The effects of ascorbic acid on viability and metabolism of osteosarcoma stem cells Utjecaj askorbinske kiseline na vijabilnost i metabolizam matičnih stanica osteosarkoma

Librenjak, Lucija

University of Zagreb
Faculty of Science
Department of Biology

Lucija Librenjak

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Lucija Librenjak

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Experimental part of this master's thesis was done at the Division of Molecular Biology of Faculty of Science, University of Zagreb as a part of a project financed by Croatian Science Foundation. The research was supervised by dr. sc. Inga Urlić, Assoc. Prof. with the assistance of dr. sc. Maja Antunović. Written thesis was submitted for an evaluation to Department of Biology of Faculty of Science, University of Zagreb to acquire the academic title of Master of molecular biology.

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Master's Thesis

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Lucija Librenjak

Rooseveltov trg 6, 10000 Zagreb, Hrvatska

Osteosarcoma is the most common primary bone malignancy and is the leading cause of tumor-induced death in children and young adults. Due to the absence of specific symptoms, this tumor is often diagnosed after it has already metastasized. Osteosarcoma is highly invasive and often shows chemoresistance. Many studies suggest that cancer stem cells play a critical role in these deadly mechanisms of cancer. Although infrequent, but with the ability to self-renew and differentiate into different cell types, this population of tumor cells supports tumor growth and heterogeneity. To successfully eradicate the whole tumor and reduce the risk of disease recurrence, anticancer therapy should also target cancer stem cells. Apart from its antioxidant properties and depending on applied concentration, ascorbic acid can also behave as a pro-oxidant and cause metabolic stress of the cell. The general aim of this thesis was to investigate the effects of ascorbic acid on viability and metabolism of osteosarcoma stem cells. We isolated cancer stem cells from the patient's biopsies using the sphere culture system and identified them as stem cells using the Hoechst 33342 exclusion assay. The cytotoxicity of high-dose ascorbic acid was confirmed by the MTT test, with osteosarcoma stem cells being the most sensitive. The Seahorse XF method showed that ascorbic acid reduces the glycolytic and aerobic metabolic potential of the cell.

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Lucija Librenjak

Rooseveltov trg 6, 10000 Zagreb, Hrvatska

Osteosarkom predstavlja najčešću primarnu malignost kosti i vodeći je uzrok smrti uzrokovanih tumorima kod djece i mladih odraslih. Zbog izostanka specifičnih simptoma ovaj se tumor često dijagnosticira nakon što je već metastazirao. Visoka stopa invazivnosti i kemorezistencije karakteristični su za osteosarkome. Mnoga istraživanja ukazuju na tumorske matične stanice kao potencijalne krivce i inicijatore tih smrtonosnih mehanizama tumora. Iako malobrojna, ova populacija stanica unutar tumorske mase svojom sposobnošću samoobnavljanja i diferencijacije u različite stanične tipove, podupire rast i heterogenost tumora. Uspješna terapija za potpunu eliminaciju tumora i smanjenje mogućnosti relapsa, trebala bi ciljati i tumorske matične stanice. Ovisno o koncentraciji, osim antioksidativnog, poznato je i prooksidativno djelovanje askorbinske kiseline koje može rezultirati metaboličkim stresom stanice. Glavni cilj ovog diplomskog rada bio je istražiti utjecaj askorbinske kiseline na vijabilnost i metabolizam matičnih stanica osteosarkoma. Iz biopsija zahvaćene kosti izolirali smo tumorske matične stanice koristeći metodu uzgoja stanica u sferama te smo dokazali njihovu matičnost pomoću metode izbacivanja Hoechst 33342 boje. Citotoksičnost visokih doza askorbinske kiseline potvrđena je MTT testom pri čemu su se najosjetljivijima pokazale upravo matične stanice osteosarkoma. Metoda Seahorse XF pokazala je da askorbinska kiselina smanjuje glikolitički i aerobni metabolički potencijal stanice.

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Ocjenitelji:
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Dr. sc. Petra Peharec Štefanić, izv. prof.
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List of abbreviations

AA	Ascorbic acid
ABC	ATP-binding cassette
AFR	Ascorbyl free radical
ALP	Alkaline phosphatase
ALDH	Aldehyde dehydrogenase
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
bFGF	Basic fibroblast growth factor
BMP-2	Bone morphogenic protein 2
CM	Collagenase medium
CSC	Cancer stem cell
CT	Computed tomography
DHA	Dehydroascorbic acid
DMEM	Dulbecco′s Modified Eagle′s Medium
ECAR	Extracellular acidification rate
EGF	Epithelial growth factor
EMT	Epithelial-mesenchymal transition
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT	Glucose transporter
GM	Growth medium
GSH	Glutathione
HEK 293	Human embryonic kidney 293 cells
HCC	Hepatocellular carcinoma
HIF	Hypoxia-inducible transcription factor
hMSC	Human mesenchymal stem cell
LDH	Lactose dehydrogenase
MC	Methyl-cellulose
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cell

OCR	Oxygen consumption rate
OS	Osteosarcoma
OS-CSC	Osteosarcoma cancer stem cell
OXPHOS	Oxidative phosphorylation
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffer saline
PDB	Paget's disease of bone
PET	Positron-emission tomography
PET/CT	Positron-emission tomography/computed tomography
ROS	Reactive oxygen species
SCGM	Stem cell growth medium
SGM	Sarcosphere growth medium
SP	Side population
SVCT	Sodium-vitamin C co-transporter
TIC	Tumor-initiating cell

1. Introduction

1.1. Cancer stem cells

Cancer stem cell (CSC) concept is based on the idea that, despite initially arising from a single mutated cell, not all cells in tumors are equal. Among others, the tumor bulk consists of dedicated cancer stem cell population that is capable of self-renewal. Just like in healthy proliferative tissues, quickly dividing stem cells fuel the growth of tumor (Clevers, 2011). Tumorigenic cancer cells that undergo processes analogous to the self-renewal and differentiation of normal stem cells were first described as "cancer stem cells" by Reya, Morrison, Clarke, and Weissman (2001). This small, phenotypically distinct subset of cancer cells has extensive proliferative potential and the ability to form a new tumour. To distinguish these cells from normal stem cells and to accentuate their ability to initiate tumor occurrence, the term "tumor-initiating cells (TICs)" is sometimes preferred by the researchers (Wei and Lewis, 2015). Nevertheless, prominent parallels can be found between healthy and cancer stem cells. The common features and differences between normal somatic stem cells and CSCs are shown **Table 1** (Rahman *et al.*, 2016).

Table 1. Features of normal and cancer stem cells. Certain features are shared between normal and cancer stem cells (A.), but some properties are unique for each of the cell type (B.) (Rahman *et al.*, 2016)

	A. Common features found in normal and cancer stem cells
1	Capacity for asymmetric divisions (self-renewal) that produces a quiescent stem cell and a dedicated progenitor
2	Regulation of self-renewability by similar signalling pathways (Wnt, Sonic Hedgehog, MAPK and Notch) and at the epigenetic level by BMI-1
3	Capacity to arrange a hierarchy of cellular derivatives that includes progenitors and differentiating cells
4	Extended telomeres and telomerase activity
5	Predilection for growth factor independence through secretion of growth factors and cytokines
6	Stimulation of angiogenesis through secretion of angiopoietic factors
7	Expression of similar surface receptors (e.g., CXCR4, CD133, a6 integrin, c-kit, c-met, LIF-R)

B. Normal stem cell properties versus CSC properties	
Normal stem cell	***Cancer stem cell***
Extensive but limited self-renewal capacity	Extensive and indefinite self-renewal capacity
Organogenic capacity	Tumorigenic capacity
Highly regulated self-renewal and differentiation	Highly dysregulated self-renewal and differentiation
Rare in normal adult tissues	Infrequent or rare within tumors
Can be identified based on surface markers	Similar types of surface markers as ordinary stem cells in the same tissue
Normal karyotype	Abnormal karyotype
Quiescent most of the time	Less mitotically active than other cancer cells
Capacity to generate normal progeny with limited proliferative potential	Phenotypically diverse progeny

For decades, it was believed that most often cancer develops as a clone from a single cell-of-origin after it undergoes at least five genetic mutations to gain cancer hallmarks. It was also proposed that each cancer cell in tumor has similar tumorigenic capacity (Wang *et al.*, 2015). According to this theory, cancer is a clear example of a Darwinian system. That is, the progression of tumor is following natural selection of the fittest variants (Greaves and Maley, 2012). This concept is known as the clonal evolution (stochastic) theory and is shown on **Figure 1**. However, there are some observations that are not in favour of this theory which is also considered overly simplistic. Currently accepted theory of cancer initiation states that the status of different cells in tumor is not equal and proposes a hierarchy with rare undifferentiated CSCs at the top. Because CSCs represent a reservoir of self-sustaining cells that maintain the whole tumor, this carcinogenesis theory is referred to as CSC theory (**Figure 2.**) (Wang *et al.*, 2015).

The acquisition of CSC-like traits by cancer cells is intertwined with the process of epithelial-mesenchymal transition (EMT). By undergoing EMT, CSCs adopt characteristics that promote tumor progression, recurrence, metastasis, and resistance to therapy (Chang, 2016). Chemoresistance and recurrence following chemotherapy is mediated by CSC slow rate of division, drug efflux by the ATP-binding cassette (ABC) transporters, high expression of aldehyde dehydrogenases (ALDHs), resistance to apoptosis, a well-tuned DNA damage sensor and repair mechanisms, and CSCs niche contribution (Zhao, 2016). Because multiple lines of CSC self-defence are hindering conventional cancer therapies, CSCs are a subject of intense research that aims at finding an efficient targeting strategy.

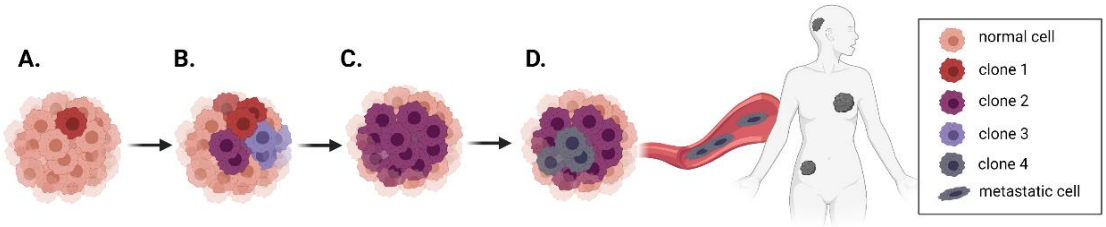

Figure 1. Clonal evolution model. Various mutations can hit a single normal cell and cause its transformation into neoplastic (A.). With these mutations, the neoplastic cell gains selective growth advantages over unchanged normal cells that will promote its further proliferation (clone 1). However, this clone is genetically instable, and more clones (clone 2, 3) are occurring (B.). Some of the generated clones cannot survive selective pressures (clone 1, 3) while some (clone 2) adapt, continue to proliferate, and expand to become the predominant population (C.). It is possible that even more persistent and aggressive variant will appear (clone 4) (D.). Adapted from Wang *et al.*, 2015. Created with BioRender.com.

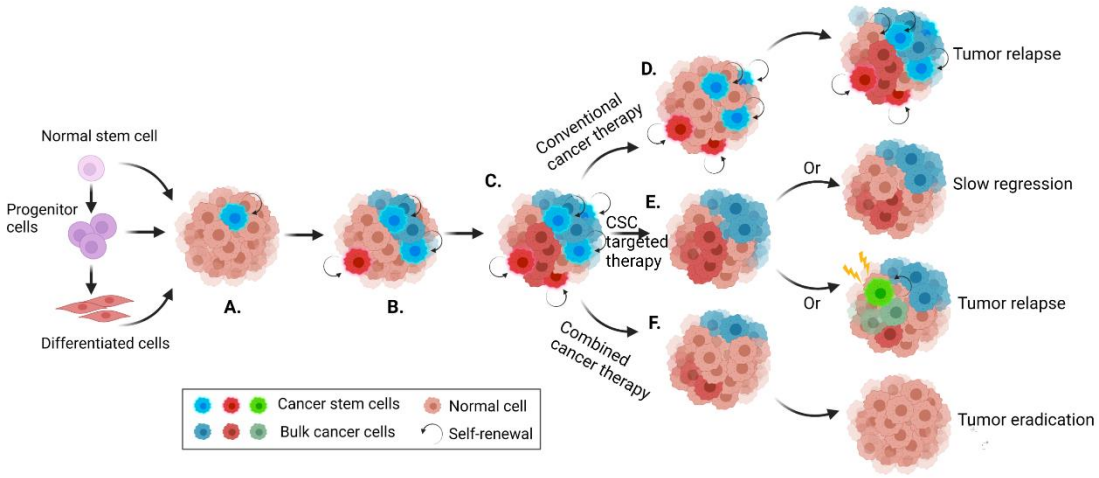

Figure 2. Current cancer stem cell theory. By gaining certain mutations, normal stem cells, progenitor cells and/or differentiated cells can transform into cancer stem cell (CSC) (A.). CSC divides into daughter CSC and differentiated cancer cells. New CSC can arise from mutated CSC or differentiated cancer cell (B.). Multiple CSC clones can coexist and contribute to the tumor heterogeneity. Conventional chemotherapy kills differentiated cancer cells, but not the CSCs which leads to tumor relapse (D.). CSC targeted therapy can lead to slow regression or tumor relapse if new CSC is generated from the mutated differentiated cancer cell (E.). Combined cancer therapy can eliminate both differentiated cancer cells and CSCs and may result in tumor eradication (F.). Adapted from Wang *et al.*, 2015. Created with BioRender.com.

1.2. Osteosarcoma

Osteosarcoma (OS) is the most frequent form of bone cancer, predominantly occurring during the rapid growth phase of long bones. The most commonly affected primary site is the metaphysis of lower long bones, in particular the distal femur and proximal tibia. Patients over 25 exhibit more various sites of primary disease (Lindsey *et al.*, 2017). Due to the intense linear bone growth, the first incidence peak of OS bimodal age distribution appears in adolescents age group of 15-19 years. The second highest peak in adults (75-79 years) has been associated with pre-existing Paget's disease of bone (PDB), prior radiation therapy and increased bone resorption by osteoclasts. Males are affected more frequently than females regardless of the age (Czarnecka *et al.*, 2020). OS has high tendency to metastasize, mostly to the lungs, which usually happens during the first or second decade of patient's life. Approximately 20% of them have radiologically detectable lung metastases by the moment of diagnosis (Müller and Silvan, 2019). It was only in 1970s, after the addition of chemotherapy to surgical resection, that the five-year overall survival increased from ~20% to 60–70% for young patients with localized disease. Relative survival decreases after the age of 60, but it is greater in female than in male individuals for all age groups (Gianferante *et al.*, 2017). Patients with metastatic disease still have the five-year survival rate as low as 10–20% (Arif *et al.*, 2019).

The aetiology of most OS cases remains unknown. Overall OS infrequency has made it difficult to carry out association studies of environmental exposures and OS with sufficient statistical power (Gianferante *et al.*, 2017). Linear relationship between the local dose of radiation and the risk of OS to occur as a second malignant neoplasm marked therapeutic radiation as a risk factor (Havaudra *et al.*, 1998). Timing of the disease with the highest incidence on the onset of puberty evokes an association with factors related to growth and development (Gianferante *et al.*, 2017). However, most of the OS cases develop in the absence of stated risk factors and the contribution of other environmental exposures has yet not been identified (Savage and Mirabello, 2011). Many inherited cancer predisposition syndromes are associated with OS. Two familiar examples are Li-Fraumeni syndrome and hereditary retinoblastoma (Czarnecka *et al.*, 2020). High median somatic mutation rate, chromosomal aneuploidy, copy number variation and structural variation have also been observed in OS genome and could potentially predispose an individual to OS (Chen *et al.*, 2014).

Both diagnosis and treatment often require multidisciplinary approach due to the complex nature of the disease. Low incidence and the absence of specific symptoms make OS difficult to diagnose (Lindsey *et al.*, 2017). Traditionally, medical imaging techniques are used to non-invasively diagnose OS. In general, pathway to OS diagnosis starts with X-ray and continues with combination of positron-emission tomography (PET) scanning, magnetic resonance imaging (MRI) and computed tomography (CT) scanning. Recently, positron-emission tomography/computed tomography (PET/CT) has been introduced into clinical practice due to its higher sensitivity (Zhang and Guan, 2018). Serum markers, such as alkaline phosphatase (ALP) and lactose dehydrogenase (LDH) correlate positively with tumour volume and can be useful in diagnosing and tracking OS progression and recurrence. To confirm the diagnosis and direct the treatment, biopsy with microscopic examination are preformed (Lindsey *et al.,* 2017). The current standard treatment for both localized and metastatic OS includes surgical resection combined with neoadjuvant and/or adjuvant chemotherapy. By means of surgery, either amputation of the limb or limb salvage can be done to remove primary tumours (Simpson *et al.*, 2017). Preoperative treatment, i.e., neoadjuvant chemotherapy should cause necrosis and size reduction of primary tumour and pulmonary metastasis, if existing. Adjuvant chemotherapy has shown positive results in decreasing postsurgical recurrence. Both neoadjuvant and adjuvant chemotherapy protocols use high-dose doxorubicin, cisplatin and methotrexate (Ferguson and Turner, 2018).

1.3. Cancer stem cells in osteosarcoma

For multiple types of cancer, it has been shown that their tumorigenic growth state is maintained mostly by a small fraction of CSCs, and OS is not an exception. Actually, being rich in growth factors and adult stem/progenitor cells and with a great capacity for regeneration, OS is a likely candidate for CSCs isolation (Gibbs *et al.*, 2011). Indeed, recent studies have reported the existence of osteosarcoma cancer stem cells (OS-CSCs). Gibbs *et al.* (2005) were the first to isolate OS-CSCs from OS biopsies and MG-63 OS cell line. Isolated cells displayed several surface markers of mesenchymal stem cells (Stro-1, CD105, and CD44) and expressed the marker genes of embryonic stem cell pluripotency (Oct 3/4 and Nanog). Additionally, Sox2 expression was also proven to be important for OS-CSC self-renewal (Basu-Roy *et al.*, 2012).

Isolating CSCs, establishing CSC cultures and studying their biology are crucial steps in searching for novel therapies that could completely eradicate the whole tumor. Putative CSC subpopulations can be isolated from OS biopsies by several methods. The most easily operated one is culturing the cells under nonadherent conditions, also used by Gibbs *et al.* (2005). The medium should be serum-free but should contain several factors such as epithelial growth factor (EGF), basic fibroblast growth factor (bFGF). Growth factors support CSCs proliferation in ultra-low attachment plate (Abbaszadegan *et al.*, 2017). Differentiated cells cannot survive in these conditions, while immature cells grow slowly forming clusters called spheres (Cammareri *et al.*, 2008). Even though the sphere culture is most widely used technique for CSC isolation, limitations of this method have been reported. A great disadvantage is the observation of sphere formation within non-stem populations (Pastrana *et al.*, 2011). Hence, additional tests should be evaluated and included into protocols for OS-CSC specific isolation and identification. Like all CSCs in general, OS-CSCs can also be identified by assays that assess chemoresistance-related properties of stem cells such as the ability to efflux dyes or to exhibit high ALDH1 activity (Murase *et al.*, 2009; Honoki, 2010). By using "side population" (SP) method it is possible to identify the cell population that is capable of the Hoechst 33342 dye efflux by ABC family of transporters. Assumption that SP cells are enriched with CSCs is suggested by the studies that revealed stem-cell-like characteristics of SP cells (Patrawala *et al.*, 2005; Chiba *et al.*, 2006; Ho *et al.*, 2007). Another method which can be used for CSC identification is confirming the presence of certain cellular markers. CSCs in most cases express similar types of surface markers as ordinary stem cells in the same tissue. CD44 and CD133 surface markers are used for identification and isolation of CSCs in general (Abbaszadegan *et al.*, 2017). Co-expression of nestin and the CD133 surface molecule is suggested to be a marker for OS-CSCs (Veselska et al., 2008).

Despite the fact that OS has a high tendency to metastasize and reoccur, and the evidence of OS-CSC existence, only a small number of attempts to target this population has been reported. In general, there are two main strategy paths for CSC eradication and examples of each can be found for OS-CSCs. "Destemming" strategy includes either promoting CSC differentiation or inhibiting self-renewal to enhance chemosensitivity of CSCs (Wang *et al.*, 2015). Being exposed to differentiation therapy CSCs lose their self-renewal ability and undergo differentiation. It has been shown that bone morphogenic protein 2 (BMP-2) suppressed tumor growth by inducing the differentiation of OS-CSCs (Wang *et al.*, 2011). As for the self-renewal, Yu *et al.* (2016) observed that blocking the Notch pathway caused a

significant depletion in OS sphere formation. Also, inhibition of this self-renewal pathway sensitized OS cells to cisplatin (Dubey *et al.*, 2013). Possible downside of self-renewal inhibition is the interference with function of healthy stem cells as many signalling pathways are common for both healthy and cancer stem cells. Second strategy utilizes directly targeting one of the molecular mechanisms specific for CSC. Targeted therapies can therefore aim for adhesion molecules, antibody-accessible surface components, signalling intermediates, survival pathway elements, chromatin modifiers or metabolism (Wang *et al.*, 2015). Peng and Jiang (2018) provided evidence that resveratrol, a natural small polyphenolic compound eliminates OS-CSCs by STAT3 pathway inhibition. Resveratrol even suppressed OS cells growth *in vivo*. There are also certain indications that dual inhibition of metabolic pathways can reduce the viability of OS-CSCs (Kishi *et al.*, 2019a), but hardly any metabolism-targeting studies are done so far. More research is needed to gain a better insight into OS-CSC biology to find a novel therapy which would aim for their eradication.

1.4. Features of osteosarcoma metabolism

A general feature of cancer cells is predominant metabolic redirection towards the glycolysis to promote proliferation and long-term maintenance. Therefore, the hallmarks of OS metabolism are increased glucose uptake and fermentation of glucose to produce lactate in hypoxic conditions. The uptake of enormous amounts of glucose by cancer cells was first observed in the 1920s by German physiologist Otto Warburg. This discovery is today known as the Warburg Effect and is still a subject of intense research (Liberti *et al.*, 2017).

In healthy cells, the glycolysis includes glucose conversion into pyruvate in the absence of oxygen (anaerobic metabolism). In the process of lactic acid fermentation, pyruvate is converted into lactate. In the presence of oxygen, generated pyruvate is converted into acetyl-CoA. When acetyl-CoA enters the Krebs cycle, NADH and $FADH_2$ are produced. NADH and $FADH_2$ transfer electrons to the electron transport chain located in the inner mitochondrial membrane with oxygen being the terminal electron acceptor. Electrochemical proton gradient generated over the inner mitochondrial membrane drives the synthesis of adenosine triphosphate (ATP). In normal conditions, oxidative phosphorylation (OXPHOS) gains 18 x more ATP molecules than the glycolysis (Lauren Pecorino, 2016). Although glycolysis is less efficient in the ATP generation, the production of lactate from glucose is 10-100 times faster than the complete glucose oxidation in the mitochondria. Also, the excess of glucose is used as

the source of carbon. Carbon is needed for biosynthesis of nucleotides, lipids and proteins – building blocks for the new cancer cells. Finally, acidification of tumor microenvironment is considered to allow enhanced invasiveness (Liberti *et al.*, 2017).

However, the Warburg effect came into question after some observations, for example that glycolysis contributes less than 50% for energy production in many malignant cell lines. Therefore, some studies have now proposed a new hypothesis, the reverse Warburg effect. This phenomenon was also observed in OS cells. Actually, Bonuccelli *et al.* (2014) demonstrated that aerobic glycolysis is induced in mesenchymal stem cells (MSCs) by neighbouring OS cells, while the same OS cells increased their mitochondrial activity. MSCs behaved as "feeder cells" because the lactate produced in these cells was transported into the OS cells.

As for the CSC metabolic properties, recent studies showed opposing results. For many tumors including hepatocellular carcinoma (HCC) (Zhou *et al.*, 2011), breast cancer (Ciavardelli *et al.*, 2014), ovarian cancer (Liao *et al.*, 2014) and OS (Mizushima *et al.*, 2020) it was shown that glycolytic metabolism is predominant. According to other studies, CSCs prefer mitochondrial oxidative metabolism (Lagadinou *et al.*, 2012; Pastò *et al.*, 2014; Vlashi *et al.*, 2014). It is suggested that CSCs can switch between glycolysis and OXPHOS in the presence of oxygen (Snyder *et al.*, 2018).

Nevertheless, there is no doubt that both differentiated cancer cells and CSCs in general can exhibit metabolic plasticity. Consequently, the combinatory inhibition of both OXPHOS and glycolysis could be a strong candidate when targeting cancer metabolism. A promising example of metabolic strategy for CSC eradication is the use of doxycycline and ascorbic acid (AA) in breast cancer (De Francesco *et al.*, 2017).

1.5. Targeting cancer metabolism with ascorbic acid

Ascorbic acid (AA), also referred to as the vitamin C, acts as an antioxidant and cofactor for several enzymatic reactions, such as collagen synthesis, carnitine synthesis, norepinephrine synthesis, hypoxia-inducible transcription factor (HIF) regulation and histone demethylation (Verrax and Buc Calderon, 2008; Du *et al.*, 2012). Humans are not able to synthesize vitamin C, but due to many of its essential roles in our organism, it is recommended to acquire this vitamin from external sources. The idea of using vitamin C to selectively kill cancer cells seems counterintuitive but has been proposed and investigated for a few decades now, but the

knowledge on how this nutrient affects cancer cells is still incomplete (Roa *et al.*, 2020). However, it was observed that choice of both the method of application and applied concentration are crucial. In the 1970s, Pauling and Cameron showed that the patients who were intravenously treated with ascorbate had a greater mean survival time comparing to the controls (Cameron and Pauling, 1978). The results appeared to be unreproducible when vitamin C was applied orally (Creagan *et al.*, 1979). Plasma concentration of vitamin C dramatically differs depending on the way of its administration while response to the treatment varies depending on the concentration. Opposing effects of low ("physiologic") versus high ("pharmacologic") concentrations of vitamin C concentrations have been shown. Yang *et al.* (2017) have demonstrated that vitamin C at high concentrations exhibited significant cytotoxicity against melanoma cell lines but significantly increased the cell growth at low concentrations.

Vitamin C exists in two redox states, ascorbic acid (AA), and its oxidized form, dehydroascorbic acid (DHA) (**Figure 3.**). Two states differ in their mechanism of transport into the cell. DHA is transported by glucose transporters (GLUTs), while the AA is transported by sodium-vitamin C co-transporters (SVCT) (Roa *et al.*, 2020).

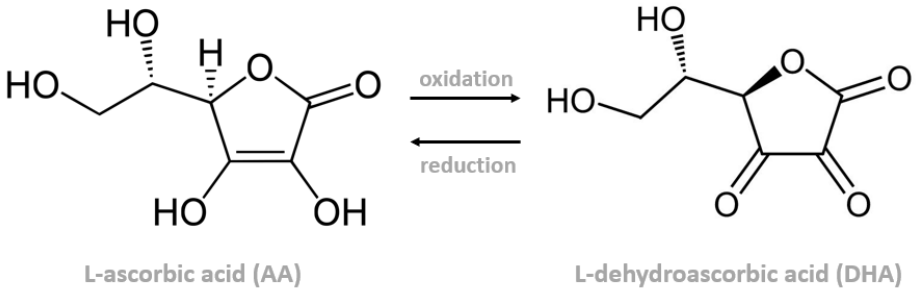

Figure 3. Two vitamin C redox states. Reduced form - ascorbic acid (AA) and oxidized form - dehydroascorbic acid (DHA). Adapted from Morales Gonzlez *et al.*, 2012.

There is no confirmed molecular mechanism by which pharmacologic dose of vitamin C affects cancer cells, but it is accepted that its cytotoxicity is mediated by generation of sustainable ascorbate radical and H_2O_2 (Chen *et al.*, 2005). Previous studies have suggested mechanisms like nonapoptotic cell death (Chen *et al.*, 2005), caspase-dependent and caspase-independent apoptosis, cell cycle arrest (Lin *et al.*, 2006), genotoxic (DNA damage) and metabolic (ATP depletion) stress (Chen *et al.*, 2005). Chen *et al.* (2007) gathered three possible pathways that can lead to ATP depletion and cause toxicity in cells (**Figure 4.**). Pharmacologic AA concentration produces extracellular H_2O_2 which can diffuse into cells and (i.) induce DNA damage which will activate Poly (ADP-ribose) polymerase (PARP). By catabolizing NAD^+,

activated PARP depletes substrate for NADH formation. (ii.) H_2O_2 may supress the energy production via glycolysis either by inactivation of the essential glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or by redirecting glucose to pentose shunt. (iii.) H_2O_2 may directly damage mitochondria.

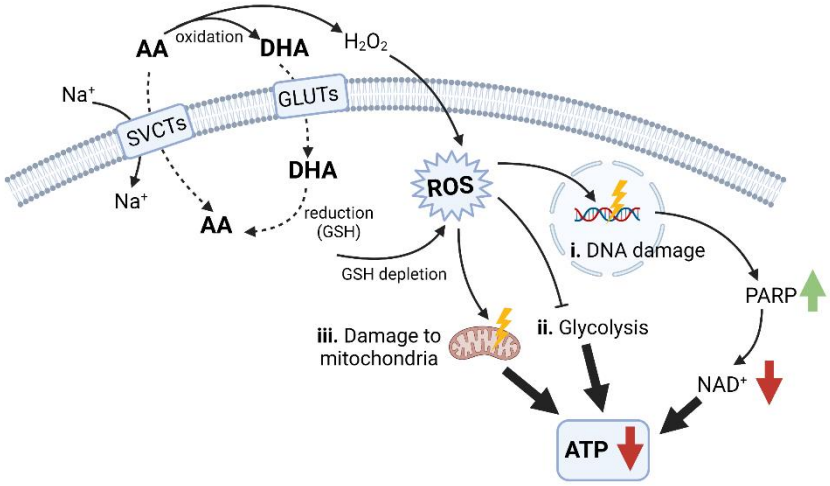

Figure 4. Vitamin C mechanism of action. Reduced form (AA) of vitamin C is taken up from plasma by sodium-dependent vitamin C transporters (SVCTs), while its oxidized form (DHA) can be transported via glucose transporters (GLUTs). Inside the cell, glutathione (GSH) mediates DHA reduction to AA. Depletion of GSH can lead to reactive oxygen species (ROS) generation. External H_2O_2 formed from AA diffuses into the cell and contributes to ROS accumulation. Internal H_2O_2 can cause ATP depletion via three mechanisms. (i.) By damaging DNA which will activate PARP and activated PARP will catabolize NAD^+, (ii.) by inhibiting glycolysis and/or (iii.) by directly damaging mitochondria. Adapted from Chen *et al.*, 2005 and Cimmino *et al.*, 2018. Created with BioRender.com.

According to what can be found in literature and the fact that OS-CSCs have not been a subject of similar studies, we hope to attain a deeper knowledge of how pharmacological AA-dose affects their viability and their glycolytic i.e., mitochondrial metabolism.

2. Objective of the study

The general aim of this research is to study how AA affects the OS-CSCs. It is based around the idea of finding a strategy to selectively target this population and improve the effectiveness of classical anticancer therapies. Chemoresistance is a major problem in cancer therapy. Due to their property of slow proliferation and cellular processes that involve drug efflux and drug enzymatic inactivation, CSCs counteract the effect of chemotherapeutic compounds. Targeting CSC hallmarks e.g., cell metabolic features, could diminish the risk of tumor metastasis and cancer recurrence.

It is known that, apart from its antioxidant properties, AA has a cytotoxic effect on cancer cells when applied in higher concentration. We want to investigate if AA could induce the state of metabolic stress that would lead to death in OS-CSCs.

The specific goals set for this study are following:

1. To isolate OS-CSCs from patient's biopsies and confirm their stemness by sarcosphere assay and dye exclusion assay
2. To study the response of parental OS cell line and isolated OS-CSCs to different AA concentrations relative to the controls, and to determine the IC_{50} value by MTT assay
3. To interpret metabolic phenotype maps generated by using Seahorse technology for experimental (parental OS cell line, OS-CSCs) and control groups (human mesenchymal stem cells (hMSCs), U2OS OS cell line) with and without AA treatment.

It is expected that the obtained results will show a negative effect of AA on both the viability and metabolism of OS-CSCs.

3. Materials and methods

Osteosarcoma samples were collected from the Children's Hospital Zagreb. The research was approved by the Ethics Committee of the same hospital (Register Number: 02-23/34-4-71, date: 8th November 2017).

3.1. Osteosarcoma cells isolation

The biopsy was placed in a 100 mm Petri dish with a small volume of phosphate buffer saline (PBS, Gibco). OS tissue samples were cut into pieces of size 0.5 to 1 mm, transferred to a conical tube with 10 mL of collagenase medium (CM, 20% FBS, 1% Pen/Strep, 3.75 mg/mL collagenase type II in Coon's modified Ham's F12 medium) for the enzymatic digestion. Conical tubes were incubated for 2 hours in a 37 °C, 5% CO_2 incubator on the orbital shaker. Conical tubes were centrifuged (300 x *g* for 10 min) to pellet the fragments. The supernatant was removed by aspiration. Pellet fragments were suspended with 10 mL growth medium (GM, 10% fetal bovine serum (FBS, Gibco), 1% Penicillin/Streptomycin (Lonza) in Coon's modified Ham's F12 medium (Biological Industries)). The resulting cell suspension was transferred into a T75 flask. Cells were incubated in a 37 °C, 5% CO_2 incubator and the GM was replaced with fresh complete GM every 3 days.

Attached and passaged cells represented the parental OS cell line.

3.2. Sarcosphere assay to isolate osteosarcoma stem cells

3.2.1. Establishment of sarcosphere assay

Parental OS cells i.e., cells that were previously isolated from patient's tumors were used for the sarcosphere assay. The medium was removed by aspiration and OS cells were washed with PBS. To dissociate the cell monolayer, 3 mL of the trypsin-EDTA (Sigma) was added at the room temperature. The plate was shaken gently, trypsin was immediately removed by aspiration and added again. Cells were incubated for 4 minutes at 37 ºC, 5% CO_2. Trypsin activity was blocked by adding 10 mL of growth medium. Cells were counted using the hemocytometer.

Cells were seeded in the 6-well ultra-low attachment plate at the cell density of 4 x 10^4 cells/well in 5 mL of sarcosphere growth medium supplemented with 2% methyl-cellulose (MC, Sigma). MC was prepared in advance by dissolving in miliQ-H2O at 4 ºC for 3 days, autoclaved and stored at 4 ºC. Sarcosphere growth medium was prepared (SGM, 20% knockout serum replacement (Gibco), 1% Pen/Strep, 0.01% EGF (Gibco), 0.01% bFGF (Gibco) in Coon's modified Ham's F12 medium (Biological Industries)). MC and SGM were combined in the 1:1 ratio. Every 3 days, fresh aliquotes of bFGF (Gibco) and EGF (Gibco) were added to each well to the final concentration of 10 ng/mL. Sarcosphere growth was observed by microscope.

3.2.2. Sarcospheres isolation

After approximately 31 days it was necessary to isolate the formed sarcospheres. Sarcospheres were transferred into 40 μm nylon cell strainers (Thermo Fisher Scientific, USA) and MC-SGM was left to drip off using only gravitational force. Wells from which the sarcospheres were taken were washed twice with 5 mL of GM which was then also gently filtered through the cell strainers. Filter unit was washed with extra 10 mL of GM to eliminate the single cells. Cell strainer was transferred to a Petri dish, rotated for 180º, washed with 10 mL of GM and gently shaken to release the sarcospheres. Microscopic observation of the cell strainer was done to confirm that there are no spheres left behind on the filter membrane. Isolated sarcospheres were grown in GM and incubated at 37 ºC and 5% CO_2. Under adherent conditions sarcospheres formed a monolayer of cells that represented the first generation of OS-CSC line.

Whole procedure of establishing and isolating sarcospheres was repeated and following experiments were done on the second generation of OS-CSC line.

3.3. Hoechst 33342 dye exclusion assay

Hoechst 33342 dye exlusion assay was performed on the second generation of OS-CSC lines from two patients, hMSCs and U2OS cell line. Cells were seeded on the 96-well plate at the cell density of 6 x 10^4 cells/well in appropriate culture medium (**Table 2.**). Cells were incubated for 24 hours at 37 ºC and 5% CO_2.

After the cells attached, 5 µg/mL of Hoechst 33342 (Sigma) dye in a total of 100 µL appropriate culture medium was added to each well and incubated for 90 minutes at 37 ºC and 5% CO_2. Control cells were left without treatment. Due to the Hoechst dye light sensitivity, plates were covered with foil during the incubation period. After 90 minutes, supernatant was removed, and the wells were rinsed with 100 µL of appropriate culture medium. The medium was aspirated and replaced with 100 µL of PBS. The absorbances of the samples were measured in a GloMax microplate reader (Promega) (excitation 360 nm, emission 450 nm). Results were analysed using R programming language.

Table 2. Cell types and belonging culture media used in the study.

Cell type	Basic cell culture media	Supplementation
U2OS	10% fetal bovine serum (FBS, Gibco), 1% Penicillin/Streptomycin (Lonza) in Dulbecco′s Modified Eagle′s Medium (DMEM) – high glucose (Sigma)	-
HEK 293	10% fetal bovine serum (FBS, Gibco), 1% Penicillin/Streptomycin (Lonza) in Dulbecco′s Modified Eagle′s Medium (DMEM) – high glucose (Sigma)	-
hMSC	10% fetal bovine serum (FBS, Gibco), 1% Penicillin/Streptomycin (Lonza) in Dulbecco′s Modified Eagle′s Medium (DMEM) – low glucose (Sigma)	0.01% bFGF (Gibco)
Parental osteosarcoma cell line	growth medium (GM, 10% fetal bovine serum (FBS, Gibco), 1% Penicillin/Streptomycin (Lonza) in Coon's modified Ham's F12 medium (Biological Industries))	-
Osteosarcoma stem cell line – 1st generation	stem cell growth medium (SCGM, 10% fetal bovine serum (FBS, Gibco), 1% Penicillin/Streptomycin (Lonza) in Coon's modified Ham's F12 medium (Biological Industries))	0.01% bFGF (Gibco)
Osteosarcoma stem cell line – 2nd generation	stem cell growth medium (SCGM, 10% fetal bovine serum (FBS, Gibco), 1% Penicillin/Streptomycin (Lonza) in Coon's modified Ham's F12 medium (Biological Industries))	0.01% bFGF (Gibco)

3.4. MTT viability assay

MTT viability test was performed on parental OS cell lines and the second generation of OS-CSC lines from three patients, human embryonic kidney 293 cells (HEK 293), hMSC and U2OS cell lines. Cells were seeded on the 96-well plate at the cell density of 6 x 10^4 cells/well in appropriate culture medium (**Table 2.**). Prior to AA (Sigma) treatment, cells were allowed to attach for 24 h (37 ºC and 5% CO_2). AA was added to the wells in concentrations ranging from 5 to 30 mM in triplicates. In wells containing control cells, the medium was

changed without the addition of AA. Cells were incubated for 24 hours at 37 ºC and 5% CO_2. After the incubation period, the medium was removed by aspiration. Cells were washed twice with PBS and 40 µL of 1x MTT solution (Sigma) made up in medium was added to each well. Cells were incubated for 3 hours (37 ºC, 5% CO_2) protected from light. Then, 170 µL of dimethyl sulfoxide (DMSO, Roth) was added to each well and the plate was incubated again for 30 minutes (37 ºC, 5% CO_2). The metabolically active cells reduced the MTT into formazan crystals, which were then dissolved in DMSO. The absorbance of the solution in each well was measured in a GloMax microplate reader (Promega) at 560 nm. Analysis was done using R programming language. Cell viability was calculated as a percentage of untreated control and the half maximal inhibition concentration (IC_{50}) as the concentration by which the 50% of cells are dead. By using a predictive modelling technique built-in R package *drc*, dose-response models were constructed. Independent variable is referred to as the AA concentration, whilst the dependent variable is referred to as response i.e., cell viability.

3.5. Determination of extracellular acidification and glycolytic rate using Seahorse XF^{e}24

3.5.1. Seahorse XF Cell Energy Phenotype Test

Agilent Seahorse XF assays were performed in an Agilent Seahorse 24-well XF Cell Culture Microplate in conjunction with a Sensor Cartridge following the Seahorse XF workflow shown on **Figure 5**. Two days prior to the assay, cells were seeded on the 24-well plate at the cell density of 6 x 10^4 cells/well in 100 µL of appropriate culture medium (**Table 2.**). Only medium was put in the background correction wells. Plate was allowed to rest at the room temperature in the tissue culture hood for 1 hour. After cells have adhered, additional 150 µL of medium was slowly added to each well. Cells were incubated overnight at 37 ºC and 5% CO_2. 24 hours prior to measuring, cells were exposed to 10 mM AA, while control ones were left untreated.

Seahorse XF Cell Energy Phenotype Test Kit was used. Essential part of the XF assay platform is the Sensor Cartridge spotted with sensor material that must be hydrated a day prior to the assay. Each well of the Utility Plate was filled with 1 mL of XF Calibrant. Hydro Booster was placed on top of the Utility Plate. The Sensor Cartridge was lowered through the openings on the Hydro Booster plate, into the Utility Plate submerging the sensors in XF Calibrant.

The whole platform with Sensor Cartridge was placed in a non-CO_2 37 ºC incubator overnight. Hydro Booster was discarded prior to the measuring.

Assay medium was prepared by supplementing Seahorse XF Base Medium with 1 mM pyruvate (Gibco), 2 mM glutamine (Gibco), and 10 mM glucose (Fluka Analytical) and kept at 37 °C until ready to use. Just prior to starting the assay, cells were washed twice in the way that all but 50 µL of culture medium was removed from each well to prevent the cells from drying out. Then, assay medium was added to the volume of 1 mL and lastly, to the final volume of 500 µL. Plate with seeded cells was placed in a 37°C incubator without CO_2 for one hour prior to the assay. During the incubation time, stressor mix (included in the kit) was prepared. Both oligomycin and FCCP were solubilized in the assay medium to the final concentration of 100 µM and then combined in appropriate amounts to create one 10x solution. Stressor mix was loaded into every port A of hydrated sensor cartridge in the volume of 56 µL. The loaded Sensor Cartridge with the Utility Plate was placed into the Seahorse XF^e Analyzer, and the Sensor Cartridge calibration was initiated. After the calibration, the Sensor Cartridge remained inside the XF^e Analyzer while the Utility Plate was ejected, and the Cell Culture Microplate was put instead of it.

Seahorse XF Cell Energy Phenotype assay was run following the manufacturer's instructions. The assay results were analysed using Wave Desktop software. Normalization of XF assay was applied on total cellular protein level measured by bicinchoninic acid (BCA) assay.

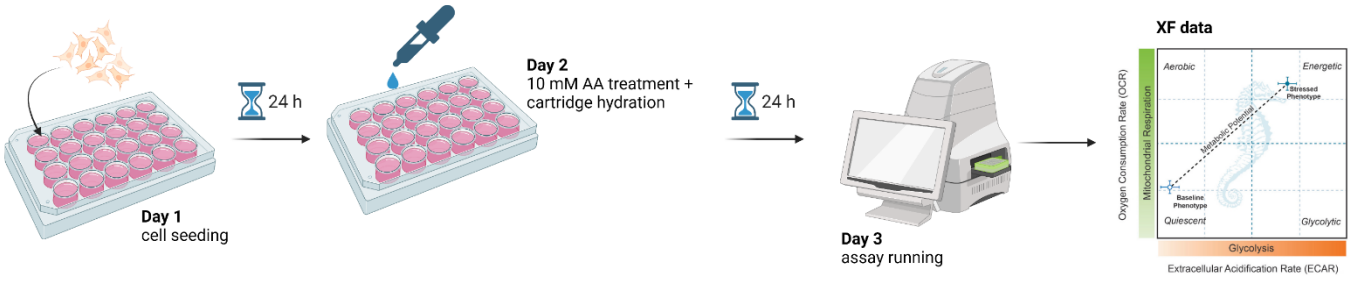

Figure 5. Simplified Agilent Seahorse XF Cell Energy Phenotype Test workflow. On the first day, cells were seeded on the 24-well plate. A day prior to measuring, cells were exposed to 10 mM AA or left untreated. Sensor Cartridge was hydrated. On the third day, Energy Phenotype assay was run using the Seahorse XF technology.

3.5.2. Seahorse data normalisation on total protein concentration

3.5.2.1. Protein extraction

The first step was cell lysis. The medium from each well containing Seahorse sample was transferred to a clean tube and washed with 2 mL of PBS which was transferred as well. 1 mL of trypsin-EDTA solution was added to each well and incubated for 4 min at 37 ºC. Trypsin was neutralized with 1 mL of medium and transferred to the tubes. Wells were once again washed with 2 mL of PBS and transferred to the tubes. The tubes were centrifuged for 10 min at 450 x *g*, 4 ºC. The supernatant was aspirated. In a separate tube, CellLytic™ M (Sigma, USA) reagent was mixed with Protease Inhibitor Cocktail in 100:1 ratio. Volumes were calculated based on the number of samples. 30 µL of CellLytic™ M and Protease Inhibitor Cocktail mix was added to each pellet, resuspended well, transferred to the clean tubes and frozen at -20 ºC.

The freeze-thaw method was used for protein extraction. Cell lysates were defrosted on the ice, transferred to liquid nitrogen and defrosted again. The process was repeated 3 times vortexing the samples each time before freezing. Tubes were centrifuged 15 min at 20 000 x *g* at 4 ºC. The supernatant containing isolated proteins was transferred to the clean tubes.

3.5.2.2. Bicinchoninic acid (BCA) assay for determination of protein concentration

Bicinchoninic acid (BCA) assay (Santa Cruz) was used to quantify the amount of proteins extracted from Seahorse samples. Prior to the assay, reagent A and B were mixed in 50:1 ratio and kept in the dark. Standards and samples were diluted 5 times in miliQ water. 25 µL of diluted samples was added to each well in 96-well plate in duplicates. Then, 200 µL of Reagent AB was added to every well containing a sample. Plate was incubated at 37ºC for 30 minutes, and then 5 minutes at room temperature. Absorbance of all wells was measured in a GloMax microplate reader (Promega) at 562 nm. Measured absorbance is a direct function of protein amount. The unknown concentration of each sample was calculated by comparing its absorbance value to a standard curve.

4. Results

4.1. Assessing stemness of the cells isolated from patient's tumors

OS-CSCs should have several stemness properties that permit the identification of this particular cellular subset in the tumor bulk. One of these properties is the ability to form spheres which is often used as a technique to isolate CSCs from the heterogenous tumor mass. The stem-like phenotype can be further confirmed by the dye exclusion method.

4.1.1. Sarcosphere assay

By culturing OS cells in ultra-low attachment plates and nutritionally deficient serum-free conditions, differentiated OS cells underwent apoptosis, while OS-CSCs survived and continued to proliferate. At the beginning of assay, cells appeared isolated from one another. On day 7, small spherical colonies formed by daughter stem cells were visible. 14 days after the start of the assay, bigger colonies could be observed floating in the medium or slightly settled down into the bottom of the well (**Figure 6.A.**). After 28-30 days of culture, several large (~200 μm in diameter) colonies were present in each well and were ready for the isolation by filtering through the cell strainers (**Figure 6.B.**). After reintroducing adherent conditions, isolated sarcospheres showed adherent expansion following a monolayer formation (**Figure 6.C.**).

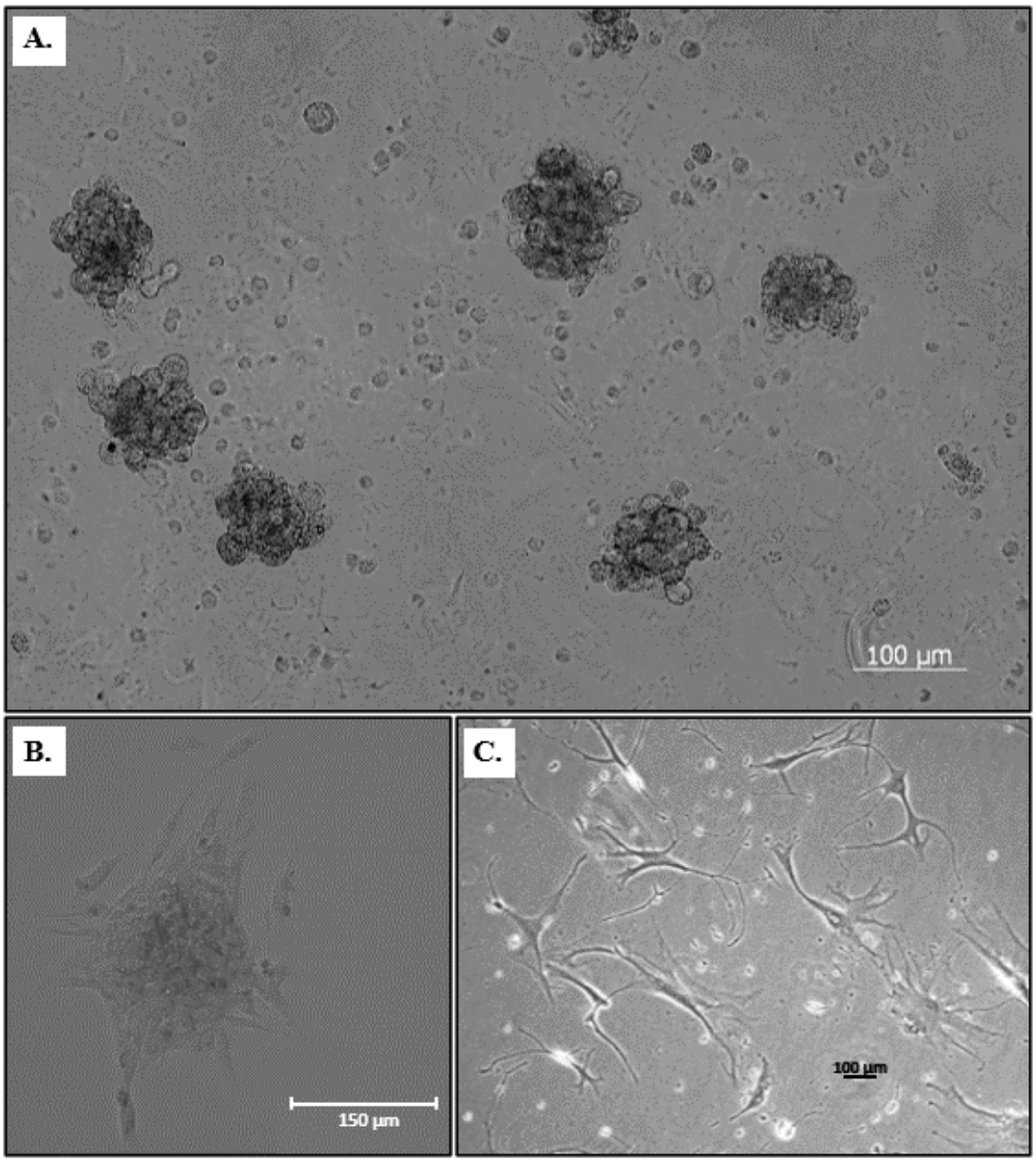

Figure 6. Culturing of osteosarcoma stem sphere-forming cells. Non-adherent conditions supported the growth of osteosarcoma stem cells (OS-CSCs) and the cells proliferated forming sarcospheres (A.). After approx. 30 days, sarcospheres were isolated (B.) and exposed to adherent conditions where they attached and started forming a monolayer (C.).

4.1.2. Hoechst 33342 dye exclusion assay

Staining the cells with Hoechst 33342 dye was done to examine the presence of side population (SP) cells in the following samples: U2OS, hMSCs and isolated OS-CSC lines. SP cells are the ones that share characteristics of CSCs considering the strength of dye efflux they show. Dye retention i.e., indirectly – dye efflux, was quantified by measuring fluorescence of the dye that remained inside of the cells after washing with PBS. Hoechst profile of each sample is represented as a percentage of the fluorescence measured for negative control and is shown on **Figure 7**. The highest fluorescence intensity i.e., the weakest dye efflux was observed in U2OS cells that were used as a negative control in each of the experiments. As expected, hMSCs showed low fluorescence because most of the dye was ejected from the cells. Due to the high expression of ABC transporters, stem cells have the ability to efficiently secret various chemicals, including the Hoechst dye, instead of degrading them in the cytoplasm. Lower fluorescence therefore assumes higher percentage of stem-like SP cells. Low fluorescence was also measured in OS-CSC lines derived from the two patient's tumor, resembling the hMSC pattern.

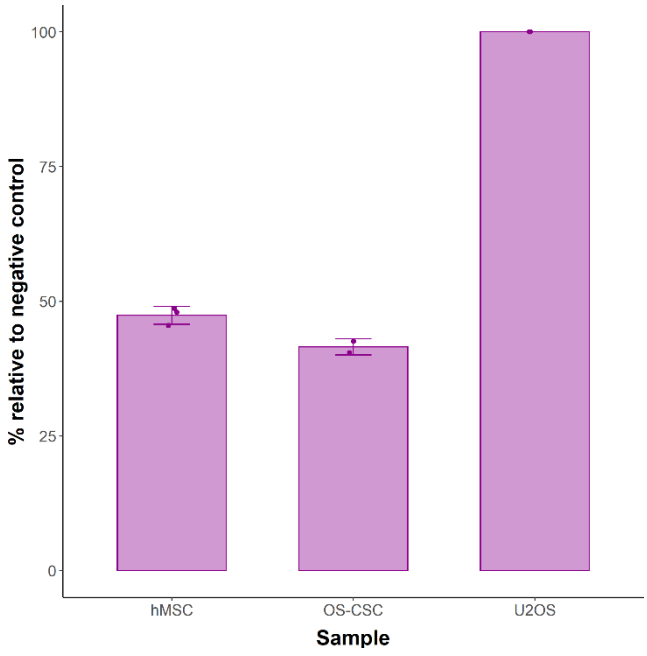

Figure 7. Hoechst profiles generated by dye exclusion assay. Fluorescence intensities of Hoechst-treated human mesenchymal stem cells (hMSC), osteosarcoma cancer stem cells (OS-CSC) and U2OS osteosarcoma cell line shown as a percentage of fluorescence measured for negative control. Results are shown as a mean value from two independent experiments ± standard deviation.

4.2. Ascorbic acid effect on cell viability

MTT assay was used to study AA effect on parental OS cell line and OS-CSC viability, and to evaluate how toxic it is for those cells compared to HEK 293, hMSC and U2OS cell lines. To draw any conclusions about AA cytotoxicity, it was also important to determine the concentration of AA which exhibited 50% cell viability i.e., that is sufficient to kill 50% of the cell population (IC_{50}). Regression analysis was done to investigate a relationship between a dependent (cell viability) and independent variable (AA concentration).

Percentage of live HEK 293, hMSC and U2OS cells relative to AA concentration is shown on **Figure 8.A.**, while the response to different AA concentrations for the same samples is represented by the dose response curves on **Figure 8.B.** Percentage of live HEK 293 cells remained around or above 100% until the AA concentration reached 10 mM. For the next 2 concentrations, cell viability decreased for 10-20%. Dose response curve shows a mild decline between 15 mM and 25 mM AA with the IC_{50} value being around 19 mM AA. For the last 2 concentrations percentage of live cells was approximately 0%. hMSC viability decreased already after 5 mM AA and varied around 50% for the concentrations between 10 mM and 25 mM AA until it dropped to almost 0% when treated with highest concentration. This cell line is the only one that does not show an expected S-shaped dose response curve and instead assumes a sort of negative linear connection between viability and concentration. IC_{50} is estimated around 18 mM. U2OS cells had an almost constant viability of approximately 80% until 20 mM AA which drastically dropped as the concentration increased to 30 mM. IC_{50} can be read out at 25.6 mM – the highest value among 3 control cell types.

All the parental OS cell line and OS-CSC samples derived from 3 different patient's tumors were grouped as parental i.e., CSC, respectively. Percentage of live parental OS cells and CSCs relative to AA concentration is shown on **Figure 9.A.**, while the response to different AA concentrations for the same samples is represented by the dose response curves on **Figure 9.B.** When treated with AA, for parental OS samples cell viability was close or higher than 100% until the concentration of 15 mM. Notable decrease is observed towards the next concentration, while IC_{50} value was reached in between, at 17.1 mM. For OS-CSC samples, percentage of viability was set down below 100% already at 5 mM, but the strongest cytotoxic effect of AA was observed between 10 mM and 20 mM. Half of this cell population would be dead at the concentration of 15.5 mM. From 20 mM to 30 mM almost no live cells were recorded for either of the two groups.

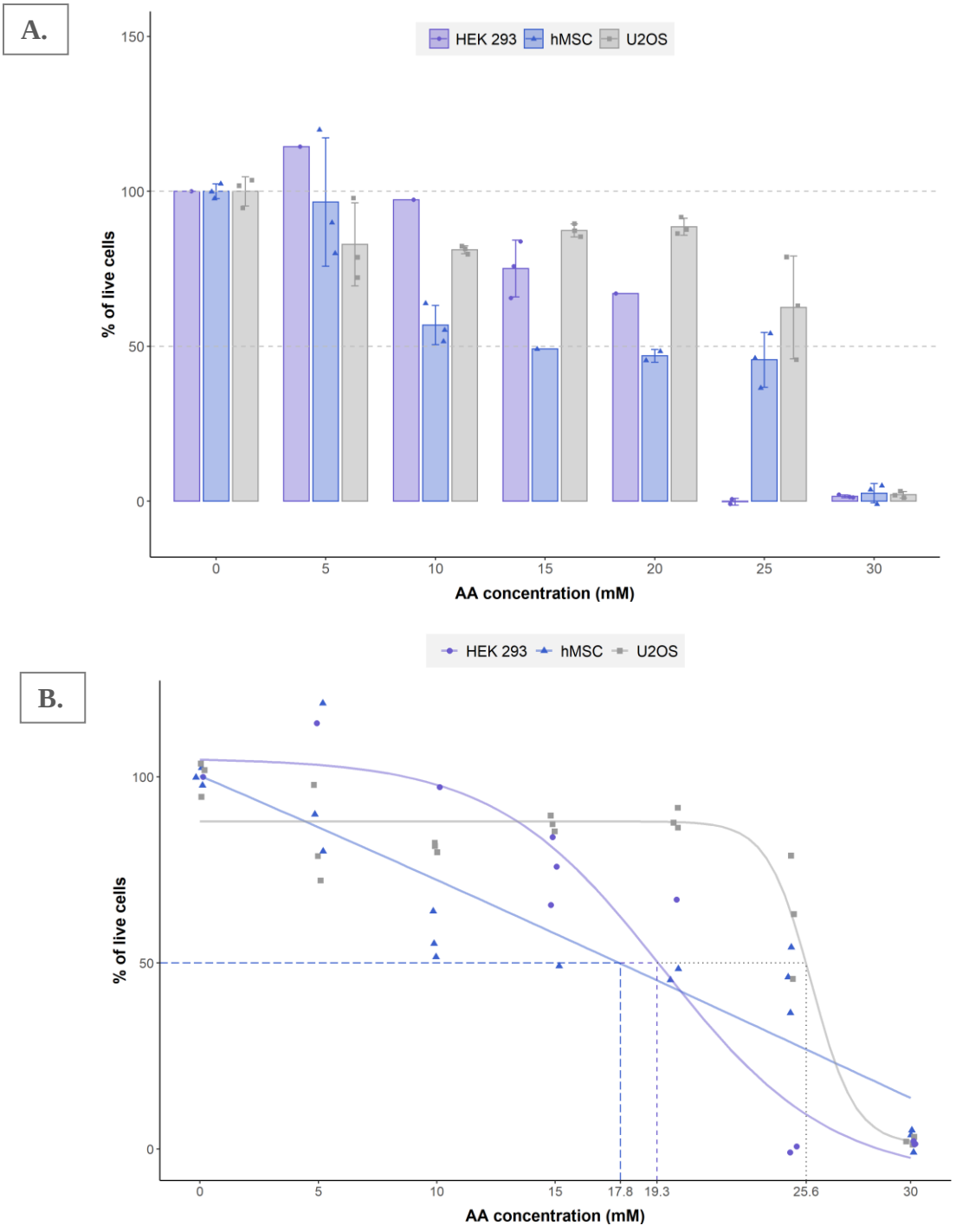

Figure 8. Ascorbic acid effect on HEK 293, hMSC and U2OS viability. Cell viability is expressed as a mean percentage of live cells (± standard deviation) as measured for 7 different ascorbic acid concentrations by MTT assay (A.) and as predicted by regression analysis; IC_{50} value for each of the cell type is indicated (B.). HEK 293 - Human embryonic kidney 293 cells, hMSC – human mesenchymal stem cells, U2OS – osteosarcoma cell line.

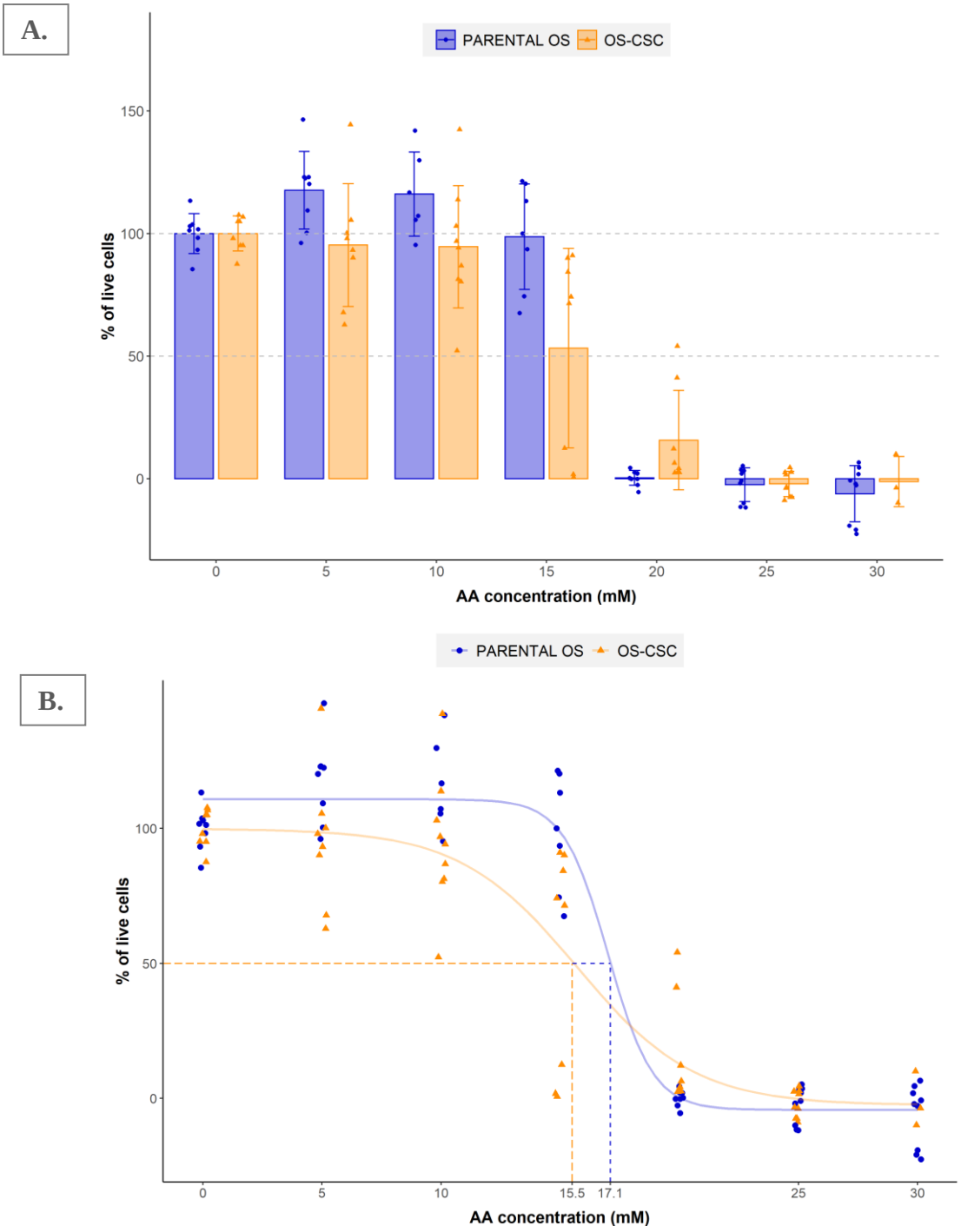

Figure 9. Ascorbic acid effect on parental osteosarcoma and osteosarcoma stem cells viability. Cell viability is expressed as a mean percentage of live cells (± standard deviation) as measured for 7 different ascorbic acid concentrations by MTT assay (A.) and as predicted by regression analysis; IC_{50} value for each of the cell type is indicated (B.). Parental OS – parental osteosarcoma cell line, OS-CSC – osteosarcoma stem cells.

4.3. Ascorbic acid effect on cell metabolism

Phenotype Test was used to study the AA effect on metabolism of living cells in culture. Seahorse XF measures glycolysis (expressed as the extracellular acidification rate (ECAR)) and mitochondrial respiration (expressed as the oxygen consumption rate (OCR)). ECAR value is a readout of media pH reflecting lactic acid and bicarbonate accumulation, while OCR is determined by extracellular oxygen level. ECAR and OCR were measured under baseline and stressed conditions for untreated and cells treated with 10 mM AA for 24 hours. Baseline OCR and ECAR measurements were taken initially, while final values were measured after simultaneously adding stressor mix to each well. By reading out these measurements, three key parameters of cell energy metabolism were revealed: baseline phenotype, stressed phenotype and metabolic potential for treated cells comparing to the cells that have not received AA. Metabolic potential denotes the cell response to an induced energy demand. Wells with at least one negative OCR or ECAR measurement were ejected from the analysis because negative values are not physically possible. Due to the well-to-well variability in the cell number and Seahorse XF analyser sensitivity to small changes in OCR and ECAR, the obtained data was normalized to post-assay protein harvest.

As stated in the manufacturer's user guide, highly aerobic cells are susceptible to CO_2 production when stressed. This background CO_2 effect can contribute to ECAR and therefore over-report the glycolytic contribution to metabolic potential. Cells with a baseline OCR/ECAR ratio < 4 are identified as the cells that produce CO_2 which makes a negligible contribution to ECAR. On the other hand, the stressed ECAR value of cells with a baseline OCR/ECAR ratio > 4 could include both glycolysis and mitochondrial activity.

Generated phenotype maps are shown on **Figure 10.** hMSC and U2OS cell lines were used as the control cells, while parental OS cells and OS-CSCs derived from four different patient's tumors are two experimental groups. In both baseline and stressed conditions, hMSCs exhibited a higher ECAR and lower OCR values for the cells incubated with 10 mM AA compared to untreated. When treated with AA, U2OS cells expressed higher ECAR values in baseline and stressed condition. On the other hand, OCR values for this cell type were elevated for the cells that were not exposed to AA. Parental OS cell line phenotype map displays a decrease in stressed OCR and an increase in stressed ECAR values in treated comparing to the untreated cells. Baseline values were roughly unchanged for treated versus untreated cells. 24-hour incubation of OS-CSCs with 10 mM caused just a slight decrease in baseline values.

In response to mitochondrial stressors, both ECAR and OCR values decreased in group that received AA treatment. As baseline OCR/ECAR ratio is higher than 4, the stressed ECAR for control cells could include both glycolysis and mitochondrial activity.

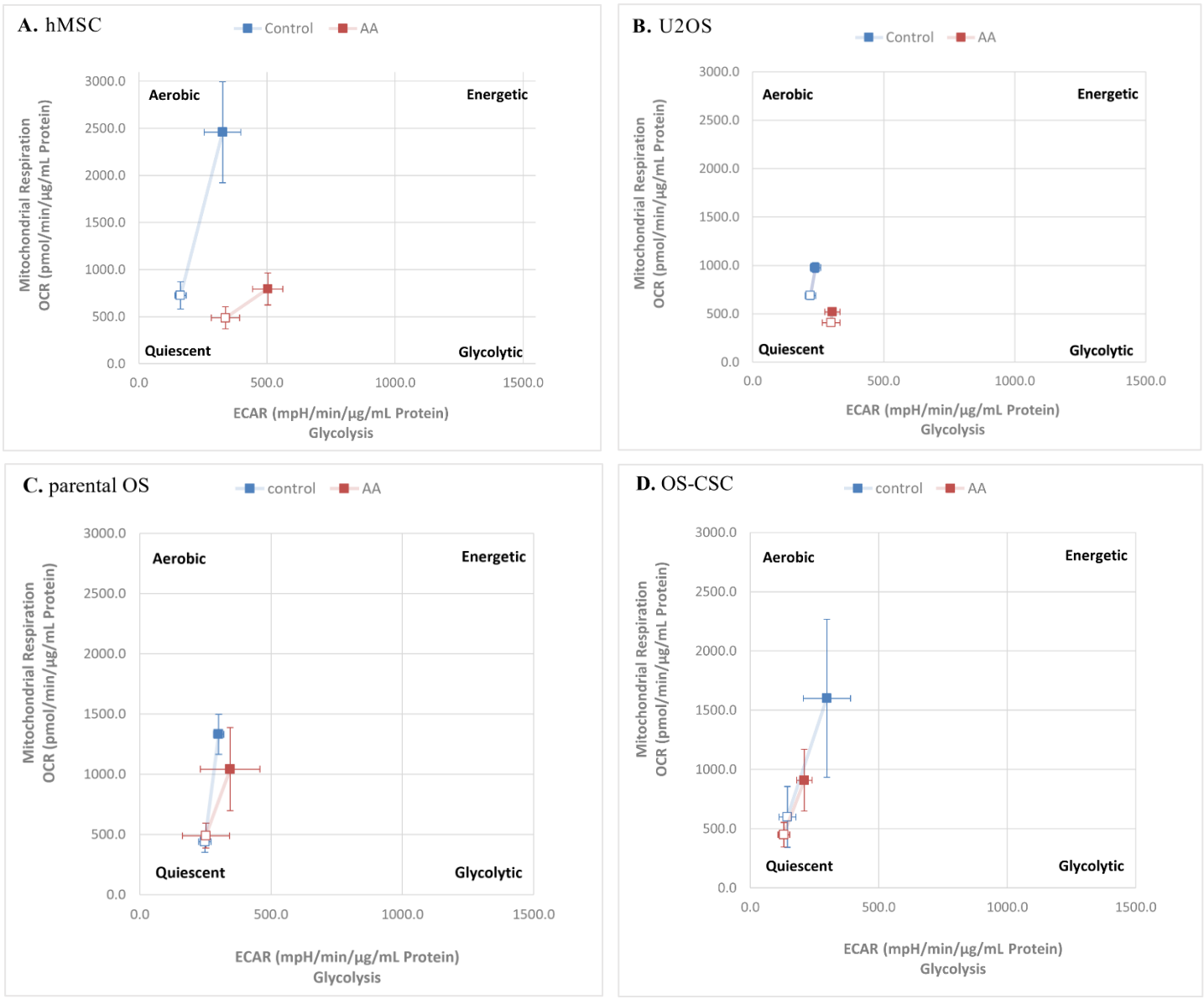

Figure 10. Ascorbic acid effect on energy phenotype of the cells. The phenotype maps generated by Seahorse XF technology display a change in oxygen consumption rate (OCR) i.e., extracellular acidification rate (ECAR) for untreated (blue) and samples treated with 10 mM ascorbic acid for 24 hours (red). OCR and ECAR values are measured in baseline (open symbols) and stressed (closed symbols) conditions for human mesenchymal stem cells (hMSC) (**A.**), U2OS osteosarcoma cell line (**B.**), parental osteosarcoma (OS) cell line (**C.**) and osteosarcoma stem cells (OS-CSC) (**D.**). All results are demonstrated as mean ± standard deviation.

Based on the baseline and stressed phenotype, metabolic potential was calculated for each of the sample group and is shown on **Figure 11**. Metabolic potential has decreased in both of its glycolytic and aerobic metabolic component in treated hMSC cells. Glycolytic potential of U2OS cells treated with AA roughly decreased, but a notable decrease in aerobic metabolic potential was observed. Treated parental OS samples exhibited almost 2 x lower aerobic metabolic potential comparing to the untreated cells. Parental OS samples are the only group of cells in which glycolytic potential appeared not to be decreased when treated with AA. A decrease in both aerobic metabolic and glycolytic potential was observed for treated OS-CSCs relative to the control.

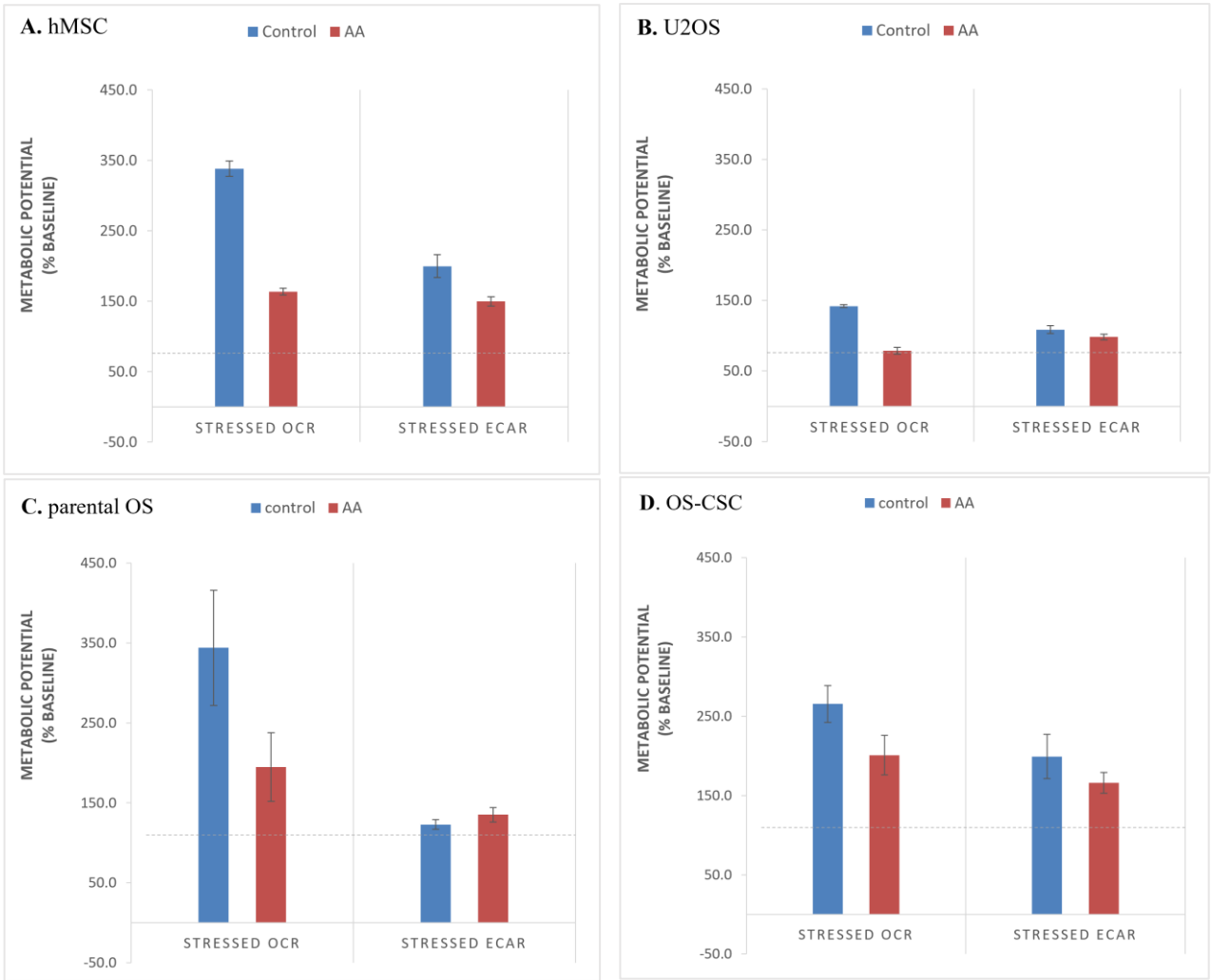

Figure 11. Ascorbic acid effect on metabolic potential of the cell. Metabolic potential is represented as a percentage increase of stressed over baseline oxygen consumption rate (OCR) i.e., extracellular acidification rate (ECAR) for untreated (blue) and samples treated with 10 mM ascorbic acid for 24 hours (red). Data for human mesenchymal stem cells (hMSC) (**A.**), U2OS osteosarcoma cell line (**B.**), parental osteosarcoma (OS) cell line (**C.**) and osteosarcoma stem cells (OS-CSC) (**D.**) is derived from the energy phenotype maps shown on Figure 10. All results are demonstrated as mean ± standard deviation.

OS-CSC group displayed the biggest deviations in measurements (**Figure 10. D.**). Being our main group of interest, we wanted to further dissect its metabolic behaviour in control and treated conditions by observing OS-CSCs from each of the patients separately (**Figure 12.**). Energy phenotypes for OS-CSCs derived from different patients differ from one another. When represented on the same scale, it is evident that patient 13 with the lowest OCR and ECAR values showed the most quiescent phenotype, while cells from patient 10 came of as the most energetic (the highest OCR and ECAR values). Patient 0 showed almost no difference in OCR and ECAR values between treated and untreated group. Patient 7 and patient 10 displayed a decrease in both glycolytic and aerobic components of metabolic potential for treated cells relative to untreated.

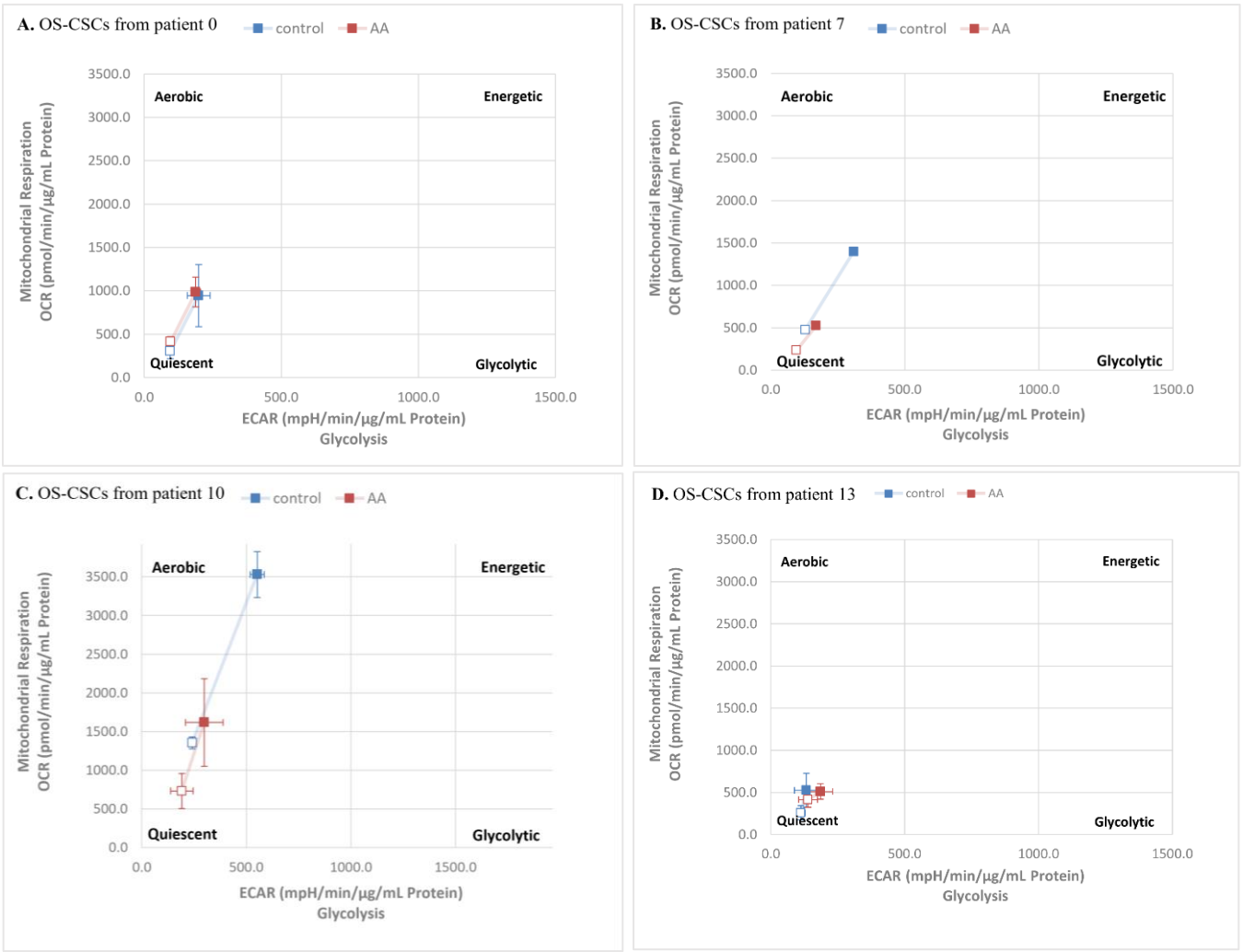

Figure 12. Phenotype maps for osteosarcoma stem cell samples derived from four different patients. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) are measured in baseline (open symbols) and stressed (closed symbols) conditions for untreated (blue) and samples treated with 10 mM ascorbic acid for 24 hours (red) using the Seahorse technology. All results are demonstrated as mean ± standard deviation.

Additionally, general bioenergetic organization of the studied cell lines was examined by comparing their baseline ECAR and OCR values (**Figure 13.**). OCR/ECAR ratio is a qualitative measure of OXPHOS versus glycolysis in terms of energy acquisition. Cell metabolism is more oxidative if the OCR/ECAR ratio is higher i.e., more glycolytic if the ratio is lower. hMSCs exhibited the highest OCR/ECAR ratio, following by the OS-CSCs and U2OS, while the lowest ratio was observed for parental OS cells.

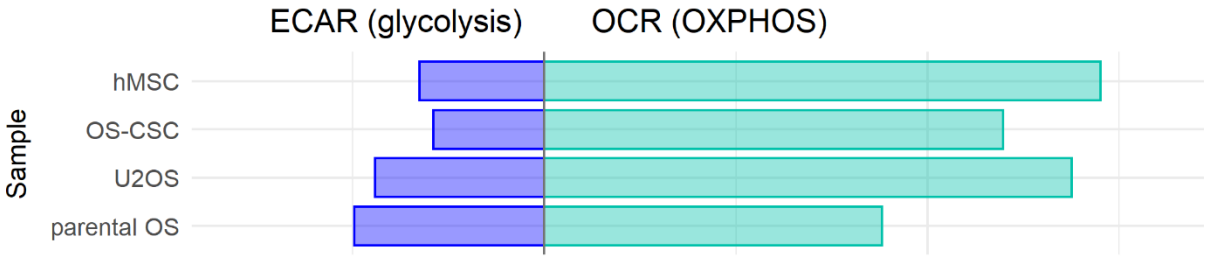

Figure 13. Bioenergetic organization of hMSC, U2OS, parental osteosarcoma cell line and osteosarcoma stem cells. Baseline oxygen consumption rate (OCR) and baseline extracellular acidification rate (ECAR) generated by Seahorse XF technology represent a measure of glycolysis i.e., oxidative phosphorylation (OXPHOS). Following samples are ranked by OCR to ECAR ratio along the y axis: hMSC – human mesenchymal stem cells, OS-CSC – osteosarcoma stem cells, U2OS osteosarcoma cell line and parental OS – parental osteosarcoma cell line.

5. Discussion

Using the sarcosphere culture system, OS-CSCs were isolated from patient's biopsies. Stemness of these cells was confirmed by first- and second-generation growth under nonadherent conditions, and by the Hoechst 33342 dye uptake assay. Compared to HEK 293, hMSC, U2OS and parental OS cells, OS-CSCs exhibited the highest sensitivity to AA as observed by the MTT assay. IC_{50} value of this experimental group was evaluated at 15.5 mM. Seahorse XF technology showed a decrease in metabolic potential of the cell when treated with 10 mM AA.

Cancer stem cells (CSCs) represent a subset of tumor cells that are able to initiate tumor formation and metastatic dissemination, maintain the population of tumorigenic cells by self-renewal, as a class of pluripotent cells – can contribute to tumor heterogeneity and are resistant to multidrug chemotherapy (Abbaszadegan *et al.*, 2017). Listed features can interfere with cancer therapies. Therefore, it is critical to develop novel targeting therapies for a complete tumor eradication. First steps for a better understanding of CSC biology are isolation of this subpopulation from the tumor bulk and establishment of CSC culture. CSCs in OS were first identified by Gibbs *et al.* (2005). They found that ~1 in 100–1000 OS cells could generate a spherical colony and were able of self-renewal. OS-CSC line in this study was generated following Palmini *et al.* protocol (2016). The self-renewal ability of isolated cells was confirmed by second-generation growth of sarcospheres. Due to the reported limitations of sphere assay (Pastrana *et al.*, 2011), additional test was done to identify isolated cells as "stem cells". Overexpression of ABC transporters is the characteristic of healthy stem cells (Zhou *et al.*, 2001; Kim *et al.*, 2002; Scharenberg *et al.*, 2002), but was also confirmed for the CSCs (Chuthapisith *et al.*, 2010; Frank *et al.*, 2011; Eyre *et al.*, 2014). This property was used to establish a dye exclusion assay and identify a small subset of cells designated as a "side population (SP)". SP cells have stem-cell-like characteristics and possess a high ability to exclude fluorescent DNA-binding dye, Hoechst 33342 (Yang *et al.*, 2011). For the purpose of this study, exact dye was used for an incubation with hMSC, U2OS and previously established OS-CSC line. hMSC represent a cell population that is doubtlessly enriched with stem cells and has stem-like properties (Ullah *et al.*, 2015). On the other hand, there is evidence that U2OS cell line consists of no SP cells (Tang *et al.*, 2011). While the dye uptake occurs universally in all cell types, efflux is more restrictive. OS-CSCs resembled a similar hMSC pattern but behaved differently from U2OS.

This observation, together with the successful growth of second sarcosphere generation, can be used to assess the stemness of the cells and confirm that OS-CSCs samples were enriched with CSCs. By using FACS, Yi *et al.* (2015) also identified a small population of SP cells (~2.1%) in human OS samples which were found to exclude the Hoechst 33342 dye and exhibit high levels of self-renewal and invasion.

Finally, after isolation and identification of CSCs, testing effect of presumptive targeting compound can be conducted. Even though there are some indications that ascorbic acid (AA) can induce terminal differentiation (Valenti *et al.*, 2014; Peng *et al.*, 2018), the aim of this research was to study its cytotoxic effect. With this intention, MTT assay was performed. Cytotoxicity of AA was proven for all the examined cell types. Cell type resistance to ascorbic acid was following: U2OS > HEK293 > hMSC > parental OS cell line > OS-CSC. With the lowest IC_{50} value of 15.5 mM, OS-CSCs showed the highest sensitivity to the AA treatment. Even though differences between IC_{50} values for different cell types are not notable, this result indicates a certain selectivity of AA effect. Fernandes *et al.* (2017) observed antiproliferative, antidifferentiation, and apoptotic effects of AA on G292 OS cell line, but by using quite lower concentration of 1 mM. The lowest concentration used in this study was of 5 mM. This concentration showed almost no effect on cell viability either of the parental OS cells or OS-CSCs, while at the same time percentage of live U2OS cells decreased for approximately 20%. Likewise, a higher AA concentration (≥10 mM) was needed to decrease cell viability of breast cancer cells (Lee *et al.*, 2019). As for the selectivity, Hong *et al.* (2013) reported that a selective killing effect of AA in human breast cancer cells is influenced by sodium-dependent vitamin C transporter 2 (SVCT-2), overexpressed in tumor tissues. Beside from observing the same in HCC samples, Lv *et al.* (2018) showed that SVCT-2 was preferentially elevated in liver CSCs. The observation that cancer tissues overexpress GLUT transporters could indicate that DHA uptake is increased as well. Glut-1 protein was actually revealed to be overexpressed in majority of OS tissues examined by Fan *et al.* (2017). Additionally, Doskey *et al.* (2016) also reported a lower catalase activity in tumor versus healthy tissues, which means a lower capacity to metabolize H_2O_2 generated by AA treatment. Upregulation of SVCT-2 and GLUT expression, and catalase downregulation in cancer and CSCs could explain the higher sensitivity of OS-CSCs and parental OS cells but is somewhat contrary to the highest resistance observed for U2OS. Nevertheless, cytotoxicity of high-dose AA is evident, but the regulation of sensitivity to AA-induced cytotoxicity in OS should be further investigated.

After observing that AA negatively affects cancer cell viability, our curiosity moves towards understanding a mechanism of AA-cytotoxicity induction. Allegedly, one of the proposed mechanisms includes reactive oxygen species generation which leads to metabolic stress and eventually, cell death (Blaszczak *et al.*, 2019). To draw any conclusions about if and how AA affects cell metabolism, Seahorse XF Cell Energy Phenotype Test was conducted. Incubation with 10 mM AA did not show a significant selectivity towards any cell type in the terms of metabolism alternation. AA decreased both glycolytic and aerobic metabolic potential of all the cell types with the exemption of a glycolytic potential in parental OS samples. AA is already known to behave as a glycolysis inhibitor. Several "two metabolic hit" studies proved AA inhibitory effect on glycolysis (Bonuccelli *et al.*, 2017; De Francesco *et al.*, 2017, 2019). By far the most investigated mechanism of AA-mediated glycolysis inhibition is linked to GAPDH deactivation and PARP activation. PARP activation leads to further GAPDH inhibition by depleting its cofactor, NAD^+. Certain scenario results in energy crisis and cell death (Ngo *et al.*, 2019). However, there is less evidence of AA affecting mitochondrial respiration. It is suggested that H_2O_2 may directly damage mitochondria and it is known that H_2O_2 can be generated through AA administration (Chen *et al.*, 2007). A recent paper published by Bakalova *et al.* (2020) suggested a possible relationship between AA and disrupted OXPHOS. They represented a "destructive mode" of ascorbyl free radical (AFR) by which it causes an impairment of mitochondrial respiration. AFR is generated in the extracellular fluids by AA, together with H_2O_2. Evidence for glycolysis inhibition and mitochondrial respiration impairment could potentially explain metabolic alternations showed in this study.

We have also observed that CSCs from four patients caused fairly big standard deviations when presented as one and gave rise to different phenotype maps when presented separately. Not only do the CSCs contribute to the heterogeneity of the tumor bulk, but this cellular population is quite heterogenous within itself. Their variability and plasticity makes them even more resistant to the eradication (Hung *et al.*, 2019).

Finally, it appeared that cell lines from our study mostly rely on OXPHOS to generate energy. It is no surprise that healthy stem cells were the most oxidative. As for the OS-CSCs, recent reports suggest that CSCs predominantly metabolize through OXPHOS (Kishi *et al.*, 2019b) which is in accordance to our result, but is contrary to the results obtained by Mizushima *et al.* (2020). However, opposing findings are not rare when exploring CSC metabolism as can be seen by the example of breast (Ciavardelli *et al.*, 2014; Vlashi *et al.*, 2014) and glioma CSCs (Vlashi *et al.*, 2011; Zhou *et al.*, 2011). We observed that U2OS and parental OS cells rely more

on glycolysis comparing to the other cell lines examined but still prefer OXPHOS. Interestingly, this is not the first study to show that cancer cell metabolism does not always strongly exhibit the Warburg effect. Under normoxic conditions, in cervical and breast cancer cell lines OXPHOS still contributes 79% and 91% respectively in overall ATP generation (Rodríguez-Enríquez *et al.*, 2010; Hernández-Reséndiz *et al.*, 2015).

6. Conclusions

Being highly invasive with metastasis and relapse propensity, successful OS treatment remains a challenge. Growing evidence suggests that CSCs play a critical role in these deadly mechanisms of cancer. Even with numerous attempts, there are still no completely successful therapies targeting OS and there are still only few agents suggested for CSC eradication. The general aim of this research was to study if AA treatment can decrease OS-CSC viability and target their metabolism.

Several conclusions can be drawn from the study:

1. Among other cell types, patient's OS tumor bulk consists of CSC population. OS-CSCs can be isolated by sarcosphere culture system and their stemness is confirmed by additional dye exclusion assay.
2. High doses of AA have a negative effect on the cell viability. OS-CSCs show the highest sensitivity to AA treatment.
3. One of the AA mechanism of action can be via affecting the metabolic features of the cell as AA decreases both glycolytic and aerobic potential of the cell.

Nevertheless, we proved a cytotoxic effect of AA and can expect a future use of AA as an agent for supportive treatment of cancer, after being further investigated.

7. References

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Curriculum Vitae

I was born on 27th of October 1996 in Zagreb, Croatia. I have finished my high school education in the Fifth Gymnasium in Zagreb. From 2015 to 2018 I attended the undergraduate program of Molecular biology at the Faculty of Science, University of Zagreb. In 2018 I obtained a title of bacc. mol. biol. (*cum laude*) and enrolled into the Graduate program of Molecular biology at the same university. As a research intern, I took part in the following projects:

- **"Genomic aspects of rapid evolution of Italian wall lizard (*Podarcis sicula*)"**, supervisor: dr. sc. Anamaria Štambuk, Division of Zoology, Department of Biology, Faculty of Science, University of Zagreb (June 2018-June 2019).

- **"Establishing a Synthetic Locus to analyze whether Antisense Transcription can generate Bistable Transcription States"**, supervisor: dr. sc. Edda Schulz, Max Planck Institute for Molecular Genetics, Berlin (August 2020-January 2021)

- **"Selective targeting of osteosarcoma cells using ascorbic acid"**, supervisor: dr. sc. Inga Urlić, Division of Molecular Biology, Department of Biology, Faculty of Science, University of Zagreb (January 2019-June 2021)

In the academic year of 2016/2017, I was a laboratory demonstrator for an undergraduate class in Zoology. From 2016 to 2019 I participated in the workshop organization for the manifestation "Night of biology". In 2019 I was an active participant at the European student's bio congress YOUNG BM held in Madrid with the presentation on Cancer Cell Metabolism.

During my studies I have gained some valuable recognitions. In 2020 I won the Erasmus+ traineeship scholarship. I was a two-time winner of national STEM scholarship (2017, 2018) and a three-time winner of city of Zagreb scholarship for excellency (2014, 2019, 2020).