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The role of extracellular vesicles in the emergence of vemurafenib resistance in melanoma spheroids

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2 Abstract

BRAF inhibitors are a subgroup of targeted tumor therapy agents. They became promising in melanoma therapy since 20% of the melanoma cells contain a V600E point mutation in their BRAF proteins. The mutation of BRAF can cause constitutive activation of the mitogen activated protein kinase (MAPK) signaling pathway which may lead to cancer. However, resistance emerges almost always, and it decreases the effectiveness of these drugs.

Extracellular vesicles (EV-s) are produced by cells and have a broad range of functions in physiological processes. In cancer, their cargo can contain RNA molecules which activate signalling pathways responsible for proliferation. They also can carry information to distant cells for example they are crucial in pre-metastatic niche formation. Their isolation is a challenging process, and different strategies were applied which are based on their unique physical properties such as their size or chemical composition.

In this study, experiments were carried out to detect the involvement of EV-s in the emergence of resistance to vemurafenib treatment. In order to mimic the *in vivo* characteristics of tumour formation, three-dimensional spheroids of corresponding cell lines originating from surgical specimens taken before the BRAF-inhibitor treatment ('pre' cells) and after the emergence of the resistance ('post' cells) were investigated. The growth of the spheroids was recorded daily with a microscope, and the number of cells was estimated with calculations derived from the microscope images. In addition, the cell proliferation was also assessed with WST-1 assay. Results show that these two measures correlate, however their relationship is not linear. Both measures show decrease of cell proliferation in vemurafenib treated spheroids compared to the control.

The effect of EV-s on spheroids and their resistance to vemurafenib was also determined with the analysis of microscopy images and WST-1. The EV-s were collected from the supernatant of the cell cultures of the same melanoma cell pair which was applied to create spheroids, and they were separated by ultracentrifugation. The isolated sample was characterized with dynamic light scattering (DLS), flow cytometry and Qubit protein assay. All of these methods showed the presence of EV-s in the sample isolated from the supernatant of both pre and post cells.

Afterwards, isolated EVs were utilized to treat spheroids. In most cases, EV-treated spheroids had smaller size than controls, however microscopy images show that their proliferation rate is higher than vemurafenib-treated spheroids. Since EV-vemurafenib co-treatment mostly caused higher metabolic activity and proliferation of the cells, EV-s might induce resistance against vemurafenib therapy *in vivo* as well, and they can be considered as effective drug targets or markers in melanoma patients.

3 Introduction

On a molecular level, melanoma cells become malignant after the appearance of gain of function mutations of proteins involved in signalling pathways stimulating cell growth and proliferation [1]. One of the most prominent irregularities found in malignant melanocytes is the mutated form of BRAF serine/threonine kinase whose sequence changes by a missense V600E mutation which is found in 40 to 60 % of melanomas [2]. This mutation leads to the constitutive stimulation of MAPK/ERK signalling pathway which results in unregulated cell proliferation.

Kinase inhibitors are promising agents to fight against melanoma with targeted treatment [3]. One of the most promising examples of these agents is vemurafenib which inhibits the gain-of-function V600E-mutated form of BRAF [4]. However, in most patients, melanoma becomes resistant to the treatment [2]. The reason of resistance among others can be the reactivation of MAPK/ERK signalling pathway or the emergence of secondary mutations of NRAS and MEK1 proteins [5].

Extracellular vesicles (EV-s) are lipid bound entities produced by plenty of cell types, and they are secreted into the extracellular space [6]. They carry molecules which influence the activity of target cells, e. g. RNA molecules which induce cell proliferation [7]. They also might have role in the development of resistance against vemurafenib which was already studied with *in vitro* treatment of melanoma cells [8]. The study of extracellular vesicles involves their isolation and characterization. A number of methods were developed to isolate EV-s including ultracentrifugation, density gradient ultracentrifugation and size exclusion chromatography [9].

In general, *in vitro* studies in cell biology involve 2-dimensional cell cultures to mimic the physiology of cell lines, this model has a number of limitations including the altered way of mitosis, lack of concentration gradient of nutrients and other compounds, homogeneous conditions and change of cell morphology [10]. In order to overcome these limitations, 3-dimensional cell cultures called spheroids are grown [11]. These models are applicable to examine cell-cell interactions, drug response and metastasis formation.

In this essay, mechanisms of two cell lines are introduced which were isolated from the biopsies of a 59-year-old female patient with melanoma. The patient got vemurafenib treatment to cure her disease, and the biopsies were taken before and during the therapy [12]. The cell lines are called Mel Pt-4 pre and Mel Pt-4 post. We assume that Mel Pt-4 post cells become resistant to

vemurafenib, and they can produce extracellular vesicles which can carry molecules that can transmit resistance to the target cells.

First of all, the cell lines were utilized to form 3-D spheroids. This process includes the creation of specific circumstances: cells were placed in U-well plates, and they were collected with centrifugation. The plates were coated with poly (2-hydroxethyl methacrylate) (pHEMA) to prevent their adhesion onto the surface of the wells. Afterwards, they were treated with vemurafenib, and their proliferation was followed on microscopy images. Their metabolic activity was also measured with WST-1 assay which is a tetrazolium-based method often used to assess the proliferation and viability of 3D cell cultures [13]. Their proliferation was also quantified with the processing of the microscopy images. The images of spheroids were characterized with calculated intensity, a measure which is described in this study in detail. It provides better and more reliable data about cell proliferation than area or circularity. The results of calculated intensity and the absorbance measured by the WST-1 assay clearly show the effect of vemurafenib treatment on the proliferation of Mel Pt-4 pre and Mel Pt-4 post cells.

Secondly, the same cell lines were used to isolate extracellular vesicles. This required the ultracentrifugation of the supernatants of the cells. The isolated samples were analysed with Qubit Protein Assay, Dynamic Light Scattering (DLS) and flow cytometry, and all methods detected the presence of the EV-s. Afterwards, the samples were used to treat spheroids to assess whether EV-s can modify the proliferation of cells in these conditions. Cells were also co-treated with Vemurafenib and EV-is isolated from Mel Pt-4 pre and post cells.

Results show that EV-s can modify the growth of spheroids. EV-s isolated from both cell lines enhanced the proliferation of Mel Pt-4 pre cells when they were added to medium containing vemurafenib compared to those wells which only had vemurafenib content. However, when Mel Pt-4 post cells were treated, the opposite effect occurred, and spheroids remained smaller if EV-s were added to vemurafenib-treated cells. This may suggest that EV-s can carry molecules which make Mel Pt-4 pre cells more resistant, however they cannot affect Mel Pt-4 post cells which are already carry resistance.

4 Overview of literature

4.1 Pathophysiology and therapeutic possibilities of melanoma

Melanoma is a type of skin cancers which emerges from melanocytes when they are exposed to an oncogenic stimulus, most frequently chronic ultraviolet radiation [14]. Although its occurrence is relatively low, only 10 % of all skin cancers is melanoma, it is responsible for 65 % of skin cancer related deaths. The melanocytes start to proliferate at the dermoepidermal junction, and their position is scattered. In later phases, melanocytes invade the upper layers of the epidermis, and they also appear in the papillary dermis. The direction of the growth can be radial which has no metastatic potential and vertical which may lead to metastasis since melanocytes invade other skin layers and further tissues.

The spread of melanoma continues with ulceration [15]. As metastasis emerges, the motion of melanocytes is driven by chemotaxis and the lymph flow. In the metastatic sites, melanoma cells are resistant to the attack of the immune system, and their extravasation is coordinated among other proteins by adhesion molecules and metalloproteases. In order to predict the outcome of the disease, a number of independent descriptors are considered including tumour thickness, mitotic rate, subtype or neurotropism (perineural or intraneural invasion) [16].

On a molecular level, the genetic alteration of melanocytes starts with the appearance of gain of function mutations [1]. In this level, tumor suppressive mechanisms serve as barriers against further invasion, however the loss of function of these genes can lead to malignancy. Primary oncogenic events include point mutations and kinase fusions of those proteins which affect MAP-kinase, PI3K-kinase and Protein kinase C pathways e. g. NRAS, HRAS or BRAF. Compared to nevi, telomerase activity elevates in the melanoma metastases.

BRAF became a primary target of therapies since its mutation is present in 40 to 60 % of melanomas [2]. In addition, a valine was inserted into a glutamic acid in 90 % of BRAF mutations at 600th residue (V600E mutation), so an inhibitor, vemurafenib was developed to block BRAF proteins with V600E mutation. As a result, 81 % of the patients (26 of 32) had a response to the treatment, but only 2 of them had complete response. Though in most cases early response is observable, resistance against the treatment often appears, and its mechanism is under research.

4.2 Understanding targeted therapy resistance

As it was described earlier, BRAF V600E mutation is common in melanoma cells which triggers MAPK/ERK pathway responsible for cell growth and proliferation, so inhibitors of mutated variant of BRAF are effective agents. However, as the treatment continues, MAPK/ERK pathway reactivates, and the activation of alternative kinase pathways e. g. phosphatase and tensin homolog (PTEN), neurofibrin 1 (NF-1) and RAS signalling also compensate the inhibition [17]. The appearance of BRAF V600E mutation is individual from UV radiation, and it often causes melanoma in young patients.

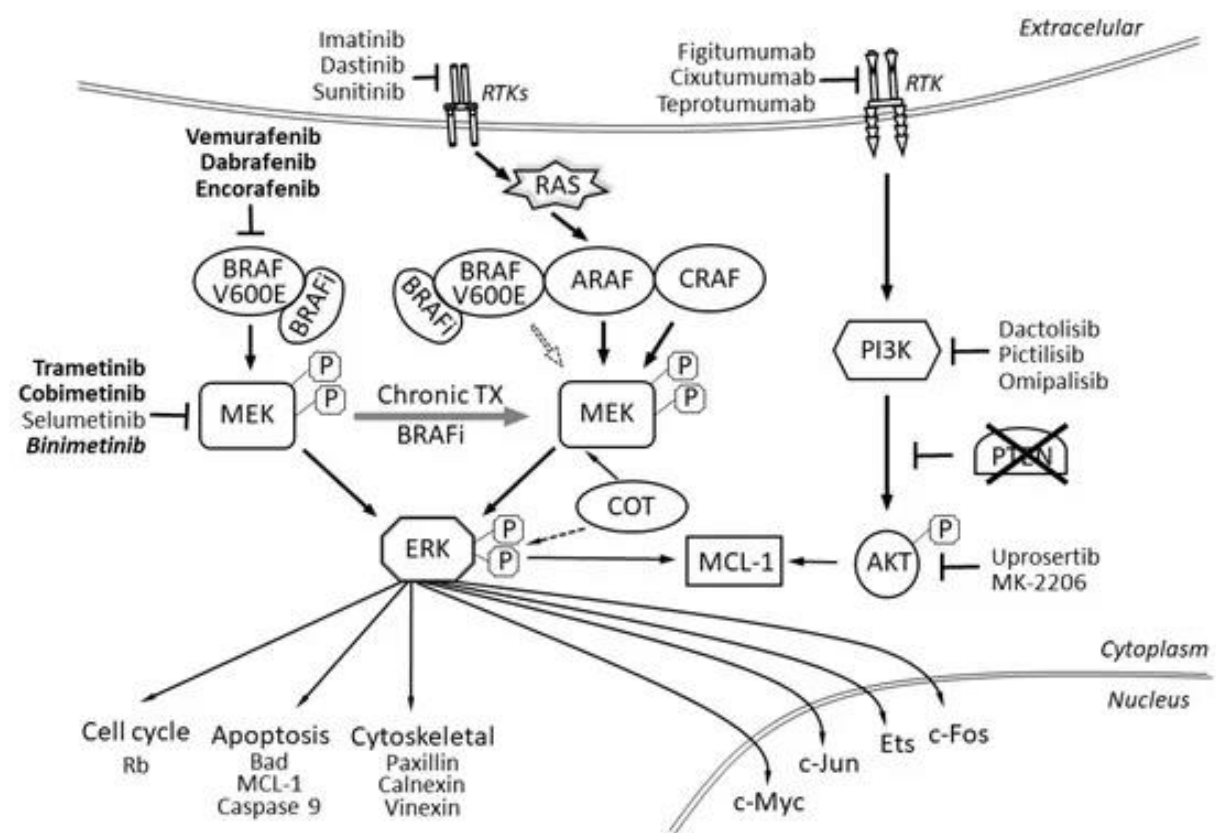


Figure 1 shows the signalling pathways which may have a crucial role in the emergence of BRAF inhibitor resistance [17]. It includes BRAF isoforms, ARAF and CRAF and other members of the MAPK/ERK and PI3K signalling pathways. The image also shows possible (normal) and accepted (bold) inhibitors to treat melanoma.

Before the development of targeted therapies, classic chemotherapy remained ineffective against melanoma, and the first V600E inhibitor which was approved by Food and Drug Administration was vemurafenib. The reason why therapy leads to resistance is the alternative splicing or overexpression of the BRAF protein which results in dimerization of BRAF also in

the presence of the inhibitor which becomes active independently from the interaction with RAS. Due to tumour heterogeneity, some cells have wild type of BRAF, and their CRAF expression is shown to be higher which results in BRAF-CRAF heterodimers. Resistance may occur by the hyperactivation of Ras which becomes capable of activating BRAF isoforms: ARAF and CRAF and MAPK/ERK pathway can work again. Loss of function mutations of tumour suppressors or the lack of their expression may also lead to resistance, e. g. if functional PTEN is missing, P13K/AKT signal transduction pathway causes cell proliferation and growth. The network of protein which are involved in therapy resistance of melanoma are shown on Figure 1.

4.3 *In vitro* research of melanoma with spheroids

Although the traditional way of modelling melanoma *in vitro* is two-dimensional cell culture with cells interacting with the surface of the wells, new 3D models were developed to mimic the three-dimensional network of tumour cell-cell interactions [18]. These models simulate better the interactions and metabolism between the tumour cells and their microenvironment. One of the methods applicable to create 3D models of melanoma are spheroids. The tumours grown this way become heterogeneous, for example the activity of ERK is higher at the periphery of the spheroids which indicates higher proliferative activity of the peripheral cells. In addition, in spheroids an increased fraction of multipotent stem cells is identified in spheroid population which express the hematopoietic CD20 marker [19]. When spheroids were separated into individual cells, and they were relocated into human embryonic stem cell (hESC) medium or melanocyte differentiation medium, their proliferation rate increased, and their pigmentation was lower when they were compared to differentiated melanocytes. Melanoma spheroid cells could also differentiate into mesenchymal lineages similarly to the cells of neural crest of embryonic development.

Numerous methods exist to characterize proliferation of spheroids [20]. In a study, cross-sectional area was used as a parameter to describe spheroids. In ImageJ, the microscopy images of different cross-sections of the spheroids were converted into black and white images with Yen thresholding algorithm, and maximal cross-sectional area was found with an ImageJ macro. They found that the capability of formation of spheroids was independent from cell lineage or genetic composition. They also applied CellTiter-Glo 3D Cell Viability Assay

(Promega) to measure ATP synthesis to describe the effect of different treatments. Although the compactness of the spheroids was described, it was not determined with quantitative units.

In another research, the effectiveness of methylcellulose and collagen additive materials to form spheroids in hanging drop platforms was described with compactness and circularity measures [21]. In order to increase reliability and reproducibility of cell viability assays, circular and compact spheroids should be created. The importance of circularity derives from diffusion coefficient of different compounds in the medium. Circularity and compactness were quantified by individual observers through blind tests on a 1-5 scale. These subjective measures might be simple, but they lack the capability to determine the number of cells in a spheroid. In addition, the article does not provide any data about the differences of the evaluations of the two individuals.

One of the most common methods to create spheroids is pellet culture [11]. Cell suspensions are centrifuged, and they are collected at the bottom of the wells. With this technique, large number of cells can aggregate rapidly. Initial size is also well controlled if this method is used, however cells are exposed to high shear stress which may damage them. In case of liquid overlay methods, low-adhesive surfaces are composed of agarose or poly (2-hydroxethyl methacrylate) (pHEMA). Assays for spheroid growth include brightfield microscopy images however it is possible that spheroids do not become spherical, and their diameter is shorter in the z-axis than expected. Additionally, tetrazolium salt WST-1 colorimetric dyes are possible tools to estimate spheroid growth. Confocal microscopy makes also possible to scan spheroid layers and create 3D images from the cell aggregates.

4.4 Extracellular vesicles

4.4.1 Physiology

Extracellular vesicles (EVs) are lipid bound structures which are constitutively secreted by cells into the extracellular space [6]. They have two major subgroups: they can be divided into exosomes and shed microvesicles (sMV). They have different diameter (exosomes: 30-150, sMV: 50-1300 nm) and their origin also differs: exosomes develop from late intraluminal vesicles which bud from the late endosomes. The microgenesis of sMV is induced by the ARF6 and RHOA-dependent reorganization of the actin cytoskeleton which causes the direct budding and exocytosis of a segment of the plasma membrane [22].

The cargo of the extracellular vesicles are typically microRNA molecules, and the protein composition of the vesicles is also special [23]. They primarily contain RNA-binding proteins, ribonucleoproteins, receptors of signal transduction pathways and integrins which are important to recognize target cells. Based on their protein composition, extracellular vesicles modulate the protein expression of the target cells.

4.4.2 Role in tumour pathophysiology and resistance

Extracellular vesicles can interact with the tumour microenvironment [24]. Cancer nowadays is considered as an ‘ecological’ disease because it is a result of abnormal cell-cell interactions, and not only the pathological characteristics of individual cells. For example, tumour cells can stimulate surrounding epithelial cells to induce neoangiogenesis. Extracellular vesicles of malignant cancer cells are also proved to affect benignant and stromal cells.

Metastasis only occurs if the tissue environment provides adequate conditions [25]. It includes vascular leakiness, inflammation and blood coagulation. Exosomes have crucial role in the formation of distant metastasis because they carry such signalling proteins and miRNA which modify the nutrient uptake and extracellular matrix formation of target cells e. g. MET receptor tyrosine kinase [26].

Evidence from pre-clinical and clinical studies were found that EVs can transmit the capability of drug resistance from cell-to-cell in a number of cancer types [8]. In melanoma, extracellular vesicles were identified to carry PDGFR β , a receptor tyrosine kinase which stimulates P13K signalling pathway [27] and thus provide non-ERK dependent, acquired resistance against vemurafenib to the target cells. In this research a cell line called LM-MEL-64 was treated with 1 $\mu\text{mol/l}$ vemurafenib. In every 3 days for 10 weeks, the medium was always replaced with fresh one containing the same amount of vemurafenib. This evolved cell line was called LM-MEL-64R3. Extracellular vesicles were isolated with ultracentrifugation, and their protein content was mapped with western blotting. The resistance was confirmed by the comparison of their proliferation in vemurafenib, and western blotting revealed the differences in their protein expression: LM-MEL-64R3 contained more pAKT and pERK in after treatment and control conditions too which means that their PI3K/AKT and MAPK/ERK pathway is more active. Especially, the difference of the presence of pERK was remarkable: after vemurafenib treatment its concentration decreased in LM-MEL-64 to a greater extent than in LM-MEL-64R3. The protein content of exosome pellets of the two cell lines were also different e. g. the

resistant cell line had higher content of pERK and PDGFR β . After the incubation of LM-MEL-64 cells in vemurafenib and exosomes for 15 minutes, their pAKT content was higher than those cells which did not get EV-s.

4.4.3 Isolation

Extracellular are isolated based on their specific physical and chemical characteristics [9]. As a consequence of their unique density and buoyant force, ultracentrifugation on 100-200.000 g can be applicable after the isolation of larger cell fragments with a lower acceleration [28]. In addition to acceleration, the angle of the tube should be also considered because it modifies the time and resolution of isolation. The advantage of ultracentrifugation is the fact that no additional treating compound is necessary which could contaminate the sample in further treatments. The size of the EVs is also utilized in ultrafiltration technologies which can be used together with ultracentrifugation to increase the quality of the sample (however it results lower quantity) [29]. In order to decrease the loss of filtration, the membranes are washed to collect or hydrophilic membranes are used. To isolate even more purified EV-s, density gradient ultracentrifugation is an adequate method when D₂O or saccharose is added to the liquid [30].

In order to separate EV-s from the protein complexes and lipoproteins of the urea, size exclusion chromatography (or also called gel filtration) is used [31]. The advantage of this method is its flexibility regarding resolution and sample amount. The resulting EV-sample has higher marker concentration and lower content of non-EV proteins. It also contains less chylomikrons, a subgroup of lipoproteins which are typically difficult to separate from EV-s [32].

Other methods separate EV-s based on their solubility. A superhydrophilic polymer, polyethylene-glycol (PEG) can precipitate the vesicles. Some examples of available kits are ExoQuick and Norgen Exosome Purification Kit. The disadvantage of these techniques is the co-precipitation of other molecules e. g. albumin [33].

EV-s can be isolated with affinity-based methods which are based on specific bonds between a reagent and a molecule on the surface of the EV-s [34]. The reagent can be an antibody, lectin or a lipid binding protein. These reagents are also immobilized on a membrane or a magnetic bead. The surface molecules of EV-s can be CD9, CD63, CD81 or EpCAM.

4.4.4 Characterisation

One powerful tool to confirm the EV-content of samples and measure their size is dynamic light scattering (DLS) [35]. This method calculates hydrodynamic diameter of particles with a laser light which penetrates the liquid. If the laser light interacts with a particle, its direction is modified, and it does not reach the detector. These intervals are affected by the size of the particle, since those compounds, whose hydrodynamic radius is higher, their diffusion coefficient becomes lower, and they move slower. As a result, they spend more time in the focus of the laser beam. Since scattered intensity is a stochastic signal, its function is transformed with an autocorrelator to extract useful information. The advantages of DLS are its non-invasiveness, sensitivity and simplicity. It also requires low amount of sample. A disadvantage of DLS is its sensitivity to large aggregates or dust particles which can ruin the measurement [36].

DLS was previously utilized to measure the size of isolated extracellular vesicles. In a paper the results were validated with atomic force microscopy (AFM), and it was confirmed that the distribution of vesicle diameter measured with DLS coincides with AFM results [37].

Flow cytometry is a method used to characterize single cells based on their light scattering features [38]. The signal can be generated by dyes or monoclonal antibodies attached to the surface of the cells. It consists of three main components: fluidics, optics and electronic network (detectors) and a computer. In the fluidics, particles are directed into the direction of a laser beam, and they interact with each other. The modified laser beam reaches the detector, and it is converted into digital signal.

Currently, bead-based assays are effective tools to analyse EV-s with flow cytometry [39]. The vesicles are bound on the surface of larger microbeads via chemical conjugation or antibodies. Afterwards, the detection occurs via fluorescent antibodies. The intensity of fluorescent emission is proportional to the number of EV-s on a given bead. There are some typical proteins which are used as identification markers of EV-s. These can be proteins involved in the multivesicular body (MVB) formation machinery, others which are parts of the fusion machinery and the tetraspanins (CD9, CD63, CD81) [40]. Tetraspanins are transmembrane proteins which interact with each other to form large protein networks, regulate cell motility, and they also take part in cell fusion and signalling [41]. This protein family has common residues and similar structure with four transmembrane domains.

4.5 Introduction of the cell lines

In this research, those Mel Pt-4 pre and post cell lines were utilized which were previously introduced in the article of Lai et al. [12]. These cell lines are isolated from the biopsies of a 59-year-old female patient who were exposed to vemurafenib treatment after the appearance of a lung metastasis. The biopsies were taken before and 4 weeks after the beginning the patient's treatment, and the cell lines show remarkable differences in morphology, cell proliferation and activation of signalling pathways. The proliferation of post cells became faster since their mean doubling time decreased compared to pre cells. During treatment new mutations in AKT1 emerged in addition to common mutations of pre and post cells e. g. in BRAF or EGFR. AKT1 is an isoform of Akt serine/threonine kinases which has specific functions in cell growth and angiogenesis [42].

5 Methods and measurement process

5.1 Measurements with spheroids

Cells were kept in T25 cell culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % Fetal Bovine Serum (FBS). In order to maintain the cell lines, they were passaged 2-4 daily with trypsin-EDTA. Before the experiments, cell lines were tested with MycoAlert® Mycoplasma Detection Kit to avoid concomitant effects of mycoplasma infection.

To form spheroids, U-bottom plates were used to place the cells. At least one day before plating the cells, the surface of the wells was coated with 30 μ l poly-(2-hydroxyethyl methacrylate) (poly-HEMA)-ethanol in 10:90 ratio. This layer prevents cells from adhering to the surface, therefore they form aggregates.

On day 0 of the measurement, cells were moved to suspensions with DMEM and 10 % FBS from the flasks with trypsin-EDTA, and the concentration of the suspensions was measured with LUNA-II Automated Cell Counter. In some experiments 2000, in others 500 cells were placed into the wells. The cell suspensions in the wells were diluted with DMEM and 10 % FBS so that the suspension volumes became 90 μ l. To collect the cells into the bottom of the U-well, the plates were centrifuged with 2200 rpm for 10 minutes. After centrifugation, 10 μ l collagen was added to each well which helps the formation of 3D cell aggregates. The pipetting of the collagen should be very slow and careful to avoid the disintegration of the group of cells.

The final concentration of collagen was $0.04 \frac{mg}{\mu l}$ in each well. The microscopy image of a well containing Mel Pt-4 pre cell is shown on Figure 2.

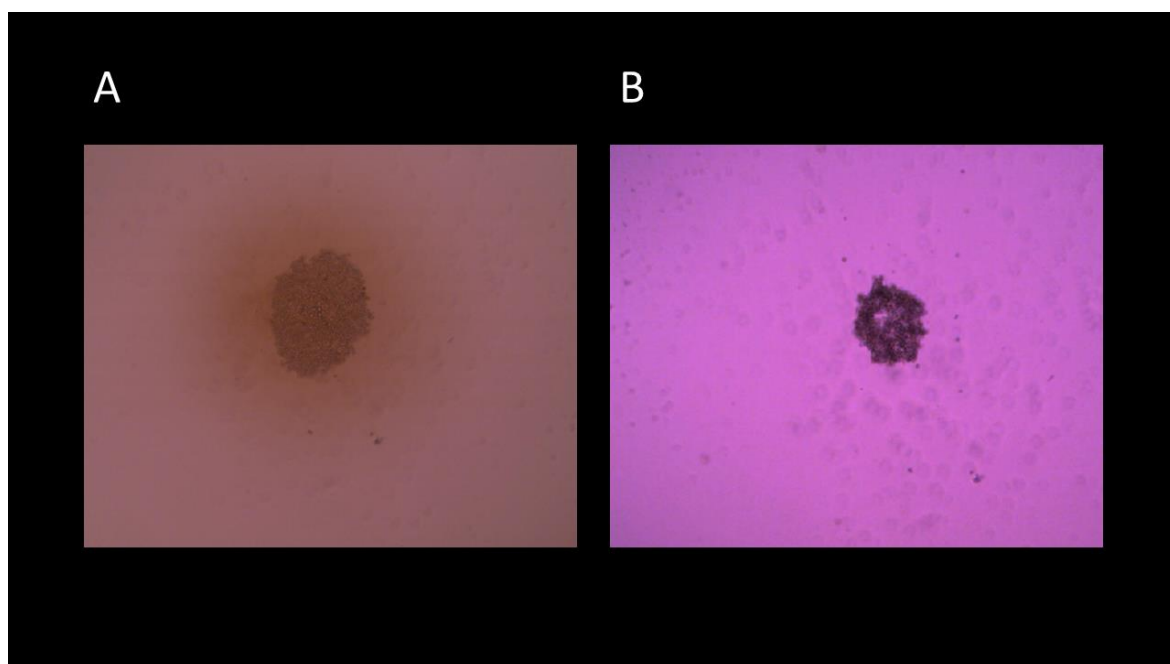


Figure 2 shows a well containing Mel Pt-4 pre cells on day 0 (A) and day 1 after treatment (B). In subfigure A the cells form one layer, and their area is barely darker than the surrounding medium. In subfigure B, cells become more condensed, and spheroid formation already has started. The colour of the medium is also different because of the addition of DMEM and 10 % FBS.

On the next day (day 1), cells were treated with vemurafenib or EV-s. They were added in $100 \mu l$ solutions of DMEM and 10 % FBS. In those experiments where EV-s were also included, EV-free FBS was utilized. The concentration of vemurafenib was $10 \mu M$ in treated cells. $100 \mu l$ DMEM and 10 % FBS was also added to those wells which were applied as controls to provide the same volume as the volume of wells with treated cells. The addition of new solutions could modify the arrangement of the spheroids; therefore, the cell aggregates were photographed with an inverse light microscope after the treatment. New photos were taken on day 3, 4, 5, 6 and 7 to follow the growth and other characteristics of spheroids.

On day 7, after taking the last photos, the measurement was terminated, and cell viability was quantified with WST-1 assay. There were at least 2 examples of each individual measurement parameter on every plate, so one of them could be used as a blank which is necessary because the solution and the cells could have a considerable absorbance, and $20 \mu M$ WST-1 was added to the others. The plates were incubated for 3.5 hours in $36^\circ C$ and 5 % CO_2 concentration, and

finally their absorbance was measured. The wavelength of the absorbed light was 440 nm, which is around the max. absorption value of WST-1.

5.2 Processing of the microscopy images

The images were analysed with the ImageJ software. Its “Wand Tool” helps to accurately select the spheroid-containing area of the images. Each selection’s integrated density was measured. If there were more spheroids in a well, their integrated density was summed. If the spheroids were not in the same plane, more images had to be taken to have all spheroids in focus. Integrated density can be a more robust and better descriptor than area because it indicates not only the spatial growth but the condensation of the spheroids too.

Since high pixel values belong to white pixels, the integrated density should be inverted which is done by subtracting its value from the integrated density of the same area with only white pixels. In addition, the background is not white which has to be subtracted as a blank. Therefore, ‘calculated intensity’ is defined with the following equation (in case of a 8-bit greyscale image):

$$CI = 255 * area - ID - (255 - median) * area = median * area - ID$$

Where *ID* is integrated density of the selected area, *area* refers to the selected and *median* refers to the oval-shaped field of vision of the microscope. Calculated intensity is influenced by the zoom level of the microscope, so all images were taken with 4x zoom level. This measure is not only more applicable than area because of the ability to detect compactness but it is more robust to the manual selection. If some background is also added to the selection, it is filtered out by the median blanking. However, it remains only robust if the selected area overestimates the size of the spheroid. Figure 3 demonstrates the robustness of calculated density compared to area and circularity. Circularity is defined as $circularity = 4 \pi \frac{area}{perimeter^2}$.

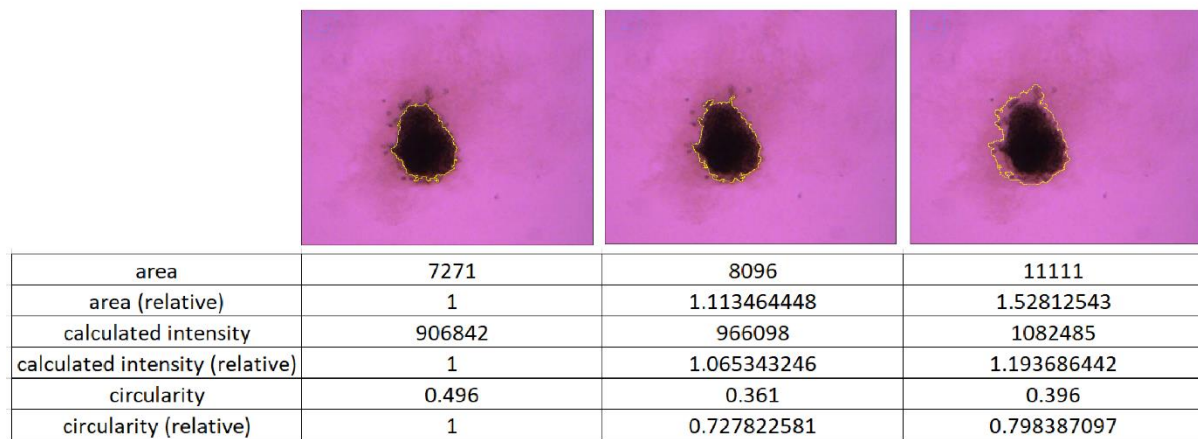


Figure 3 shows three selections of the same spheroid. All selections could be acceptable, but their area is very sensitive. However, calculated intensity changes less if the selection is slightly modified. Circularity is also shown as a possible measure of spheroids which is also more sensitive to the selection than calculated intensity.

WST-1 and calculated intensity are two measures which can be applied to estimate cell proliferation individually. Their imperfect correlation may have other reasons than measurement error, and it can have biological relevance. WST-1 absorbance is determined by mitochondrial activity of the cells, so its increase can also indicate the stimulation of metabolism. Calculated intensity is determined by the number of cells, and it is also altered by the change of cell size which can be an important factor to understand the mechanism of a treatment. The calculated intensity is also modified if the colour of the medium changes e. g. due to pH or light conditions of the laboratory, and these circumstances can be considered as measurement errors. Measurement errors are random, but biological factors have a tendency which should appear on WST-1 – day 7 calculated intensity plots which are discussed later.

5.3 Isolation of extracellular vesicles

Extracellular vesicles were isolated from the supernatant of Mel Pt-4 cells. The number of cells was elevated, and they were stored in 3 T75 cell culture flasks for 72 hours. When the supernatants were removed, the confluency of the cell flasks was 60-100%. In this period, cells were stored in 1 % EV-free FBS to create EV-samples which only contain those EV-s which were produced by the cells. The supernatants were centrifuged for 5 minutes with 500 g acceleration to remove floating cells. Finally, the supernatants were frozen and stored in refrigerator with -80 °C temperature.

Before ultracentrifugation, the cells were centrifuged once again on 5000 g, 15 min to remove those structures (e. g. apoptotic bodies) whose sedimentation coefficient is between the coefficient of cells and EV-s. The ultracentrifugation took 90 minutes, and the acceleration was 100.000 g. To maintain balance in the centrifuge, the mass of the sample was determined with an analytical balance, and PBS was added to make their mass equal. After ultracentrifugation, the EV-s got to the pellet, so the supernatant was removed. The pellet was resuspended in 300 μ l PBS.

5.4 Characterization of EV-s with Qubit protein assay, DLS and flow cytometry

Qubit® Protein Assay was utilized to determine the protein content of the samples containing EV-s. On its own, this method is not eligible to decide whether a sample contains EV-s or not, but it is a reliable tool to compare the EV content of different specimens. Qubit® protein assay is a fluorimetric method, and it utilizes a set of protein standards to set a standard curve. The fluorescence of the samples was fitted to the standard curve to calculate their unknown protein content. To measure their protein content, 10 μ l sample should be added to 190 μ l Qubit® Working Solution which is composed of Qubit® Protein Reagent and Qubit® Protein Buffer in 1:200 ratio. The protein concentration is measurable in a short range, so the sample should be occasionally diluted.

The size distribution of the isolated EV-s was measured with DLS. The device was W130i apparatus (Avid Nano Ltd., High Wycombe, UK), and the sample was loaded into a low-volume disposable cuvette (UVette, Eppendorf Austria GmbH, Wien, Austria). The device emits a diode laser whose wavelength is $\lambda = 660\text{ nm}$. The detector is located at a fixed angle of 90°.

The samples were also analysed with flow cytometry. The EV-s were bound onto the surface of 3.1 μ m aldehyde/sulphate latex beads (Thermo Fisher Scientific; 4% w/v). The mixture of the latex beads and EV-s were diluted and centrifuged in room temperature for 30 min with 320 rpm. A 200 μ l suspension was created from the pellet with the addition of PBS. Afterwards, the beads were blocked with glycine and BSA to prevent unspecific binding of primary antibodies. In the next step, the beads were centrifuged again for 1 hour with 320 rpm. This was followed by a washing process which consisted of centrifugation for 5 minutes with 3000 x g, and resuspension of the pellet in 1 ml PBS. This suspension was aliquoted into 200 μ l samples, and four kinds of primary antibodies were added to each of them: 0.5 μ l of anti-CD9, anti-CD81 and anti-CD63 antibodies and 10 μ l 10 μ L WGA-Alexa Fluor™647 from the 100 μ g/mL

stock which binds to the lipoproteins of EV-membranes. After incubation (20 min, 37 °C, 320 rpm), secondary antibodies were also added (except for WGA), and another incubation period began with the same parameters. The exact quantities of necessary compounds are summarized in Table 1.

	latex beads, 4% w/v, 3.1 µm 100x diluted	PBS (1x, filtrated)	EV sample	glycine 1M (in PBS)	2% BSA (in PBS)	Primary antibody: WGA-Alexa Fluor™647 (to 200 µL sample)	Secondary antibody
1.	60 µL	940 µL	-	-	-	-	-
2. Negative control	60 µL	940 µL	-	112 µL ($C_{final}=100\text{mM}$)	340 µL ($C_{final}=0.5\%$)	1. 0.5 µL CD9 2. 0.5 µL CD81 3. 0.5 µL CD63 4. 10 µL WGA-Alexa Fluor™647 from the 100 µg/mL stock	1. 0.75 µL Alexa488 2. 0.75 µL Alexa488 3. 0.75 µL Alexa488 4. -
3. Pre sample	60 µL	940 µL	200 µL	56 µL ($C_{final}=100\text{mM}$)	170 µL ($C_{final}=0.5\%$)	1. 0.5 µL CD9 2. 0.5 µL CD81 3. 0.5 µL CD63 4. 10 µL WGA-Alexa Fluor™647 from the 100 µg/mL stock	1. 0.75 µL Alexa488 2. 0.75 µL Alexa488 3. 0.75 µL Alexa488 4. -
4. Post sample	60 µL	940 µL	200 µL	56 µL ($C_{final}=100\text{mM}$)	170 µL ($C_{final}=0.5\%$)	1. 0.5 µL CD9 2. 0.5 µL CD81 3. 0.5 µL CD63 4. 10 µL WGA-Alexa Fluor™647 from the 100 µg/mL stock	1. 0.75 µL Alexa488 2. 0.75 µL Alexa488 3. 0.75 µL Alexa488 4. -

Table 1 summarizes the quantities of different compounds utilized in the sample preparation of flow cytometry measurement

The evaluation of the flow cytometry results started with the gating of forward scatter height function (FSC-H), and only those values were included in the spectrum of fluorescent isocyanate channel which are members of the largest peak. Afterwards, the spectrum of the negative control and EV-containing sample was compared: their normalised X-median value was compared (10.000 was divided with the number of all instances and it was multiplied with X-median) with a division. This ratio is called fold change, and it provides information, how strong was the signal emitted by the secondary antibodies compared to controls.

6 Results

6.1 Calculated intensity and wst-1 results of vemurafenib-treated cells compared to controls

The growth of spheroids was followed as described in chapter 5.1. The wells with the cells were in four independent plates. Measurements with 2000 cells had 10 technical repetition of control and 10 of vemurafenib-treated spheroids. In case of 500 cells, 8 repetitions were carried out. Not all of them could be assessed with WST-1 since one of them should have been used as a blank on each plate, therefore 6 and 4 technical repetitions were done from this measurement, respectively. Although the WST-1-painted wells were randomly chosen, some outlier could make the results inconsistent if the two measures were compared. Calculated intensity measurements were taken with two cell lines: Mel Pt-4 pre and post in two initial quantities: 500 and 2000 cells. These measurements indicate the reliability of calculated intensity and WST-1 to estimate proliferation, and they also help to determine whether 3D structure modified the resistance of cells compared to 2D models. In all measurements, the concentration of vemurafenib was $10\ \mu M$ which is approximately the same amount as the IC₅₀ value of vemurafenib treatment in measurements with the same cell lines in two-dimensional configurations.

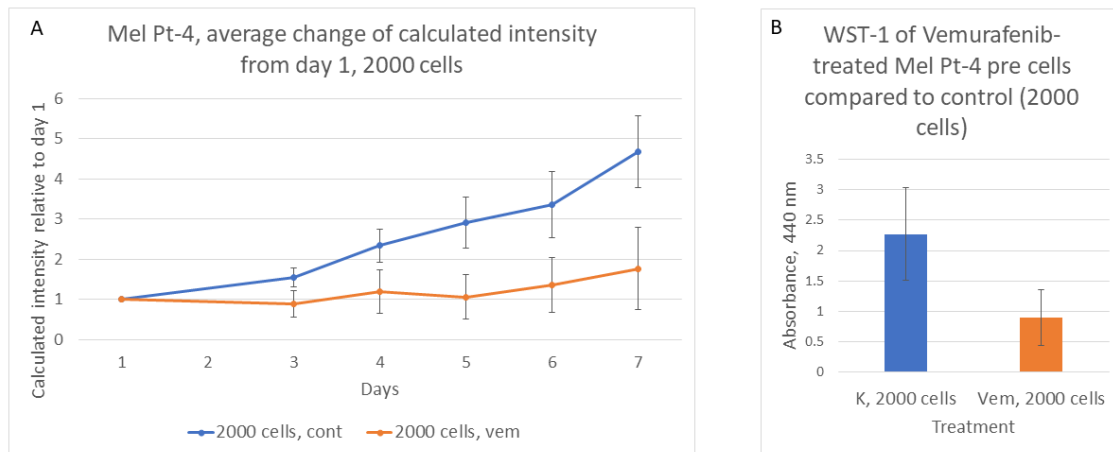


Figure 4 - Calculated intensity and WST-1 values of Mel Pt-4 pre cells treated with vemurafenib compared to control. Initial cell quantity was 2000 cells. The decrease of cell proliferation is clearly shown by both methods. Subfigure A shows calculated intensity of the cells in days 1 and 3-7. Since the calculated intensity was compared to day 1, its value is 1 on day 1 in all cases. The calculated intensity value is only 37.8 % of treated cells compared to control, and WST-1 absorbance also decreased by the treatment: its value is only 39.4 %. The two measures consistently show the effect of the vemurafenib treatment.

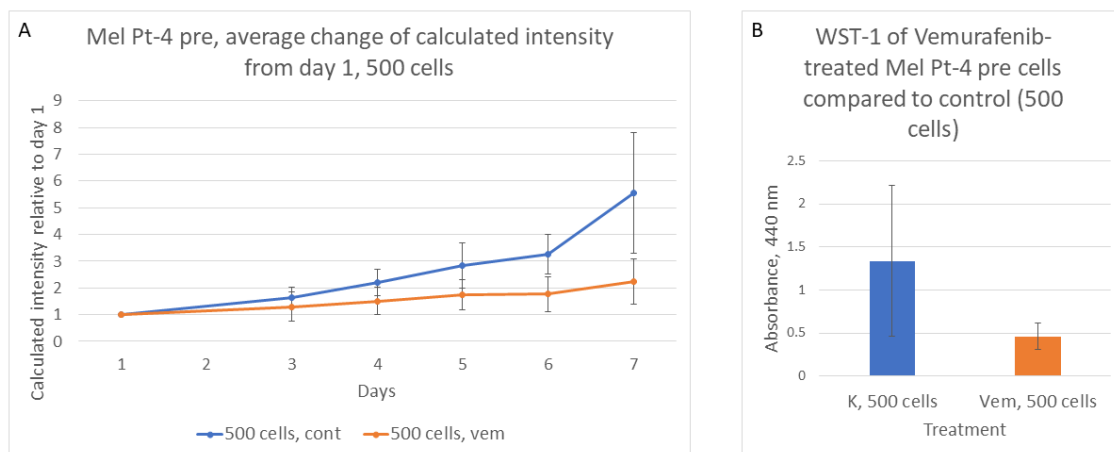


Figure 5 - Calculated intensity and WST-1 values of Mel Pt-4 pre cells treated with vemurafenib compared to control. Initial cell quantity was 500 cells. Both methods indicate the decrease of proliferation by the vemurafenib, however control samples have high standard deviation by both methods. The change of calculated intensity is somewhat higher than the values of 2000 cell measurements, but the decrease of proliferation compared to control is similar: 40.2 % based on change of calculated intensity and 34.5 % is shown by the WST-1 results.

Figure 4 and Figure 5 show the effect of vemurafenib treatment of Mel Pt-4 pre cells. Results of wells with 2000 and 500 initial cells were plotted individually. The calculated intensities of day 7 and WST-1 measurements show similar drop of vemurafenib-treated cells. Initial cell number does not seem to have a major affect to the growth of spheroids or to the treatment, because the results of 500 and 2000 cells are similar.

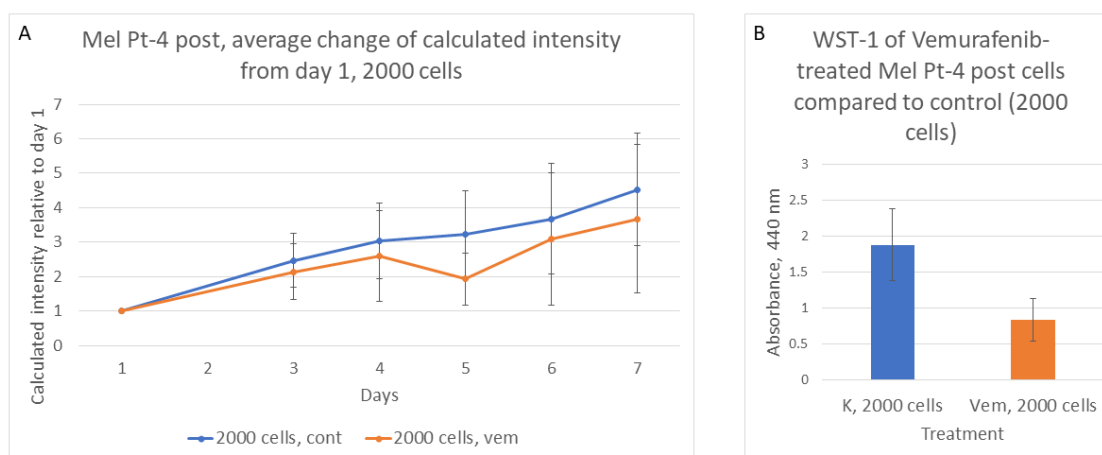


Figure 6 Calculated intensity and WST-1 values of Mel Pt-4 post cells treated with vemurafenib compared to control. Initial cell quantity was 2000 cells. Although both methods indicate the decrease of cell proliferation when vemurafenib treatment was applied, the results are inconclusive because the calculated intensity results show high and overlapping standard deviation. WST-1 results clearly show the effect of treatment. The average proliferation of treated spheroids is 81.0 % according to the calculated intensity results, and 44.2 % based on WST-1 results.

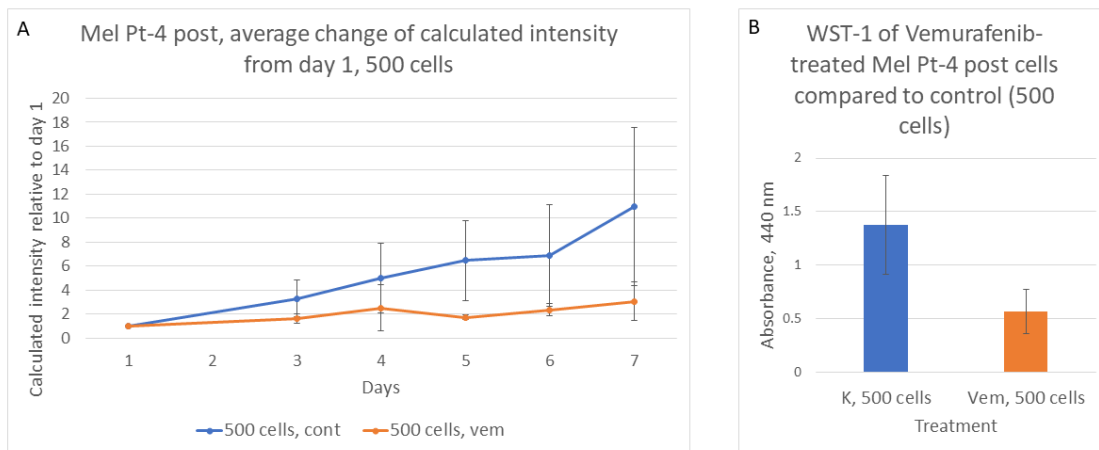


Figure 7 Calculated intensity and WST-1 values of Mel Pt-4 post cells treated with vemurafenib compared to control. Initial cell quantity was 500 cells. Both methods show clear decrease of proliferation with vemurafenib compared to control samples. The mean relative calculated intensity of vemurafenib treatment is 27.9 % of the controls, and the mean WST-1 value is 41.4 %. The calculated intensity of control cells has high standard deviation.

The effect of vemurafenib treatment of Mel Pt-4 post cells is shown by Figure 6 and Figure 7. The decrease of proliferation based on WST-1 results is similar in measurements with 500 and 2000 initial cells but calculated intensity results show difference. These results are less reliable due to their high standard deviation caused by a few outlier results. Calculated intensity results indicate that the growth of 4 post cells is faster than pre cells in controls and also in treated cells, but it is not confirmed by the WST-1 results.

6.2 Characterization of EV-s with Qubit protein assay, DLS, flow cytometry

Qubit protein assay showed big difference between the protein content of EV-samples isolated from pre and post cells: pre EV had $77.1 \mu\text{g/ml}$ and post EV had $309.8 \mu\text{g/ml}$ protein content. The volume of both samples was approximately $400 \mu\text{l}$ (measured with pipette tips). The volume of the spheroid wells was $200 \mu\text{l}$ and each of them was treated with $10 \mu\text{g/ml}$ EV, so $25.9 \mu\text{l}$ of pre EV sample and $6.45 \mu\text{l}$ of post EV was added to each wells.

DLS results are shown on Figure 8. The mean and mode diameter of the pre and post samples are similar (mean pre: 146.15 nm , mean post: 156.22 nm), and they are in the range of the typically measured exosome diameter which is $30 - 150 \text{ nm}$. Based on intensity and mass

distribution figures, the diameter of the particles is in a certain range between approx. 50 – 500 nm. The diameter distribution of the samples is high because the standard deviation almost exceeds mean (stdev pre: 101.38 nm, stdev post: 87.49 nm).

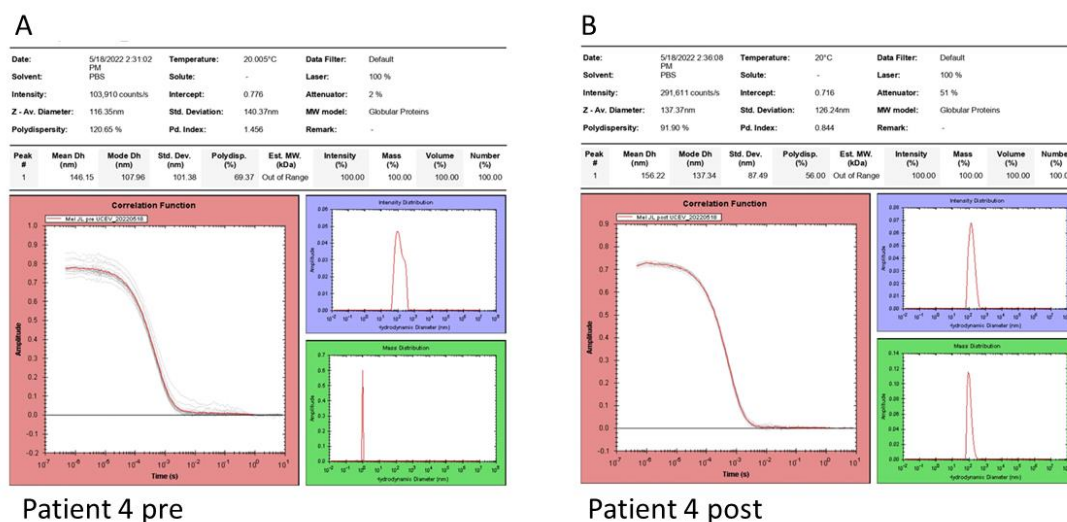


Figure 8 shows the basic statistical description of DLS results. Red boxes show the correlation functions calculated from the intensity functions of the DLS. Intensity distribution and mass distribution are shown in the blue and green boxes respectively. The presence of EV-s was detected in both samples.

Flow cytometry also detected the presence of EV-s in the samples. Compared to negative controls, the median of their FITC-function shifted which indicates higher intensity of light emitted by the fluorescent activity of the secondary antibodies. Flow cytometry measured higher concentration in the post sample which coincides with the Qubit protein assay measurement. Figure 9 shows the detailed results.

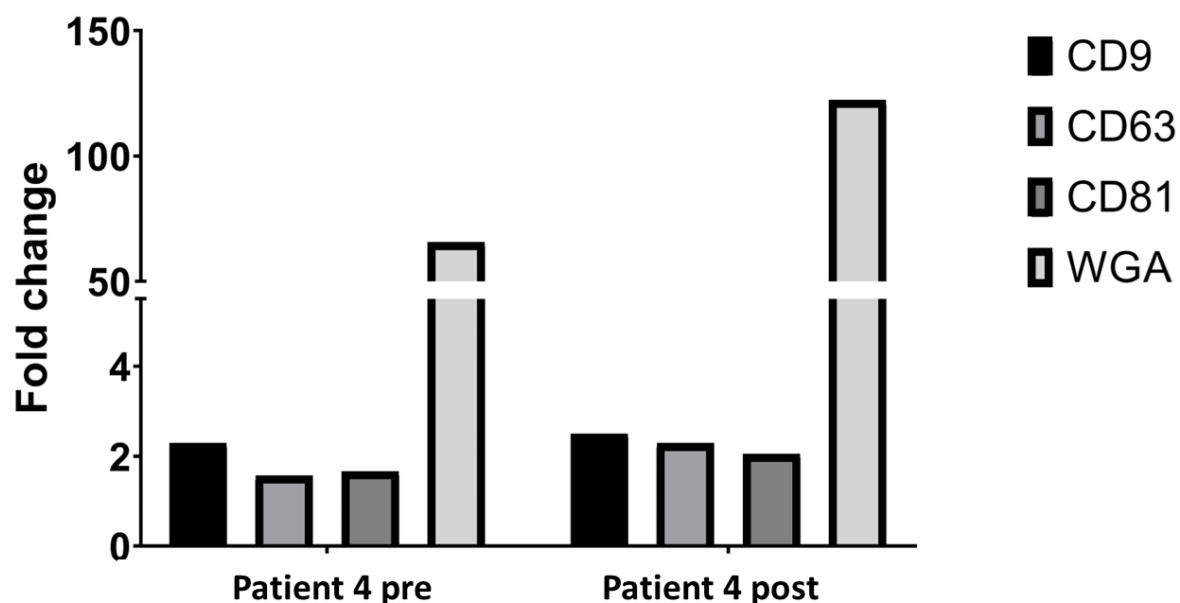


Figure 9 shows the fold change of EV-samples compared to the negative control. The tetraspanin-antibody and WGA signal was higher in post cells which is similar to the result of Qubit protein assay.

6.3 Effect of EV-s on vemurafenib treatment of pre and post cells

The isolated EV-s were utilized to set measurements to test, how EV-s can modify the growth of spheroid on their own, or together with vemurafenib. Based on the Qubit results, samples with 10 $\mu\text{g/ml}$ protein content were added to the wells. 6 kinds of treatments were carried out with each cell line with 2000 initial cells: control, vemurafenib, Mel Pt-4 pre EV, Mel Pt-4 post EV, Mel Pt-4 pre EV + vemurafenib and Mel Pt-4 post EV + vemurafenib. Measurements were also done with 500 initial cells with Mel Pt-4 post cells but, in this case, EV-s were only added together with vemurafenib. From the measurements calculated intensity and WST-1 data were extracted.

Figure 10 - Figure 11 show the relative calculated intensity results and WST-1 values of EV-treated Mel Pt-4 pre cells. Based on these results, pre EV treatment resulted in barely higher growth than vemurafenib treatment so these EV-s have inhibitory effect, and post EV-treated cells grew in the similar pace as controls. Both EV-s increased the proliferation compared to those values when only Vemurafenib was added. Their stimulatory effect is shown by the calculated intensity and the WST-1 absorbance values. Measurements of EV-vemurafenib co-treatment had high standard deviation with both EV-s.

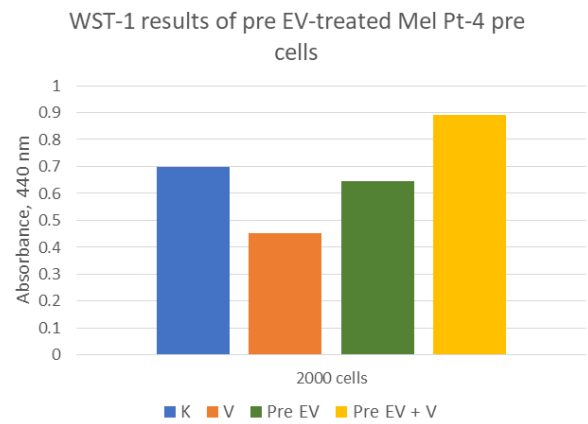
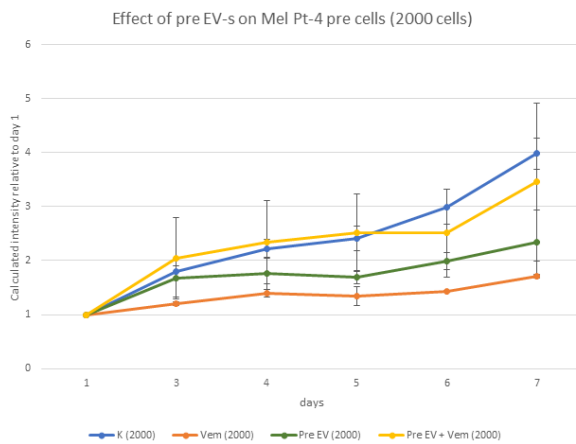


Figure 10 - Effect of patient 1 pre EV on Mel Pt-4 pre cells with 2000 initial cells. The graph shows relative intensity compared to day 1 and WST-1 absorbance. As a comparison, relative intensities of control and only vemurafenib measurements are also shown. Based on the results, pre EV inhibits the growth of the spheroids. EV + vemurafenib treatment has controversial results: it has high standard deviation but it seems to inhibit the cytostatic effect of vemurafenib.

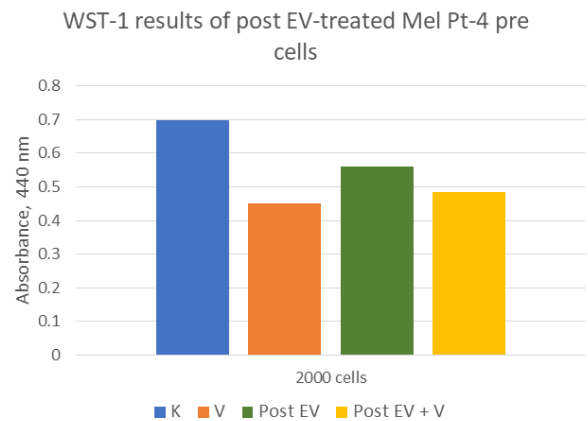
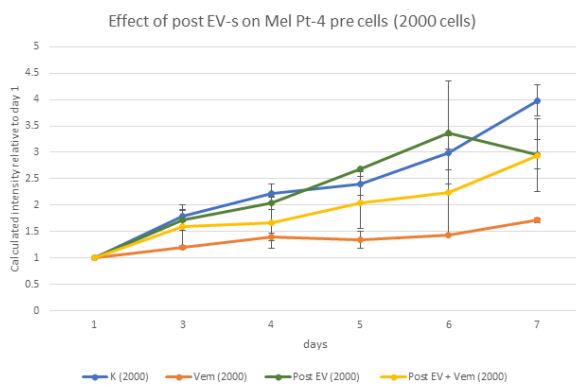


Figure 11 - Effect of patient 1 post EV on patient 1 pre cells with 2000 initial cells. The graph shows relative intensity compared to day 1 and WST-1 absorbance. As a comparison, relative intensities of control and only vemurafenib measurements are also shown. Based on the results, post EV does not particularly modify the growth of the spheroids and the proliferation remains similar to controls. EV + vemurafenib treatment has higher relative calculated intensity than vemurafenib at day 7 but the standard deviation is high, and WST-1 does not decisively confirm the resistance induced by the post EV-s.

WST-1 measurements of Mel Pt-4 pre with 2000 cells show differences from calculated intensity results: the pre EV-treated well had higher WST-1 results than those cells which interacted with post-EV. Although pre EV + vemurafenib treatment stimulated the growth of spheroids, according to this result in contrast to post EV + vemurafenib, the difference might be only due to the arbitrary selection of the well: calculated intensity results show that the difference was high between these two measurements and in case of pre EV + Vem the well with higher intensity, in case of post EV + Vem the sample with lower intensity was selected. Similarly, to calculated intensity results measurements with 500 cells show incongruent values, probably due to unequal initial cell numbers; consequently, this measurement should be repeated to assess the effects of EV-s more accurately with this initial cell number.

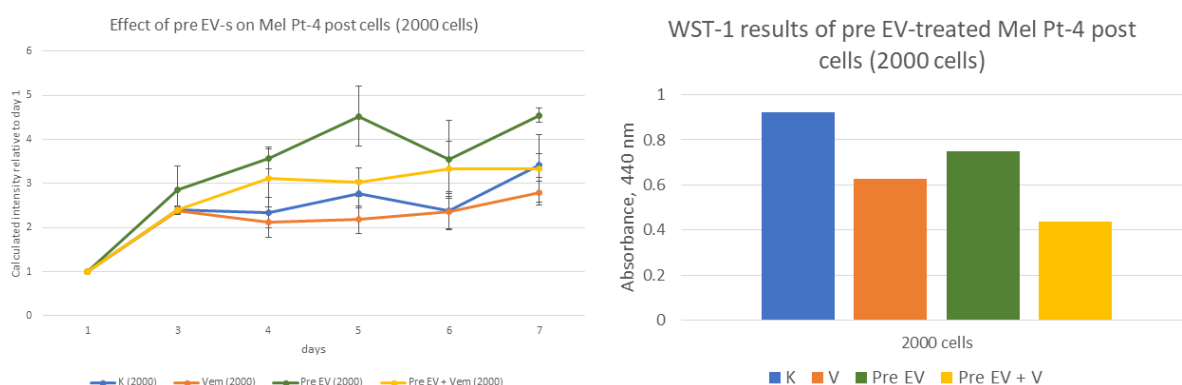


Figure 12 Effect of Mel Pt-4 pre EV on Mel Pt-4 post cells with 2000 initial cells. The graph shows relative intensity compared to day 1. As a comparison, relative intensities of control and only vemurafenib measurements are also shown. The intensity of control and vemurafenib-treated cells is close to each other, and EV-s increased the growth of spheroids. The two measurements with pre EV and vemurafenib combined show varied results. WST-1 results show differences: the values of control and vemurafenib treatment can be clearly distinguishable, and pre EV-s have an inhibitory effect both in the presence and absence of vemurafenib.

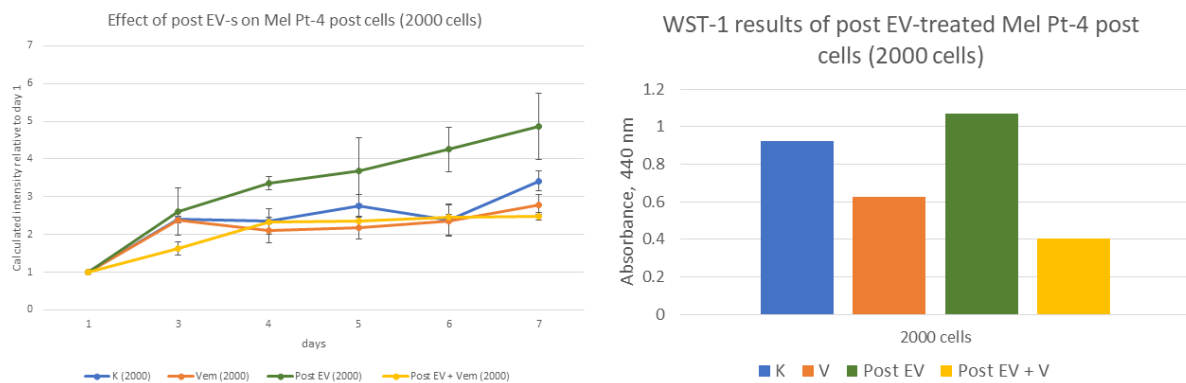


Figure 13 Effect of Mel Pt-4 post EV on Mel Pt-4 pre cells with 2000 initial cells. The graph shows relative intensity compared to day 1. As a comparison, relative intensities of control and only vemurafenib measurements are also shown. Although post EV-treated cells increased the proliferation, this effect is not visible if vemurafenib was also added. WST-1 values show similar results: the stimulation of EV-s is only present if no vemurafenib was added.

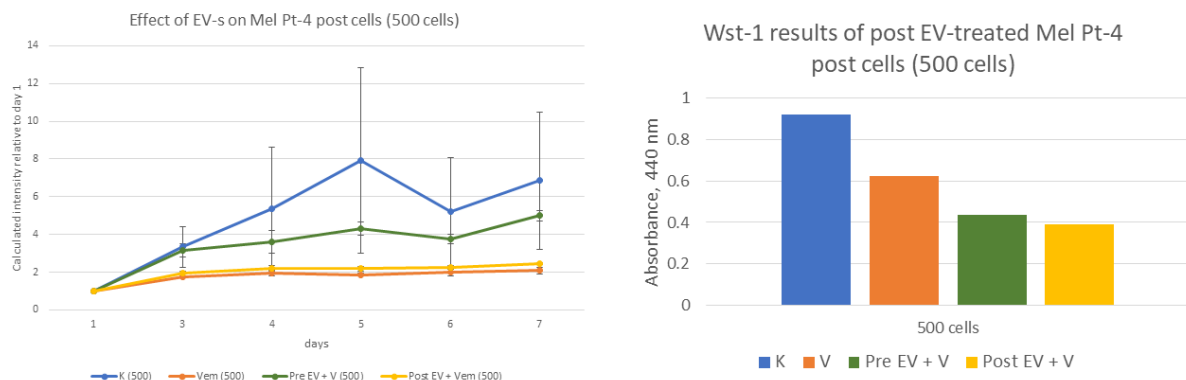


Figure 14 Effect of Mel Pt-4 pre and post EV-s on Mel Pt-4 post cells with 500 initial cells. The graph shows relative intensity compared to day 1. Similar effects are shown here which were visible in results of experiments with 2000 cells: pre EV increased cell growth in the presence of vemurafenib but post EV did have no effect if vemurafenib was also present. Both EV-s caused a decrease in the WST-1 results.

On Figure 12 - Figure 14 one can see the calculated intensity results of Mel Pt-4 post cells. Measurements with 500 and 2000 cells show congruent results since in both cases vemurafenib only mildly decreases the growth of the spheroids, and Pre EV can decrease their effect. Both EV-s seem to increase proliferation but only pre EV-s can keep this effect in the presence of vemurafenib.

WST-1 results show that the EV-s decrease metabolic activity if vemurafenib is present. However, without vemurafenib this decrease does not appear, and the measured values were around the level of control. This means that the excitatory effect of pre EV-s with vemurafenib does not appear if mitochondrial activity is examined. Measurements with 2000 and 500 cells show congruent results.

6.4 Relationship between calculated intensity and WST-1 results

Since both calculated intensity and WST-1 are used to estimate cell number, their comparison gives us valuable information of their reliability and biological relevance. On Figure 15 all WST-1 and day 7 calculated intensity results of Mel Pt-4 pre and post measurements are shown. Although their relationship is far from linear (R^2 is small), the correlation of these values is clear ($p < 0.05$). The R^2 and the p-value of the trend lines is summarized in Table 2.

Treatment	control	vemurafenib
R-squared	0.544	0.697
p-value	0.0002	<0.0001

Table 2 shows the R-squared and p-value of the trend lines fitted to all vemurafenib and control calculated intensity – WST1 results. Although the relationship of the two values is not linear, they are correlated.

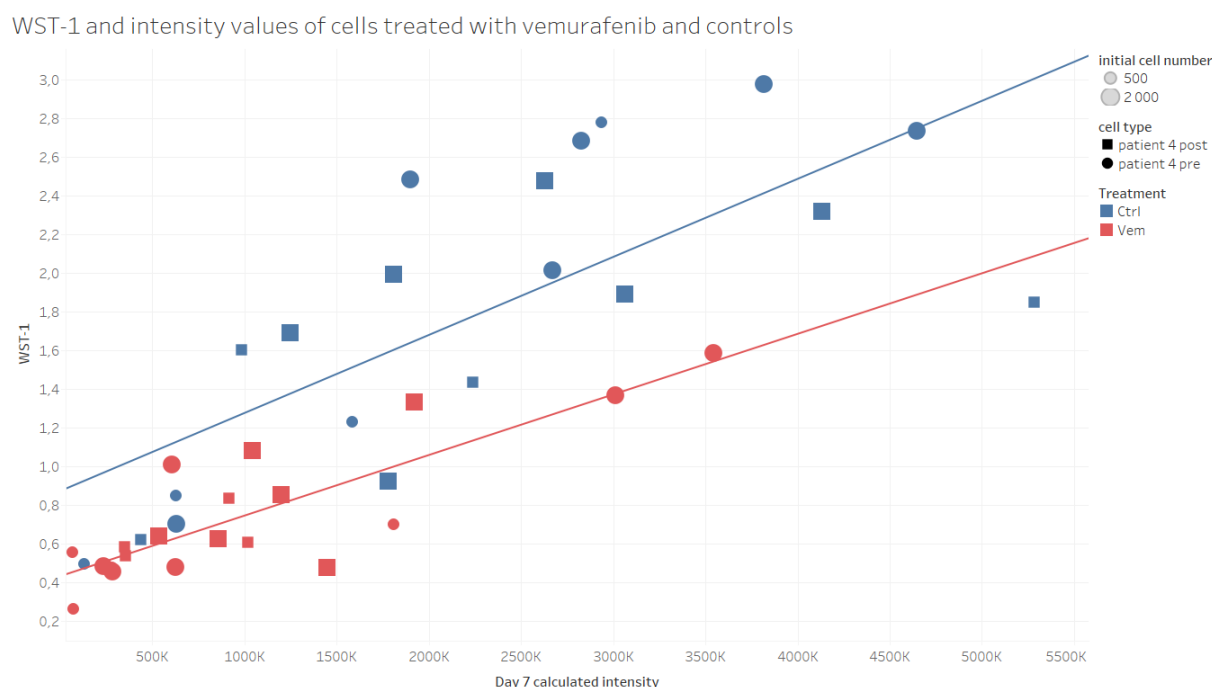


Figure 15 shows day 7 calculated intensity and WST-1 values of all control and vemurafenib treatments included in this research. Trend lines of vemurafenib-treated and control samples are also shown individually

The calculated intensity-WST1 function of measurements about EV-treatments is also plotted on Figure 16 and Figure 17. In case of Mel Pt-4 pre cells, the correlation of the values is not visible because of the outlier value of Pre EV + vemurafenib treatment. In general, EV-treatments caused a decrease in WST-1 and day 7 intensity values, but post EV-vemurafenib co-treatment elevated proliferation compared to vemurafenib according to both measures. However, EV-s could not increase proliferation of vemurafenib-treated post cells.

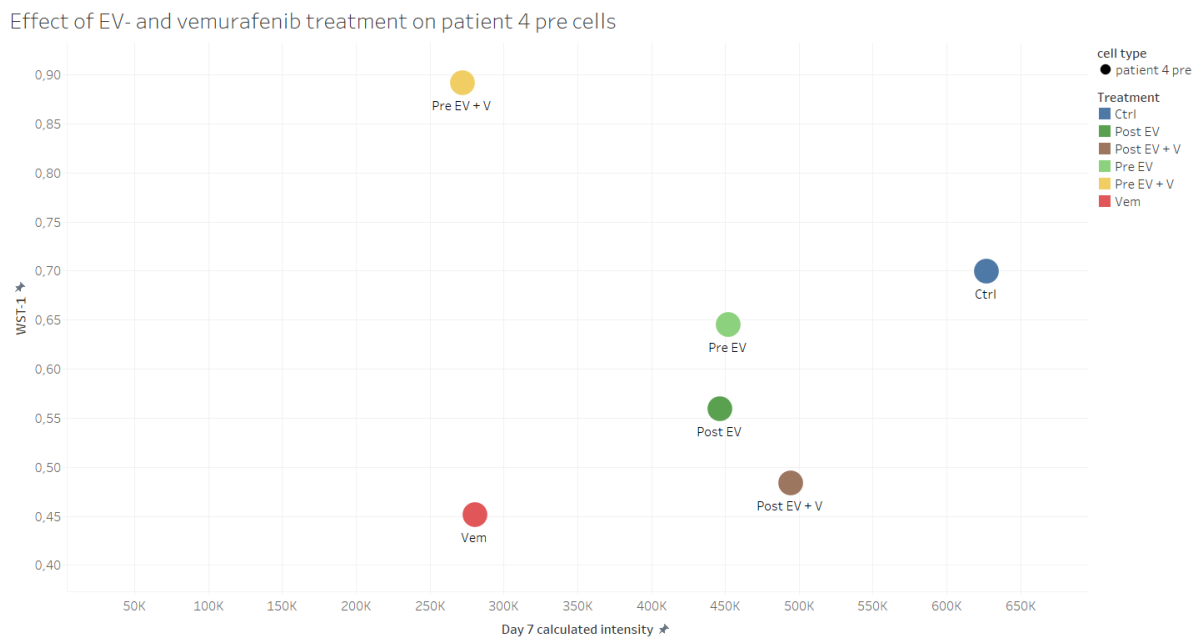


Figure 16 shows the day 7 calculated-intensity – WST-1 function of Mel Pt-4 pre cells. EV-s decreased proliferation compared to control; however higher cell number was indicated compared to Vemurafenib treatment. It is remarkable that the values of post EV and post EV + vemurafenib are close to each other which can be a consequence of resistance against vemurafenib caused by post EV-s

Effect of EV- and vemurafenib treatment on patient 4 post cells

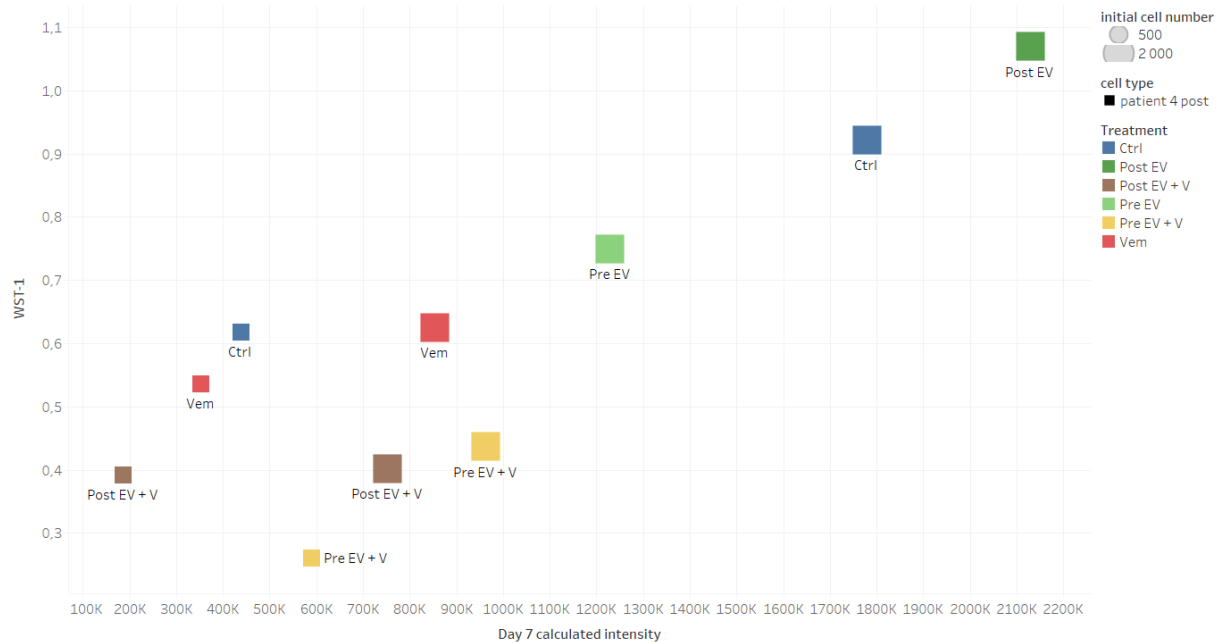


Figure 17 shows the day 7 calculated intensity – WST-1 value of Mel Pt-4 post cells. Measurements with 2000 and 500 initial cells are also indicated. Neither WST-1 nor day 7 calculated intensity values show any resistance effect of pre and post EV-s with vemurafenib treatment.

7 Discussion

In this research, spheroids were characterized with two quantitative parameters: calculated intensity and WST-1. Calculated intensity could be an efficient tool to use microscopy images to estimate cell proliferation next to area or a grading system of compactness which was used in other articles [43]. Its advantages are robustness to the area selection and its reproducibility. In order to advance the image processing, a clustering algorithm to select the contours of the spheroids would be appropriate to make the evaluation more automatic and objective [44]. Another disadvantage of this method is the inability to determine the width of black spheroids. Although the colour of the cell aggregate intuitively deepens with respect to its width, the mathematical relation of pixel colour and spheroid width was not evaluated in detail, instead a linear relationship was assumed. We might assume that width could reach a given threshold when the colour of the spheroid can not become darker. The spatial structure of the spheroids could be further analysed with microscopy techniques which are able to create three-dimensional images [45].

The reliability of the measurements could be increased if initial spheroid size was standardized. The initial cell numbers in independent measurements varied due to the inaccuracy of the cell

counter, but this error could be minimized with the measurement of more samples from the solution with the cell counter. The initial variability could have biological reasons, not only technical inaccuracies: it is likely that tumour heterogeneity could influence spheroid formation. The further growth of spheroids could also be modified by their initial compactness.

In some cases, the borders of spheroids were not well-defined. In these wells, cells were also located in the surrounding of spheroids but in lower density. This makes the characterization of the spheroids with image processing more challenging. When the area of the spheroids was selected, these scattered cells were excluded.

In further measurements WST-1 also could be applied more effectively because the cell number did not modify background absorbance, so all wells with the same treatment could have been stained. After WST-1 staining, we did two measurements, and between them the spheroids were disaggregated with suspending the medium. We experienced no large difference between these measurements. Therefore, we can assume that those staining molecules which remained hidden between the cells did not critically influence the result.

The correlation between calculated intensity and WST-1 is very promising, so these methods seem to be good descriptors of cell proliferation. The measurements with vemurafenib treatment compared to control confirm this since the treatment decreased both values. In addition, the average decrease of these values was lower in Mel Pt-4 post cells compared to Mel Pt-4 pre which suggests that the resistance of post cells also could be detected. Several reasons can induce outliers for example differences in cell size (larger cells make larger spheroids but their metabolic activity elevates less with cell growth). It was shown that vemurafenib treatment can induce autophagy which could also be stimulated by elevated metabolic activity [46].

Regarding the isolation of extracellular vesicles, the high difference between the protein content of EV samples from pre and post EV-s were remarkable which could have several reasons. First, the amount of EV-s produced by the cells could be different. Secondly, there could have been some difference in the manual steps during separation: during the resuspension after ultracentrifugation, some part of the pellet could have lost, or large protein content could be caused by the remaining supernatant. The first possibility is more likely because the difference in EV-content was also identified with the flow cytometry measurements. The physical properties of EV-s produced by the cell lines could have been also different, e. g. some vesicles could have lost due to their inappropriate sedimentation coefficient.

Our measurements suggest that EV-s can modify cell viability in spheroids. Estimated effects of EV-s to cell proliferation and activity are summarized in Table 3. Based on our results, both pre-EV-s and post EV-s can induce resistance against vemurafenib treatment in Mel Pt-4 pre cells. Without the presence of the drug, their effect was rather inhibitory in this cell line. When post cells were treated, the proliferation of vemurafenib-treated cells further decreased by the effect of EV-s. In the absence of vemurafenib, pre EV-s were rather inhibitory but post EV increased the activity of Mel Pt-4 post cells. However, further measurements are necessary to prove these hypotheses. To understand more deeply the mechanism of the EV-s, molecular biological techniques e. g. western blot of treated cell lines and EV-samples is required.

Cell line and treatment	Mel Pt-4 pre, control	Mel Pt-4 pre, vemurafenib	Mel Pt-4 post, control	Mel Pt-4 post, vemurafenib
Change of cell proliferation by pre EV-s				
Change of cell proliferation by post EV-s				

Table 3 shows estimated effects of EV-s on Mel Pt-4 pre and post cell lines. Pre EV-s show inhibitory effect except in case of Vemurafenib-treated pre-cells where the results are controversial. Post EV-s show strong activation of Mel Pt-4 pre cells under vemurafenib treatment which might induce that they neutralized the inhibition by vemurafenib.

8 Conclusion

In this study, spheroids were formed from two melanoma cell lines extracted from the biopsy of the same patient. Mel Pt-4 pre was isolated before, and Mel Pt-4 post was isolated after vemurafenib treatment. The spheroids were treated with vemurafenib, and their proliferation was assessed with image processing and WST-1 assay. Image processing included the selection of the spheroids and the application of a measure called calculated intensity. Both methods showed that the growth of the spheroids of both cell lines was decreased by vemurafenib. The relation of the results got from these two methods was examined, and their correlation was confirmed.

Afterwards, extracellular vesicles were isolated from both cell lines with ultracentrifugation. The isolated samples were characterized with three methods: Qubit protein assay, dynamic light scattering and flow cytometry. Qubit and flow cytometry results show that samples isolated from the supernatants of Mel Pt-4 post cells contain higher amount of EV-s than Mel Pt-4 pre-originated ones.

Measurements were taken with the isolated EV-s and they stimulate the growth of spheroids of Mel Pt-4 cells in vemurafenib treatment. In contrast, in EV-vemurafenib co-treatment, the growth of Mel Pt-4 post spheroids was inhibited by both EV-s compared to those measurements when only vemurafenib was added to the medium. This result suggests that EV-s can improve the resistance of Mel Pt-4 pre cells.

9 Acknowledgements

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**A 36. OTDK
BIOLÓGIA SZEKCIÓ FELHÍVÁSÁNAK
2. SZÁMÚ MELLÉKLETE**

TÉTELES SZERZŐI HOZZÁJÁRULÁS

Alulírott **Kós Tamás** nyilatkozom, hogy a 36. OTDK-ra benyújtott **The role of extracellular vesicles in the emergence of vemurafenib resistance in melanoma spheroids** című pályamunkámban leírtak esetében az alábbi munkafolyamatok elvégzésében vettem részt:

Részfeladat	A részvétel mértéke (ötfokozatú skálán[#] jellemezve)
<i>A kísérletek megtervezéséhez szükséges szakirodalom feldolgozása</i>	5
<i>Sejtenyészetek fenntartása a mérés időszakában</i>	5
<i>Szferoidok készítése és vizsgálata</i>	5
<i>A szferoidokról elkészült képek feldolgozása</i>	5
<i>Mérés kiértékelése, konklúzió levonása</i>	5

Kelt: 2023. 01. 23.

(Kérjük a dőlttel jelölt részeket értelemszerűen adják meg. A táblázat szükség szerint értelemszerűen módosítható).

Pályamunkát benyújtó hallgató (név)
alírása

A nyilatkozatban foglaltak helyességét megerősítem:

Témavezető

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