treatment of cancer, among other reasons, due to the improvement in quality of life of the patients. Immunotherapy targets immune-checkpoints located in the tumour cells that during tumour development, blocks the immune-response. However, the expression of these checkpoints is not correlated with response to the treatment (16–18) .

On the contrary, predictive biomarkers for targeted therapies, with exceptions, present a higher correlation between detection of the biomarker and response to the treatment (19,20) . The problem lies on the detection of these markers. Biopsies only represent a fraction of the tumour, and does not always content enough tumoral material to make a screening test for all the markers. Moreover, in late stages NSCLC or recurrence, very often is not feasible to biopsy the patient and the samples are based on non-invasive cytologies, resulting on inadequate tumour profile (21) . For this reason, in chapter 5.1 and chapter 5.2, we have developed two protocols to analyse ALK-EML4 translocation in exosomal RNA and EGFR mutations in exosomal DNA respectively. The results of the identification of ALK-EML4 are

very promising with a sensitivity of 64% and a specificity of 100%, however, the number of patients analysed is low, and further research must be performed in a bigger group of patients. In addition, we are willing to include other less common variants of the translocation in order to increase the detection of the assay.

On the second part of predictive biomarkers study, we have not been able to identify the mutation of EGFR in patients yet, however, the assay performed in exosomes derived from cells, indicates that we EGFR is present inside the exosomes. However, to implement these assays in the clinic, high standardization is required, and thus, we will work on the development of a simple and standard assay to isolate exosomal DNA without ultracentrifugation.

Despite the good results showed by targeted therapies, many of the patients develop a resistance to the current treatment as explained in different chapters. The mutation occurs partially due to the continuous exposure to the drug, inducing mutations in the target cells. However, it has been described that exosomes might mediate partially this resistance through different mechanisms, moreover, these exosomes might transfer other signals such as aggressiveness, progression or survival (9,22,23) . Lastly, in chapter 6, we have investigated the function of exosomes derived from primary resistant cell lines and acquired resistant cell lines. We have shown different scenarios where exosomes are inducing phenotypic changes in morphology, and survival with and without treatment. Although these changes are not significant, only about 15% of difference between exosomes incubation and non-exosomes incubation, it shows an effect, that might be possible to increase modifying the conditions. We have also shown the transference of genetic material from exosomes to cells through the detection of the T790M mutation in CRL-2868 which naturally does not contain this mutation. However, these results are not fully conclusive, molecular assays are required to analyse the real effect that exosomes are producing in the cells, such as reduction of the apoptosis or epithelial-mesenchymal markers to analyse the phenotype of the cells. Furthermore, we are willing to start experiments on 3D co-culture of resistant and non-resistant cells, one of the them transfected with a fluorescent lentiviral vector in order to evaluate how the different cells evolve in the presence of a treatment. This would allow us to mirror more similarly a real tumour situation and observe how the clonality of the tumour evolves.

CONCLUDING REMARKS

The main objective of these project was to contribute to the improve the knowledge of exosomes in cancer, trying to ameliorate the methods of exosomes observation, investigating the exosomes as predictive biomarkers for targeted therapies and studying them as possible mediators between tumoral cells, especially in lung cancer.

We have been able to described a protocol to observe live exosomes being internalized by the cells, and improved other protocols used for exosomes imaging.

We have described for the first time the presence of ALK-EML4 translocation in RNA derived from exosomes, and study the tissue concordance, finding a good correlation between them. Moreover, we have put our efforts on correlate the EGFR status from patients detected on tissue with exosomal DNA. The results on the cell derived exosomes indicates that it will be possible to assess the EGFR status through exosomal DNA analysis.

To conclude the thesis, we have studied how exosomes alter the phenotype of the recipient cells. We have shown a change in the resistance of sensitive cells after treatment with exosomes derived from resistant cells. In addition, these exosomes induce morphological changes in the treated cells, which moreover, induce survival in starving situations and seem to delay the wound closure after making a scratch. This might prove, that cells exposed to exosomes might undergo epithelial-mesenchymal changes in their phenotype making them more aggressive and resistant to the treatment.

In conclusion, we are proud to contribute with this translational research to increase the knowledge in this exciting field, including a potential clinical application.

BIBLIOGRAPHY

1. Johnsen KB, Gudbergsson JM, Skov MN, Pilgaard L, Moos T, Duroux M. A comprehensive overview of exosomes as drug delivery vehicles - endogenous nanocarriers for targeted cancer therapy. Biochim Biophys Acta. 2014 Aug;1846(1):75–87.

2. Pathan M, Fonseka P, Chitti S V, et al. Vesiclepedia 2019: a compendium of RNA, proteins, lipids and metabolites in extracellular vesicles. Nucleic Acids Res [Internet]. 2018;gky1029. Available from: http://dx.doi.org/10.1093/nar/gky1029

3. Van Deun J, Hendrix A, consortium E-T. Is your article EV-TRACKed? J Extracell vesicles [Internet]. 2017 Nov 10;6(1):1379835. Available from: https://www.ncbi.nlm.nih.gov/pubmed/29184624

4. Sokolova V, Ludwig A-K, Hornung S, et al. Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy. Colloids Surf B Biointerfaces. 2011 Oct;87(1):146–50.

5. Zaharie F, Muresan MS, Petrushev B, et al. Exosome-Carried microRNA-375 Inhibits Cell Progression and Dissemination via Bcl-2 Blocking in Colon Cancer. J Gastrointestin Liver Dis. 2015 Dec;24(4):435–43.

6. Ringuette Goulet C, Bernard G, Tremblay S, Chabaud S, Bolduc S, Pouliot F. Exosomes Induce Fibroblast Differentiation into Cancer-Associated Fibroblasts through TGFbeta Signaling. Mol Cancer Res. 2018 Jul;16(7):1196–204.

7. Hood JL, San RS, Wickline SA. Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis. Cancer Res. 2011 Jun;71(11):3792–801.

8. Lv M, Zhu X, Chen W, et al. Exosomes mediate drug resistance transfer in MCF-7 breast cancer cells and a probable mechanism is delivery of P-glycoprotein. Tumour Biol. 2014 Nov;35(11):10773–9.

9. Shimada Y, Okano T, Yenerall P, et al. Exosomes secreted from mesenchymal NSCLC cells to promote invasiveness and metastatic capability of recipient cells through UCHL1. J Clin Oncol [Internet]. 2018 May 20;36(15_suppl):e24008–e24008. Available from: https://doi.org/10.1200/JCO.2018.36.15_suppl.e24008

10. Harris DA, Patel SH, Gucek M, Hendrix A, Westbroek W, Taraska JW. Exosomes Released from Breast Cancer Carcinomas Stimulate Cell Movement. PLoS One [Internet]. 2015 Mar 23;10(3):e0117495. Available from: https://doi.org/10.1371/journal.pone.0117495

11. Ciravolo V, Huber V, Ghedini GC, et al. Potential role of HER2-overexpressing exosomes in countering trastuzumab-based therapy. J Cell Physiol. 2012 Feb;227(2):658–67.

12. Morse MA, Garst J, Osada T, et al. A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. J Transl Med [Internet]. 2005;3(1):1–8. Available from: http://dx.doi.org/10.1186/1479-5876-3-9

13. Taieb J, Chaput N, Novault S, et al. Dendritic cell derived-exosomes boost effector CD8+ T cells in the absence of tumor induced-T regulatory cells: Synergistic antitumor effects of cyclophosphamide and exosome-based vaccines. Cancer Res [Internet]. 2014 Oct 23;65(9 Supplement):623 LP-624. Available from: http://cancerres.aacrjournals.org/content/65/9_Supplement/623.4.abstract

14. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin [Internet]. 2016;66(1):7–30. Available from: http://dx.doi.org/10.3322/caac.21332

15. Noone AM, Howlader N, Krapcho M, Miller D, Brest A, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ CK. SEER Cancer Statistics Review (CSR) 1975-2015 [Internet]. 2015. Available from: https://seer.cancer.gov/csr/1975_2015/

16. Gridelli C, Ardizzoni A, Barberis M, et al. Predictive biomarkers of immunotherapy for non-small cell lung cancer: results from an Experts Panel Meeting of the Italian Association of Thoracic Oncology. Transl lung cancer Res. 2017 Jun;6(3):373–86.

17. Voong KR, Feliciano J, Becker D, Levy B. Beyond PD-L1 testing-emerging biomarkers for immunotherapy in non-small cell lung cancer. Ann Transl Med [Internet]. 2017 Sep;5(18):376. Available from: https://www.ncbi.nlm.nih.gov/pubmed/29057236

18. Hellmann MD, Ciuleanu T-E, Pluzanski A, et al. Nivolumab plus Ipilimumab in Lung Cancer with a High Tumor Mutational Burden. N Engl J Med. 2018 May;378(22):2093–104.

19. Ahmadzada T, Kao S, Reid G, Boyer M, Mahar A, Cooper WA. An Update on Predictive Biomarkers for Treatment Selection in Non-Small Cell Lung Cancer. J Clin Med [Internet]. 2018 Jun 15;7(6):153. Available from: https://www.ncbi.nlm.nih.gov/pubmed/29914100

20. Patel JN, Ersek JL, Kim ES. Lung cancer biomarkers, targeted therapies and clinical assays. Transl lung cancer Res [Internet]. 2015 Oct;4(5):503–14. Available from: https://www.ncbi.nlm.nih.gov/pubmed/26629419

21. Pisapia P, Lozano MD, Vigliar E, et al. ALK and ROS1 testing on lung cancer cytologic samples: Perspectives. Cancer Cytopathol. 2017 Nov;125(11):817–30.

22. Qin X, Yu S, Zhou L, et al. Cisplatin-resistant lung cancer cell-derived exosomes increase cisplatin resistance of recipient cells in exosomal miR-100-5p-dependent manner. Int J Nanomedicine. 2017;12:3721–33.

23. Corcoran C, Rani S, O'Brien K, et al. Docetaxel-Resistance in Prostate Cancer: Evaluating Associated Phenotypic Changes and Potential for Resistance Transfer via Exosomes. Kyprianou N, editor. PLoS One [Internet]. 2012 Dec 10 [cited 2018 Nov 17];7(12):e50999. Available from: https://dx.plos.org/10.1371/journal.pone.0050999

SCIENTIFIC PRODUCTS

Articles

- Old methods in a hot topic field: Improving exosomes visualization. **Pablo Reclusa**, Verstraelen Peter, Marzia Pucci, Isabel Pinteleon, Nathalie Claes, Simona Tarverna, Sara Bals, Christian Rolfo. (Submitted)

- EML4-ALK translocation identification in RNA exosomal cargo (ExoALK) in NSCLC Patients: a novel role for liquid biopsy. **P. Reclusa***, J.F. Laes*, U. Malapelle, Anna Valentino, D. Rocco, I. Gil-Bazo, & C. Rolfo. **TRANSLATIONAL CANCER RESEARCH**

- Amphiregulin contained in NSCLC-exosomes induces osteoclast differentiation through the activation of EGFR pathway. Taverna, S.; Pucci, M.; Giallombardo, M.; Di Bella, M.; Santarpia, M.; **Reclusa, P**.; Gil-Bazo, I.; Rolfo, C.; Alessandro, R. **SCIENTIFIC REPORTS**. 2017

- FGFR a promising druggable target in cancer: Molecular biology and new drugs. Porta, R.; Borea, R.; Coelho, A.; Khan, S.; Araújo, A.; **Reclusa, P**.; Franchina, T.; Van Der Steen, N.; Van Dam, P.; Ferri, J.; Sirera, R.; Naing, A.; Hong, D.; Rolfo, C. **Critical Reviews in Oncology/Hematology.** 2017

- Profile of the Roche cobas® EGFR mutation test v2 for non-small cell lung cancer. Malapelle, U.; Sirera, R.; Jantus-Lewintre, E.; **Reclusa, P**.; Calabuig-Fariñas, S.; Blasco, A.; Pisapia, P.; Rolfo, C.; Camps, C. **Expert Review of Molecular Diagnosis.** 2016

- Exosomal microRNAs in liquid biopsies: future biomarkers for prostate cancer. Valentino, A.; **Reclusa, P.**; Sirera, R.; Giallombardo, M.; Camps, C.; Pauwels, P.; Crispi, S.; Rolfo, C. **Clinical and traslational oncology.** 2016

- Exosomes in lung cancer liquid biopsies: Two sides of the same coin? Rolfo, C.; Giallombardo, M.; **Reclusa, P.**; Sirera, R.; Peeters, M. **Lung Cancer.** 2016

- Exosomes genetic cargo in lung cancer: A truly Pandora's box. **Reclusa, P**.; Sirera, R.; Araujo, A.; Giallombardo, M.; Valentino, A.; Sorber, L.; Bazo, I.; Pauwels, P.; Rolfo, C. **Traslational Lung Cancer Research**. 2016

- Exosomes as miRNA nanoshuttles: dual role in tumor progression. M.Pucci, **P.Reclusa**, E. Durendez, E. Jantus, M.Malarani, S.Khan, C. Rolfo, S.Taverna. **TARGETED ONCOLOGY**

- The crucial role of exosomes in premetastatic niche formation: the Trojan horse in cancer progression. S.Taverna, M. Pucci, **P. Reclusa**, E Durendez, M. Malarani, I. Gil-Bazo, A. Russo, C. Rolfo. **ONCOLOGY REPORTS**

- Exosomes in Semen: opportunities as a new tool in cancer diagnosis. M. Pucci, S. Taverna, **P. Reclusa**, E. Durendez, M. Malarani, R. Alessandro, C. Rolfo. **TRANSLATIONAL CANCER RESEARCH**

Abstracts

- Exo-ALK Proof of Concept: Exosomal Analysis of ALK Alterations in Advanced NSCLC Patients. Christian Rolfo, Jean François Laes, **Pablo Reclusa**, Anna Valentino, Maxime Lienard, Ignacio Gil-Bazo, Umberto Malapelle, Rafael Sirera, Danilo Rocco, Jan Van Meerbeeck, Patrick Pauwels, Marc Peeters. IASLC 2016 Vienna

- Evaluation of Different Exosomal RNA Isolation Methods in NSCLC Liquid Biopsies: Track: Biology and Pathogenesis Giallombardo, M.; **Reclusa, P**.; Valentino, A.; Sirera, R.; Pauwels, P.; Rolfo, C. LALCA 2016 Panamá

- Exosomal miRNA Analysis in Non-Small Cell Lung Cancer: New Liquid Biomarker? Track: Biology and Pathogenesis. **Reclusa, P**.; Giallombardo, M.; Castiglia, M.; Sorber, L.; Van Der Steen, N.; Pauwels, P.; Rolfo, C. LALCA 2016 Panamá

- Van Der Steen, N.; Rolfo, C.; Giovannetti, E.; **Reclusa, P**.; Peters, G.; Pauwels, P. cMET in NSCLC: Expression, Amplification and Mutations: Track: Biology and Pathogenesis. LALCA 2016

Oral presentations at scientific meetings

- **39th EORTC PAMM WINTER MEETING, Rome, 9th September 2018**
  "NSCLC cells sensitive to EGFR tyrosine kinase inhibitors internalize exosomes released by NSCLC resistant cells"

- **MIPRO "Molecular Imaging, Pathology, Radiotherapy & Oncology" MEETING, Antwerp, 7th July 2017**
  "Study of the cargo from Non-Small Cell Lung Cancer derived exosomes"

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