

Metaboličke promjene u matičnim stanicama sarkoma uzrokovane dehidro-L-askorbinskom kiselinom

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Sveučilište u Zagrebu
Prirodoslovno-matematički fakultet
Biološki odsjek

Zara Škibola

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stanicama sarkoma uzrokovane dehidro-L-
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Diplomski rad

Zagreb, 2023.

University of Zagreb
Faculty of Science
Department of Biology

Zara Škibola

**Metabolic changes in sarcoma stem cells
induced by dehydro-L-ascorbic acid**

Master thesis

Zagreb, 2023

Ovaj rad je izrađen u Laboratoriju zavoda za molekularnu biologiju Prirodoslovno-matematičkog fakulteta u Zagrebu, pod mentorstvom izv. prof. dr. sc. Inge Urlić te komentorstvom Maje Ledinski, mag. biol. mol. Rad je predan na ocjenu Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu radi stjecanja zvanja magistra molekularne biologije.

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Sveučilište u Zagrebu
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Metaboličke promjene u matičnim stanicama sarkoma uzrokovane dehidro-L-askorbinskom kiselinom

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Matične stanice tumora posebna su grupa tumorskih stanica sa sposobnošću samoobnavljanja i smatra se da su ključne za inicijaciju i rast tumora. Često su otporne na konvencionalne terapije što predstavlja veliki problem u liječenju. Smatra se da vitamin C ima različite antitumorske mehanizme koji se razlikuju ovisno o vrsti tumora. Vitamin C osim što narušava redoks stanje u stanicama i uzrokuje oksidacijski stres također mijenja i metabolički profil stanice. U ovom radu se istraživao učinak oksidiranog oblika vitamina C, dehidroaskorbinske kiseline (DHA), na matične stanice sarkoma. Milimolarne koncentracije DHA u mediju sa i bez bazičnog FGF-a smanjile su vijabilnost svih tretiranih staničnih linija. Također je pokazano kako tretmani s DHA mijenjaju ekspresiju gena važnih za glikolizu i oksidativnu fosforilaciju. Ekspresija gliceraldehid-3-fosfat dehidrogenaze je gledana i na razini proteina gdje je također zabilježeno da dolazi do promjena. Pošto su sarkomi vrlo heterogena skupina tumora, tri vrste matičnih stanica pokazale su različite odgovore na tretman DHA. Općenito, matične stanice hondrosarkoma i osteosarkoma su pokazale bolji odgovor od stanica rbdomiosarkoma. DHA je pokazala potencijal kao modulator metabolizma matičnih stanica sarkoma, ali za daljnji razvoj kao antitumorske terapije svakako treba uzeti u obzir specifičnu pozadinu tumora.

Ključne riječi: metabolizam sarkoma, GAPDH, PGC-1 α , vitamin C
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Master thesis

Metabolic changes in sarcoma stem cells induced by dehydro-L-ascorbic acid

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Cancer stem cells are a subpopulation of tumor cells with the ability of self-renewal and are thought to drive tumor initiation and growth. Cancer stem cells are resistant to conventional therapy which poses major problem in treatment. Vitamin C is thought to have different anti-tumor mechanisms that differ depending on the type of tumor. Apart from disrupting the redox state in cells and causing oxidative stress, vitamin C also changes the metabolic profile of the cell. In this work, the effect of the oxidized form of vitamin C, dehydroascorbic acid (DHA), on three sarcoma stem cell types was investigated. Millimolar concentrations of DHA in medium with and without basic FGF decreased the viability of all treated cell lines. This work also demonstrated that treatments with DHA alter the expression of genes important for glycolysis and oxidative phosphorylation. Effects of DHA treatment on change of glyceraldehyde-3-phosphate dehydrogenase protein expression were also shown. However, sarcomas are a very heterogeneous group of tumors, so three types of stem cells showed different responses to DHA treatment. General response to treatment was more positive in chondrosarcoma and osteosarcoma stem cells than in rhabdomyosarcoma stem cells. DHA has shown potential as a modulator of sarcoma stem cell metabolism, but for further development as an anti-tumor therapy, the specific background of the tumor must be considered.

Keywords: sarcoma metabolism, GAPDH, PGC-1 α , vitamin C
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ABBREVIATIONS LIST

2DG- 2-deoxy-D-glucose

2-HG- 2-hydroxyglutarate

3 AB-OS- human osteosarcoma cell line

AA- L-ascorbic acid

ABC- ATP-binding cassette

aKG- α -ketoglutarate

ALDH- aldehyde dehydrogenase

AMPK- AMP-activated kinase

APS- ammonium persulfate

ARMS- alveolar rhabdomyosarcoma

ATP- adenosine triphosphate

BRAF- v-raf murine sarcoma viral oncogene homolog B

BCA- bicinchoninic acid assay

bFGF- basic fibroblast growth factor

BSA- bovine serum albumin

CHS- chondrosarcoma

CSC- cancer stem cells

DHA- dehydro-L-ascorbic acid

DMEM- Dulbecco's modified Eagle's medium

DMSO- dimethyl sulfoxide

DNMT- DNA methyltransferase

ECM- extracellular matrix

EDTA- ethylenediaminetetraacetic acid

ERMS- embryonal rhabdomyosarcoma

ESC- embryonic stem cells

FBP2- fructose-1-6-biphosphatase 2

FGF- fibroblast growth factor

FOXO1- forkhead box protein O1

GAPDH- glyceraldehyde 3-phosphate dehydrogenase

GLUT- glucose transporter

GSH- glutathione

H₂O₂- hydrogen peroxide

HIF- hypoxia-inducible factor

HT29- human colorectal adenocarcinoma cell line

IDH- isocitrate dehydrogenase

IHH- Indian hedgehog

IVA- ifosfamide, vincristine, actinomycin D

KRAS- Kirsten rat sarcoma viral oncogene

LFS- Li-Fraumeni syndrome

MCF7- human breast cancer cell line

MG63- human osteosarcoma cell line

MSC- mesenchymal stem cells

MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAD- nicotinamide adenine dinucleotide

NADPH- nicotinamide adenine dinucleotide phosphate

NF1- neurofibromatosis type 1

NMR- nuclear magnetic resonance

OS- osteosarcoma

OXPHOS- oxidative phosphorylation

PARP- poly (ADP-ribose) polymerase

PAX3- paired box protein 3

PAX7- paired box protein 7

PBS- phosphate-buffered saline

PD-1- programmed cell death protein

PDAC- pancreatic ductal adenocarcinoma

PD-L1- programmed death ligand 1

PGC-1 α - peroxisome proliferator-activated receptor-gamma coactivator

PPP- pentose phosphate pathway

PTHrP- parathyroid hormone related protein

RMS- rhabdomyosarcoma

ROS- reactive oxygen species

RT-qPCR- reverse transcription quantitative polymerase chain reaction

SDS- sodium dodecyl sulfate

STS- soft-tissue sarcoma

SVCT1/2- sodium-dependent vitamin C cotransporter type 1/2

TEMED- tetramethylethylenediamine

TET- ten-eleven translocation

TGF- transforming growth factor

TIC- tumor-initiating cells

VAC- vincristine, actinomycin D, cyclophosphamide

VEGFR- vascular endothelial growth factor receptor

1. INTRODUCTION

1.1.Sarcomas

Sarcomas constitute extremely diverse group of mesenchymal neoplasms usually affecting young patients. The main categorization of sarcomas is into two groups: soft tissue sarcomas and bone sarcomas. The most important prognostic factors are the grade, size and location of the primary tumor. When starting therapy treatment, a biopsy is performed first and after the diagnosis, surgery usually follows, if possible. The course of further therapy depends on the type of sarcoma. Since the incidence of sarcomas is relatively rare, and it is highly heterogenous tumor group, further research is needed in this field (Ferrari et al., 2016).

1.1.1. Osteosarcoma

Osteosarcoma (OS) is the most common, nonhematopoietic, primary malignancy of bone. Its estimated annual incidence rate is 4-5 cases per million. Despite its rarity, OS is the most prevalent primary malignancy in adolescents after leukemia and lymphoma and also the most prevalent primary cancer of the bone in children. The incidence of OS has a bimodal distribution across ages. About 60% of the patients are under age of 25, disease usually occurs in second decade of life during puberty. The second peak of disease occurrence is observed in patients over 60 years of age. Osteosarcoma most commonly arises in the metaphysis of the long bones near the growth plates, with two-thirds of tumors emerging from lower extremity long bones. Although long bones of the extremities remain to be the most prevalent site of OS, the relative incidence of OS in non-long bone increases after 60 years of age. Craniofacial and axial tumors account for 40% of all cases after 60 years of age and less than 12% before the age of 25 (Mirabello et al., 2009). This tumor has a highly heterogeneous genetic profile, but advances in defining patient subgroups were made by investigating its biology and revealing genetic aberrations. These developments were made feasible by the emergence of thoroughly annotated tissue banks along with the advancement and broader availability of technologies for detailed molecular profiling (Gill & Gorlick, 2021).

1.1.1.1. Genetics and microenvironment of OS

Many of the most common genetic alterations detected in osteosarcomas are sporadic, somatic, loss-of-function mutations that happen in key tumor suppressor genes. In addition to somatic mutations, some familial syndromes are also known to predispose to OS development and they are commonly used to discover genetic drivers of OS by comparing them with mutations found in sporadic OS (Durfee et al., 2016). Li- Fraumeni syndrome (LFS), caused by a germline mutation in *TP53* gene is the syndrome that most frequently predisposes children to sarcomas. *TP53* encodes for p53, a master transcription

factor and about third of people with LFS develop OS. Therefore, it is no surprise that loss of tumor suppressor function of p53 also happens in sporadic cases of OS (Durfee et al., 2016). Another tumor suppressor gene, whose germline mutation causes hereditary retinoblastoma, is *RB* which codes for pRB and is often altered in sporadic cases of OS. It has been shown that loss of RecQ helicases, that unwind DNA prior to replication, also represents an increased risk of developing OS. Germline mutations in RecQ family of genes cause recessive autosomal cancer predisposition syndromes, such as Bloom's syndrome and Werner's syndrome, which are associated with increased frequency of OS development (Mohaghegh & Hickson, 2001). OS is known as very heterogenous tumor at the intra-tumoral level as well as between patients. Two very recognizable mutational processes generate heterogenous intra-tumoral profile of OS: complex chromosomal rearrangement also known as chromothripsis and localized hypermutated regions, named kataegis (Gianferante et al., 2017). Additional layer of complexity in all tumors, aside from the genetic alterations are the changes in signaling pathways that promote a specific tumor microenvironment, which has a positive effect on further tumor propagation.

Osteosarcomas grow in very complex and dynamic bone microenvironment, made up of bone cells (osteoclasts, osteoblasts, osteocytes), stromal cells (mesenchymal stem cells, fibroblasts), vascular cells (endothelial cells and pericytes), immune cells (macrophages, lymphocytes) and a mineralized extracellular matrix (ECM). A lot of growth factors are released from the degraded bone matrix and they establish a vicious cycle of bone remodeling process (Lamoureux et al., 2007). Mesenchymal stem cells (MSC) are a part of bone marrow niche and they can differentiate into osteoblasts under the control of specific transcription factors. Growth factors like transforming growth factor (TGFs), fibroblast growth factors (FGFs), or members of the wingless-type MMTV integration site family must first engage a sophisticated signal transduction cascade in order to activate those transcription factors (Corre et al., 2020). Most of these cytokines or growth factors are connected to the development of OS. TGF-1 is mostly linked to the formation of OS during the growth of primary tumors or the metastatic progression (Verrecchia & R dini, 2018). FGFs are crucial regulators of skeletal development (Ornitz & Marie, 2019). For example, FGF18 is required for the maturation of osteoblasts and FGF2 (also known as basic FGF) is essential for the growth and development of pre-osteoblasts. FGF receptors are receptor tyrosine kinases and because of the importance of FGF signaling in bone microenvironment, they could be used as a therapeutic target in OS patients (Zhou et al., 2016).

1.1.1.2. Therapy for OS

Prior to the advent of chemotherapy, OS was almost a universally fatal disease. In the 1970s, Jaffe published the first significant success of chemotherapy, showing that methotrexate was a useful agent to manage metastases in advanced disease (Jaffe, 1972). The current regimen of methotrexate, adriamycin, and cisplatin (MAP) has become standard in North America and Europe. Today, most OS patients receive neoadjuvant chemotherapy, followed by surgical resection of all detectable disease and

a regimen of adjuvant chemotherapy postoperatively. With these therapeutic regimens, the 5 year survival has reached 78% for children and young adults with localized disease, but still remains at only 20% in patients with metastasis at diagnosis or in relapse (Kansara et al., 2014). Moreover, in the last 40 years, survival has not notably improved for patients without metastases and has not improved at all for metastatic patients. Therefore, improving therapy for OS remains a constant and major goal for many worldwide research and clinical groups.

The Multi-Kinase Inhibitors (MKIs) are seen as new promising therapies in OS treatment. These molecules exert an anti-tumor activity by simultaneously targeting several kinases. Initially developed as potent vascular endothelial growth factor receptor (VEGFR) signaling inhibitors, it is now accepted that their anti-tumor action affects the inhibition of angiogenic but also non-angiogenic pathways. Targeting the immune system by the macrophage-activating agent mifamurtide resulted in the most recent improvement of OS therapy since polychemotherapy. However, stimulating the immune system by using antibodies directed against programmed cell death protein 1/programmed death-ligand 1 (PD-1/PD-L1) in OS is still debated (Gill & Gorlick, 2021).

1.1.2. Chondrosarcoma

Chondrosarcoma (CHS) is a rare malignant cartilage-forming tumor. The annual estimated incidence of CHS is one in 200,000. CHS is a heterogenous group of tumors that can be divided into central tumors when they develop in the medullary canal or peripheral tumors when they do so in the cartilage cap of an exostosis. The majority of CHS are conventional type and most of them arise in the medullary cavity of long bones. Along with conventional there are various other forms of CHS, such as dedifferentiated, mesenchymal, or clear cell subtypes, which display differential genetic and clinicopathologic characteristics. About 85% of CHS are conventional and it usually affects older people. Incidence peaks during the fifth and seventh decades of life (Ro et al., 2011). Any bone can be affected by CHS, and both axial and appendicular involvement occur often. The pelvic bones, particularly the ilium, are frequently affected. Long tubular bones typically experience damage. The proximal femur is the preferred position, with the proximal humerus, distal femur, and ribs following closely after (Murphey et al., 2003). A proper diagnosis of CHS may be challenging based only on morphologic features, so it is important to search for new markers of cell differentiation, proliferation, and signaling which may provide crucial therapeutic and prognostic information.

1.1.2.1. Genetics of CHS

The hedgehog signaling pathway is crucial in controlling chondrocyte proliferation, terminal differentiation, and endochondral bone formation. Hedgehog pathway factors are abnormally highly expressed in high-grade central chondrosarcomas. A negative regulatory feedback loop exists with

Indian hedgehog (IHH) and parathyroid hormone related protein (PTHrP) which is secreted by MSC. IHH inhibits chondrocyte differentiation and increases expression of PTHrP, which then inhibits the expression of IHH and regulates chondrocyte proliferation. PTHrP pathway induction has been linked to the development of traditional CHS (Tiet et al., 2006). Another pathway implicated in pathogenesis and progression of conventional CHS is the reactivation of antiapoptotic protein Bcl2 (Rozeman et al., 2005).

Isocitrate dehydrogenase (IDH) is an enzyme in the Krebs cycle that catalyzes oxidative decarboxylation of isocitrate to create α -ketoglutarate (aKG). There are three regulatory enzymes in it (IDH1, IDH2 and IDH3). Dedifferentiated chondrosarcomas (57%) and conventional central chondrosarcomas (50%) have both been linked to mutations in the *IDH1* and *IDH2* genes. *IDH1* or *IDH2* mutations are considered early events. They result in a decreased capacity for aKG production and an increased capacity for aKG conversion to delta-2-hydroxyglutarate (2-HG), which is thought to be an oncometabolite (Speetjens et al., 2016).

The role of p53 in CHS pathobiology is still unclear but the overexpression of the p53 protein, 17p13 changes, and the prevalence of *TP53* mutations in nearly all high-grade CHSs imply that the p53 mutation is a late event implicated in CHS progression (Bové et al., 2010). The histological grade of the tumor and the presence of metastases were both significantly correlated with overexpression or mutation of the *TP53* gene (Oshiro et al., 1998).

1.1.2.2. Therapy for CHS

The cornerstone of the treatment for chondrosarcoma is surgery. Since chondrosarcomas are typically radiotherapy- and chemotherapy-resistant, there are currently few treatment options available for patients with unresectable or metastatic disease. Conventional chemotherapy may only be beneficial for patients with advanced mesenchymal and dedifferentiated chondrosarcoma (Italiano et al., 2013). Thanks to advances in understanding of the molecular mechanisms underlying the various chondrosarcoma subtypes it is becoming possible to provide novel systemic treatment options for chondrosarcoma patients (Samuel et al., 2014). In vitro CHS cell migration and proliferation are inhibited using hedgehog inhibitors, and tumor growth in CHS xenografts is also reduced (Xiang et al., 2014). ABT-737, a BH3 mimetic that antagonizes anti-apoptotic proteins such as Bcl-2, Bcl-xl, and Bcl-w, has been shown to sensitize CHS cells to conventional chemotherapy, suggesting that it may one day be used to treat CHS patients (van Oosterwijk et al., 2012). Because *IDH1/2* mutations (mtIDH1/2) are early events in oncogenesis and are consequently present in a significant number of CHS cells, including the CSC, they are appealing therapeutic targets. In glial, cartilaginous, biliary, and myeloid tissues, these mutations cause a block in the differentiation of cells at an early stage of maturation, and mtIDH1/2 inhibitors potently reverse the metabolic effects of mtIDH1/2 and release differentiation of mtIDH1/2 cells into more mature cells. Researchers anticipate that mtIDH1/2 inhibitors could be used most

effectively in combination with cytotoxic agents because the mechanism of action is distinct from and/or complementary to that of these drugs. This will allow for the most effective CSC subgroup eradication and, as a result, long-term remissions for IDH1/2mt cancer patients (Molenaar & Wilmink, 2022).

1.1.3. Rhabdomyosarcoma

The most prevalent soft-tissue sarcoma (STS) in children, rhabdomyosarcoma (RMS), affects 4.6/million children under the age of 15 in the United States. RMS is a high-grade, malignant tumor in which cancer cells show a predilection for myogenic differentiation. It affects almost half of children with soft tissue sarcoma. There are two main RMS subtypes: embryonal RMS (ERMS) and alveolar RMS (ARMS), which are caused by essentially distinct processes. Rhabdomyosarcoma's most prevalent subtype, ERMS, affects 3.0/million U.S. children under the age of 15. The majority (46%) of embryonal rhabdomyosarcomas affect children under the age of five, and most of the cases affect children under the age of 10. Although ERMS contains cells that are histologically identical to developing striated muscle, less than 9% arise within the skeletal musculature of the extremities, it is most frequently found in the head and neck, genitourinary tract, and the retroperitoneum (Gurney et al., 1996). ARMS is a primitive malignant round cell neoplasm that cytologically resembles lymphoma. It displays partial skeletal muscle differentiation. ARMS most commonly occurs in adolescents and young adults. They occur less frequently than ERMS. ARMS commonly arise in the trunk and extremities. The prognosis of ERMS is generally favorable. In contrast, ARMS has a poorer prognosis. It is postulated that these clinical and pathologic variations between ARMS and ERMS are caused by different genetic changes in myogenic precursors and different biological mechanisms of carcinogenesis which then poses distinct clinical challenges (Skapek et al., 2019).

1.1.3.1. Genetics of RMS

ARMS and ERMS have emerged as the two major RMS subtypes based on light microscopic features of cells distributed around an open central space (ARMS) or cells resembling immature skeletal myoblasts (ERMS). The pathogenesis of those two subtypes is distinct, as ARMS tumor cells usually contain a balanced chromosomal translocation generating an oncogenic 'fusion protein' that is absent in ERMS. That balanced chromosomal translocation involves chromosomes 2 or 1 and chromosome 13 (referred to as t(2;13) and t(1;13)), originally detected by cytogenetics. Fusion gives rise to novel fusion proteins involving *PAX3* (encoding paired box protein 3) or *PAX7* (encoding paired box protein 7) and *FOXO1* (encoding forkhead box protein O1). Numerous reports highlight that children with certain genetic disorders develop RMS more frequently than unaffected peers. Syndromes that are most commonly seen in children with ERMS include Li–Fraumeni syndrome (germline mutation of *TP53*)

and neurofibromatosis type I (deletions in the *NF1* gene). Cancer predisposition syndromes appear to be more frequent in patients with ERMS than in those with ARMS. Notably, although all subtypes of RMS resemble skeletal myoblasts, the cell of origin is not well characterized. It is probable that RMS driven by different oncogenic changes at different anatomical sites may originate in different types of cells that are programmed during tumor formation (Skapek et al., 2019).

1.1.3.2. Therapy for RMS

Prior to treating RMS, the gross original tumor must be removed. This is frequently done by combining surgery and/or external beam ionizing radiation. Systemic chemotherapy has also been used as a part of curative therapy to get rid of the disseminated disease that most children with RMS are thought to have. Observations from the 1960s and 1970s, that show common regional and distant recurrence in 8 of 15 children treated without chemotherapy (Heyn et al., 1974), have contributed to the idea that most, if not all, patients have disseminated disease. In addition, RMS cells in bone marrow or peripheral blood in 12 of 16 RMS patients were found with sensitive molecular biology methods like RT-PCR, including those with localized disease (Gallego et al., 2006). The ability to categorize risk groups based on clinical, pathological, and increasingly molecular markers is a significant development in RMS management. The development of risk classification has made customized therapy possible. A foundation of intense, multi-agent chemotherapy based on alkylating agents continues to serve as the basis for systemic treatment for RMS. The conventional backbone therapy for RMS in North America is vincristine, actinomycin D, and cyclophosphamide (also known as VAC), but the norm in Europe is ifosfamide, vincristine, and actinomycin D (known as IVA). Future research should also focus on molecularly targeted treatments, which could significantly enhance outcomes, particularly for young patients with metastatic or recurrent cancer.

1.2. Cancer stem cells (CSC)

Uncontrolled proliferation of malignant cells with distinct morphologies and functions is a hallmark of cancer. Two models have been suggested to account for the cellular diversity found within tumors. The traditional, stochastic explanation for the onset and progression of cancer involves the gradual accumulation of mutations. On the other hand, hierarchical model, based on cancer stem cell hypothesis, states that the disease's onset is caused by a small population of stem cells. The tumor's hierarchical structure has a faint resemblance to that found in many normal tissues. At the top of this hierarchy are the populations of cancer stem cells (CSC), sometimes called tumor-initiating cells (TIC), which are able to self-renew, as well as produce all cell types of a particular cancer sample (Hermann et al., 2007). They are also responsible for tumor progression, metastasis, and chemotherapy-resistance (Kreso & Dick, 2014). CSC can result from several different processes, including cell fusion, mutations in stem

cells, from somatic differentiated cells with high rates of cell turnover caused by genomic instability, or changes in the microenvironment, and metabolic reprogramming. Drug resistance has been linked to CSC through several processes, including the activation of dormant or quiescent cells, an increase in drug efflux pumps and drug detoxification, an efficient activation of DNA damage repair, and resistance to apoptosis (Atashzar et al., 2020). Eliminating CSC might result in a patient's complete recovery because they are thought to be the origin of cancer cells, are in charge of metastatic spread and are also responsible for relapse after conventional therapy, which exclusively targets differentiated cancer cells and fails to eradicate CSC (Pattabiraman & Weinberg, 2014). Thus, one of the most significant difficulties facing current cancer research is the specific removal of CSC.

1.2.1. Methods to isolate CSC

The standard culture condition for CSC maintenance in vitro is growth in DMEM F12 medium supplemented with growth factors such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). EGF signaling is essential for maintaining stemness in glioma stem cells (Soeda et al., 2008) and bFGF is recognized as key mitogen for neural stem cells (Kitchens et al., 1994). Several techniques have been created to recognize and isolate CSC. Functional in vitro tests are routinely used to enrich for CSC. More descriptive assays and in vivo verification are frequently performed after this; however, the order and choice of the tests may vary. Often the first step in enriching for CSC-like cell populations is the functional in vitro test of tumor sphere formation in non-adherent and serum-free conditions. Serial transplantation of isolated putative CSC into immunocompromised mice to measure tumorigenic potential at low cell counts is the accepted technique for validating CSC candidates in vivo. The descriptive assays, such as the measurement of stem-like factor gene expression levels and identification based on cell surface markers, are usual addition to functional tests. Side population analysis (dye exclusion assay) and measuring aldehyde dehydrogenase activity (ALDH) are widely used to enrich for CSC. Undoubtedly, the most desirable and sought-after way to identify CSC is through cell surface markers. It would be much easier to isolate and treat CSC if they had a distinct cell surface marker that was only present on CSC and not on normal cells, including stem cells. Importantly, each strategy must be carefully considered because it is impossible to assume that any of these experimental settings will result in a pure CSC population or the detection of all CSC sub-populations (Brown et al., 2017).

1.2.2. CSC in osteosarcoma

CSC can evade therapy and it has been hypothesized that this is accomplished by an increase in ATP-binding cassette (ABC) multidrug efflux transporters. High ABC transporter-expressing cells keep the dyes out and show up as a side population during analysis. In primary human osteosarcoma samples, there was a greater detection rate of side population cells than in osteosarcoma cell lines (Murase et al., 2009; Yang et al., 2011). In comparison to cells from the non-side population, downstream investigation

of the side population cells revealed elevated gene expression (ABC transporters, *OCT4*, *NANOG*), enhanced sphere formation, and higher multidrug resistance to doxorubicin, methotrexate, and cisplatin.

The production and activity of the drug-detoxifying enzyme ALDH is another method by which CSC employ their chemoresistance. Honoki et al. (2010) discovered a subpopulation of ALDH1^{high} MG63 (human osteosarcoma) cells and further reported that ALDH1^{high} cells had higher expression of stem-like genes (*NANOG*, *OCT3/4*, *STAT3*, *SOX2*), higher resistance to chemotherapy and higher self-renewal ability as demonstrated by sphere formation. Importantly, human osteosarcoma samples also contained ALDH1^{high} cells, and elevated ALDH activity was further linked to the possibility of metastasis (Greco et al., 2014).

Tirino et al. (2008) demonstrated that the membrane glycoprotein CD133 may serve as a marker for CSC in osteosarcoma. They found CD133+ cells in three osteosarcoma cell lines (Saos2, MG63, U2OS). Further analysis revealed that CD133+ cells proliferated more, overexpressed *OCT3/4* and *ABCG2*, had a small side population and formed spheres in serum-free environments, in contrast to CD133- cells. Further research enabled the group to discover CD133+ cells in two primary human osteosarcomas and demonstrated stem-like gene expression, sphere formation, and side population fractions. Furthermore, CD133+ cells generated spheres and were able to produce substantial tumors in vivo (Tirino et al., 2011).

Gibbs et al. (2005) were the first to describe osteosarcoma stem cells from sphere-forming assay in 2005. As a result, numerous other labs went on to demonstrate that sphere-forming cells were also tumorigenic in immunodeficient animals and that they were more treatment resistant, indicating a CSC phenotype (Fujii et al., 2009). They demonstrated that cells separated from spheres had mesenchymal stem cell characteristics, including elevated gene expression of stemness genes *OCT3/4*, *NANOG*, and ABC transporters (Martins-Neves et al., 2012). Further investigation is required into novel strategies that will enable the identification and isolation of pure subpopulations of CSC from osteosarcoma since it appears that the enhanced CSC phenotype may depend on the applied isolation method(s).

1.2.3. CSC in chondrosarcoma

Analyses that are typically used to isolate CSC in chondrosarcoma (CHS) are analogous to those in osteosarcoma samples. A highly proliferative fraction of CHS cells isolated from primary tumor samples had elevated CD271, also one of the MSC surface markers. Comparing CD271+ and CD271- cells, it was shown that CD271+ cells were more capable of self-renewal, differentiation, drug resistance, and tumorigenicity (Wirhth et al., 2013). A fraction of CHS cells contained another crucial marker, that is a feature of embryonic stem cells (ESC) and CSC, which is CD133. In vivo, tumor growth can be initiated and supported by CD133+ cells because they exhibit stem-like characteristics. Small fraction of the CD133+ cells in CHS cell lines derived from clinical samples was capable of self-renewal, sphere formation, adipogenic and osteogenic differentiation, and showed significant tumorigenicity in vivo (Tirino et al., 2011).

The expression of typical stem-related transcription factors was verified in CHS stem cells. In CHS spheres grown from patient samples, higher levels of the *OCT3/4* and *NANOG* expression were detected. These transcription factors in normal state maintain the pluripotency of undifferentiated ESC, but their overexpression has been linked to several malignancies so they may also function as oncogenes (Gibbs et al., 2005).

Established human CHS cell lines with increased invasiveness, tumorigenicity and the capacity to form tumor spheres have been reported to have highly elevated ALDH. CHS cells with increased expression of ALDH showed a considerably lower reactive oxygen species (ROS) level than ALDH-cells (Sládek, 2003). Recent studies found, besides the well-established markers of stemness in cancer cells, some new important factors maintaining the stem-like state of CHS cells. Tumor suppressive microRNA-34 (miR-34), which is well-known for modulating target genes like Notch homolog 1 (*NOTCH1*), *C-MYC* and *KLF4*, can suppress stem-like characteristics in many cancers and it has been proposed that miR-34 may also have an impact on CHS stemness. miR-34 is typically downregulated in CHS cell lines, and its overexpression greatly decreased CHS invasive activity and its capacity to form spheroids in in vitro-maintained CHS cells (Vares et al., 2020). Finally, recent findings from in vitro and in vivo models have described a substantial role for hypoxia-inducible factors (HIFs) in CHS development and progression, including the maintenance of stem-like characteristics. HIF proteins were shown to be overexpressed in CHS, to relate to the tumor's histological grade and they were proven to be crucial in promoting tumor development, proliferation, metastasis, and stem cells' capacity for self-renewal. Additionally, it has been demonstrated that inhibiting some HIF protein family members can overcome CHS chemoresistance to treatment and enhance conventional chemotherapeutics (Kim et al., 2020).

1.2.4. CSC in rhabdomyosarcoma

Various approaches used to segregate CSC in bone sarcoma are also those used in STS with a few additional methods. As in bone sarcomas, membrane glycoprotein CD133 is reported to identify CSC in rhabdomyosarcoma (RMS). In vitro studies on samples taken from patients with RMS and in five RMS cell lines showed that CD133+ cells identify a subpopulation of cancer cells that exhibits high amounts of stemness genes, in vitro self-renewal, and elevated clonogenicity and tumorigenicity (Sana et al., 2011). Subpopulations of human embryonal RMS (ERMS) cell lines with increased ALDH activity were shown to have higher proliferation rate and clonogenicity than the ALDH1^{low} cells. It was discovered that after being treated with chemotherapeutic drugs for RMS, the survival rate of the ALDH1^{high} cells was higher than that of the ALDH1^{low} cells (Nakahata et al., 2015). Side population phenotype, characterized by higher tumorigenicity, drug efflux capability and therefore chemoresistance has been used to enrich for CSC in many cancer types. It was shown that side population with all the above-mentioned characteristics also exists in mouse models of ERMS (Rubin et al., 2011). Three

transcription factors (TFs) Oct4, Nanog, and Sox2 are known as the essential ESC factors supporting pluripotency. In many malignancies, these TFs induce and support CSC. All three ESC pluripotency factors were found to be highly expressed in putative CSC in RMS (Riggi et al., 2010). Epigenetic changes are thought to be important in the establishment and maintenance of CSC. DNA methyltransferase (DNMT) hyperactivation has been suggested to be necessary for CSC maintenance. Knock-down of *DNMT3B* in ERMS alters the phenotypes of tumor cells by restoring the myogenic program, hence it is thought to be crucial for maintaining a less differentiated phenotype (Megiorni et al., 2016). Nestin is a cytoplasmic protein that belongs to intermediate filaments. It was found to be a marker of neural stem cells as well as CSC in tumors of mesenchymal origin. It has been demonstrated that RMS cells express nestin (Ishiwata et al., 2011). Nestin coexpression with other stem cell markers, such as CD133, was identified as a CSC phenotype and cells expressing those markers have a higher tumorigenic potential (Sana et al., 2011). In pediatric sarcomas, elevated nestin expression has been linked to a poor prognosis, suggesting that this protein may also be used as a prognostic factor (Zambo et al., 2016).

1.3.Cancer metabolism

Although the first reports of the metabolic changes that are typical of tumors date back over a century, the study of cancer metabolism has recently attracted new attention. New biochemical and molecular biology methods have identified novel hallmarks of tumorigenesis-associated metabolic reprogramming that span all stages of cell-metabolite interaction. Some of the principal characteristics of cancer metabolism are increased uptake of glucose and rate of aerobic glycolysis (known as Warburg effect), utilization of opportunistic forms of nutrient acquisition, use of glycolysis and Krebs cycle intermediates for biosynthetic processes, elevated requirement for nitrogen, changes in gene regulation driven by metabolites and metabolic intercommunication with the microenvironment. Researchers are continuously exploring how these characteristics function in the establishment and maintenance of the tumorigenic state and how that knowledge might be used in creation of novel therapeutics (Pavlova & Thompson, 2016).

1.3.1. Cancer stem cell metabolism

The main source of energy for cancer stem cells is still under debate, with some studies pointing to glycolysis and others to mitochondrial metabolism. Additionally, it appears that cancer stem cells can switch their energy production from one pathway to another or acquire intermediate metabolic phenotypes to modify their metabolism in response to microenvironmental changes. The original theory was that CSC should have a metabolic profile similar to that of normal tissue hierarchy, in which

multipotent stem cells are primarily glycolytic and differentiated somatic cells are dependent on oxidative phosphorylation (OXPHOS) (Folmes et al, 2012).

The concept of glycolysis-driven stemness was subsequently extended to CSC in several studies, and research using breast cancer cell lines, hepatocellular carcinoma, and nasopharyngeal carcinoma did corroborate this idea. Unexpectedly, CSC from different cancer types have shown that OXPHOS is the predominant method of energy production. This has been convincingly demonstrated so far for side population cells in lung cancer, sphere-forming and CD133+ cells for both glioblastoma and pancreatic ductal adenocarcinoma (PDAC), and ROS^{low} quiescent leukemia stem cells.

Aside from being a significant source of ATP for cancer cells, mitochondria also play a role in regulating several signaling pathways, such as the release of cytochrome C to start apoptosis, the release of ROS, and the creation of metabolites such acetyl-CoA, which controls protein acetylation. Therefore, regardless of the underlying metabolic state in individual cells, mitochondria also seem to control stemness features. Increased mitochondrial biogenesis does indeed seem to be a crucial component of CSC functioning in both glycolytic and OXPHOS-dependent CSC (Sancho et al., 2016). Increased cancer research has shifted its focus to understanding the role of cancer stem cell metabolism in carcinogenesis, and significant work is being done to identify potential treatment targets.

1.3.2. Sarcoma metabolomics

Sarcomas have aberrant metabolic activity patterns like other tumors do, although these have not yet been well studied or linked to particular gene alterations. Most sarcoma metabolomics investigations have utilized cell lines and not patient-derived cells because of bigger flexibility in handling the cells and examining their metabolic status. The text will now focus on some traits of bone and soft-tissue sarcomas.

1.3.2.1. Osteosarcoma metabolomics

There is little information on the metabolism of bone sarcomas because of their rarity and the unique characteristics of bone metabolism (Lv et al., 2020). Different metabolic markers were examined using mice OS models, and relationships between metabolic adaptations, tumor growth, and metastasis were discovered (Hua et al., 2011). Different markers showed differential expression between the nonmetastatic state and the development of lung metastases. OS most likely experiences a general metabolic decrease throughout the pulmonary metastases stage because of hypoxia and a switch from lipids to amino acids and carbohydrates as energy source (Hua et al., 2011).

Concurrently, during the metastatic phase (supporting the de novo DNA synthesis required for the development of lung metastases), levels of DNA precursors such as uridine and uracil were increased

and levels of PPP intermediates such as glucose, glucose phosphate, and gluconolactone decreased (Dean et al., 2018; Hua et al., 2011). Significantly decreased quantities of metabolites from the inositol pathway were also discovered in a study of OS highly metastatic human and mouse cell lines. When the competitive glycolysis inhibitor, 2-deoxy-D-glucose (2DG) was added to human, canine, and mouse OS cell lines in vitro, the metastatic phenotype was considerably lowered, with significant reductions in cathepsin L (a lysosomal cysteine protease capable of degrading the extracellular matrix), α -actin, and β -tubulin, which resulted in reduced cell migration (Dean et al., 2018). Recent publications of intriguing investigations on OS CSC are noteworthy, such as paper published in 2014 by Palorini et al. They found that 3AB-OS (human osteosarcoma cell line) CSC are more dependent on high glycolysis and less dependent on OXPHOS for energy production and survival than OS MG63 cells (non-CSC). In contrast to OS MG63 cells, 3AB-OS CSC exhibit increased lactate dehydrogenase expression and lactate buildup in the culture medium. In line with this, 3AB-OS CSC showed decreased mitochondrial respiration, increased sensitivity to glucose depletion, increased sensitivity to glycolysis inhibition, and decreased sensitivity to oxidative phosphorylation inhibitors (Palorini et al., 2014).

1.3.2.2. Chondrosarcoma metabolomics

Previous research suggests that CHS exhibits metabolic adaptations, including upregulation of glycolysis and downregulation of OXPHOS in high versus low-grade CHS (Rozeman et al., 2005), hyperactivation of the mTOR pathway and subsequent metabolic adaptations (Zhang et al., 2013), and missense and heterozygous *IDH 1/2* mutations causing accumulation of the 2-hydroxyglutarate (2-HG) oncometabolite. As a result of its buildup, tumor suppressor genes are downregulated, cellular differentiation is inhibited, and carcinogenesis is increased (Pavlova & Thompson, 2016). Further research revealed that inhibiting mutant *IDH 1/2* significantly reduced 2-HG production, reversed histone and DNA hypermethylation, promoted cellular differentiation, significantly reduced colony formation and migration in human CHS cells, and significantly inhibited colony formation and migration (Li et al., 2015).

1.3.2.3. Soft-tissue sarcoma metabolomics

There is a lack of comprehensive information on the metabolome of soft-tissue sarcomas (STS). Sarcomas have mutations in many oncogenes and tumor suppressors involved in the control of metabolic pathways. A hypoxic tumor microenvironment, characteristic of sarcomas also affects metabolism and is associated with a worse prognosis (Sadri & Zhang, 2013). Elevated glucose intake and turnover are seen in sarcoma cells. Glycolysis is counterbalanced by gluconeogenesis, and gluconeogenic enzymes may be important factors in the control of tumor cell development. One of these gluconeogenic enzymes is fructose-1-6-biphosphatase 2 (FBP2), and Huangyang et al. (2020) demonstrated that this enzyme's

expression is suppressed in a wide range of STS subtypes. Another well-known metabolic adaptation of cancer cells is increased glutamine absorption. Lee et al. (2020) used a variety of STS cell lines and have shown in a metabolomic investigation that certain STS types both exhibit substantial glutaminase expression and significant glutamine dependence. The alteration of arginine metabolism, especially the overexpression of protein arginine methyltransferase, may also contribute to the development of sarcomas. An arginine methyltransferase inhibitor's application on a murine sarcoma has shown encouraging therapeutic potential and had anti-tumor effects (Zhang et al., 2018).

1.4.Vitamin C in cancer research

Vitamin C can exist in two different states. In reduced form as L- ascorbic acid (AA) or oxidized form as dehydro-L-ascorbic acid (DHA). AA is transported into cells via sodium-dependent vitamin C cotransporter type 1 and 2 (SVCT1 and SVCT2) and DHA enters the cells via glucose transporters: GLUT1, GLUT2, GLUT3, GLUT4 AND GLUT8. SVCT1 is mainly distributed in epithelial tissues while SVCT2 is ubiquitous and can be found in brain, thyroid, heart, bone, muscle (Ferrada et al., 2019). For a long time, DHA was employed to effectively trap and store AA within cells, because when DHA enters the cells, it is quickly reduced to AA at the expense of cellular antioxidants. Different experimental methods investigating vitamin C effect on tumor cells have produced complex and diverse outcomes. Even though some discoveries state that vitamin C antagonizes conventional antineoplastic drugs, a lot of investigators found that administration of vitamin C (AA or DHA) in millimolar concentrations can result in the induction of cell death (Chen et al., 2005; Tian et al., 2014; Yun et al., 2015b).

1.4.1 Vitamin C as anti-tumor drug

An essential natural antioxidant vitamin C has a dubious past as a anti-tumor therapy. Clinical trials carried out by Pauling and Cameron in the 1970s showed the effectiveness of intravenous ascorbate in enhancing the survival of cancer patients with terminal disease. The failure of subsequent double-blind, placebo-controlled trials utilizing oral vitamin C at the Mayo Clinic to demonstrate any benefit, however, led to harsh criticism of these studies. Later, it was determined that the primary cause of the disparity was the method of vitamin C administration. Compared to later trials using oral vitamin C, the initial reported research using intravenous vitamin C produces significantly greater plasma concentrations and those higher doses are needed for treatment of cancer. Physicians and practitioners of complementary and alternative medicine have utilized high dose intravenous vitamin C for many years, with little to no known negative effects (Padayatty et al., 2010). According to studies conducted on animals, giving vitamin C along with a variety of chemotherapeutic drugs (such as gemcitabine, paclitaxel, carboplatin, melphalan, carfilzomib, bortezomib, cisplatin, and temozolomide) decreased the

growth of xenograft tumors, including those in a pancreatic tumor model that was resistant to chemotherapy, and increased survival (Espey et al., 2011). Intravenous vitamin C in combination with a variety of chemotherapeutic drugs has not been associated with any negative side effects in human trials, and in many cases, lower off-target toxicity and better health-related quality of life have been noted. Numerous in vitro, preclinical, and clinical investigations were conducted after the findings about different pharmacokinetics of oral and intravenous vitamin C. Pre-clinical investigations have shown efficacy of intravenous vitamin C, and in vitro research has offered helpful insights into probable mechanisms of action (Carr & Cook, 2018).

1.4.2. Mechanism of action of vitamin C

The so-called 'pro-oxidant' activity of vitamin C is currently one of the most accepted anti-tumor mechanisms. This phrase, nevertheless, can be deceptive because vitamin C always functions as an antioxidant by donating electrons; the 'pro-oxidant' activity follows and is thus a side consequence. In cell culture medium or buffers, transition metal ions like ferric and cupric cations can be reduced by vitamin C (in the form of the ascorbate anion). By reducing oxygen to the superoxide radical, which can combine with itself to form hydrogen peroxide (H_2O_2), the reduced transition metal ions can subsequently produce hydrogen peroxide. However, it is still unclear if transition metal ions that are catalytically accessible can increase oxidative stress in vivo because iron and copper are typically found in transport and storage proteins (e.g., transferrin and ferritin). It has been proposed that the extracellular fluid of the tumor microenvironment contains transition metal ions that are more readily available for catalysis. In vitro research has demonstrated that the addition of high (millimolar) vitamin C content in cell culture medium shows varying cytotoxicity against different cancer cell types but not on normal cultured cells (Chen et al., 2005).

Numerous studies in both cell culture and preclinical settings have demonstrated that vitamin C supplementation has regulatory effects on several transcription factors and cell signaling pathways, with subsequent effects on the pathways for cell cycle, angiogenesis, and cell death (Parrow et al., 2013). Thus, vitamin C supplementation, especially at lower dosages, has an impact on gene regulation. Vitamin C has been known as a cofactor for metalloenzymes with different roles. It is long known that vitamin C serves as cofactor for three hydroxylases necessary for the stabilization of collagen structure. Animal models show that after the treatment with vitamin C tumor stroma becomes richer in collagen and tumor invasion and metastases decrease (Polireddy et al., 2017). Another example where vitamin C serves as cofactor are HIF hydroxylases. HIF-1 α is a transcription factor that is constitutively expressed and controls many genes involved in survival. HIF-1 α is modified by hydroxylases in normoxic circumstances, preventing coactivator binding and designating HIF for proteasomal destruction. HIF-1 α is increased in the hypoxic core of solid tumors because the substrates and cofactors necessary for hydroxylase-dependent downregulation are not present.

Through its role as a cofactor for DNA and histone demethylases, which are members of the same family of enzymes as the collagen and HIF hydroxylases, vitamin C has recently been linked to epigenetic control. The ten-eleven translocation (TET) dioxygenases, which hydroxylate DNA's methylated cytosine moieties, require vitamin C as a cofactor. Thus, vitamin C controls the epigenome through assisting DNA and histone demethylases (Gillberg et al., 2018). Numerous findings state that cancer, chronic inflammation, and oxidative stress are all intimately related. Due to its capacity to scavenge a variety of reactive oxygen species, vitamin C is a powerful antioxidant both in plasma and within cells, protecting crucial biomolecules from oxidative damage. Typically, oxidative stress markers and inflammatory markers, such as different cytokines are increased in cancer patients. Vitamin C works to reduce inflammation by altering cytokine levels and in few cancer models and patients decreased levels of the pro-inflammatory cytokines interleukin-6 and interleukin-1 following the ingestion of vitamin C were found (Mikirova et al., 2013).

1.4.3. Metabolic effects of DHA

Historically, DHA has received little attention in the literature because it was thought that it has extremely short half-life, so it was only used to deliver AA into the cells that didn't express SVCTs by uptake of DHA via GLUTs. After import, DHA is reduced to AA at the cost of glutathione (GSH), thioredoxin, and nicotinamide adenine dinucleotide phosphate (NADPH). After AA performs its antioxidant function it oxidized to DHA. However, using ¹³C-NMR it was found that half-life is not that short, around 50 minutes (Himmelreich et al., 1998), DHA may therefore be stable to perform physiological tasks, such as acting as a hexokinase inhibitor (Fiorani et al., 1996) or to form complex with GSH (Regulus et al., 2010). Recently, research has emphasized the importance of vitamin C on regulating metabolic pathways, primarily on glycolysis and/or Krebs cycle. One group showed metabolic alterations in both pathways on breast cancer cell line (MCF7) and colorectal adenocarcinoma cell line (HT29) after vitamin C treatment (Uetaki et al., 2015). They found that glycolytic metabolites upstream of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) increased, while downstream metabolites lowered in concentration, and they also found increase in citrate and cis-aconitate in Krebs cycle. Vitamin C generated H₂O₂ which then depleted cellular NAD⁺ levels and led to cell death. They also confirmed results from earlier research (Chen et al., 2005) that vitamin C- induced H₂O₂ damages DNA and increases poly (ADP-ribose) polymerase (PARP) activation, which consequently causes depletion of ATP and consumption of NAD⁺. Another group focused on the metabolic effects of DHA on colon cancer cells with *KRAS* and *BRAF* mutations (Figure 1) and they found significant alterations in glycolysis (Yun et al., 2015).

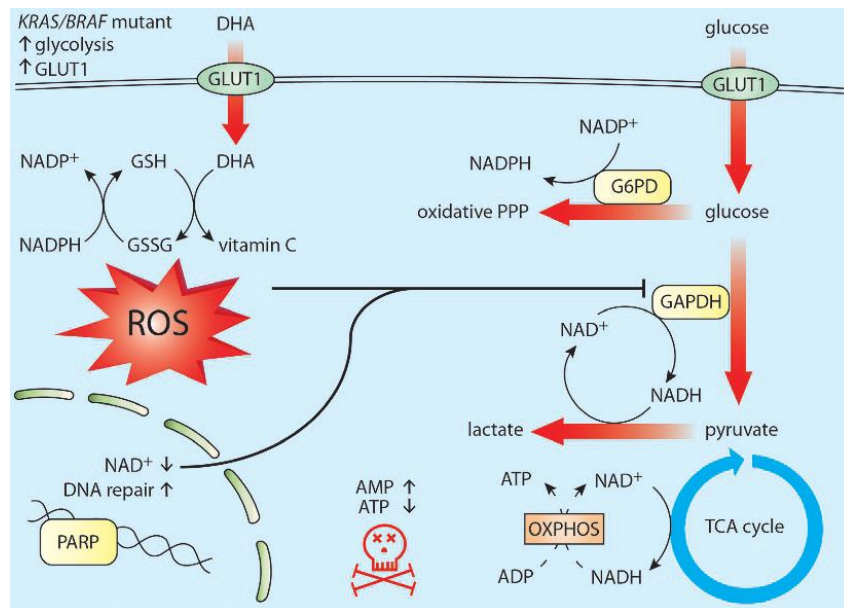


Figure 1. The mechanistic overview of vitamin C toxicity in cancer cells with *KRAS* and *BRAF* mutations. GSSG: oxidized form of glutathione; NADP⁺: nicotinamide adenine dinucleotide phosphate; NADPH: reduced nicotinamide adenine dinucleotide phosphate; NADH: reduced nicotinamide adenine dinucleotide; NAD⁺: oxidized nicotinamide adenine dinucleotide; TCA: citric acid cycle also known as the tricarboxylic acid or the Krebs cycle. DHA: dehydroascorbic acid; GLUT1: glucose transporter 1; *KRAS*: Kirsten rat sarcoma viral oncogene; *BRAF*: v-ras murine sarcoma viral oncogene homolog B; GSH: reduced form of glutathione; GSSG: oxidized form of glutathione; ROS: reactive oxygen species; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; ATP: adenosine 5'-triphosphate. Picture taken from Van Der Reest & Gottlieb (2016).

Aside from metabolic changes caused by DHA they also found that treatment inhibited proliferation and colony formation. Like the previous group they also noted accumulation of metabolites upstream of GAPDH and depletion of those downstream. They found that inhibition of GAPDH occurs by two different ways and that it shifts glycolytic flux into the oxidative pentose phosphate pathway (PPP) which is necessary to restore levels of cellular NADPH that is consumed to reduce DHA to AA. Vitamin C treatment significantly increased endogenous ROS in *KRAS* and *BRAF* mutant cells, and ROS are known to target the cysteine in GAPDH's active site. Reversible S-glutathionylation of the active-site cysteine can result in the oxidized cysteine which then forms mixed disulfide with GSH (Cys-GSH). They also proposed that further inhibition of GAPDH is caused by depletion of its substrate NAD⁺ that is a consequence of activation of PARP due to the increased levels of ROS and consequently DNA damage. All these changes conclusively lead to decreased ATP levels, energy crisis and cell death.

2. RESEARCH AIM

A small fraction of tumor cells known as cancer stem cells (CSC) have the ability to self-renew, differentiate, and induce tumors in vivo. Emerging research which shows that CSC are resistant to traditional chemotherapy and that they are very likely the cause of cancer metastasis has reinforced the clinical relevance of CSC. Vitamin C has proved to be cytotoxic for malignant cells. Dehydroascorbic acid (DHA) is oxidized form of vitamin C that enters the cell via glucose transporters which are often highly expressed in cancer cells. The methods cancer cells use to change their energy metabolism have recently come under the spotlight but there is still not a lot of information available about the sarcoma metabolome. Further study of the sarcoma metabolic landscape will enable the identification of new treatment targets and this paper will examine possible effects of DHA on sarcoma stem cell metabolism.

Specific research aims:

- 1) To examine the influence of DHA on the viability of patient-derived sarcoma stem cells, human embryonic kidney cell line HEK293 and human osteosarcoma cell line U2OS
- 2) To examine changes in the expression of genes important for metabolism (*GAPDH*, *PPARGC1A*) after seven days treatment of patient-derived sarcoma stem cells with 1mM DHA in medium with and without bFGF
- 3) To examine the change in the protein expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) after seven days treatment of patient-derived sarcoma stem cells with 1mM DHA in medium with and without bFGF

3. MATERIALS AND METHODS

3.1. Materials

Cells

- human embryonic kidney cell line HEK293
- human osteosarcoma cell line U2OS
- patient-derived chondrosarcoma stem cells
- patient-derived osteosarcoma stem cells
- patient-derived rhabdomyosarcoma stem cells

Cell culture and treatment

- High Glucose Dulbecco's modified Eagle's medium (DMEM) (Capricorn Scientific, Ebsdorfergrund, Germany)
- Modified Coons F-12 medium with L-glutamine, 0.863 mg/L zinc sulphate, without NaHCO₃ (Capricorn Scientific, Ebsdorfergrund, Germany)
- penicillin - streptomycin (1%) (Capricorn Scientific, Ebsdorfergrund, Germany)
- fetal bovine serum (FBS) (Capricorn Scientific, Ebsdorfergrund, Germany)
- phosphate buffered saline (PBS) (Capricorn Scientific, Ebsdorfergrund, Germany)
- 0,25% trypsin (Sigma-Aldrich, St. Louis, Missouri, USA)
- Trypan Blue (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- Dehydroascorbic acid powder (Sigma-Aldrich, St. Louis, Missouri, USA)
- basic fibroblast growth factor (bFGF) (Sigma-Aldrich, St. Louis, Missouri, USA)

MTT test

- MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St. Louis, Missouri, USA)
- dimethyl sulfoxide DMSO (Sigma-Aldrich, St. Louis, Missouri, USA)

Isolation of RNA and protein

- Quick DNA/RNA Miniprep plus Kit (Zymo Research Irvine, California, USA)
- CelLytic MT Cell Lysis Reagent (Sigma-Aldrich, St. Louis, Missouri, USA)

Western Blot

- BCA Protein Assay Kit (Santa Cruz Biotechnology, Dallas, Texas, USA)
- TGX Stain-Free™ FastCast™ Acrylamide Solutions (Bio-Rad, Hercules, California, USA)
- 10x Tris/Glycine/SDS (Bio-Rad, Hercules, California, USA)
- TEMED (Carl Roth GmbH, Karlsruhe, Germany)
- Tween 20 (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- Ammonium persulphate (Sigma-Aldrich, St. Louis, Missouri, USA)
- Tris(hydroxymethyl)aminomethane (Sigma-Aldrich, St. Louis, Missouri, USA)
- Glycine (Carl Roth GmbH, Karlsruhe, Germany)
- Methanol (Sigma-Aldrich, St. Louis, Missouri, USA)
- Clarity Western ECL Substrate (Bio-Rad, Hercules, California, USA)
- GAPDH (D16H11) XP® Rabbit monoclonal antibody (Cell Signaling Technology, Massachusetts, USA)
- Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling Technology, Massachusetts, USA)

RT-qPCR

- Deoxyribonuclease I (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, Massachusetts, USA)
- SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, California, USA)

Laboratory equipment

- HERAsafe HS18 Safety Bench Hood Cabinet (Thermo Heraeus, Hanau, Germany)
- Axiovert 40 CFL Inverted Phase Contrast Microscope (Zeiss, Oberkochen, Germany)
- Incubator (Panasonic, Osaka, Japan)
- GloMax Multi Detection Plate Reader (Promega, Madison, Wisconsin, USA)
- Precistern Water Bath (JP Selecta, Barcelona, Spain)
- Centrifuge Universal 320R (Hettich, Tuttlingen, Germany)
- Thermoblock (Eppendorf, Hamburg, Germany)

- NanoVue Plus™ (Biochrom, Cambridge, United Kingdom)
- CFX Opus 96 Real-Time PCR (Bio-Rad, Hercules, California, USA)
- Mini-PROTEAN Tetra Cell (Bio-Rad, Hercules, California, USA)
- Trans-Blot® Cell (Bio-Rad, Hercules, California, USA)
- Bürker-Türk- counting chamber (Marienfeld, Lauda-Königshofen, Germany)
- Petri dishes (Sarstedt, Nümbrecht, Germany)
- Test tubes (Sarstedt, Nümbrecht, Germany)
- 96- well plate (Sarstedt, Nümbrecht, Germany)
- 6-well plate (Sarstedt, Nümbrecht, Germany)

3.2. Methods

3.2.1. Cell culture

For this experiment I used commercially available cell lines HEK293 (human embryonic kidney) and U2OS (human bone osteosarcoma epithelial cells) and patient-derived sarcoma stem cells which were isolated by sphere formation assay. The samples were from three types of sarcomas: conventional osteosarcoma, primary chondrosarcoma, and rhabdomyosarcoma. Cells from tumor biopsy samples from patients in Children's Hospital Zagreb were used with the permission of Ethics Committee and the voluntary consent of patients and guardians. Briefly, samples from patients were cultured in non-adherent conditions and the cells that formed spheres were then isolated. Those cells were again cultured in non-adherent conditions and that second generation of spheres was put in adherent culture to allow propagation of isolated cancer stem cells which were then used for these experiments.

Cell culture work was done in sterile conditions in laminar flow hood. I cultured HEK293 and U2OS cell lines in High Glucose DMEM with L-glutamine and Sodium Pyruvate with the addition of 10% fetal bovine serum (FBS) and 1% penicillin- streptomycin (pen/strep). To grow patient-derived cancer stem cells in culture I used Coon's modification of Ham's F12 medium with L-glutamine (F12 DMEM), 10% FBS and 1% pen/strep. This medium also contained 0.1% bFGF because it was shown that bFGF is needed for normal growth of stem cells and maintenance of multipotency. The cells were grown in tissue culture treated Petri dishes in incubator at 37 °C and CO₂ set to 5%. In order to passage the cells, firstly I aspirated the existing medium and washed the plate with 2 mL PBS. Then, I aspirated the PBS and added 2 mL trypsin and incubated the cells for 4 minutes at 37 °C, 5% CO₂. After that I checked on microscope if the cells detached and then inactivated the trypsin with 4 mL of corresponding medium with FBS depending on the cell line. When I needed to plate the cells in certain number, for MTT assay or for seven days treatment with 1 mM DHA, I diluted part of the cell suspension with trypan blue in 1:1 ratio and then counted live cells on Bürker-Türk Counting Chambers under Zeiss Axiovert 40 CFL microscope. After counting the cells and calculating the concentration in cell suspension I diluted the necessary volume of cell suspension with the corresponding medium.

3.2.2. Cell viability determination using MTT assay

The MTT assay was done for 5 cell lines: HEK293, U2OS and three patient-derived sarcoma stem cell lines. In all cases the cells were plated on 96-well plate in concentration 10⁴ cells/mL in 100 µL of corresponding medium. For HEK293 and U2OS that was High Glucose DMEM and for patient-derived sarcoma stem cells that was F12 DMEM with 0.1% bFGF. All cells were plated in triplicates. As negative control I used cells that were not treated that followed the same protocol as the rest of the wells except the treatment part. As blank I used empty wells. After plating, cells were incubated for 24 hours at 37 °C and 5% CO₂.

I treated the cells with DHA in following concentrations: 0.05, 0.1, 0.3, 0.5, 1, 5 and 10 mM. Concentrations in millimolar range were chosen because it was shown that vitamin C shows antitumor effects in higher concentrations given intravenously. 10 mM DHA stock solution was made from DHA powder and then it was diluted in corresponding medium (depending on the cells treated) to get the final concentrations for treatment. To prepare 10 mL of 10 mM DHA I weighed 0.0174 g of DHA powder and dissolved it in 10 mL of miliQ water with 10% FBS and 1% pen/strep. To dissolve the DHA powder, I put the tube with miliQ water, FBS and DHA in water bath on 60 °C for 5 minutes until the solution became transparent. Then I added the necessary amount of corresponding medium powder (High Glucose DMEM or F12 DMEM) and NaHCO₃ (for the volume of 10 mL) to the solution and resuspended. In laminar flow hood I filtered the solution through sterile syringe filter with pore size 0.22 µm and then I got sterile 10 mM DHA in adequate medium depending on the cells I treated. I added 0.1% bFGF to half of prepared 10 mM DHA stock solution. From that stock solution I made dilutions in corresponding medium to make the wanted concentrations of DHA for treatment. I treated the cells with 200 µL of treatment or medium for negative control. Treated cells were then incubated for 72 hours at 37 °C and 5% CO₂.

After 72 hours I aspirated the medium from the wells and washed the cells three times with PBS. Then I added 40 µL of MTT prepared in corresponding medium in concentration of 0.5 mg/mL and put the 96-plate to incubate for 4 hours on 37 °C and 5% CO₂. After the incubation I put 170 µL of DMSO per well to dissolve the formed formazan crystals. To improve dissolution, I gently shook the 96-well plate on vortex for 2 minutes. I read the absorption on GloMax Microplate Reader at 560 nm.

3.2.3. Gene expression analysis of *GAPDH* and *PPARGC1A* by RT-qPCR

In order to examine the effect of DHA on changes in gene expression of sarcoma stem cells I treated the cells with 1 mM DHA in duration of seven days. First, I plated the cells in 6-well plates in concentration 5×10^4 cells/ mL in 1 mL of Coon's modification of Ham's F12 medium. I also plated same cells in the same medium with the addition of 0.1% bFGF. Cells were left to incubate for 24 hours on 37 °C and 5% CO₂. Next day I treated the cells. To prepare 1 mM DHA solution I started by making the stock solution of 10 mM DHA as described in protocol for MTT treatment (Section 3.2.2.). After obtaining 10 mM DHA I separated half of it and added 0.1% bFGF. Then, I had 10 mM DHA in medium with and without bFGF. To prepare 1 mM DHA I diluted stock solution with the corresponding medium. I aspirated the medium from the cells and added 2 mL of prepared 1 mM DHA. Control cells weren't treated, I just aspirated old medium and added 2 mL of fresh one, with and without bFGF. After the treatment or, in case of control cells, change of the medium, the cells were put in incubator. The treatment lasted for seven days with the aspiration of old treatment/medium and addition of fresh 1 mM DHA treatment or medium every two days.

After seven days of DHA treatment, I isolated RNA (and proteins) from the cells with Quick-DNA/RNA Miniprep Plus Kit (Zymo Research) following the manufacturer instructions. First, I needed to detach the cells from 6-well plate, so after aspirating treatment or medium I washed the cells with PBS and then added 2 mL of trypsin. I put the cells in incubator for 4 minutes and then neutralized trypsin with 4 mL of corresponding medium. I put the cells from every well into separate tube and centrifuged it for 1 minute on 500 x g on 25 °C. Then, I removed supernatant and resuspended the cell precipitate in buffer that shields RNA from degradation. After brief vortex, I added lysis buffer. Lysed sample was then centrifuged, and 100% ethanol was added to the flow-through. Again, I centrifuged the sample and used the resulting flow-through to isolate the proteins in next step. I added RNA Prep Buffer to the column and centrifuged. After that I discarded flow-through and washed the column two times with washing buffer. To elute RNA, I added DNase/RNase-Free Water and centrifuged the tube. The samples were then stored on -20 °C.

I measured concentration and purity of previously isolated RNA from samples using NanoVue Plus Spectrophotometer (Biochrom). Values of ratios A260/A280 and A260/A230 indicated that I have successfully isolated good quality RNA. Then I proceeded to do DNase I treatment to get rid of all eventually left-over DNA in samples. In each reaction I used 350 ng of RNA, 1 µL of DNase I, RNase-free (Thermo Scientific), 1 µL of 10x reaction buffer with MgCl₂ that comes with kit and added the necessary miliQ water to the final volume of 10 µL. Samples were then incubated at 37 °C for 30 minutes. After that I added 1 µL of 50 mM EDTA and incubated for 10 minutes at 65 °C to inactivate the enzyme. I used thusly prepared RNA in the next step to get cDNA.

For the quantitative conversion of RNA to single-stranded cDNA I used the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). In each 20 µL reaction I added all of the previously treated RNA, 2 µL of 10x buffer, 0.8 µL of 25x dNTPs, 1 µL MultiScribe Reverse Transcriptase, 2 µL 10x random primers, 1 µL RNase inhibitor and miliQ water up to final volume. One sample was prepared without reverse transcriptase and was later used as no-RT control in qPCR. Protocol setup for reverse transcription was 10 minutes at 25 °C, 120 minutes at 37 °C, 5 minutes at 85 °C and then at 4 °C until next step. This procedure was performed in CFX Opus 96 Real-Time PCR system.

Primer pairs for genes of interest (*GAPDH*, *PPARGC1A*) and endogenous control (*ACTB*) were ordered from Macrogen Europe. Sequences are given in Table 1. β-actin gene was used as endogenous control to normalize the results. To prepare working mixture of forward and reverse primers first I dissolved lyophilized primers in 250 µL miliQ water each and then I took 25 µL of forward and 25 µL of reverse primer and added 950 µL of miliQ water. I added 20 µL of miliQ water to each cDNA sample and then continued to prepare reaction mix for qPCR reactions. I used SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Per one 20 µL reaction I mixed 10 µL SsoAdvanced Universal SYBR Green

Supermix, 4 μ L of prepared primer mixture, 2 μ L of cDNA sample and 4 μ L miliQ water. In addition to samples, two controls were also used, one was no-RT (NRT) control (to evaluate residual genomic DNA) and the other was no-template control (NTC) to evaluate purity of reagents. Thermal cycling protocol for qPCR was polymerase activation and DNA denaturation for 30 seconds at 95 °C, then 40 cycles of 15 seconds denaturation at 95 °C and annealing/extension for 30 seconds at 60 °C. After 40 cycles protocol to get the melting curve was 5 seconds at 65 °C and after that increase of temperature in steps of 0.5 °C up to 95 °C. qPCR step was also performed in CFX Opus 96 Real-Time PCR system.

Table 1. Sequences of primer pairs for genes of interest (*GAPDH*, *PPARGCIA*) and endogenous control (*ACTB*) used in qPCR. F-forward primer, R-reverse primer.

Gene	Sequence 5'-3'
<i>ACT</i>	F: CAC CAT TGG CAA TGA GCG GTT C R: AGG TCT TTG CGG ATG TCC ACG
<i>GAPDH</i>	F: TCA AGG CTG AGA ACG GGA AG R: CGC CCC ACT TGA TTT TGG AG
<i>PPARGCIA</i>	F: CCA AAG GAT GCG CTC TCG TTC A R: CGG TGT CTG TAG TGG CTT GAC T

3.2.4. Western blot detection of GAPDH

After seven days DHA treatment, along with RNA, I also isolated the proteins. To isolate them I used flow-through from one step of RNA isolation protocol. I added 4 volumes of ice-cold acetone to the flow-through and after resuspending it, I left it to incubate on ice for 30 minutes. After incubation, I centrifuged the samples at maximal speed at 4 °C for 10 minutes. I discarded the supernatant and added 400 μ L of 100% ethanol to the precipitate. Then I centrifuged the sample for 1 min, 13000 x g on room temperature. I discarded the supernatant and left the protein pellet to dry for 10 minutes, room temperature. After that, I resuspended the pellet in 50 μ L of Cell Lytic Buffer (Sigma-Aldrich) with 1% Protein Inhibitor. Proteins were then stored on -20 °C.

I used Bicinchronic Acid (BCA) Protein Assay Kit (Santa Cruz Biotechnology) to determine protein concentration in samples. I prepared a set of bovine serum albumin (BSA) standards dilutions in PBS in range 125-2000 μ g/mL to make a standard curve from BSA stock solution 2 mg/mL. The samples were diluted 2x with miliQ water. Standards and diluted samples were pipetted onto 96-well plate in duplicates of 10 μ L. To prepare the working solution I used Reagent A and Reagent B from the kit in a 50:1 ratio. To each well I added 200 μ L of prepared working solution and then covered the 96-well plate with parafilm. I incubated the plate at 37 °C for 30 minutes. After half an hour I removed the

plate from incubator and let it cool at room temperature for 5 minutes. Finally, I measured the absorbance of all wells in a microplate reader GloMax at 560 nm. After plotting the standard curve using the data from the standard sample dilutions, I used the curve to determine the concentration of each sample.

To prepare the gels for electrophoresis I used TGX Stain-Free FastCast Acrylamide Solutions (Bio-Rad). I mixed 4 mL of Resolver A and 4 mL of Resolver B and added 4 μ L TEMED and 40 μ L 5% APS and casted the resolving gel in 1.5 mm Bio-Rad Glass Plate. To prepare the stacking gel I used 1.5 mL Stacker A and 1.5 mL Stacker B and then added, right before casting, 3 μ L TEMED and 15 μ L 5% APS. I put the well comb and let the gel polymerize for 30 minutes. To prepare the running buffer I used premade 10x Tris/Glycine/SDS (Bio-Rad) and diluted it to 1x with distilled water.

I calculated the volume of the sample needed to have 10 μ g of protein and then mixed it with appropriate volume of premixed 4x Laemmli Sample Buffer (Bio-Rad). The samples were then incubated for 5 minutes at 95 °C using Eppendorf Thermomixer R. The samples were then centrifuged and left on ice before loading the gel. I assembled the gel in Mini-Protean TETRA System (Bio-Rad), added 1x Running Buffer enough to cover the wells and removed the comb. Then I loaded the samples in wells. In first well I put 3 μ L of Blue Protein Standard Broad Range (New England Biolabs). The electrophoresis was run on 180 V for 100 minutes. During the run-time of SDS-PAGE I prepared the transfer buffer according to the recipe: 3.03g Tris(hydroxymethyl)aminomethane, 14.4 g glycine, 200 mL methanol and distilled water to 1 L. Then I soaked the nitrocellulose membrane (GE Healthcare), filter paper and foam pad (Bio-Rad) necessary for the transfer in transfer buffer for 15 minutes.

When the SDS-PAGE had finished, I opened the glass plate and gently removed stacking gel portion of the gel. I assembled the transfer cassette on cathode side. First, I put one presoaked foam pad and filter paper, then I put the gel and nitrocellulose membrane over it making sure there's no bubbles. Then I added another presoaked filter paper and foam pad and carefully removed any formed bubbles with roller. I closed the cassette holder and put it in Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). I also put ice block next to the cassette holder in Transfer Cell for optimal cooling. I poured the transfer buffer until the marked sign and let the electrophoresis transfer run for 1 hour at 350 mA.

After the completion of transfer, I opened the cassette and marked the first well by cutting the corner of the membrane and then went to check efficacy of transfer on ChemiDoc Imaging System (Bio-Rad). I briefly washed the membrane in 1x TBST pH 7.4 (recipe in Table 2) and then visualized the transfer using option Stain Free Blot. After I confirmed that the transfer was successful, I blocked the membrane in 5% non-fat milk in 1x TBST for 1 hour. After the incubation in non-fat milk, I washed the membrane a couple times in 1x TBST and then put the membrane to incubate in primary antibody GAPDH (D16H11) XP® Rabbit mAb (Cell Signaling Technology). Antibody was prepared in 5% non-fat milk in 1x TBST in ratio 1:5000. Membrane was left to incubate in anti-GAPDH antibody overnight on 4°C on shaker. The next day I washed the membrane 3 times with 1x TBST for 5 minutes each wash

and then put the membrane to incubate with secondary Anti-Rabbit IgG HRP-linked Antibody (Cell Signaling Technology). I prepared secondary antibody in 0.5% non-fat milk in 1x TBST in ratio 1:2500. I let the membrane incubate with secondary antibody for 1 hour on room temperature on shaker. After the incubation, I briefly washed the membrane in 1x TBST and then visualized the membrane with Clarity Western ECL Substrate (Bio-Rad). I prepared 5 mL of substrate solution by mixing kit components in 1:1 ratio and then added substrate to the blot and incubated for 5 minutes. I placed the membrane in plastic sheet to protect it from drying. To visualize the signals on the membrane, I used ChemiDoc Imaging System (Bio-Rad), option Chemiluminescence.

Table 2. Tris- buffered saline wit 0.1% Tween20 detergent (TBST) 1x recipe

Tris base	1.211 g
NaCl	4.383 g
Tween20	0.1%
Total volume	500 mL

4. RESULTS

4.1. Analysis of cell viability using MTT test

MTT test was used to measure cell viability after treatment with DHA for 72 hours. I treated patient-derived sarcoma stem cells and two commercially available cell lines: HEK293 and U2OS. Treatment was prepared in concentration range from 0.05 to 10 mM. For patient-derived stem cells, I prepared DHA treatments in medium with and without bFGF. After 72 hours I measured the cell viability by adding MTT solution, dissolving the formed formazan crystals in DMSO and then measuring the absorbance. All the values were normalized to non-treated control. Those non-treated cells were taken as negative control and they denote 100% cell viability.

Decrease in cell viability when using 5- and 10-mM concentrations of DHA is noticeable in U2OS cells, while in HEK293 cells only the highest used concentration lowered cell viability (Figure 2). Comparing between the commercial cell lines, HEK293 which are human embryonic kidney cells show almost no response to treatment at all and cell viability in U2OS, osteosarcoma cell line decreased after 5 mM and 10 mM DHA treatment.

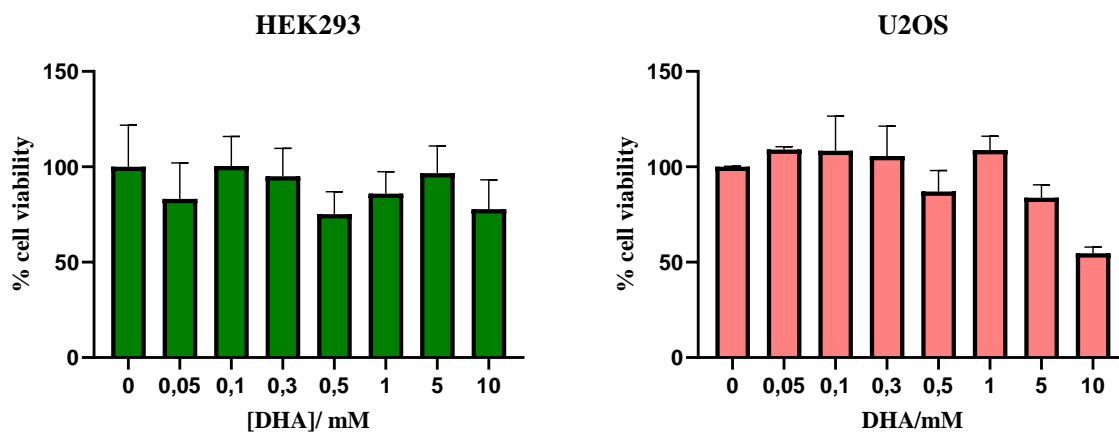


Figure 2. Cell viability of HEK293 (human embryonic kidney) cells and U2OS cells (osteosarcoma) after treatment with DHA for 72 hours determined using MTT test. Data are expressed as percentage of negative control, mean \pm standard deviation, $n = 3$.

Results show that the cell viability in all three sarcoma stem cell types is drastically lowered upon treatment with 5 mM and 10 mM DHA (Figure 3). The effect is visible in both treatments and is not dependent on bFGF presence. DHA treatment on chondrosarcoma stem cells decreased their viability only at 5 mM and 10 mM concentrations and there is no difference depending on the presence or absence of bFGF in medium across the whole range. It is noticeable that osteosarcoma stem cells treated with 5 mM DHA have the lowest cell viability across all samples. There is also a big decrease in cell viability with 10 mM DHA treatment. Treatment in medium without bFGF shows higher viability than the same concentration of DHA prepared in medium with bFGF, except in the case of 1 mM DHA.

Rhabdomyosarcoma stem cells show lower viability when the treatment was prepared in medium without bFGF, but with one exception at 1 mM DHA where the treatment with bFGF significantly decreased cell viability. Significant decreasing of cell viability was also caused by 5 mM and 10 mM DHA. Inhibition of cell growth in commercial osteosarcoma cell line U2OS was not as prominent as in patient derived sarcoma stem lines, no matter the sarcoma type.

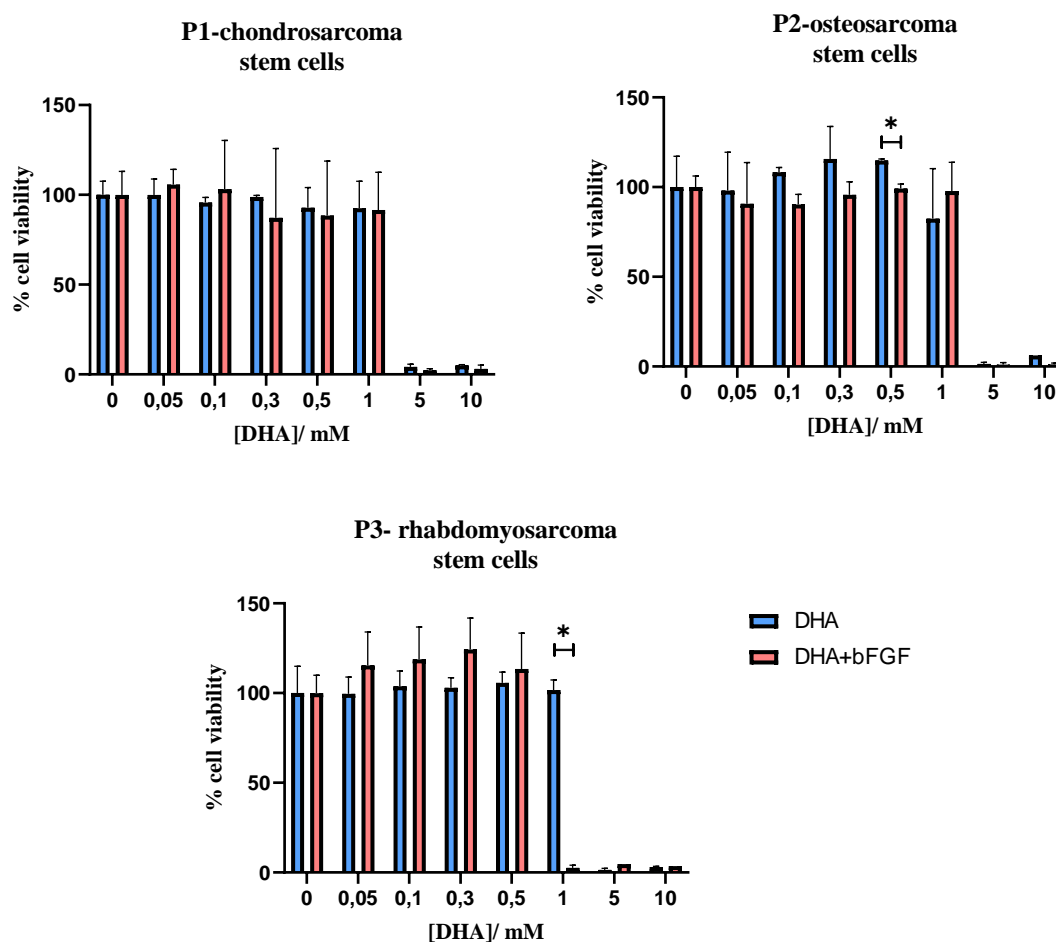


Figure 3. Cell viability of patient-derived sarcoma stem cells after treatments with DHA for 72 hours determined by using MTT test. Two groups of DHA treatment were prepared, one with supplementation of bFGF in culture medium and one without bFGF. Data are expressed as percentage of negative control, mean \pm standard deviation, n = 3. The data were statistically processed with multiple unpaired t-test in program GraphPad Prism 9.5. Statistical significance: * $p \leq 0.05$.

4.2. Changes in gene expression of metabolically relevant genes

In order to examine if any changes in metabolism occur when sarcoma stem cells are treated with oxidized form of vitamin C, dehydroascorbic acid, I measured gene expression of *GAPDH* and *PPARGCIA* by quantitative PCR. *GAPDH* codes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a crucial enzyme in glycolysis and *PPARGCIA* for peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 α) which is a master regulator of mitochondria biogenesis. Upon validation of the no-template and no-RT controls, qPCR data were analyzed using the $\Delta\Delta C_q$ method. Control group of cells treated in media with bFGF was chosen as a group for normalization of the data after calculating fold change ($2^{-\Delta\Delta C_q}$). That group was chosen for normalization because presence of bFGF is known to be necessary in natural growth conditions. As endogenous control I used the *ACTB* gene that codes for β -actin which is cytoskeletal protein.

Chondrosarcoma stem cells show significant decrease in expression of *GAPDH* (Figure 4) compared to untreated control. The effect is most visible in the treatment with medium without bFGF and both treatments with DHA gave similar fold change results, with the bFGF variant showing slight increase in comparison to DHA in medium without it. Osteosarcoma stem cells show significant decrease in *GAPDH* expression in both treatments without bFGF- medium or 1 mM DHA. Treatment with DHA in the medium with bFGF shows expression similar to untreated control group. Rhabdomyosarcoma stem cells show the biggest increase in *GAPDH* expression after treatment with DHA in medium with bFGF, slight increase is noticeable after treatment with DHA alone and modest decrease is noticeable in the sample where the cells were grown in medium without bFGF. Looking across all samples the most prominent decrease after DHA treatments occurred in the chondrosarcoma stem cells which have the lowest expression of *GAPDH*. Also, chondrosarcoma stem cells are the only one that don't show considerable difference between DHA treatments in different media. In other two samples 2x increase in expression of *GAPDH* is noticeable in DHA with bFGF treatment group compared to DHA without bFGF. The expression decreased in all three types of sarcoma stem cells when they were grown in medium without bFGF.

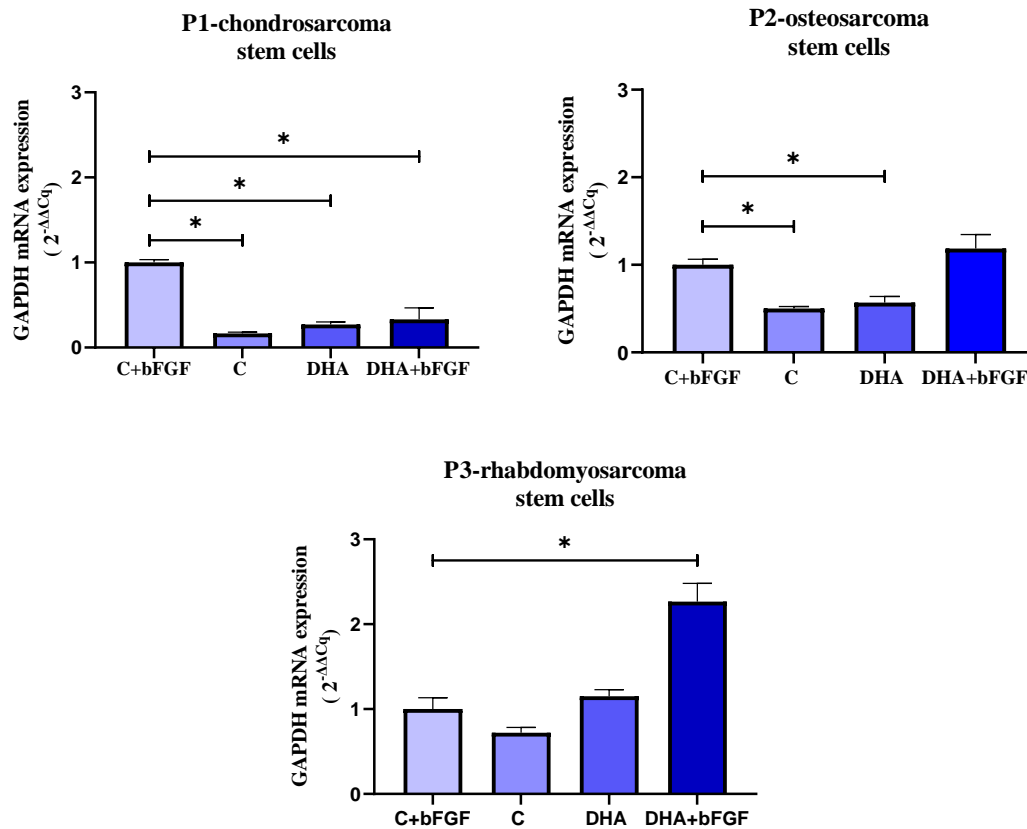


Figure 4. Fold change ($2^{-\Delta\Delta Cq}$) in the expression of *GAPDH* after 7 days treatment with 1 mM DHA. Treatment was prepared in the medium without bFGF (DHA), and in the medium with bFGF (DHA+bFGF) and it was changed every two days. Control cells that were grown in medium with bFGF were chosen as untreated control (C+bFGF) and the gene for β -actin was used as endogenous control. C-cells that were grown in medium without bFGF. The data are presented as the mean \pm standard deviation, n=3. Multiple unpaired t-test was used to determine the significance of differences between groups. Statistical significance: * $p \leq 0.05$.

The expression of gene that codes for PGC-1 α (Figure 5) shows significant decrease in chondrosarcoma stem cells after any of treatments. Osteosarcoma stem cells after treatment with DHA in the medium without bFGF also show decrease in expression of *PPARGCIA*, as well as after treatment with medium without bFGF, but the expression increased after treatment with DHA in the medium with bFGF. However, the biggest increase in expression is visible in rhabdomyosarcoma stem cells that were treated with DHA in the medium with bFGF. Other two treatments on that sarcoma stem cell type caused decrease of *PPARGCIA* expression. While the chondrosarcoma stem cells show decreased expression of *PPARGCIA* when they are treated in medium with bFGF compared to the medium without it, other two sarcoma types show the opposite: an increase of expression after treatment with DHA in the medium with bFGF compared to treatment with DHA alone. In all three sarcoma stem cell types expression of *PPARGCIA* was significantly decreased after seven days of incubation in the medium without bFGF.

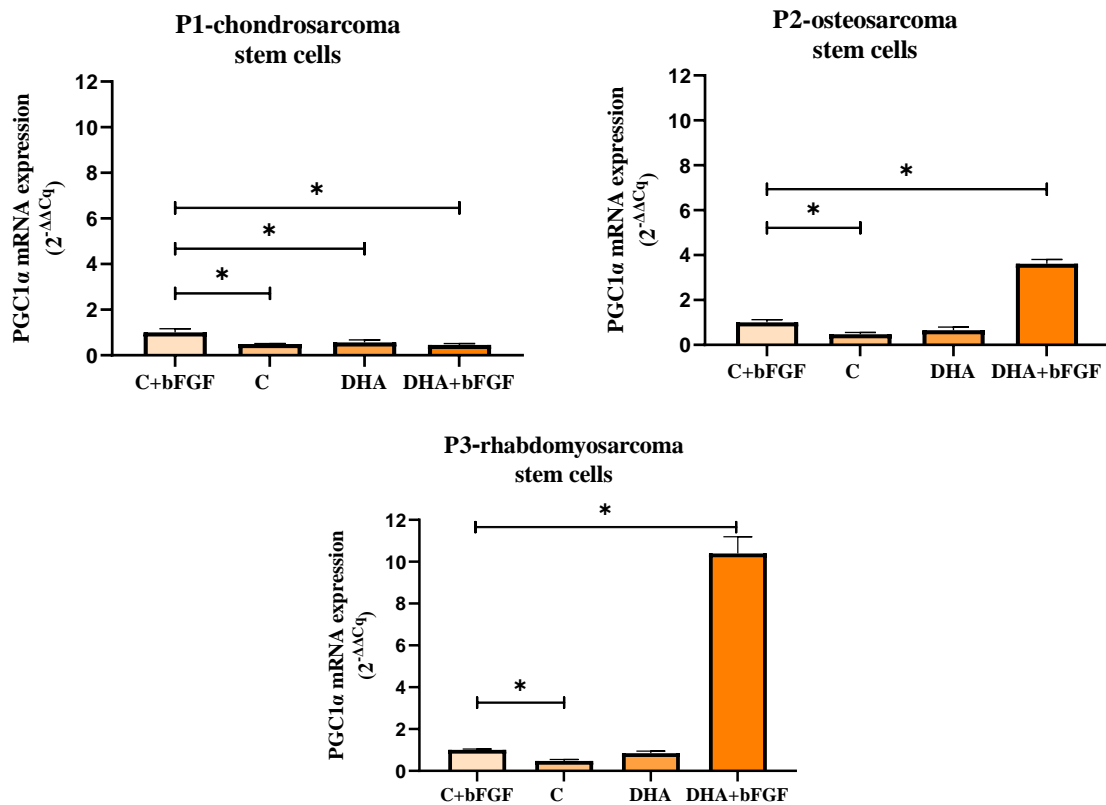


Figure 5. Fold change ($2^{-\Delta\Delta Cq}$) in the expression of *PPARGC1A* (that codes for PGC-1 α) after 7 days treatment with 1 mM DHA. Treatment was prepared in the medium without bFGF (DHA), and in the medium with bFGF (DHA+bFGF) and it was changed every two days. Control cells that were grown in medium with bFGF were chosen as untreated control (C+bFGF) and the gene for β -actin was used as endogenous control. C-cells that were grown in medium without bFGF. The data are presented as the mean \pm standard deviation, n=3. Multiple unpaired t-test was used to determine the significance of differences between groups. Statistical significance: * $p \leq 0.05$.

4.3. Changes in protein expression of GAPDH

To confirm the changes in gene expression of *GAPDH* on protein level I did Western Blot with the isolated proteins from three different types of sarcoma stem cells. *GAPDH* catalyzes the sixth step in glycolysis and is regulated by many transcriptional and posttranscriptional mechanisms, so I wanted to see how DHA affects its expression. I isolated the proteins after seven days 1mM DHA treatment with and without bFGF in the medium.

As can be seen on Figure 6 in all the wells protein separated nicely on the gel and then transferred fully to the membrane. To normalize the results, I used total protein content method instead of housekeeping gene and then with the normalized volumes of GAPDH signals for each treatment I calculated the fold change. I divided normalized volume of each sample type with the normalized volume of corresponding untreated control (cells grown medium with bFGF) to get the fold change. That group was chosen for normalization for the same reasons as in qPCR calculations.

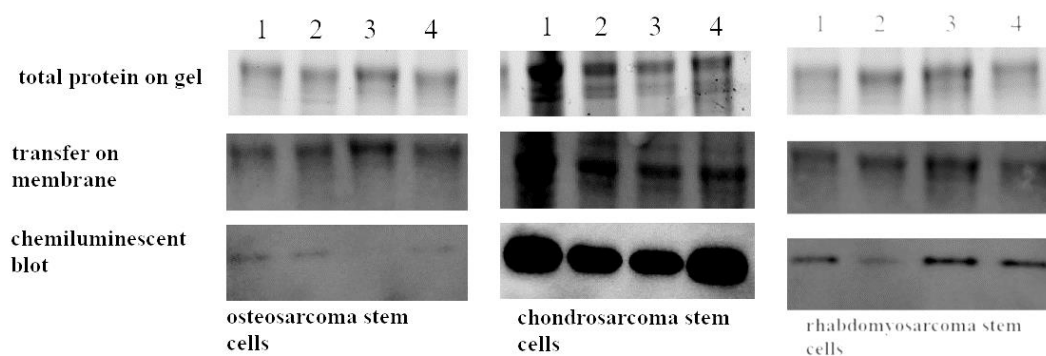


Figure 6. Expression of GAPDH protein after 7-day treatment with 1 mM DHA in medium with and without bFGF. Treatment was changed every two days. 1- culture medium without bFGF, 2- culture medium with bFGF, 3- 1 mM DHA in medium without bFGF, 4 - 1 mM DHA in medium with 0.1% bFGF. Pictures taken in Bio-Rad ChemiDoc Imaging System.

Results of fold change in protein expression of GAPDH are presented in Figure 7. The expression of GAPDH after 1 mM DHA treatment shows decrease in chondrosarcoma stem cells, but it increased after treatment with DHA in the medium with bFGF. Growth in medium without bFGF didn't show any effect on expression of GAPDH in chondrosarcoma stem cells. The protein expression does not follow the gene expression in this sarcoma stem cell type except in the case of DHA treatment in medium without bFGF where the expression was decreased. Osteosarcoma stem cells show increase in GAPDH expression after growth in medium without bFGF, but the expression decreased after both DHA treatments. Such effect is absent in qPCR results where only treatment with DHA without bFGF caused

decrease in expression. In the case of rhabdomyosarcoma stem cells there is a noticeable increase in GAPDH expression in all three treatment groups. That is quite in line with the results for *GAPDH* gene expression where, in the case of this sarcoma stem cell type, there was also observable increase, predominantly after DHA with bFGF treatment. When comparing across the samples rhabdomyosarcoma stem cells have the highest expression of GAPDH regardless of the treatment and the greatest efficacy on decreasing GAPDH expression is noticeable in osteosarcoma stem cells. In all three sarcoma stem cell types treatment with 1 mM DHA in the medium with bFGF increased the GAPDH expression in contrast to 1 mM DHA treatment.

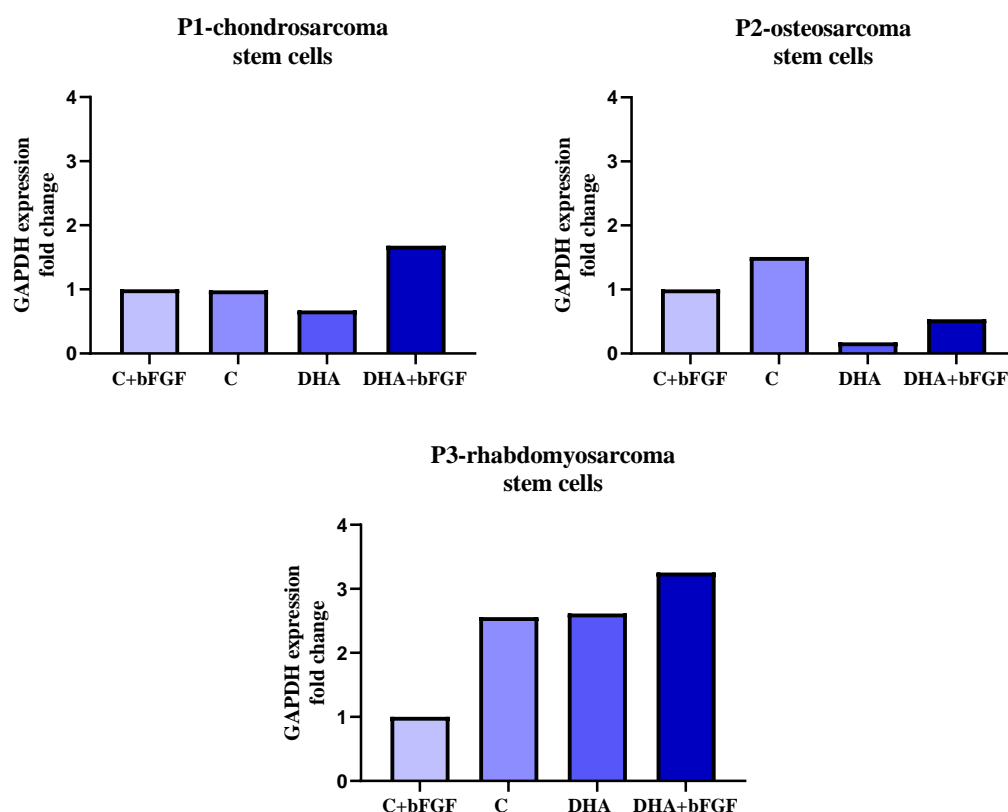


Figure 7. Fold change of GAPDH expression after 7-day treatment with 1 mM DHA or 1 mm DHA in medium with bFGF. The treatments were changed every two days and then proteins were isolated. Instead of Western Blot loading control, total protein on the gel was used for normalization. All signals have their background subtracted and the fold change was calculated by dividing normalized volume of the signal for every treatment with the normalized volume of untreated control that are cells grown in medium with bFGF (C+bFGF). C-medium without bFGF, DHA- 1 mM DHA in medium without bFGF, DHA+bFGF- 1 mM DHA in medium with bFGF.

5. DISCUSSION

Cancer stem cells (CSC) are a subtype of tumor cells that exhibit some similarities to mesenchymal stem cells. The spread of tumors, resistance to treatments, the occurrence of metastasis and relapse are all caused by CSC. CSC have been found in sarcomas, a rare and highly heterogeneous group of malignancies (Martínez-Delgado et al., 2020). Since Pauling and Cameron first proved the anti-tumor effect of intravenously delivered vitamin C in the 1970s, there has been a disputable history around the use of vitamin C in tumor therapy. After many disagreements, research from today demonstrates that tumor cells are preferentially targeted by therapeutic amounts, in millimolar range, of vitamin C in vitro without harming normal cells (Lv et al., 2018). Since it has been demonstrated that high vitamin C concentrations are necessary to see its anti-tumor potential, DHA concentrations for MTT assay were chosen in range from 0.05 to 10 mM. Results from MTT assay done on human embryonic kidney (HEK293) and osteosarcoma (U2OS) cell line support the claim that vitamin C preferentially kills tumor cells. Even though an obvious trend can't be noticed, cell viability decreased more in U2OS cell line after treatment with high concentrations DHA (5 mM and 10 mM) than in HEK293.

MTT assay with DHA in the range from 0.05 to 10 mM was also done on three patient-derived cancer stem cells originating from chondrosarcoma (P1), osteosarcoma (P2) and rhabdomyosarcoma (P3). This treatment with DHA was done in two different types of culture medium: one that contained 0.1% bFGF and the other one without it. bFGF is necessary in culture medium for embryonic and induced pluripotent stem cells because it maintains their capacity for self-renewal, rapid proliferation, and multilineage differentiation. This growth factor activates different signaling pathways and is also used in culture medium for cancer stem cells to keep their multipotency (Onuma et al., 2015). All three sarcoma stem cell types showed significant decrease after treatment with 5 mM and 10 mM DHA when almost all cells in the sample were killed. In lower concentrations DHA had little impact on cell viability, except in the case of rhabdomyosarcoma stem cells where 1 mM DHA in medium with bFGF showed noticeable decrease in cell viability. On the other hand, treatment with 1 mM DHA in medium without bFGF did not affect cell viability and that difference between two treatments of the same DHA concentration is statistically relevant but biological relevance could be doubted because it would be expected that treatment without bFGF would decrease cell viability. In osteosarcoma sample, decrease of cell viability happened after treatment with 0.5 mM DHA in medium with bFGF but even though it is statistically relevant, cell viability after treatment with 0.5 mM DHA exceeds 100%, which was the viability of untreated control, so it is most likely not biologically relevant. Explanation for cell viability exceeding the untreated control could be that the treated cells are metabolizing the MTT more rapidly and therefore the absorbance value is larger.

Many studies showed that vitamin C kills cancer cells when applied in millimolar concentrations. Studies demonstrated synthetic-lethal metabolic strategy on CSC combining doxycycline and vitamin C (Fiorillo et al., 2019; Francesco et al., 2017) but also the treatment of vitamin C alone successfully eradicated liver CSC (Lv et al., 2018). Fukui et al. (2015) attributed the noticed cytotoxic effects of vitamin C on pancreas cancer cells to its pro-drug function which induces oxidative stress in the cell and suppresses proliferation of malignant cells. They proposed another mechanism of vitamin C-induced cell death which is activation of caspase-independent apoptosis pathway. The effect of vitamin C on pancreatic cancer cells was also studied by Du et al. (2010) who found that treatment reduced viability in all tested cell lines, but didn't affect immortalized pancreatic ductal epithelial cell line. It is important to note that they used 5 mM and 10 mM concentrations of vitamin C that also showed significant effects on decreasing cell viability in this experiment on sarcoma stem cells. Those are considerably high concentrations, but they can be achieved in patients by intravenous dosing. Millimolar concentrations of vitamin C were also used by Chen et al. (2005), they found that 2 mM concentration resulted in almost 100% cell death of lymphoma cells, but in contrast did not affect normal lymphocytes and monocytes. Some experiments do not clearly state which form of vitamin C was used, so it is not possible to know if oxidized or reduced vitamin C mediated the effect on cells. However, part of researchers clearly state that they used DHA. One of those examples is the study by Rivière et al. (2006) which suggests that Jurkat cell death was a consequence of DNA damage induced by DHA. The relevance of DHA in reducing cell viability was also confirmed in a study on *KRAS* and *BRAF* mutant colorectal cancer cells which were selectively killed by high concentrations of oxidized vitamin C (Yun et al., 2015).

Cancer cells modify their cellular metabolism to sustain their rapid proliferation rate. For the biosynthesis of ATP and other building blocks, cancer cells preferentially use glycolysis, which is less effective than oxidative phosphorylation, a process known as Warburg effect. However, CSC display a distinctive metabolic profile that, depending on the kind of cancer, can be heavily glycolytic or OXPHOS reliant, as opposed to differentiated bulk tumor cells that rely on glycolysis (Sancho et al., 2016).

To see what the effect of DHA on sarcoma stem cell glycolysis is, *GAPDH* gene expression was measured. *GAPDH* was chosen because it is an essential regulator of glycolysis oftentimes overexpressed in various cancer types and its expression is controlled by transcription factors which are frequently mutated in cancer such as HIF-1 α , p53 or AP-1. Additionally, *GAPDH* gene has insulin (IRE) and hypoxia response elements (HRE) in its promoter (Zhang et al., 2015). Results of this experiment show changes in gene expression of *GAPDH* after seven days treatment with 1 mM DHA, but they vary between sarcoma stem cell types. In sample P1, two DHA treatments, as well as treatment with culture medium without bFGF resulted in decreasing of *GAPDH* expression and all those results are statistically significant when compared to untreated control (cells that were grown in culture medium with bFGF).

Sample P2 also showed statistically relevant decrease in *GAPDH* expression after treatment with DHA without bFGF and medium without bFGF but showed slight increase after treatment with DHA and bFGF. The third sample's results don't quite match the results from previous two sarcoma stem cell types, because here only treatment with medium without bFGF caused noticeable decrease of *GAPDH* expression whereas other two treatments resulted in increased expression. It could be explained by extreme heterogeneity of sarcoma cells which also affects their metabolism.

Sarcomas are metabolically diverse, therefore some cancer cells in well-perfused areas consume glucose and maintain glycolysis and OXPHOS, whereas cells in poorly perfused areas rely on alternative carbon sources (Esperança-Martins et al., 2021). In addition, correlation of *GAPDH* overexpression with increased HIF-1 α protein levels has been suggested. Vitamin C is known to activate vitamin C-dependent hydroxylases which then downregulate HIF-1 α under normal oxygen conditions. However, in hypoxic conditions inhibition of HIF-1 α hydroxylation occurs and consequently the expression of genes that have HRE in their promoters, such as *GAPDH*, increases. Hypoxic microenvironment could be the explanation why the rhabdomyosarcoma stem cell sample shows increase in *GAPDH* expression after treatment with DHA (Satheesh et al., 2020).

The idea that cancer stem cells primarily rely on glycolytic metabolism (as do the normal stem cells) for generation of ATP has been put forth in the past, and it has been demonstrated to be true in several cancer types (i.e., breast, nasopharyngeal and hepatocellular stem cells). However, studies on certain other malignancies (i.e., glioblastoma and leukemia stem cells) presented evidence that OXPHOS was the preferred method of energy production (Sancho et al., 2016). Although, the mechanistic insight into reported OXPHOS phenotype is still to elucidate, proteins regulating mitochondrial biogenesis and structure may have a significant role in maintaining stemness features and functionality. Research by Sancho et al. (2015) speaks in favor of this thesis. They showed that in pancreatic ductal adenocarcinoma stem cells, PGC-1 α , a master regulator of mitochondrial biogenesis, was crucial for maintaining stemness properties, OXPHOS functionality and maximum in vivo tumorigenic capacity.

To see how DHA treatments affected sarcoma stem cells OXPHOS pathway, qPCR for gene *PPARGCIA*, that encodes for PGC-1 α , was performed. In sample P1 treatments with DHA, as well as medium without bFGF reduced *PPARGCIA* expression and the levels of PGC-1 α mRNA are similar after all treatments. In sample P2, treatment with DHA and bFGF caused significant increase of *PPARGCIA* expression, whereas two other treatments led to decrease of expression. These results for *PPARGCIA* expression are analogous to the results for *GAPDH* expression in the same sample, which could mean that for osteosarcoma stem cells, DHA treatment in medium with bFGF does not affect either glycolysis or OXPHOS, but DHA treatment without bFGF decreases flux in both processes. Considerable increase of *PPARGCIA* expression is also visible in sample P3 after treatment with DHA

in the medium with bFGF and this treatment also augmented expression of *GAPDH*. However, in contrast to *GAPDH* expression that increased after DHA treatment without bFGF, PGC-1 α mRNA levels decreased after the same treatment which could suggest that this specific rhabdomyosarcoma sample relies more on glycolysis as energy source after application of DHA.

PGC-1 α is a transcriptional coactivator crucial for regulating mitochondrial biogenesis and oxidative metabolism. Environmental and physiological stimuli control *PPARGC1A* expression and activity. *PPARGC1A* expression is primarily induced by temperature, more specifically by the reaction to cold. *PPARGC1A* is extremely sensitive to the cell's energy level. The AMP-activated protein kinase (AMPK), a sensor of cellular AMP levels, also controls PGC-1 α transcription. It is known that exercise causes activation of AMPK in muscle tissue, which upregulates mitochondrial biogenesis and metabolism. Altered cellular metabolism is characteristic of cancer cells so it's no surprise that PGC-1 α is involved in modifications of cancer cells (Bost & Kaminski, 2019). Its function in cancer is very diverse and depends on cancer type. For example, high PGC-1 α in breast cancer is related to metastasis and overall poor prognosis (Klimcakova et al., 2012), while high PGC-1 α in prostate cancer implicates completely opposite prognosis; it acts as suppressor of tumor propagation and lowers tumor aggressiveness (Torrano et al., 2016). PGC-1 α inhibits forming of metastasis in melanoma as well but surprisingly it enables proliferation and survival of cancer cells (Luo et al., 2016). Due to this duality of function, PGC-1 α effect on sarcoma cells needs to be thoroughly examined because conclusions cannot be drawn by comparing with other cancer types since they all respond individually. Research and analyses of PGC-1 α expression could be of extreme importance especially for CSC because it is suggested that their maintenance depends on mitochondrial function, no matter the primary energy producing process (Sancho et al., 2016).

Many different types of human cancer show elevated GAPDH levels, which are frequently associated with decreased survival. GAPDH dysregulation in malignancies reveals the ambiguous functions of this enzyme in determining cell fate. GAPDH inhibition causes cease in glycolysis and induces energetic crisis in cell which then leads to cell death. GAPDH is regulated at transcriptional level and also by posttranslational mechanisms (Zhang et al., 2015). For that reason, along with qPCR, to check how the seven days treatment with DHA (in medium with and without bFGF) affected GAPDH on protein level, Western Blot was performed.

Obtained results from sample P1 show that DHA treatment in medium without bFGF caused decrease of GAPDH protein expression compared to untreated control, this is in line with results of *GAPDH* gene expression. However, GAPDH expression increased after DHA treatment in medium with bFGF even though gene expression after this treatment was decreased. Discrepancy between gene expression and protein levels could arise from the fact that GAPDH is regulated by posttranslational mechanisms. Nonetheless, the difference in protein levels after two types of DHA treatment could be

explained by previously mentioned study (Onuma et al., 2015) that refer to bFGF as necessary growth factor for normal functioning of stem cells and also by study which showed that bFGF signaling enhances aerobic glycolysis (Bradley, 2018). Therefore, it could be that the effect of DHA treatment on decreasing GAPDH expression is further enhanced by removal of bFGF from culture medium. Both DHA treatments in P2 sample also show the trend observed in P1 sample, which is decreased level of GAPDH after treatment with DHA in medium without bFGF and higher expression after DHA treatment with bFGF. *GAPDH* gene expression also decreased after DHA treatment. As opposed to P1 where protein level after DHA with bFGF treatment exceeds untreated control, in this case the protein expression after DHA with bFGF treatment is lower than the untreated control, which suggests that osteosarcoma stem cells responded more favorably to DHA in general. Sample P3, again, differs from two other sarcoma stem cell types. The gene expression of *GAPDH* was most increased after treatment with DHA in the medium with bFGF and that is the case for protein expression as well. Increase also occurred after treatments with DHA in the medium without FGF or just the culture medium without bFGF, but the fold change is lower than after DHA with bFGF treatment. This could mean that for rhabdomyosarcoma stem cells, greater impact on GAPDH expression comes from removal of bFGF from culture medium than the addition of DHA treatment itself.

Inhibitory effects of high doses of vitamin C on GAPDH were also observed in study by Uetaki et al. (2015) in which they showed that vitamin C in concentrations > 1 mM significantly alters the metabolomic profile of breast cancer cells. They found that levels of glycolytic metabolites upstream of GAPDH increased while the downstream ones decreased. In addition to glycolysis intermediates accumulation, upstream intermediates of Krebs cycle, citrate and cis-aconitate, also accumulated. They confirmed that altered energy flux in glycolysis and Krebs cycle is a result of vitamin C mediated H_2O_2 generation and that significant decrease in ATP levels in cells is observed. Their study showed that the observed cell death after vitamin C treatment inactivated GAPDH by depleting NAD^+ , which is in agreement with studies by Colussi et al. (2000) and Yun et al. (2015). The proposed mechanism by which DHA treatment led to inhibition of GAPDH is S-glutathionylation as a consequence of ROS targeting cysteine residues in GAPDH (Yun et al., 2015). Additionally, they suggested another mechanism that causes loss of GAPDH activity which is NAD^+ depletion. They found that levels of NAD^+ , which is a substrate for GAPDH, were diminished after DHA treatment and explained that as a consequence of PARP (which also uses NAD^+) activation due to DNA damage caused by vitamin C-induced ROS. As well as previously mentioned study, Yun et al. (2015) also showed accumulation of glycolysis intermediates upstream of GAPDH.

Obtained results show that DHA affects glucose metabolism as well as OXPHOS in sarcoma stem cells. They confirm that vitamin C can induce alterations in metabolism of cancer cells which is meaningful for further research of anti-tumor properties of vitamin C. Nonetheless, development of novel therapeutic strategies that would include vitamin C needs to acknowledge severe heterogeneity of

tumors and their specific genetic and metabolic background, which greatly influences the response to treatment.

6. CONCLUSION

Cancer stem cells can initiate and drive tumor growth, cause metastasis and confer resistance to conventional chemotherapy. Because of their properties they are recognized as key targets for development of novel therapeutic strategies. A lot of studies have shown that pharmacologic concentrations of vitamin C selectively kill various cancer cell lines, however it is still not unambiguously determined whether ascorbate has a negative impact on CSC.

The aim of this work was to elucidate the effects of oxidized form of vitamin C, dehydroascorbic acid (DHA), on metabolic profile of patient-derived sarcoma stem cells. Used sarcoma stem cells were isolated from patients with chondrosarcoma, osteosarcoma, and rhabdomyosarcoma and all three types of cells showed reduced cell viability after treatment with millimolar doses of DHA. In contrast, HEK293 cells showed greater resistance to high concentrations of DHA, which supports previous studies that state how vitamin C is not toxic to normal cells and shows minimal negative side effects when given as adjuvant therapy in patients. To gain better insight into the metabolic alterations after DHA treatments prepared in culture medium with and without bFGF, gene expression of *GAPDH* and *PPARGC1A* was examined as well as GAPDH protein levels. bFGF is standard supplement in culture medium for maintaining CSC in vitro, so by preparing two types of DHA treatment its role was also examined. *GAPDH* encodes for glycolytic enzyme GAPDH, and many papers suggest that its activity is inhibited by vitamin C. After DHA treatments chondrosarcoma stem cells showed decrease of *GAPDH* expression and Western Blot confirmed that DHA treatment decreases the expression of GAPDH. However, treatment with DHA and bFGF caused increased levels of protein. Same can be said about osteosarcoma stem cells, where treatment with DHA also decreased expression, while treatment with DHA and bFGF increased it which could suggest that bFGF supports glycolytic metabolism of these cells. Rise in *GAPDH* expression also occurred in rhabdomyosarcoma stem cells, here after both DHA treatments. *PPARGC1A* encodes for PGC-1 α , a master regulator of mitochondrial biogenesis and oxidative phosphorylation (OXPHOS). Most noticeable increase in its expression was observed after DHA with bFGF treatment in rhabdomyosarcoma stem cells, which showed unfavorable response to all treatments.

These results confirm high heterogeneity of sarcomas and show how some types of stem cells are more dependent on FGF signaling, some have higher glycolytic flux, while others depend more on OXPHOS. Vitamin C has potential as anti-tumor treatment that targets metabolism, nevertheless genetic background of tumor needs to be considered in order to demonstrate positive effects in patients.

7. LITERATURE

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8. BIOGRAPHY

I was born in Zadar in 1999. After finishing general grammar school in Zadar, in 2017 I enrolled Undergraduate Degree in Molecular Biology on Faculty of Science in Zagreb. In my first year I took part in scientific event *Noć biologije* (Night of biology) that is organized annually to promote interest for science in wider audience. Last year of my undergraduate degree I did laboratory practice in Division of Molecular Biology under the mentorship of Associate Professor Ivana Ivančić Baće, PhD. In 2020 I enrolled Graduate Degree in Molecular Biology on the same faculty. That year I did laboratory practice in The Croatian Institute for Brain Research. First, I did laboratory practice in Laboratory for Neuro-Oncology under (laboratory leader: Professor Pećina Šlaus, PhD) and continued my practice in Laboratory for Neuro-Chemistry (laboratory leader: Professor Kalanj-Bognar, PhD). In 2020 I received University of Zagreb Rector's Award for student scientific paper *Computational investigation of properties of non-proteinogenic amino acid norvaline within the elements of the secondary protein structure*. My mentor for that paper was Associate Professor Aleksandra Maršavelski and main methodology used for that research was Molecular Dynamics Simulations. In the third semester of my graduate studies, I went on an ERASMUS+ student exchange in Girona, Spain, where I also did a laboratory internship in the research group for Targeted Antitumor Therapies (laboratory leader: Professor Silvia Barrabes, PhD), which is focused on developing new strategies for a more effective and selective treatment of cancer.