University of Crete, Biology Department
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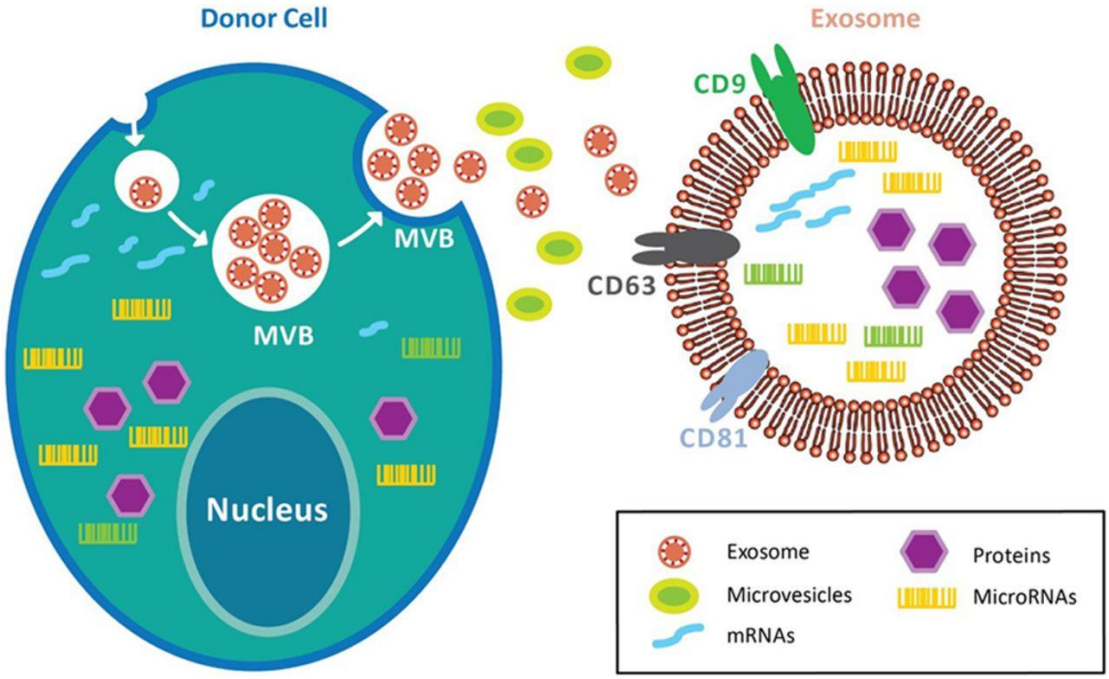
Study of exosomal pathways in Hodgkin and non-Hodgkin lymphoma cells after p53 re-activation



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To my parents

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Contents

Περίληψη

Η δράση των μονοπατιών της βιοσύνθεσης και έκκρισης των εξωσωμάτων είναι σημαντική στη διατήρηση της κυτταρικής ομοιόστασης, διαδραματίζοντας σημαντικό ρόλο στην καρκινογένεση. Συνεπώς, η αποκρυπτογράφηση του πρωτεϊνωματικού προφίλ της οδού σε λεμφώματα-φορείς άγριου-τύπου (wt)-p53 στοχεύει στη διερεύνηση της λεμφωματικής βιοπαθολογίας. Στην παρούσα μελέτη, στόχος μας ήταν ο εντοπισμός των διαφορικά εκφρασμένων πρωτεϊνών που σχετίζονται με την εξωσωμική οδό, η αξιολόγηση της λειτουργικής τους σχέσης και η συμβολή τους στη θεραπευτική απόκριση της επαν-ενεργοποίησης της p53 μέσω της Nutlin-3a (N3α) σε διαφορετικούς υποτύπους λεμφώματος. Κυτταρικές σειρές-μοντέλα ανθρώπινου λεμφώματος μετά από N3α υποβλήθηκαν σε συγκριτική πρωτεϊνωματική και βιοπληροφορική ανάλυση. Από τις συνολικά 4037 ταυτοποιημένες πρωτεΐνες, εφαρμόζοντας λειτουργική ανάλυση μονοπατιών, 77 πρωτεΐνες προσδιορίστηκαν ότι σχετίζονται με την εξωσωμική οδό. Επιπλέον, η ανάλυση δικτύου πρωτεϊνικών αλληλεπιδράσεων αποκάλυψε το βαθμό της λειτουργικής συσχέτισης και αλληλεπίδρασης μεταξύ των πρωτεϊνών, υποδεικνύοντας πιθανούς δείκτες στόχευσης κατά της εξέλιξης των λεμφωμάτων. Συνολικά, η μελέτη μας σκοπεύει στην αποσαφήνιση του ρόλου της βιογένεσης και έκκρισης των εξωσωμάτων στη λεμφωματογένεση και στην ανοχή των λεμφωμάτων σε θεραπευτικές προσεγγίσεις που στοχεύουν στην ενεργοποίηση της p53.

Abstract

The role of exosomal pathways is important for the maintenance of cellular homeostasis, especially in tumorigenesis. Thus, the deciphering of the proteome of lymphoma wild type p53 would help us understand the patho-biology of lymphoma. In this study, our purpose was to identify the differentially expressed proteins which are associated with exosomes, the evaluation of their interaction and their therapeutic role after p53 re-activation with Nutlin (N3a) in three different type of lymphoma. *In vitro* human lymphoma cell line model systems -/+ N3a were subjected to comparative proteomic and advanced bioinformatics analysis. In total, 4037 proteins were identified and 77 proteins of them were associated with exosomes. Moreover, the analysis of the protein interaction network revealed their functional association and interaction between the proteins, indicating possible markers for targeting in human lymphomas. In conclusion, our study aims to investigate the role of biogenesis and secretion of exosomes in lymphomas and the tolerance of lymphoma in therapeutic approaches after p53 activation.

1.Introduction

1.1 Exosomes

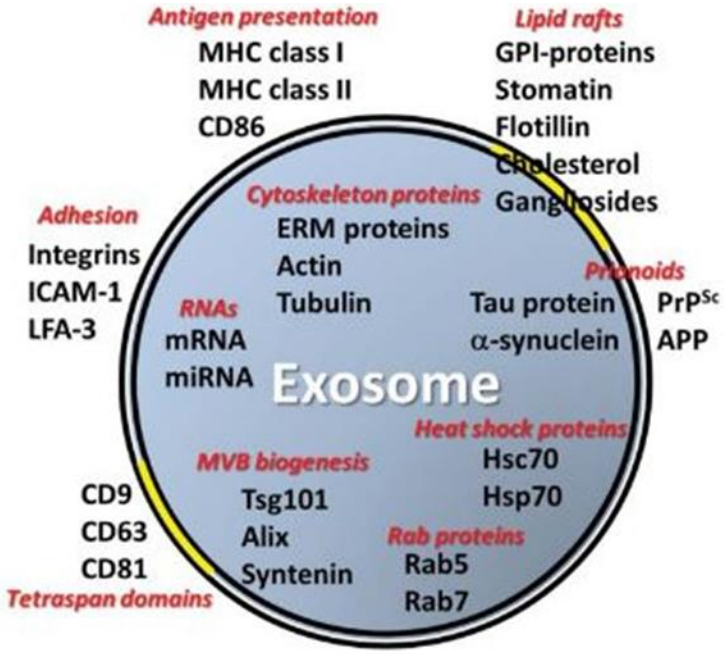
Exosomes were first discovered about 50 years ago. Although they were initially thought to be not essential products released via shedding of the plasma membrane, the interest on the subject was exploded this past decade[1]. Many different types of membrane-enclosed structures, which are called Extracellular Vesicles (EVs) are secreted from cells. It has been shown that EVs contain proteins and nucleic acids that could be transferred to recipient cells and change their physiological state[2]. There are three major types of vesicles that cells release into their extracellular microenvironment and these are exosomes, apoptotic bodies and microvesicles [3].

Exosomes are lipid bilayer structures. Their size ranges from 30 nm to 100 nm and their density from 1.13 to 1.19 g/ml. They are "cup-" or "dish-" shaped and released from the cell after membrane fusion [4]. Exosomes are mostly used to remove the rejected materials from the cells. In addition, they play an important role in immune response and in cells' communication. Researchers found that exosomes' proteins and nucleic acids have a functional role when they are transferred to another cell[5]. Blood, urine and saliva are some of the biological fluids that exosomes can be found. Thus, they could be used as possible biomarkers for various diseases [6].

1.1.1 Structure and function of exosomes

Exosomes consist of a lipid bilayer which surrounds their cytosol. The presence of lipids is important because they shape exosomes, as well as they participate in their function. Double-stranded DNA has been found in exosomes derived from cancer cells and it speculates the mutational status of the parental cells. In addition, mRNA and miRNA are also detected in exosomes. Different proteomic methods have been used to characterize the protein composition of tumor cell-derived exosomes. All this information can be found in ExoCarta, a database for exosomes' contents. These contents are different between physiological and pathological conditions. The composition of exosomes differentiates due to the selective sorting of the cargo into exosomes [5].

Exosomes contain proteins, which participate in membrane transport and fusion. Some of these proteins are Rabs, GTPases, annexins and flotillins. Moreover, Alix and tumor susceptibility gene Tsg101 are found in exosomes participating in biogenesis. Researchers are also found integrins and tetraspanins playing a functional role. e.g CD9, CD81, CD82, CD83 and CD63. However, the most common proteins among exosomes are the HSP90, HSP70 and CD63. Other groups of frequently found proteins are the cytoskeleton proteins (e.g β-actin, tubulins, myosin, cofilin), proteins which are related with the metabolism (e.g. glyceraldehyde 3-phosphate dehydrogenase) and major histocompatibility complex molecules e.g. MHC class I and II [7].



Picture 1 : Contents of exosomes
Blanc, L. & Vidal, M. New insights into the function of Rab GTPases in the context of exosomal secretion. *Small GTPases* (2018)

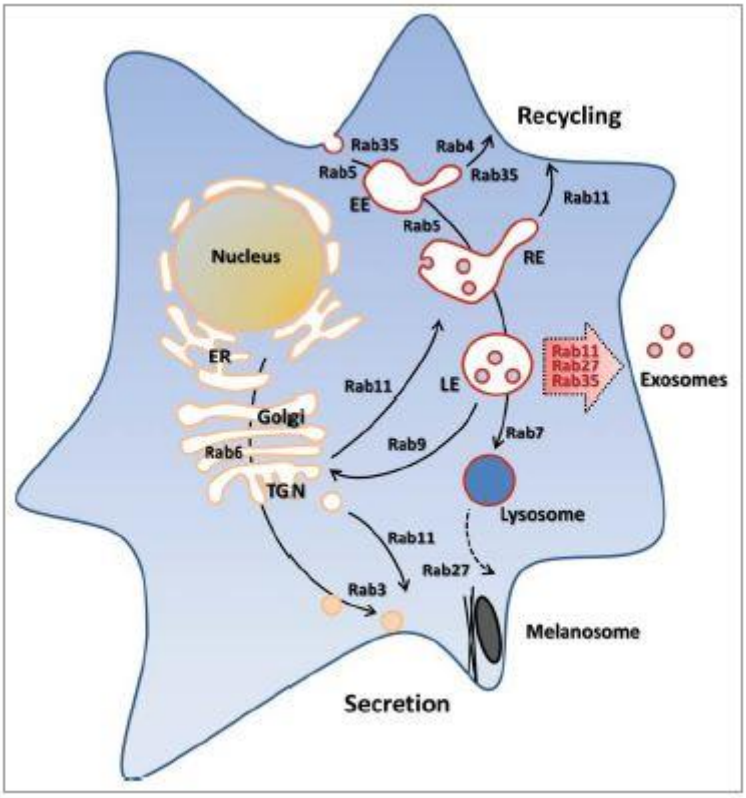
1.1.2 Important proteins of exosomes

Rab5a

Ras GTpases family contains the Rab GTPases. They function as molecular button with an ON (GTP) and OFF (GDP) state. They regulate a distinct intracellular transport step. Rab proteins are involved in endocytic and secretion pathways, as well as membrane transport and fusion [8]. Rab proteins have been shown to participate in the regulation of cancer progression.

Rab5 participates in endocytosis and vesicle transport [9]. EGFR-containing endosomes are regulated by Rab5 protein, and as a result, apoptosis is inhibited and te cell proliferation, angiogenesis, and invasion are induced[10]. Rab5 increases the rates of internalization and recycling of β1 integrins, stimulates focal adhesion disassembly and promotes the activation of Rac1, leading to cell migration[11].

More specifically, Rab5 was found to be induced in breast and ovarian cancers, promoting cancer growth and invasion. Hepatocellular carcinoma samples had also increased levels of Rab5a. Rab5a regulated the invasive tumor growth of breast cancer. Moreover, lung and gastric cancer with elevated amounts of Rab5a showed enhanced metastasis. Oncoproteins promote cancer, and thus Rab5a may belong to this type of proteins [9], [12].



Picture2: GTPase regulation.
Blanc, L. & Vidal, M. New insights into the function of Rab GTPases in the context of exosomal secretion. *Small GTPases* (2018)

HSPs

Stress conditions are the main factor, which activates a family of proteins called Heat Shock proteins (HSPs). Their main purpose is to act like chaperons. However, it has been found that they participate in cardiovascular function, as well as in antigen presentation. HSPs can also be found in vaccines as an immune response booster [7]. They can be found both in cytosol in small amounts, as well as in the extracellular environment when there is no cellular necrosis[13]. In general, chaperones prevent other proteins to misfold and aggregate. The most abundant members of this family are HSP90 and HSP70 [14].

HSP90

HSP90 is a protein-folding chaperone that shapes the manifestations of genetic variation in model organisms. HSP90 is present when there is a big necessity for growth. This event ensures that the cell will have a protein–folding buffer that will provide support for the function of proteins with critical control, such as protein

quality control and trafficking, signal transduction, apoptosis, cell-cycle regulation, gene silencing, and genome maintenance. The amount of HSP90 increases in stress and hypoxia conditions [14]. Secretion also increases following stimulation with various growth factors, including transforming growth factor alpha (TGFα) and epidermal growth factor (EGF)[15]. However, sometimes HSP90 levels may not be satisfying for the folding and function of mutant protein variants. Client specific cofactors , which are limited under stress, are required HSP90's activation [14].

Researchers showed that tumor invasiveness and metastasis could be affected by extracellular Hsp90 (eHsp90). Proteins are not only transported from the endoplasmic reticulum (ER) to the Golgi apparatus via vesicles, but also to the plasma membrane, in case of secretion. Studies showed that treatment of cells with dimethyl amiloride (DMA), in order to block the exosomal secretion pathway, significantly eliminated the amount of secreted eHsp90. In addition, translocation of Hsp90 to the cell surface has been associated with increased Hsp90 secretion, and phospholipase C gamma (PLCγ) and protein kinase C gamma (PKCγ) are needed for the translocation of Hsp90 to the plasma membrane. However, the mechanism, which Hsp90 is loaded onto exosomes for secretion, is not yet fully understood.

HSP90 secretion occurs when the Thr-90 is phosphorylated and the EEVD motif is cleaved. HSP90 ability of binding client proteins and its localization into the cell maybe be affected from post-translational modifications, such as phosphorylation and acetylation. These proteins may play a critical role for HSP90's targeting to exosomes and for exosomal cargo selection [15].

Researchers have found two isoforms of HSP90, HSP90α (84kDa) and HSP90β (86kDa). eHsp90 includes both isoforms α and β as well as some cochaperone complexes e.g. Hop, Hsp40, Hsp70, and p23. Hsp90N (75kDa) is a variant of the cytoplasmic HSP90 and appears to be membrane-associated. Hsp90 is also able to interact with the calcium–binding protein calmodulin. In addition, a pore complex is important for HSP90 to be transferred into the nucleus. When Hsp90 is inhibited, the endocytic pathway is activated, the sorting pathway of exosomes changes and the result is the accumulation of endosomes within multivesicular bodies and lysosomes, independent of cargo identity. Thus, Hsp90 may affect exosome secretion [15,16].
Studies have showed that HSP90 inhibitors are very effective. BL cells were led to apoptosis after HSP90 inhibition from the interfere of the spleen tyrosine kinase (SYK)-mediated tonic BCR signaling[17]. Human HSP90 levels are often increased and usually found in many types of cancer, such as breast cancer, fibrosarcoma, glioblastoma, melanoma and prostate cancer[15].

Gene name	Protein name	Old names	Human gene ID	Cellular location
HSPC1[a]	HSPC1	HSP90AA1; HSPN; LAP2; HSP86; HSPC1; HSPCA; HSP89; HSP90; HSP90A; HSP90N; HSPCAL1; HSPCAL4; FLJ31884	3320	cytosol
HSPC2[a]	HSPC2	HSP90AA2; HSPCA; HSPCAL3; HSP90ALPHA	3324	cytosol
HSPC3[a]	HSPC3	HSP90AB1; HSPC2; HSPCB; D6S182; HSP90B; FLJ26984; HSP90-BETA	3326	cytosol
HSPC4[a]	HSPC4	HSP90B1;ECGP; GP96; TRA1; GRP94; endoplasmin	7184	endoplasmic reticulum
HSPC5[a]	HSPC5	TRAP1; HSP75; HSP90L	10131	mitochondrial matrix

Table 1: Human HSP90 family of chaperons[18].

HSP70

The HSP70 family includes at least 13 members. Four of them are the major proteins and these are the HSC70, the GRP70 which is localized in the endoplasmic reticulum, the mitochondrial mtHSP70 and the stress-inducible HSP70.

Protein	UniProt ID	Alternative names	Cellular localization	Length (aa)	Stress-inducible
HspA1A	P0DMV8	Hsp70-1, Hsp72, HspA1, Hsp70-1A, Hsp70i	Cytosol, nucleus, cell membrane, extracellular exosomes	641	Yes
HspA1B	P0DMV9	Hsp70-2, Hsp70-1B	Cytosol, nucleus, extracellular exosomes	641	Yes
HspA1L	P34931	Hsp70-1L, Hsp70-hom, Hsp70-1t, Hum70t	Cytosol, nucleus	641	No
HspA2	P54652	Heat shock 70kD protein 2, Hsp70.2	Cytosol, nucleus, cell membrane, exosomes	639	No
HspA5	P11021	Hsp70-5, BiP, Grp78, Mif-2	ER, extracellular exosomes	654	No

HspA6	P17066	Hsp70-6, Hsp70B'	Cytosol, extracellular exosomes	643	Yes
HspA7	P48741	Hsp70-7, Hsp70B	Blood microparticles, extracellular exosomes	367	Yes
HspA8	P11142	Hsp70-8, Hsc70, Hsc71, Hsp71, Hsp73	Cytosol, nucleus, cell membrane, extracellular exosomes	646	No
HspA9	P38646	Hsp70-9, Grp75, HspA9B, MOT, MOT2, PBP74, mot-2, mtHsp70, mortalin	Mitochondria, nucleus	679	No
HspA12A	O43301	Hsp70-12A, FLJ13874, KIAA0417	Intracellular, extracellular exosomes	675	No
HspA12B	B7ZLP2	Hsp70-12B, RP23-32L15.1, 2700081N06Rik	Endothelial cells, intracellular, blood plasma	685	No
HspA13	P48723	Hsp70-13, Stch	ER, extracellular exosomes, microsomes	471	No
HspA14	Q0VDF9	Hsp70-14, Hsp70L1	Cytosol, membrane	509	Yes

Table 2: Human HSP70 family of chaperons[18,19].

HSC70 can be found in every organ and tissue. The role of HSP70, similar to HSP90, is to act like a chaperone. Some of HSP70's roles are to prevent protein misfolding and to transport proteins across the membrane of the cell in normal conditions.

HSP70 is found under different stressful conditions. Transcriptional heat shock factor 1 (HSF1) regulates the HSP70 synthesis. Heat shock elements (HSEs) are essential for this purpose. The presence of HSP70 under stressful conditions helps cells to deal with increased concentrations of unfolded or denatured proteins. Death receptors signaling and executors of cell death program are being affected by HSP70, regulating apoptosis in different ways (extrinsic and intrinsic). Bcl-2 and Bax, which target p53, have been found to be affected by HSP70. Moreover, when apoptosis is

inhibited by HSP70, the cytochrome c is not able to be released while caspase-3 is activated.

When it comes to cancer, HSP70 often protects cells undergoing transformation from oncogenic stress, induced by overexpression of oncogenes. Tumor derived exosomes have HSP70 on their plasma membrane. There is a great interest for the possible immune-adjuvant effect of exosome HSPs, which can enhance the maturation of dendritic and NK cells. In addition, HSP70 suppresses cellular senescence, something that is important in the proper response to anti-cancer therapy [20].

The connection between the overexpression of HSP70 and the aggressiveness of different types of cancer was proved by many proteomic approaches [21]. Cancer development and chemotherapeutic resistance are some of the results of HSP70 high expression levels. Thus, HSP70 is more presented on the cytoplasmic membrane of cancer cells [22].

1.2 Biogenesis, secretion, and uptake of exosomes

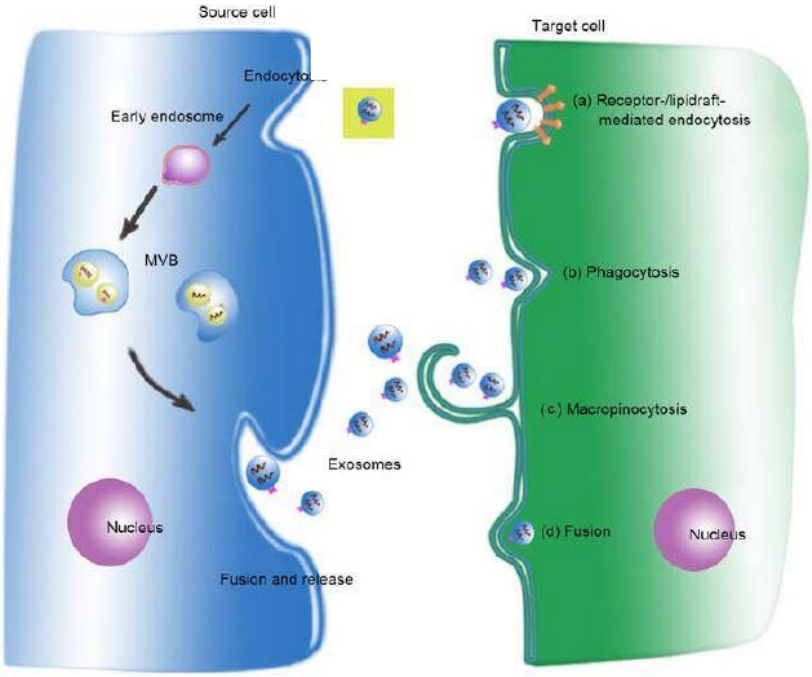
Biogenesis of exosomes takes place at the plasma membrane. The way that proteins' cargo is organized is not clear yet. Exosome biogenesis use the endosomal sorting complex required for transport (ESCRT) and the multimolecular machinery for their formation. Protein complexes from the cytosol built the ESCRT machinery. The set of these proteins is recruited from some membrane proteins, which have been ubiquitinated on their domain that is in contact with the cytosol. Tsg101 recognizes the proteins, which have been ubiquitinated in this pathway. However, there is another way for vesicles to be formed without the ESCRT machinery. ESCRT-independent pathway requires the tetraspanin CD63. Researchers have showed that this formation maybe speed up due to the properties of lipids, such as ceramide. In addition, biogenesis of exosomes is been controlled by the syndecan heparin sulfate proteoglycans and their cytoplasmic adaptor syntetin[1,4].

Various mechanisms involving the ESCRT machinery, a ceramide- based mechanism, tetraspanism, tetraspanin proteins, Alix or Hsc70 have been described as potential mechanisms that are driving protein sorting and budding events[8]. Rab GTPases control their degradation and their secretion.

The final release takes place when vesicles are fused with the cellular plasma membrane. SNAREs arbitrate this process. In addition, tumor derived exosomes are increased when the expression of p53, PKM2 and TSAP6 is induced. Secretion of exosomes has also been affected by the accumulation of intracellular Ca^{2+} and differences in the microenvironmental PH.

The uptake of the exosomes is carried out by several molecular mechanisms. The most common is endocytosis, which consists of a range of molecular pathways, such as calthrin-mediated endocytosis, coveolin-mediated endocytosis, phagocytosis and macropinocytosis. Direct cell surface membrane fusion of exosomes is another way

of uptake, which can take place in low PH. Moreover, studies have showed that lipid rafts participate in exosome internalization and thatthe uptake of exosomes by recipient cells is an energy-dependent process. In ovarian cancer cells, there are endocytic pathways that inhibit exosomes inserted into the cell at 4˚C [4].



Picture 3: Biogenesis, release, content and uptake of exosomes.
Guo, W. *Et al.* Exosomes: New players in cancer (Review). *Oncology Reports* (2017)

1.3 Biological role of exosomes in cancer

Exosomes, which are released from cancer cells have been found to be implicated in tumor growth, tumorigenesis, angiogenesis, immune escape, therapeutic resistance and tumor metastasis. However, the role and the functions of exosomes still remain unknown. Nevertheless, they have come out as a potential source of information to identify cancer and to provide information for tumor progression and metastasis.

Many biological fluids contain exosomes, e.g. urine, breast milk, blood, amniotic fluid, etc. Thus, exosomes could be used as biomarkers of biological fluids helping the diagnosis of cancer e.g. prostate, pancreatic, breast, and ovarian cancers, glioblastoma and melanoma. Moreover, lipids and metabolites could offer information about the biology of cancer. DNA in exosomes may supply information about cancer-specific mutations, while proteins may aid in the detection of cancer. For instance, tumorigenesis may be promoted from oncoproteins. The purpose of exosome release is to affect normal and tumor cells through communication [23]. At the same time, tumor cells release exosomes to reprogram their surroundings to be tumor permissive and even tumor promoting. Furthermore, exosomes modulate tumor antigen presentation and polarize tumor immunity. For instance, exosomes derived from dendritic cells can activate T and B cells, and perhaps, those derived

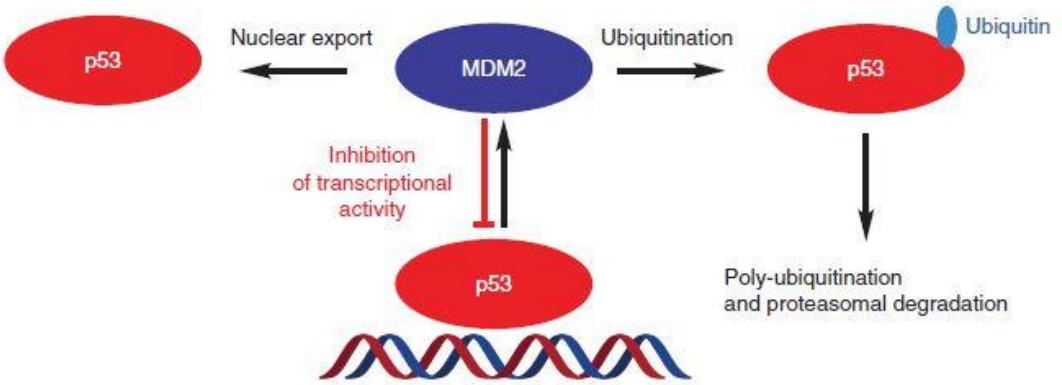
from cancer cells could directly activate NK cells via presentation of the stress protein HSP70. Increased exosome production due to intratumoral hypoxia stimulates angiogenesis in endothelial cells. Exosome-mediated cell signaling induces invasive features of cancer cells associated with metastasis. Thus, understanding the precise physiological function of exosomes will be critical to determining their role in cancer [4, 24].

1.3.1 Lymphoma

Lymphomas are a group of haematological malignancies derived from lymphocytes and occur predominantly in lymph nodes or other lymphoid structures. More than 50 different types of lymphoma have been described. They are clinically and biologically heterogeneous and have overlapping diagnostic features[25]. There are two main types of lymphomas, Hodgkin Lymphoma and Non-Hodgkin Lymphoma. Hodgkin lymphoma (HL), usually derived from B- lymphocytes, includes five different types of lymphoma and involves the Reed-Sternberg cells, which are giant, multinucleated abnormal lymphocytes with a unique morphology and immunophenotype[26]. Non-Hodgkin lymphoma (NHL) derive from B-, T-, or NK-lymphocytes and includes at least 60 types, some of which are more common than others. Non-Hodgkin Lymphomas do not involve Reed-Sternberg cells.

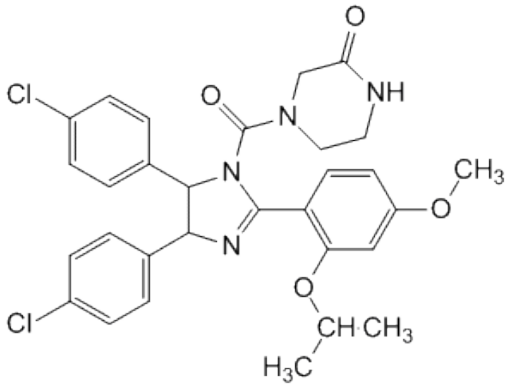
Our study focuses on one type of HL (classical Hodgkin Lymphoma (cHL)) and two of NHL, Mantle Cell Lymphoma (MCL) and Anaplastic Large Cell Lymphoma (ALCL). cHL contains the Reed-Sternberg cells and it is affected by factors which are related with family, exposure to viruses and immune response inhibition [27]. Mantle Cell Lymphoma (MCL) belongs to the Non-Hodgkin Lymphomas and it is very aggressive. Its most common characteristic is the t(11;14)(q13;q32) translocation, resulting in the induction of cyclin D1 expression levels. Many pathways are also deregulated, such as NOTC1, BCR, Mtor, BAFF-R, and WNT signaling[28, 29]. ALCL is a Non-Hodgkin lymphoma and it is characterized by strong CD30 immunostaining and recurrent chromosomal translocations involving the Anaplastic tyrosine Kinase (ALK) gene. ALK is translocated between 16 and 17 exons, and the most frequent chromosomal rearrangement is NPM1/ALK[30].

p53 regulates many cellular processes and its role is to act as a transcription factor. Some of these processes are DNA repair, apoptosis, cell-cycle progression, metabolism, and senescence. However, its main function is to be a tumor suppressor gene. The expression and activity of p53 is highly controlled. An oncogene called MDM2 forms a negative-feedback loop with p53 negatively regulating it, preventing cell from apoptosis. p53 activates MDM2, while the latter protein inhibits p53by MDM2-binding, reducing its transcriptional activity. Furthermore, MDM2 can export p53 from the nucleus to the cytoplasm, or target p53 with ubiquitination for proteasomal degradation [31, 32].



Picture 4: Regulation of p53 from MDM2 [Chène, P. Inhibition of the p53-MDM2 interaction: targeting a protein-protein interface. *Mol. Cancer Res.* (2004)]

p53 mutations can accelerate tumorigenesis. Mutations of p53 gene have been found in different types of cancer, including lymphoma[33] [34] . Interaction of MDM2 with p53 can be antagonized by a small lipophilic molecule called Nutlin-3a (N3a), that binds to the MDM2's p53-binding pocket, leading to p53 re-activation [35].



Picture 5: The structure of Nutlin-3.
(https://en.wikipedia.org/wiki/Nutlin)

1.3.2 Exosomes in Lymphoma

B lymphocytes can interact with exosomes, which contain molecules, such as ICAM-1 and MHC class II. This interaction provokes antigen-specific effector responses. Surface Proteoglycans (HSPGs), such as Syndecans and Glypicans may aid exosomes uptake from B cells[36]. Studies have showed that B-lymphocytes, as well as dendritic cells, release exosomes in normal physiology. These exosomes have multiple immune functions, which lead scientists searching for possible ways to use them as vaccinations against malignant diseases. In addition, exosomes released from lymphomas originated by B cells, can be used for early diagnostic applications. For instance, exosomal B cell antigens may inhibit immunotherapy. The presence of CD20 on exosomes facilitates the treatment with rituximab at B cell lymphomas [37, 38]. Tumor associated mRNA in exosomes could be used as a prognostic value in patients with B cell NHL, while BCL-6 and c-MYC expression indicated worse progression in

the pretreated samples [39]. The complex MyD88L265P, IRAK4 and IRAK1/2 kinases activates NF-kB and induces secretion of interleukin-6 (IL-6), IL-10, interferon b and immunoglobulin M by lymphoma B cells[40]. Mantle cell lymphoma (MCL) exosomes in both healthy and diseased B-lymphocytes used clathrin and caveolin for fusion and when they were applied to B lymphocytes, NK cells and T lymphocytes, they were only incorporated into B lymphocytes [36].

Lymphomas, which are associated with Epstein Barr Virus (EBV) infection, release exosomes, which contain molecules essential for the adaptive immune response. This virus can modify the contents of exosomes, especially LMP-1 expression. In addition, EBV induces proliferation, migration, invasion and B cell differentiation [41].

DLBCL exosomes mainly suppress the immune system, while they promote proliferation of the cell, invasion, angiogenesis, and migration. The cells which are affected from migration are human fibroblasts and stromal cells [39]. Moreover, exosomes with mutated MYD88 showed to reprogram the marrow microenvironment [42]. Furthermore, exosomes in the presence of p-ALK may be responsible for tumorigenesis and resistance to ALK Inhibitors[43]. Studies have also showed that T cells could be stimulated by exosomes with antigen-presenting cells (APC)[36]. Thus, it is believed that exosomes released from lymphomas may be of great importance to be further studied for their role in the disease.

1.4 Functional Proteomics in Lymphoma

Mass spectrometry (MS) is a modern method for the quantitation and identification of proteins with high accuracy. There are two ways for protein analysis: “bottom-up” approach and “top-down” approach. In bottom up approach, proteins are separated through electrophoresis according to their molecular weight, being digested into peptides, which are separated by liquid chromatography and identified by mass spectromentry. In top-down approach, an intact protein is purified and then analysed with mass spectrometry [44]. Proper protein sample preparation is integral for a successful MS-based proteomics experiment (e.g. proteins should be solubilized with detergents for in depth proteome analysis, MS-incompatible components, such as detergents, should be removed or replaced when possible, before LC-MS/MS analysis).

Filter-aided sample preparation (FASP) is used for protein digestion and removal of the detergents. After digestion, peptides are eluted pure, free of the high-molecular weight interfering substances. The use of the filter is the great advantage of FASP method, which combines the benefits of “in gel” and “in solution” digestion. Even if SDS solubilizes the proteome, including hydrophobic membrane proteins, it should be removed from the sample because it decreases the resolution and the signal. FASP followed by desalting method allows removal of SDS, salt, nucleic acids and lipids prior to on-filter digestion of solubilized proteins.

After digestion and before the peptides are inserted in the mass spectrometer (MS), they cross a Microscale Capillary High-Performance Liquid Chromatography column. The peptides are eluted from these columns depending on their hydrophobicity by

using an organic solvent. Molecules entering the MS should be charged and dry. Peptides become ions because they gain or lose protons. ESI is a “soft” ionization method that allows the formation of ions without sample corruption, which is important in order to discover the accurate mass information about the proteins[45].

1.5 Scope of Thesis

Our purpose was to identify the differentially expressed proteins, which are associated with exosomes, and to evaluate their interaction and their therapeutic role after p53 re-activation with (N3a in three different types of lymphoma.

The results of this study will help better understand the exosomal pathways, such as biogenesis, secretion and uptake of exosomes. In addition, we will be able to identify proteins, which may have a role as biomarkers, assisting us to detect the disease at an early stage and investigate their role in lymphomagenesis.

2. Materials and Methods

2.1 Cell lines and growth conditions

Three cell lines were used, from different types of lymphoma. The cell lines were **MDA-V, JMP-1, SUP-M2,** kindly provided to our lab by Prof. E. Drakos and his colleagues from the University of Texas MD Anderson Cancer Center, Houston, TX, USA.

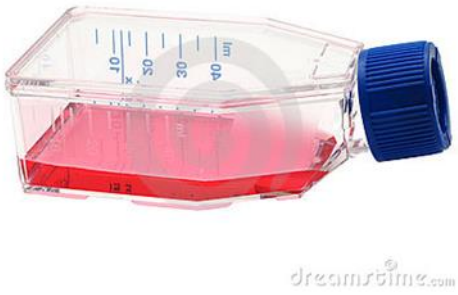
MDA-V belongs to Classic Hodgkin lymphoma (cHL) and it is a classic B-cell Hodgkin lymphoma. It was created from a 45 year old untreated male patient's lymph nodes with EBV-positive, who was diagnosed with stage I cHL with nodular sclerosis.[46]

JMP-1 (M-1) is a B-cell Non-Hodgkin Lymphoma (NHL) and belongs to Mantle Cell Lymphoma family (MCL). It was derived from peripheral blood sample of a 58 years old man, with relapsing mantle cell lymphoma. The patient was diagnosed with blastoid mantle lymphoma in leukemic phase and had the chromosomal rearrangement t (11; 14) (q13; q32). [47]

SUP-M2 is a T-cell Non-Hodgkin lymphoma (NHL) cell line. It belongs to Anaplastic lymphoma from Large Cells (ALCL), ALK-positive family, bearing the translocation t (2;5), which leads to the creation of mutant NPM-ALK gene. They express CD30+. These round cells can grow either individually or in small concentrations (piles). SUP-M2 was derived from cerebrospinal fluid of a 5-year-old girl, who was considered to be suffering from uncontrolled "Malignant Histocytosis". [48]

Cell growth was performed in 84% RPMI 1640 (ThermoScientificLSG, Gibco) with 1% L-glutamine, 15% fetal bovine serum (ThermoScientificLSG, Gibco) heat inactivated (56ºC for 30 '), and 1% penicillin- streptomycin (ThermoScientificLSG). The medium was filtered using 0,2mm filters to avoid contamination.

Cells were subcultured in flasks (KISKER, Germany, 25cm^2&75 cm^2), handled under aseptic conditions and experiments were performed in 6-well plates (in triplicates). Incubation was held in an incubator (Shel lab) with wet air atmosphere of 95% oxygen and 5% CO_2 and a temperature of 37 ° C.



Picture 6:Flask 25 cm^2
(https://www.dreamstime.com/photos-images/tissue-culture-flask-red-liquid.html)

Picture 7 :Cell Culture's incubator.
(https://cellbiology.med.unsw.edu.au/cellbiology/index.php/2012_Lab_9)

Cells were cryopreserved submerged in liquid nitrogen or stored in a freezer of -80° C. The composition of freezing medium was 60% RPMI with FBS, 30% FBS and 10% DMSO which is used to reduced ice formation and prevent cell death during the procedure. Cryovials were used for cell-storage at low temperatures.

Cells were thawed when needed, requiring quick handling in order to minimize the toxic effects of DMSO, measuring the success of the cryopreservation by calculating the percentage of alive cells and able to recover normal function once thawed. A controlled rate of freezing and rapid thawing was necessary for optimal cryopreservation and cell-recovery.

2.2 Viability testing with Trypan Blue Exclusion Method

For the viability test, cells were stained with Trypan blue dye (TB) (1:1, cell suspension:TB) and placed on a hemocytometer which is a counting-chamber device for cells. Alive cells have intact membranes and do not absorb the dye, while in dead cells the membrane is traversed. Thus, dead cells appear with blue color, while live cells are not coloured[49]. After measuring the number of alive and cells in a phase contrast optical microscope, followed calculation of the cell viability. The viability measurement was repeated every other day for the renewal of the medium

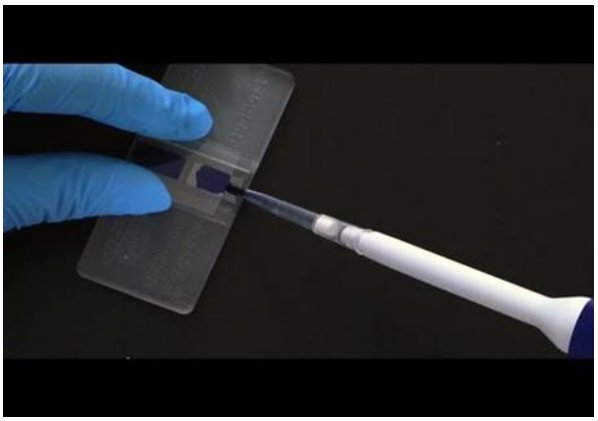
containing growth factors and for maintaining the cell culture at a density of 10^6cells/ml.

$$\textbf{\textit{\% Viability}} = \#Alive\ cells/\ (\#\ (Alive + Dead)\ cells)*100$$

The total number of cells was calculated by the following equation:

$$\textbf{\textit{Concentration of cells/ml}} = Number\ of\ cells\ *Dilution\ Factor*10^{4\#}$$

#10^4 corresponds to hemocytometer volume



Picture 8:.Hemocytometer
(https://www.youtube.com/watch?v=pP0xERLUhyc)

2.3 SDS-PAGE electrophoresis

SDS-PAGE helps us separate proteins according to their molecular weight. The proteins move through the pores of the gel depending on their size. A protein marker of proteins with known molecular weight is used to identify the proteins.
All the electrophoreses were performed under reducing conditions, in order not to retain complexes in the gel. Gels of 10% and 12% concentration of acrylamide were used.

Reagents	10% Running gel (10ml)	12% Running Gel	5% Stacking Gel
dH_20	4 ml	3,3 ml	3,4 ml
Acrylamide	3,3 ml	4 ml	0,83 ml
SDS 10 %	100 µl	100 µl	50 µl
APS 10%	100 µl	100 µl	50µl
Tris- HCl	2,5 ml (1,5M Tris-HCl pH 8,8)	2,5 ml1,5M Tris-HCl pH 8,8	0,63 ml (1M Tris-HCl pH 6,8)
TEMED	4 µl	4 µl	5 µl

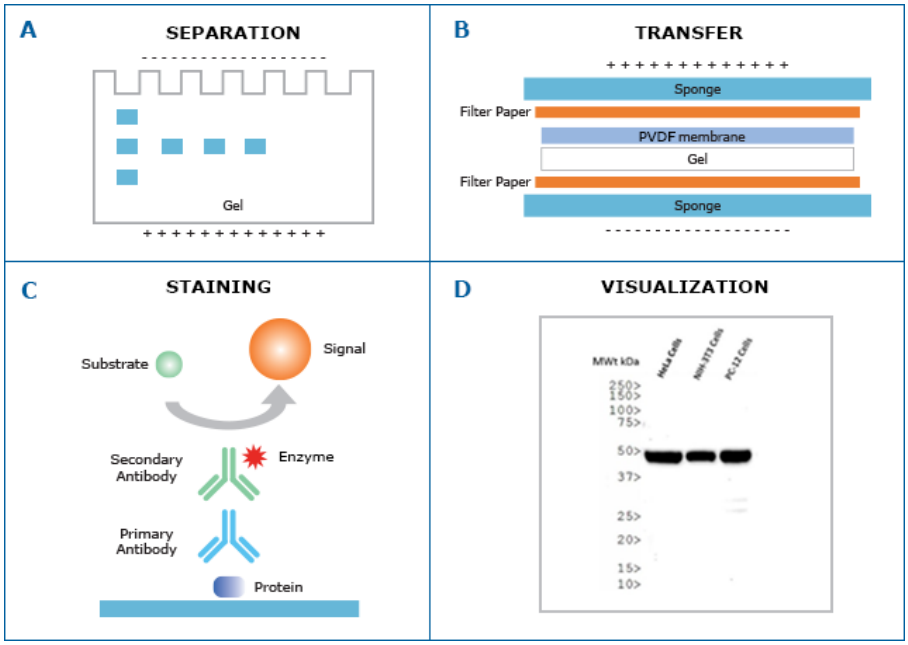
The first step was to prepare polyacrylamide gel according to standard protocol and the molecular weight of proteins we wished to separate. We filled the container with running buffer 1x. Before loading, samples were heated at 95˚C for 5 min. Samples and the suitable marker were loaded and the gel run at 80 mA for 10-15 min and in the time that the sample leaves the stacking gel we continued at 120 mA. At this point, the gel could either be transferred to a membrane (Western protocol) or stained with Coomassie Blue R250 (proteomics analysis).

In our case, two types of samples were used: cell lysates and cell supernatant. The cells were lysed using the Ripa 1x Buffer, the supernatants were precipitated according to the methanol-ethanol protocol. Then the protein pellets were lysed with the FASP Buffer and FASP protocol was followed, as mentioned below. After lysing, samples were diluted in Sample Buffer 5x. The concentration of protein and sample buffer in each sample was determined by performing Bradford and BCA protein assays.

Cells lysis protocol and protocol for precipitation can be found in the appendix.

2.4 Western Blot

Western Blot is used to identify specific proteins and relies on the specificity of the antibody for the protein of interest. It is held in two parts, the transfer of the proteins on a PVDF/nitrocellulose membrane and the incubation of the membrane with the suitable antibody. This technique is important for determining protein expression, sub-cellular localization (via cell fractionation), post-translational modifications (e.g. ubiquitination, phosphorylation, etc.), protein processing, and protein-protein interactions when coupled with immunoprecipitation[50].



Picture 9 : Western Blot assay.
(https://www.novusbio.com/application/western-blotting)

The protocol includes SDS-PAGE running of the samples as the first step. In case of PVDF, membrane is wetted with methanol for 1-2 min and after that it is washed with ddH2O for 2 min, twice. Membrane is soaked in transfer buffer for 10 min and gel for 20 min. Next step is the setting up of the transfer from the membrane to the gel. The transfer sandwich is placed in a transfer chamber. The device is adjusted at 400 mA for 120 min for 1.5 mm gels. When transfer is over, the membrane is washed for 15 min with TBS buffer and the gel for 30 min in fixation buffer. Membrane is blocked for 1 hr with 5% milk in TBS-T with shaking before the overnight incubation in 4°C with the antibody. Gel is incubated with Coomassie overnight, post-transfer, to check the efficiency of the transfer. The next day the membrane is washed with TBS for 10 min (3X), incubated for 1 hour with the secondary antibody on a shaker and then is washed 3 times with TBS-T for 10 min. Detection of horseradish peroxidase (HRP) enzyme activity from antibodies of interest was able to occur after the incubation of the membrane with an ECL (enhanced chemiluminescent) substrate for 3-5 min. The membrane was exposed for 1-20 min, depending on the specific substrate used and the enzyme-to-substrate ratio in the system.

In our case, **3** different antibodies related with exosomes were used and **Anti-Actin antibody** as loading control (Clone a.a. 50-70, clone C4, Cat.No. MAB1501, Merck-Milipore):

HSP90 90kDa (Cat.No.4874 Cell signalling), which is monoclonal and detects endogenous levels of total HSP90 protein, a and b isoforms. This antibody does not cross-react with other HSPs. The dilution used was 1:1000 in 5% BSA in TBS-T.

HSP70 70kDa (Clone D69) (Cat.No.4876 Cell signalling), which detects endogenous levels of total HSP70 protein. The dilution used was 1:200 in 5% BSA in TBS-T.

Rab 5 25kDa (D-11, Cat.No. 46692, Santa Cruz), which is recommended for detection of Rab 5A, 5B and 5C. The dilution used was 1:500 in 5% BSA in TBS-T.

2.5 Protein Quantification Assays

The colorimetric detection and protein quantification of total protein in every sample was performed with Bradford and BCA methods. The use of each method depended on the compatibility of the reagent of Bradford or BCA with the chemical compounds of each cell lysate or supernatant, according to the manufacturer's instructions. Final protein concentration of unknown samples was calculated according to standard curve, taking into account any dilutions performed to the samples.

2.5.1 BCA Assay

The BCA (bicinchoninic acid) assay is based on the following principle:

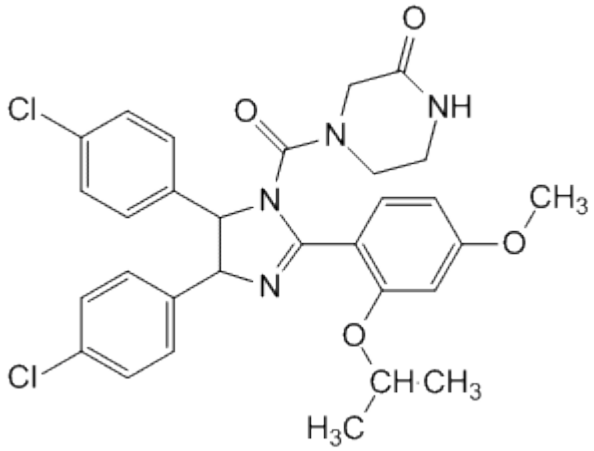
Protein + Cu^{++} => Tetra dentate – Cu^{+} complex
Cu^{+} + 2BCA => BCA-Cu^{+} complex (purple at A_{562})

Proteins in alkaline environment react with Cu ions and Cu^{2+} is reduced in Cu^{+}. After that Cu^{+} reacts with two BCA molecules creating a compound, which absorbs in 562nm. A protein range of 20 μg/ml – 2000 μg/ml is able to be identified[51].

At first, BCA working reagent was prepared in a dilution of 50:1 ratio of Reagent A and B respectively. Standard proteins of different concentrations of BSA were prepared. 200 μl of working reagent and 25 μl of sample were transferred in a 96-well plate. This step was followed for each standard protein and unknown sample. The plate was incubated in 37 ˚C for 30 minutes. The absorbance was measured in a photometer at 562nm.

2.5.2 Bradford Assay

Bradford assay is very fast and sensitive quantitative technique defining the whole concentration of proteins in biological samples. The method depends on the ability of proteins to interact with the Coomasie Brilliant Blue G-250 pigment in acid aqueous solutions.



Picture 10 : Coomassie Brilliant Blue G-250 structure.
(https://www.goldbio.com/product/3758/coomassie-brilliant-blue-g-250)

The pigment binds to the Arginine, Lysine and Hydrophobic amino acids. After the binding, the pigment takes its blue unprotonated form and the wavelength of its maximum absorbance changes from 465 to 595 nm [52].

In order to measure the absorbance of the sample, Bradford 5X reagent was diluted 1:5 in ddH_20. A series of BSA dilutions were prepared to create a standard curve. 1 ml of Bradford solution and 20 μl of sample were transferred into a plastic cuvette to make the measurement.

2.6 Methods used in Proteomics

In Gel Digestion of Coomassie stained denaturated gels Including S-S reduction and S-H alkylation

The bands of our interest were excised with a small lancet and transferred into 1,5ml tubes. In order to remove the dye, 2 washes with 100μl 50% ACN/water and then 100μl 50mm ABS were performed. This step was repeated twice with 15 min shaking during each wash. We reduced the gel pieces with 100μl 10mM DTT for 45min at 56oC. After the removal of DTT (Sigma Adrich), the samples were alkylated with 100μl 55mM IAA for 45min at room temperature in the dark. The IAA (Sigma Adrich) was removed and gel washing with 100μl 50% ACN/water and then 100μl 50mm ABS was performed. This step was repeated twice with 15 min shaking during of each wash. The proteins were digested with 100μl Trypsin (20μg/20μl of storage buffer) over night at 37°C. The next day, we transferred the supernatant into new tubes and we covered the gel pieces with 100 μl of NanoPure water, with constantshaking for 20 min. After that, water as transferred into the new tube and the gels were covered with 50μl of 50% ACN/water with constant shaking for 20 min. Next, all supernatants were transferred into the new tube and 50μl of 0.1% TFA-50% ACN/water were added into the tubes and gel pieces were left with shaking for 20 min. Finally, the supernatants were transferred into the new tube and samples were evaporated till dry powder by using a Speed Vac. The lyophilized samples were stored at 4°C for the nanoLC-MS/MS analysis [53].

Filter Aided Sample Preparation Method (FASP)

The following protocol describes the gel – free sample preparation and proteolysis steps that we followed performing the FASP protocol. 30 μl of each protein extract was mixed with 200μl 8M UA in a tube, transferred onto a filter unit (Microcon-30, Merck Milipore) which was centrifuged at 14.000 g at 20 °C for 20 min. Then, the flow-through was discarded from the collection tube. Next, 100 μL of iodoacetamide (Sigma Adrich) solution was added onto the filter unit, which was incubated for 20 min in the dark. The tube was centrifuged at 14.000 g at 20 °C for 10 min. The resulting concentrate was diluted with 100 μL of 8 M urea and was centrifuged again at 14.000 g at 20 °C for 15 min. This step was repeated twice, and the precipitate was diluted with 100 μL ABC and centrifuged again at 14.000 g at 20 °C for 15 min, which was also performed twice. The concentrate was then subjected to trypsin digestion (40 μl ABS with trypsin, enzyme to protein ratio 1:100) in a wet chamber for 12 h at 37 °C. The digestion solution was collected by centrifugation, and the filter device was rinsed with 40 μL of ABS and centrifuged again. This step was also performed twice. The resulting solutions were acidified with trifluoroacetic acid (TFA) and dried under vacuum [54].

Desalting

In order to remove salts and other contaminants and allow them to be washed off the sample, we used the desalting protocol. After the acidifying of the samples, peptides were desalted using homemade C18 (Empore 2215) pipette tips. Activation,

equilibration, peptide washes, and elution were followed. Tips containing C18 resin were activated by 90% acetonitrile (ACN), and then equilibrated by 5% aqueous solution of formic acid (FA). Subsequently, the peptide-rich solution was slowly aspirated and dispensed by tip for 3 cycles (after this step, peptides were bound to the C18 tips). Desalting was conducted through two washes with 5% FA. The desalted peptides were eluted using 20 µL 90% ACN/5%FA through 3-5 washes, and the elution product was dried under vacuum.

2.7 Nanoscale liquid chromatography coupled online with MS

Identification and relative quantification of proteins was done by reversed phase nano-flow chromatography coupled with electrospray ionization system and mass spectrometer (nLC-ESI-MS/MS). The peptides were dissolved in 10µl of formic acid aqueous solution of 0.5% and separated on reverse phase column (Reprosil Pur C18 AQ, particle size= 3 micrometers, pore size= 120 Angstrom (Dr. Maisch), inner diameter= 75µm, length= 15cm). The flow of small-scale elution liquid chromatography was 300 nl/min. Peptides separated and gradually eluted in water-ACN and entered the mass spectrometer.

2.8 Functional Analysis and pathway analysis

Visualization of MS-based proteomics data is an important part in high-throughput technologies. Identification, characterization, abundance, location, modifications, structure and interactions of proteins are some of the information derived. Bioinformatics analysis starts with SEQUEST, a program which helps us to compare the observed MS_2 spectra to theoretical spectra derived from in silico enzymatic digestion of a database. Databases search software packages can search only those peptides that have a mass within a narrow tolerance window, around the observed m/z of its precursor peptide ion. Thus, a visualization of the connections between spectra and peptides is created.

Popular tools allowing researchers to combine results from multiple search engines are Scaffold and Proteome Discoverer (PD). After that, the visualization of the connections between peptides and proteins is important to be created. However, a peptide can map to more than one protein and this is a major problem, known as the protein inference problem [55, 56].

Post translation modifications (PTMs) and their site assignment scores can support the interpretation of biological activity. Analysis of protein and peptide identifications needs the detection and visualization of PTMs. Moreover, the modification sites are often presented on the sequence or spectrum. Scaffold is used for this purpose.

Perseus is used to present the expression level of proteins in different conditions as parallel line charts (different cell lines on the *x*-axis, expression level on the *y* axis). Proteins which have similar regulation will be linked [57].
The identified proteins of all samples were further analyzed using the STRING database in order to predicted protein–protein interactions, to classify proteins by

GO enriched biological processes and cellular components [58], and to map proteins by KEGG pathways [59]. All bioinformatics analysis was performed using Uniprot Human database.

The Gene Ontology website provides information for functional genomics using ontologies to describe biological knowledge and it is edited as the biological information expands. Molecular function, cellular component and biological process are the 3 main elements of GO activity [60] [58].

String is a database which predicts interactions among proteins. It uses data that are derived from literature and experiments, text analytics, from interactions that came after the study of genomes, as well as from interactions which were observed after the study of model organisms [61].

KEGG assembles many databases with information about genomes, disease and biological pathways. It is very useful for bioinformatics and proteomic data analysis [59].

3. Results

3.1 Exosome related protein Proteome in 3 human lymphoma cells lines+/- N3a

The total number of proteins, which were identified and quantified in the three cell lines of our study was 4037 proteins, and 77 proteins of them were associated with exosomes. In MDAV cell line (HL) 2 unique proteins detected were associated with exosomes, while in JMP1 (MCL) 8 and in SUPM2 (ALCL) 5 proteins. The number of the proteins, which were common in all 3 cell lines, was 51, as we can see in Figure1 below.

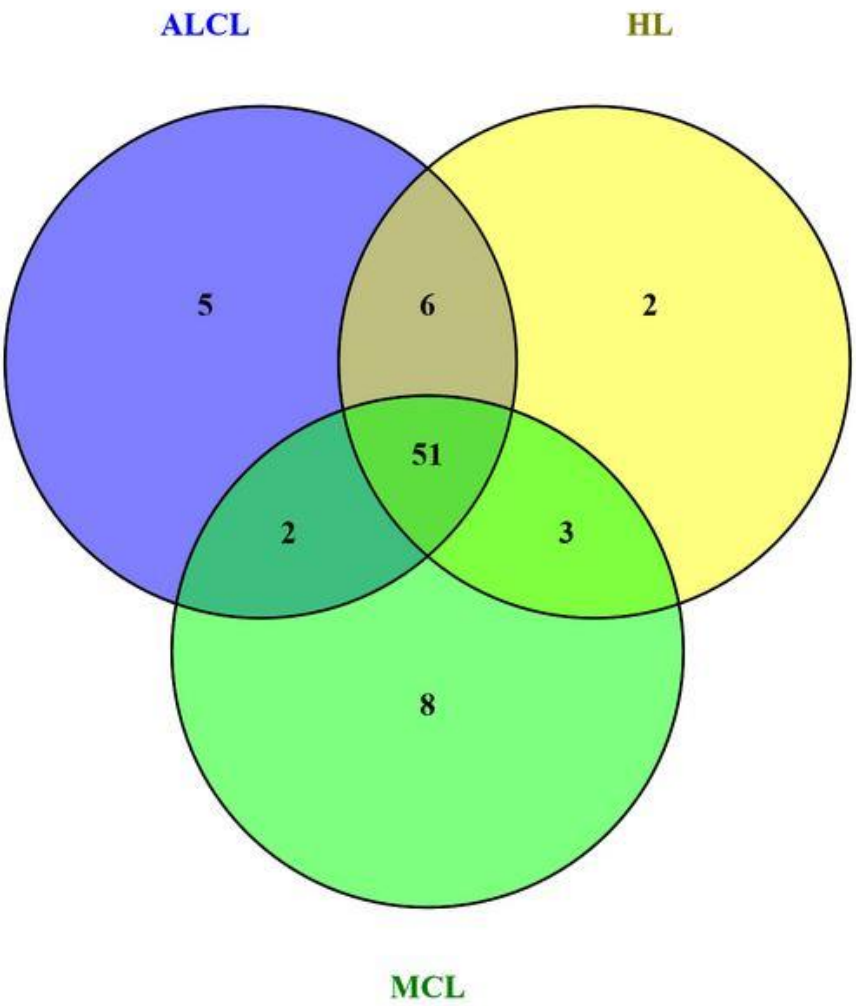


Figure 1: Total number of proteins for the three cell lines. On the diagram we can see the unique proteins of each cell line, proteins that were common for two cell lines and proteins that were common for all of them.

3.2 Protein Profiles of the 3 lymphoma cell lines using hierarchical clustering.

Heat map is a table, which contains colors instead of numbers, showing the expression levels of proteins in the 3 cell lines on the x axis, and proteins as dendrograms on the y axis. The color gradient indicates the lowest and the highest value in protein expression. The green color indicates low expression, the red color high expression, the black color no change and the grey color that this protein is not identified. Heat maps help us visualize large amounts of data and identify similar values in areas with similar color.

Clustering analysis is used in order to group objects into similar categories. Each row is considered as a cluster. In hierarchical clustering the two most similar clusters are combined and continue to combine until all objects are in the same cluster. This type of clustering creates a tree, which is called 'dendrogram'. This tree shows the hierarchy of the clusters. Hierachical clustering represents abundance values of proteins (rows) and the 3 cell lines (columns). Perseus was used in order to create a hierarchical cluster for exosome related proteins.

In our case, the proteins were clustered using simple unsupervised hierarchical clustering. HL and MCL cell lines were separated from ALCL cell line in one main branch of the dendrogram. In addition, HL had the most up regulated proteins and ALCL the most down-regulated proteins.

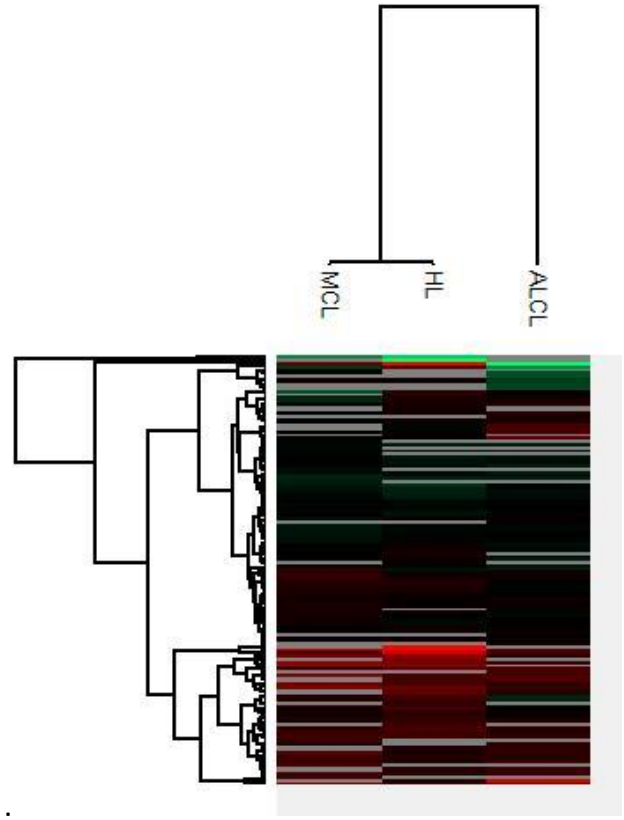


Figure 2: Heat map of proteins in our 3 cell lines. The first column from the left represents JMP-1, the second MDAV and the third SUPM2

3.3 Exosomal pathways

The exosomal proteins of the 3 cell lines of our study were further analyzed using the STRING database, in order to predict protein–protein interactions, classify proteins by GO enriched biological processes and cellular components and map proteins by KEGG pathways. All bioinformatic analyses were performed using Uniprot Human database.

Proteomic results were further processed, in order to investigate the identification of a variety of proteins with a role in exosomal pathways and the predicted protein-protein interactions in the 3 cell lines of our study, after p53 re-activation with N3a. In particular, MS-based proteome analysis revealed 77 proteins associated with exosomes, showing important differences in their expression levels. Among them, 43 proteins were involved in **exocytocis** (e.g. HSP90B, Rab3B&D, Rab5C, Rab6A, Rab8A&B, Rab10, Rab11B, Rab14, Rab18, Rab27A). Rab3D was only found in MDA-V (HL) and overexpressed after treatment with N3a, while Rab3B detected in JMP-1 (MCL) was reduced after N3a. Rab27A was overexpressed in MDA-V (HL) and JMP-1 (MCL), whereas SUP-M2 (ALCL) showed no difference in its expression levels. Rab8B expression levels were higher in SUP-M2 (ALCL) and MDA-V (HL) but not in JMP-1 (MCL). In addition, 11 proteins were associated with **endosomal transport** (Rab6A&C, Rab7A, Rab8A, Rab9A, Rab10, Rab11B, Rab13, Rab14, Rab35) and 6 proteins with **endosome organization** (Rab5B, Rab27A, VPS11, VPS18, VPS33B, CHMP2A). Rab13 was only detected in MDA-V (HL) and its expression levels were high. VPS33A was overexpressed in all the 3 cell lines. Calnexin, Rab9, HSP90b, Rab14 were also associated with **endocytosis**. Calnexin was overexpressed in JMP-1 (MCL) after N3a treatment, while Rab9 expression levels were higher in MDA-V (HL) and JMP-1 (MCL). Finally, 5 proteins associated with **endosome to lysosome transport** were also detected (VPS18, VPS33A, VPS11, VAMP7, Rab7A). VAMP7 was overexpressed in all 3 cell lines.

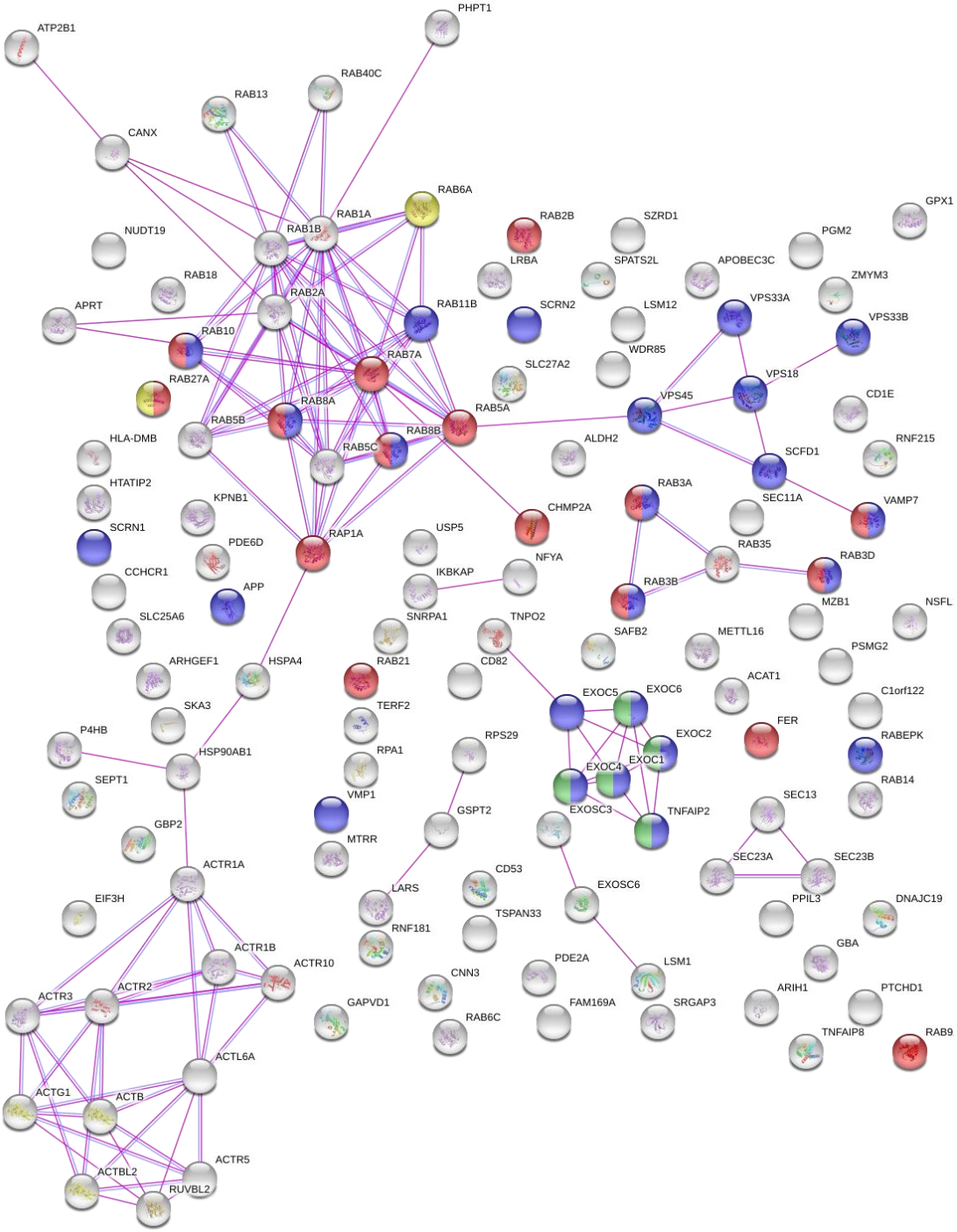


Figure 3: Protein-protein interactions in exosome-related proteins with STRING.

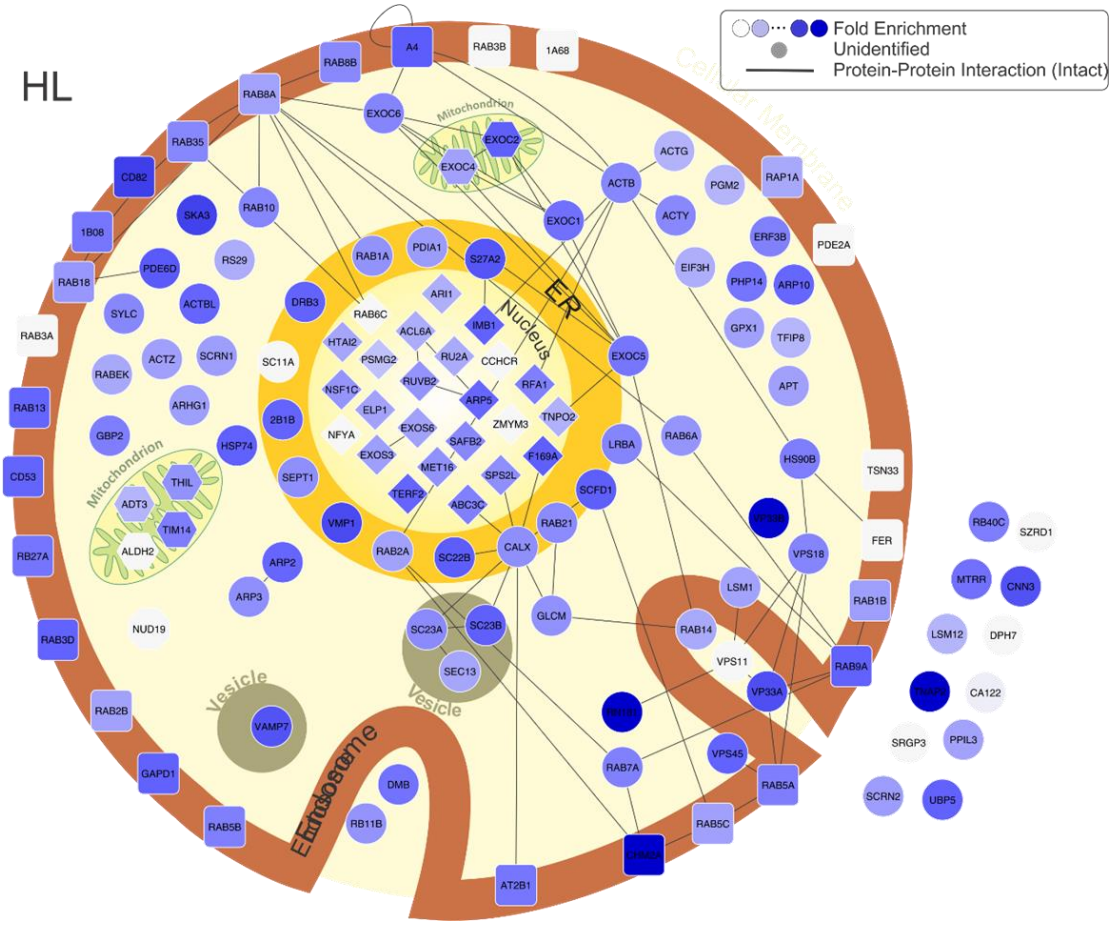


Figure 4: Protein-protein interactions among proteins of exosomal pathway and autophagy in Hodgkin Lymphoma (HL). The darker the color, the bigger the fold enrichment is. Grey color indicates that this protein is not identified. The black lines show possible interactions. The shapes indicate the location of the protein: membrane proteins are square, cytoplasmic proteins are circles, nuclear are rhomboid, mitochondrial are polygon.

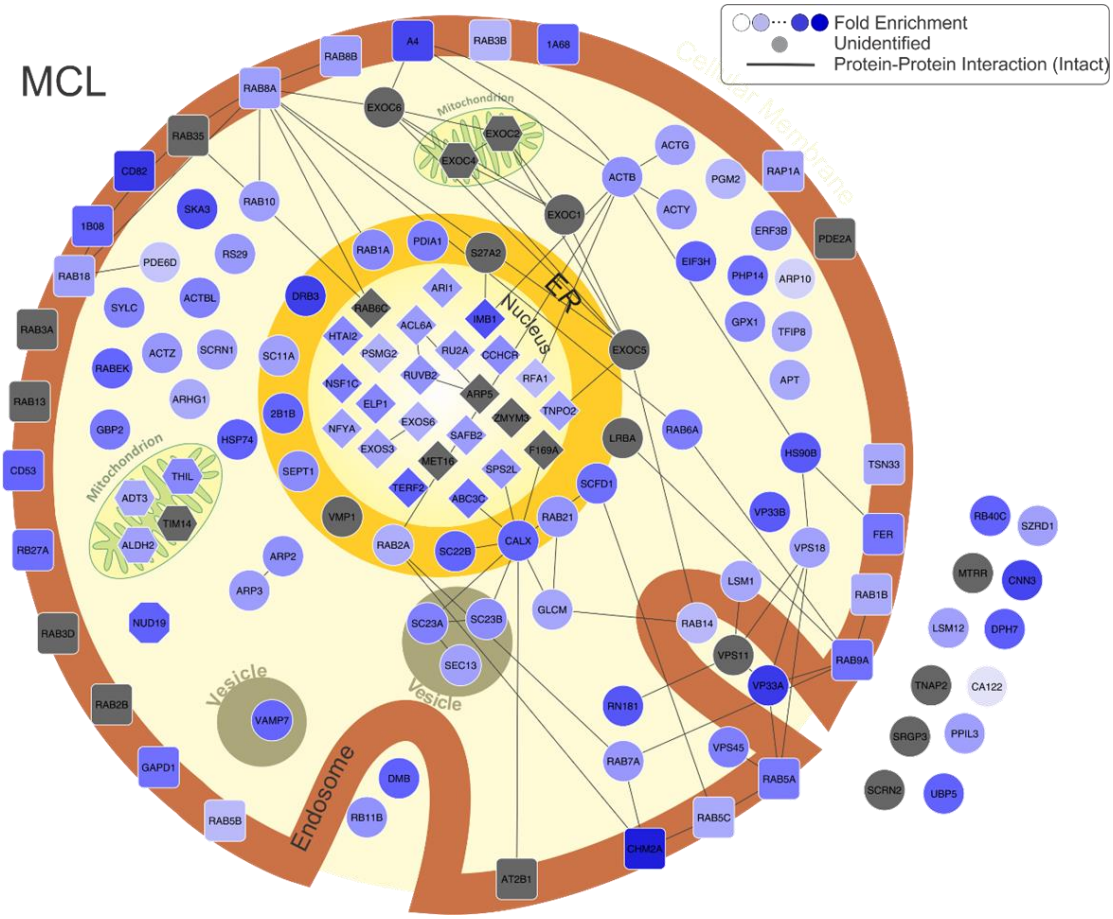


Figure 5: Protein-protein interactions among proteins of exosomal pathway and autophagy in Mantle Cell Lymphoma (MCL). The darker the color, the bigger the fold enrichment. Grey color indicates that this protein is not identified. The black lines show possible interactions. The shapes indicate the location of the protein, e.g membrane proteins are square, cytoplasmic proteins are circles, nuclear are rhomboid, mitochondrial are polygon.

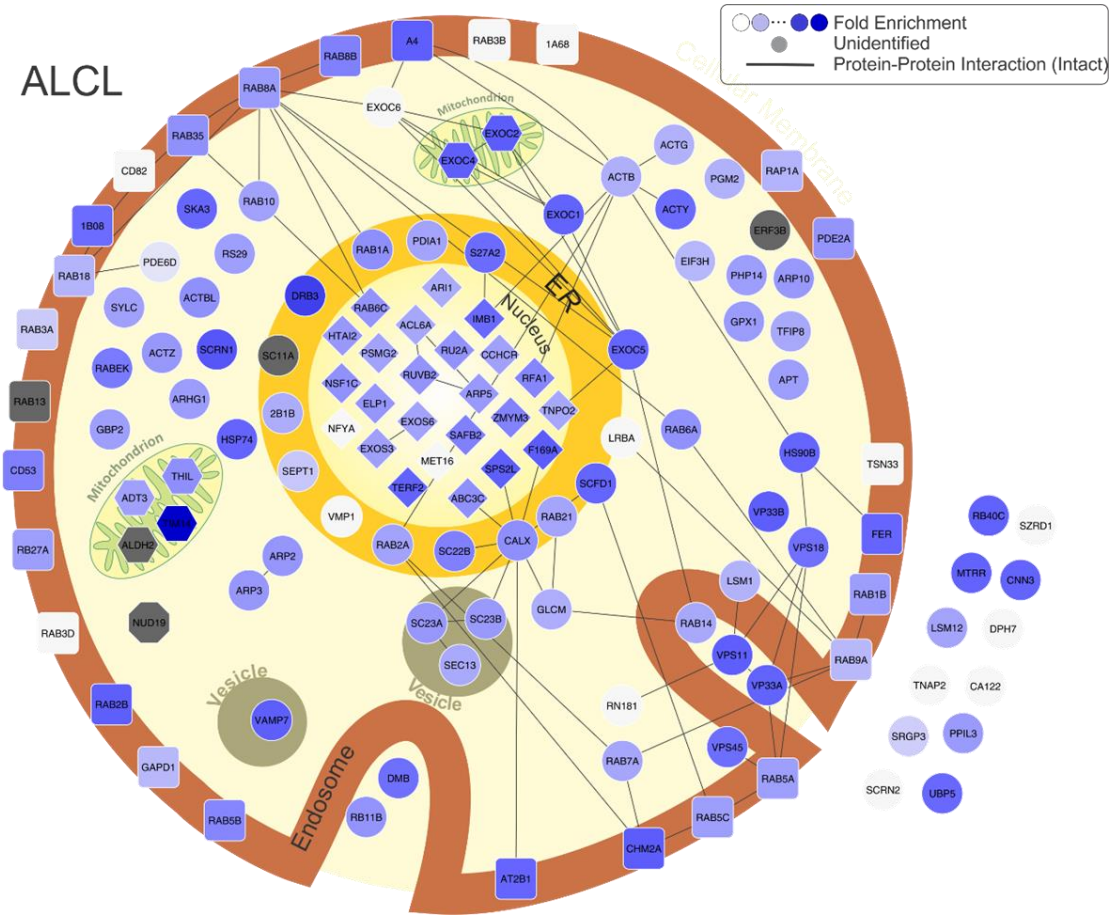


Figure 6: Protein-protein interactions among proteins of exosomal pathway and autophagy in Anaplastic Large B cell Lymphoma (ALCL). The darker the color, the bigger the fold enrichment. Grey color indicates that this protein is not identified. The black lines show possible interactions. The shapes indicate the location of the protein, e.g membrane proteins are square, cytoplasmic proteins are circles, nuclear are rhomboid, mitochondrial are polygon.

3.4 Exosomal Protein Identification in HL/NHL cell lines

In order to investigate the differences before and after the p53 re-activation induced by Nutlin-3a (N3a) in exosomal pathways, we used protein immunoblotting in 3 lymphoma cell lines, one Hodgkin (MDAV) and two Non-Hodgkin (JMP-1, SUP-M2), after treatment with N3a. Both cell lysates and secreted material from each cell line were examined. 50 μg of protein per sample were loaded in 12% or 10% polyacrylamide gels, which were constructed according to the molecular weight of the protein (s) of interest.

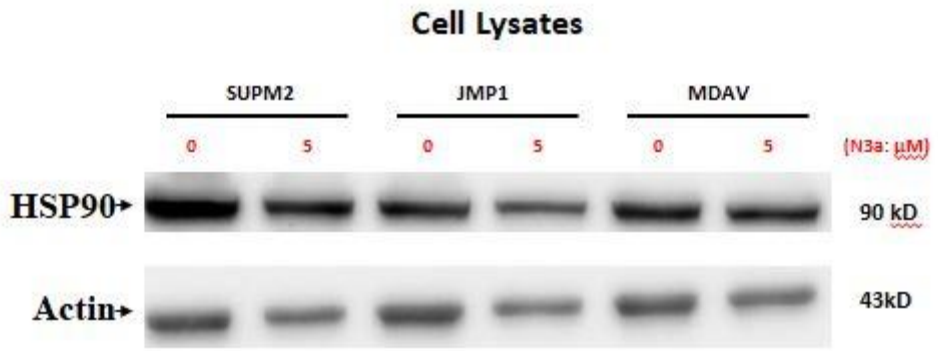


Figure 7: Detection of the N3a effect in exosomal pathways with protein immunoblot (Western Blot) in cell lysates. Differences of HSP90 expression among the 3 cell lines.

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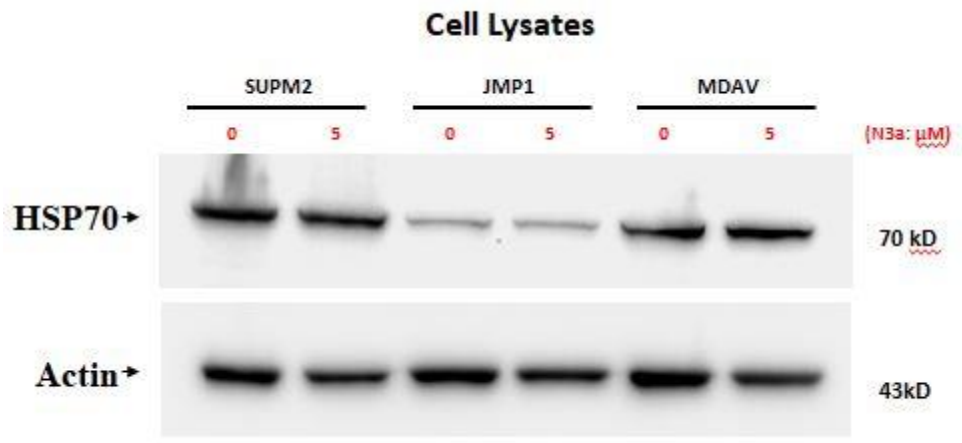

Figure 8: Detection of the N3a effect in exosomal pathways with protein immunoblot (Western Blot, WB) in cell lysates. Differences of HSP70 expression among the 3 cell lines.

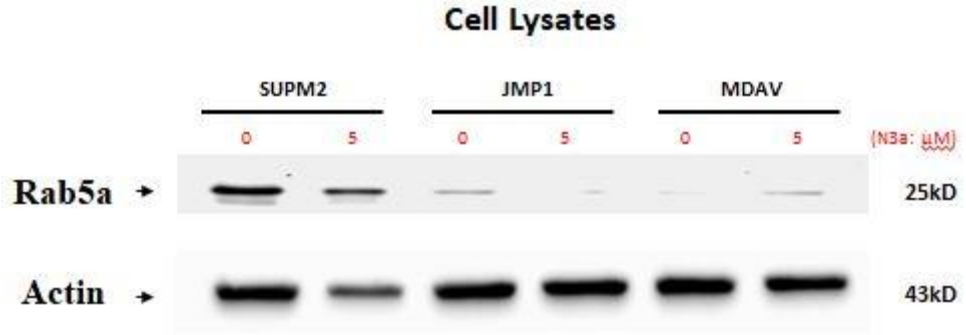

Figure 9: Detection of the N3a effect in exosomal pathways with protein immunoblot (WB) in cell lysates. Differences of Rab5 expression among the 3 cell lines.

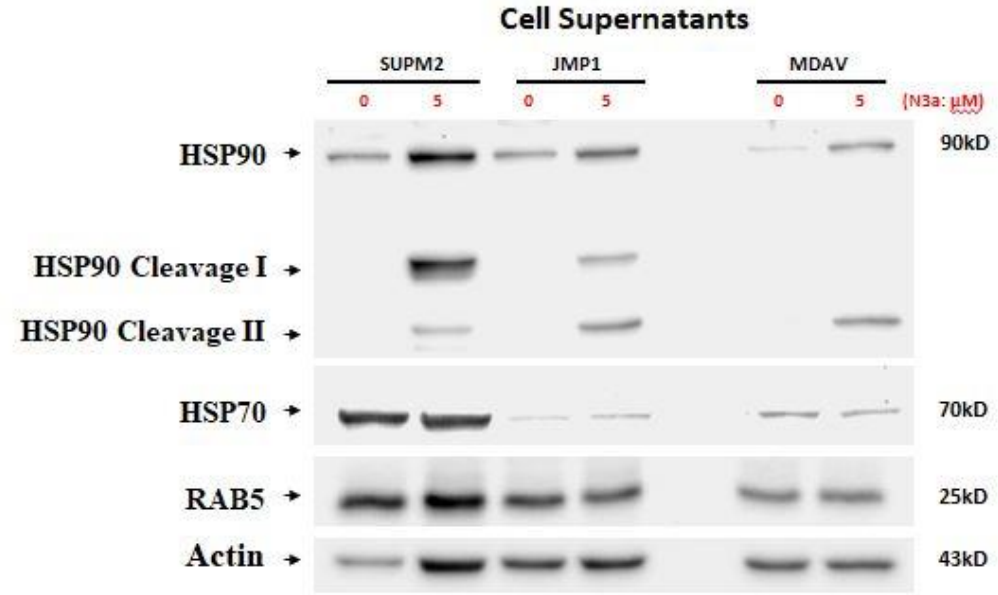

Figure 10: Detection of the N3a effect in exosomal pathways with protein immunoblot (WB) in cell's secreted material. Differences of HSP90, HSP70, Rab5 expression levels can be seen among the 3 cell lines.

Western blot analysis of samples indicated the effect p53-reactivation via N3a had in common markers of exosomes, such as Hsp90, HSP70, and RAB5 (Figure 10). Whole cell lysates were used as a positive control, while β-actin was used as loading control.

4. Discussion

Exosome Characterization in 3 human lymphoma cells lines+/-N3a

According to the experimental data, the common proteins between the three cell lines (HL/MCL/ALCL) represent 66% of the total proteins that were identified. As we can see from *Figure 1*, 51 out of the 4037 total proteins were common. In addition, common proteins related with exosomes, seemed to be more between HL and ALCL, while HL had the fewest unique proteins.

Protein Profiles of the 3 lymphoma cell lines using hierarchical clustering

Hierarchical clustering is a method of cluster analysis, which builds a hierarchy of clusters. According to Figure 2, there was no great difference among the 3 cell lines. However, there was an up-regulation in many proteins after the treatment with N3a in all 3 cell lines. B cell lines were clearly separated from the T cell line. The 3 lymphoma subtypes are independent entities and they have different biology. There was an up-regulation in many proteins after the treatment with N3a, in all the 3 cell lines of our study. HL had the most up-regulated proteins and the ALCL the most down-regulated. This knowledge indicates the unique and specific signature of each subtype and is of a great importance for therapeutic purposes.

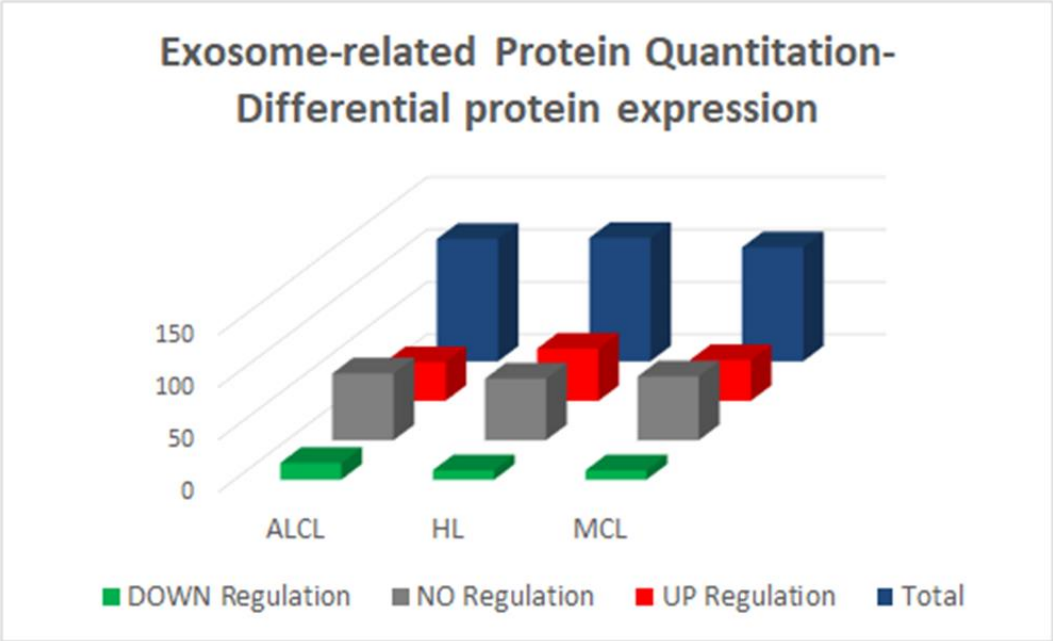

Figure 11: Exosome Related Protein Quantification-Differential protein expression

Exosomal proteomic profiling

Proteomic results showed that **Rab3D** was only found in HL and overexpressed after treatment with N3a, while **Rab3B** detected in MCL was reduced after N3a. Rab3D is involved in exocytosis but its role in cancer is not known yet. Rab3B has been found

increased in some cancer types, reducing tumor cell apoptosis [62]. Thus, the reduction of Rab3B in MCL after N3a may suggest that it leads cells to apoptosis.

Rab27A was overexpressed in HL andMCL, whereas ALCL showed no difference in its expression levels. Rab27A enhances tumor growth through regulation of cytokines release [62]. Therefore, Rab27 may promote cell growth of HL and MCL.

Rab8B expression levels were higher in ALCL and HL, but not in MCL. Its role is to help vesicles get fused with the target membrane [PubMed 9030196]. Therefore, vesicle fusion may be higher in ALCL and HL cell line.

Rab13 was only detected in HL and its expression levels were high. According to the human protein atlas, Rab13 regulates membrane trafficking and endosomes recycling.

VPS33A was overexpressed in all the 3 cell lines**.** Interaction of VPS33A with HOPS and VPS16 leads to endosome- and autophagosome–lysosome fusion[63]. Thus, endosome-lysosome fusion is suggested to be increased in all the HL/NHLs cells of our study.

Rab9 expression levels were higher in HL and MCL. Rab9A affects the secretion of exosome[64], therefore our results suggest that the release of exosomes in HL and MCL could be very different of those in ALCL.

VAMP7 was overexpressed in all 3 types of lymphoma. It has been showed that this protein participates in exocytic SNARE complex enhancing tumor aggressiveness and exosome secretion[65]**.**

Exosomal Protein Identification using Immunoblot Assay

In our experiments, cells of the 3 cell lines were treated for 24h with N3a. Then, the cells and their secreted material were separated and used for detection of the expression levels of 3 exosome related proteins, HSP90, HSP70 and Rab5, before and after N3a-treatment.

As it has been previously mentioned, heat shock proteins are activated in response to stress[7]. The amount of HSP90 increases under stress and hypoxia conditions, and extracellular Hsp90 has an important role in the regulation of tumor invasiveness and metastasis[14]. Researchers have found that HSP90 affects the cell growth and survival of leukemia cells, as well as of MCL cells. Overexpression of HSP90 has been detected in most of the Non-Hodgkin Lymphomas[66]. In the cell lysates of SUP-M2 and JMP-1, Hsp90 seemed to be more expressed in the control samples (without N3a) than the treated (with N3a), while in MDA-V there was not a great difference between control and treated samples. In the secreted material, treated samples showed higher levels of Hsp90 than in control. Furthermore, Hsp90 was found to be cleaved after N3a; 3 bands were detected, a ~90kDa band which indicated HSP90 and two cleavages. Secreted Hsp90 includes both of its isoforms, Hsp90α and

Hsp90β[15]. Hsp90 seemed to be more expressed in SUP-M2 after treatment with N3a, in a little smaller amount in JMP-1 and less in MDA-V, which had only one cleavage of the protein. Studies have showed that oxidative stress conditions can cleave Hsp90[67]. The cleavage of HSP90 results in 55 kDa or 70 kDa fragments, depending on the type of stimulus and on the involved mechanism. According to literature, HSP90 down-regulation by cleavage leads to client protein decrease and cell death[68]. Thus, our results indicate that HSP90 is more activated in MLC and in ALCL inside the cell before treatment, and in the secreted material after treatment.

HSP70 is also activated in stress conditions to protect cells undergoing transformation from oncogenic stress induced by overexpression of oncogenes. HSP70 is also found on the plasma membrane of tumor-derived exosomes[20]. HSP70 is overexpressed in many cancer cells and confers resistance to chemotherapeutic drugs, promoting cancer development[22]. Leukemia cells[69] and other haematological malignancies overexpress HSP70, which is translated as apoptosis inhibition[70]. In our results, Hsp70 showed a marginal increase in response to N3a treatment in the cell lysates of our 3 cell lines. In the secreted material, Hsp70 showed no differences between the 3 cell lines. Therefore, Hsp70 is slightly activated after treatment inside the cell and in the secreted material remains the same, before and after treatment.

Rab proteins are associated with endocytosis and vesicle transport[8]. Rab proteins have been shown to participate in the regulation of cancer progression. They promote cancer growth and invasion. Rab5a may have a significant role in cancer development by acting like an oncoprotein[9,12]. Inside the cell, Rab5 appeared to be increased in MDA-V and reduced in both JMP-1 and SUP-M2 after treatment. In the secreted material, Rab5 was expressed in higher levels in SUP-M2 and MDA-V after N3a, while there was no great difference in JMP-1 after treatment.

5. Conclusions

In conclusion, according to the proteomic data 77 proteins related with exosomes were identified. There was an up-regulation in many proteins after the treatment with N3a, in all the 3 cell lines of our study. HL had the most up-regulated and ALCL the most down-regulated proteins.

Rab3B in MCL after treatment with N3a could be related with apoptosis and Rab27 could promote cell growth in HL and MCL cell lines. Vesicle fusion could be higher in ALCL and HL cell line, whose trafficking may be regulated by Rab13. The endosome-lysosome fusion is possibly increased in all 3 cell lines after treatment, as well as tumor aggressiveness and exosome secretion.

HSP90 was more activated in MCL and in ALCL inside the cell before treatment and in the secreted material after treatment. Thus, exosomal HSP90 may promote tumorigenesis and metastasis in MCL and ALCL lymphomas, and the HSP90 cleavages in the secreted material may indicate an oxidative microenvironment and a possible way of communication suggesting cell death. Hsp70, on the other hand, was slightly activated after treatment inside the cell, while in the secreted material it remained the same, before and after treatment with N3a.

Rab5, inside the cell, was found to be increased in HL and reduced in both MCL and ALCL after treatment. On the contrary, in the secreted material, Rab5 was expressed in higher levels in ALCL and HL, and it showed no significant difference in MCL after treatment. Therefore, our results suggest that secreted Rab5 may stimulate tumor growth and invasion in HL and ALCL lymphomas.

6. Bibliography

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7. APPENDIX

Cell lysis Protocol

The samples were centrifuged at 2-3,000 rpm for 10'. Then, the supernatant was removed and 1 ml of lysis buffer was added. Two lysis buffers were used: RIPA1X and RIPA 5X. Their components are showed at the table below.

RIPA 1X

Reagents (Stock)	Volume per 50 mL of solution (v/v)	Final concentration
1 M Tris, pH 8.0	2.5 mL	50 mM Tris
5 M NaCl	1.5 mL	150 mM NaCl
100% NP-40	0.5mL	1% **(v/v)** NP-40
10% sodium deoxycholate	5 mL	1% (w/v) Na-DOC
10% SDS	0.5 mL	0.1% (w/v) SDS
ddH2O	40 mL	

RIPA 5X

Reagents (Stock)	Volume per 10 mL of solution (v/v)	Final concentration
1 M Tris, pH 8.0	1.25 mL	125 mM Tris
5 M NaCl	1.5 mL	750 mM NaCl
100% NP-40	0.5mL	5% **(v/v)** NP-40
10% sodium deoxycholate	5 mL	5% (w/v) Na-DOC
10% SDS	0.5 mL	0.5% (w/v) SDS
ddH2O	1.25 mL	

Per 1ml lysis buffer 1mM of PMSF and DTT, while phosphatate inhibitors (cocktail I/cocktail II, Sigma) and protease inhibitors (Sigma) were added, according to manufacturer instructions. Depending on the sample pellet size, 60-300 µl of lysis buffer was added. Then, samples were vortexed for 10" and sonicated 30''. They were shaked for 20' at 4^0C. After that, they were centrifuged at 14,000 rpm for 15'at 4^0C. The supernatant was selected into a new tube and stored in ice for protein concentration measurement or the samples were freezed at -80^0C.

Methanol-Ethanol Precipitation (Precipitation Protocol)

Sample aliquots of 500 µL were transferred in 2 ml tubes. 750 µl methanol and 750 µl ethanol were added. The samples were vortexed for 15 min at room temperature. Then, they were centrifuged at 13,500 *g* for 10 min at 4 °C. The samples were cooled in fridge at -20°C for 30 minutes to further precipitate proteins. The supernatant was transferred in a new tube and the pellet was evaporated to dryness in a Speed Vac concentrator. The samples stored -20°C, waiting for LC-MS/MS or they were further used in FASP protocol.

Ultracentrifugation for exosome isolation

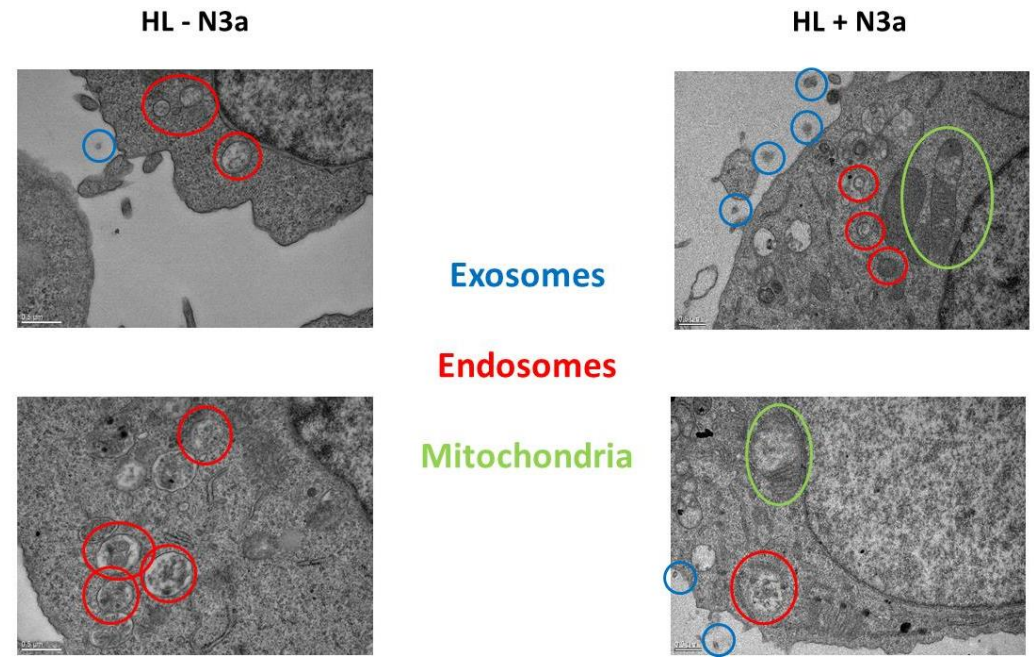
The supernatant was collected from the cell culture after 24h. The fluid was centrifuged at 2.000 x g, for 30 min at 4 ˚C, to remove cells, dead cells and cellular debris. The supernatant was pipetted off and transferred into polyallomer tubes or polycarbonate bottles appropriate for the ultracentrifugation rotor. In this step we had to ensure that none of the pellet was collected, contaminating the supernatant. We left behind ~0.5 cm of liquid above the pellet. After that, we marked one side of each ultracentrifuge tube with a waterproof marker, oriented the tube in the rotor with the mark facing up, and centrifuged 30 min at 10,000 × g at 4 ˚C. The mark was a reference for the location of a pellet at the end of the centrifugation. When the centrifugation was over, the supernatant was transferred to a fresh tube or bottle of the same size. An ultracentrifugation for 100 min at 100,000 × g at 4 ˚C was performed. For this high-speed centrifugation, all tubes should be at least three-quarters full (adding PBS, if necessary). Centrifugation time is calculated to allow a full hour at 100,000 × g, i.e., ~10 min for the centrifuge to reach 100,000 × g plus 1 hr at the final speed. A longer time (up to 3 hr) will not damage the exosomes. After that, supernatant was removed completely. The pellet was resuspended in 1 ml PBS and centrifuged for 1 hr at 100,000 × g at 4 ˚C, to remove contaminating protein aggregates. At the end, supernatant was removed and the pellet resuspended in 50 to 100 µl of RIPA 5X buffer.

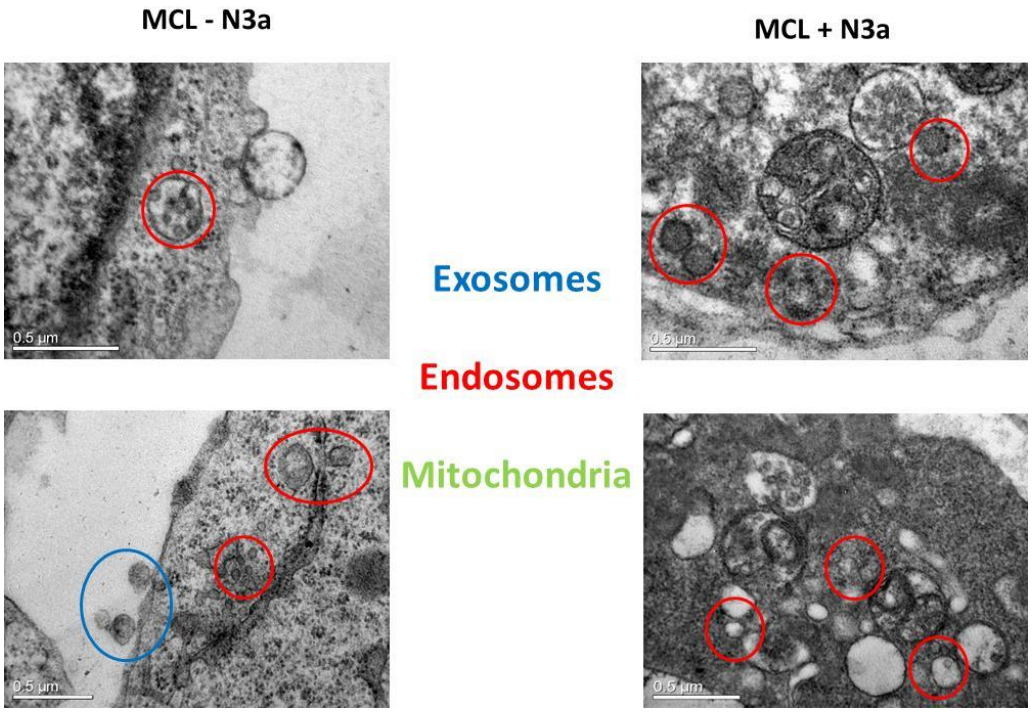
The ultracentrifugation used was SORVALL WX Ultra 80 of Thermo.

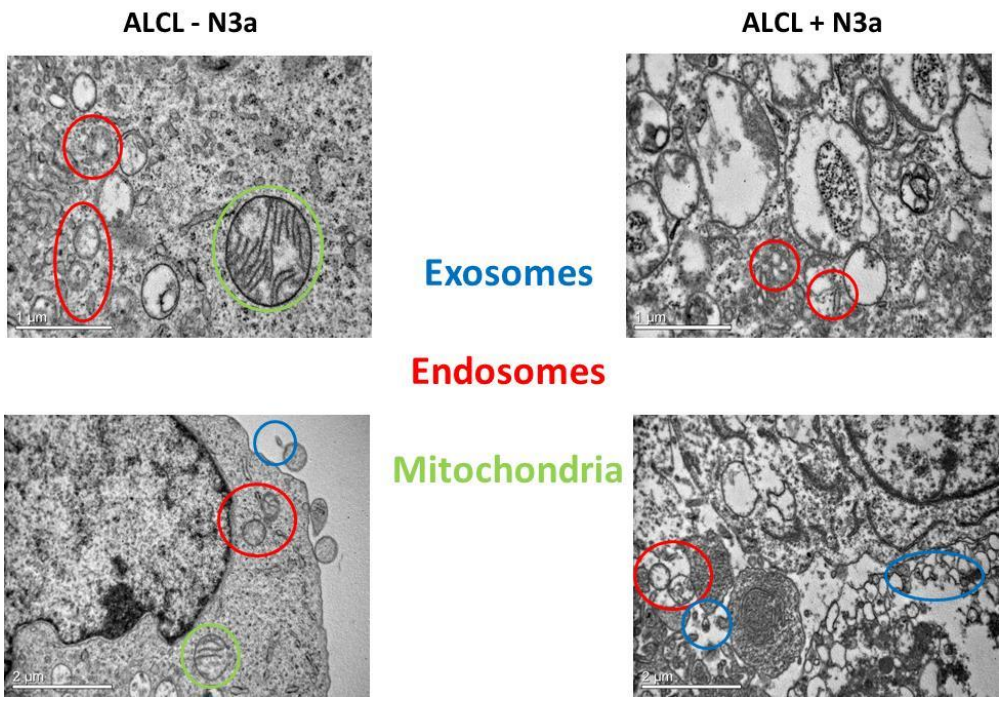
TEM protocol/ TEM imaging of the biological samples

Cells are fixed in 2.5% glutaraldehyde for 24 h at 4°C, washed in 0.1 M sodium cacodylate buffer (PH=7.4), post –fixed in 2% OsO4 in 0.1 M sodium cacodylate buffer for 60 min at 4°C, dehydrated in increasing concentrations of alcohol. The samples are impregnated with prophylene oxide and embedded in epoxy resin embedding media. Then ultra-thin sections are cut with LKB ultratome V-2088 and are placed on a TEM grid, post-stained with uranyl acetate and lead citrate. Images are obtained using on a LaB6 JEOL 2100 electron microscope operating at an accelerating voltage of 80 kV.

TEM Images


HL - N3a
HL + N3a
Exosomes
Endosomes
Mitochondria


MCL - N3a
MCL + N3a
Exosomes
Endosomes
Mitochondria
0.5 µm


ALCL - N3a
ALCL + N3a
Exosomes
Endosomes
Mitochondria