

## Title page

Title: Ferroptosis, acyl starvation and breast cancer

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## Abbreviations

$\alpha$ KG –  $\alpha$ -ketoglutarate, AOA – amino-oxyacetate, ACLS4 – acyl-CoA synthetase long-chain family member 4, BSO – DL-buthionine-[S,R]- sulfoximine, CARS – carboxylic acid reductase, CBS – cystathionine  $\beta$ -synthase, CGL – cystathionine gamma-lyase, DMT1 – divalent metal transporter 1, FABP – fatty-acid-binding protein, FANCD2 – Fanconi anemia complementation group D2, CD36 – fatty acid translocase, Fum – fumarate, GCLC – glutamate-cysteine ligase, Gln – glutamine, Glu – glutamate, GLS2 – glutaminase, GR –

glutathione-disulfide reductase, GSH – glutathione, GSS – glutathione synthetase, GSSG – glutathione disulfide, GOT – glutamic-oxaloacetic transaminase, GPX4 – glutathione peroxidase 4, Hcy – homocysteine, HMG-CoA – 3-hydroxy-3-methylglutaryl coenzyme-A, HMGCR – 3-hydroxy-3-methylglutaryl-coenzyme A reductase, HMOX1 – heme oxygenase 1, HSBP1 – heat shock protein beta-1, IREB2 – iron response element-binding protein 2, IRP – iron regulatory protein, LD – lipid droplets, LIP – labile iron pool, LOX3 – lipoxygenase, LPCAT3 – lysophosphatidylcholine acyltransferase, Mal – malate, MAT – methionine adenosyltransferase, MT – methyl transferase, MUFA – monounsaturated fatty acids, NCOA4 – nuclear receptor activator 4, OXPHOS – mitochondrial oxidative phosphorylation system, PLOH – hydroxyl lipid, PLOO<sup>•</sup> – phospholipid peroxy radical, PLOOH – phospholipid hydroperoxide, POR – cytochrome P450 reductase, PUFA – polyunsaturated fatty acids, PUFA-PL – phospholipid containing polyunsaturated fatty acid chain, RCD – regulated cell death, RLS3 – ras-selective lethal small molecule, RTA – radical-trapping antioxidants, ROS – reactive oxygen species, SAHN – S-adenosylhomocysteine/Methylthioadenosine nucleosidase, SCD1 – Stearoyl-CoA desaturase ( $\Delta$ -9-desaturase), SGLT2 – sodium-glucose cotransporter 2, SLC1A5 – neutral amino acid transporter belonging to the SLC1 family, SLC3A2 – glutamate/cystine antiporter solute carrier family 3 member 2, SLC7A11 – glutamate/cystine antiporter solute carrier family 7 member 11, SREBP1 – sterol regulatory element-binding protein 1, STEAP3 – Metalloreductase, Suc – succinate, TCA – tricarboxylic acid cycle, TFRC – transferrin receptor, TXNRD1 – thioredoxin reductase, VDAC – voltage-dependent anion channel.

## Abstract

To maintain their growth rate, cancer cells must secure a supply of fatty acids, which are necessary for building cell membranes and maintaining energy processes. This is one of the reasons why tissues with intensive fatty acid metabolism, such as the mammary gland, are more likely to develop tumours. One natural or induced defence process against cancer is ferroptosis which interferes with normal fatty acid metabolism. This leads to the oxidation of polyunsaturated fatty acids, which causes a rearrangement of the metabolism and damages cell membranes. As a consequence of this oxidation there is a shortage of normal polyunsaturated fatty acids, which disturbs the complicated metabolism of fatty acids. This imbalance in metabolism, resulting from the deficiency of properly structured fatty acids, is called, by these authors, "acyl starvation". When cancer cells are exposed to alternating hypoxia and reoxygenation, they often develop resistance to neoadjuvant therapies. Blocking the stearoyl-CoA desaturase – fatty-acid-binding protein4 – fatty acid translocase axis appears to be a promising pathway in the treatment of breast cancer. On the one hand, the inhibition of desaturase leads to the formation of toxic phospholipid hydroperoxides in ferroptosis, while, on the other hand, the inhibition of fatty-acid-binding protein4 and translocase leads to a reduced uptake of fatty acids and disruption to the cellular transport of fatty acids resulting in intracellular acyl starvation. The disruption in the metabolism of fatty acids in cancer cells may augment the effectiveness of neoadjuvant therapy.

## Significance Statement

Regulation of the metabolism of FAs in cancer cells seems to be a promising therapeutic direction. Studies show that the induction of ferroptosis in cancer cells, combined with use of classic neoadjuvant therapies, effectively inhibits the proliferation of these cells. We link the process of ferroptosis with apoptosis and SCD1-FABP4-CD36 axis and propose the term ‘acyl starvation’ for the processes leading to FAs deficiency, dysregulation of FAs metabolism in cancer cells and, most importantly, the appearance of incorrect proportions FAs.

## Introduction

In addition to the growth, differentiation and proliferation of cells, the fourth process that completes the cycle of life is the death of the cells. The cause of death may be the activation of self-destruction mechanisms or the occurrence of disturbances in the cell metabolism caused by external or intracellular factors. An example of regulated cell death (RCD) is ferroptosis - iron-dependent lipid peroxidation resulting from disruption to intracellular iron metabolism. As a result, the redox potential of the cell is disturbed, which leads to the oxidation of cellular lipids (Jiang et al., 2021). This causes damage to the cell membranes, a loss of stimuli, an increase in the entropy of the cell and, eventually, its death. The key compounds in the regulation of ferroptosis are glutathione and glutathione peroxidase 4 (GPX4). Glutathione is the most common reductant in mammalian cells. It is, first of all, a cofactor of GPX4, a selenoperoxidase, whose main role is to protect cells against oxidation by peroxides generated during biochemical processes such as hydrogen peroxide ( $H_2O_2$ ) and phospholipid hydroperoxide (PLOOH) among others. Glutathione deficiency or a decrease in GPX4 activity exacerbate ferroptosis (Dixon et al., 2012). The mechanism of ferroptosis regulation, however, is much more complicated. There are numerous metabolic pathways involved, such as the transsulfuration pathway, the mevalonate (MVA) pathway, the lipid peroxidation pathway, the Stearoyl-Co-enzyme A (CoA) desaturase-1 (SCD1) pathway, the fatty acid membrane transport pathway, the transferrin/ferritin pathway, the  $x_c^-$  cistine/Glu antiporter system and many others. These processes directly or indirectly regulate the level of cellular glutathione and thus intensify or inhibit ferroptosis.

In recent years, ferroptosis has become a subject of interest for oncologists, especially in terms of supporting hormone or drug therapy. Ferroptosis interferes with many biochemical pathways but, most importantly, it interferes with the normal metabolism of fatty acids, which are used in large quantities for the construction of cell membranes and energy processes of rapidly dividing tumour cells. Therefore, tissues with intensive fatty acid metabolism, such as the mammary gland, are more likely to develop tumours. It has been observed that cancer cells tend to become resistant to neoadjuvant therapies. The reason for the successful survival of cancer cells is the ease with which they adapt to metabolic and oxidative stress. Although the mechanism is not fully understood, it is known that resistance is connected with the occurrence of hypoxia in tumour cells. There are two types of hypoxia. Chronic hypoxia is located mainly around the necrotic areas of the cancer tumour and is the result of the increasing distance between the proliferating cancer cells and the blood vessel (diffusion-limited hypoxia) (Rofstad et al., 2007). Cyclic (transient, acute) hypoxia is characterised by the occurrence of alternating states of hypoxia and reoxygenation, and it appears in different areas of the tumour as a result of changes in blood flow, the presence of non-functional vessels and the process of vascular remodelling during angiogenesis (perfusion-limited hypoxia/intermittent hypoxia) (Carmona-Bozo et al., 2021). Both types of hypoxia lead to the cancer cells becoming more aggressive and have an adverse effect on the success of the therapy. (Muz et al., 2015). In studies involving various drugs, small molecule-induced ferroptosis has been shown to have a strong inhibitory effect on tumour growth in a drug-resistant environment, which may increase the sensitivity of the tumour to chemotherapeutic treatment (Luis et al., 2021). The question is, however, how does ferroptosis reduce the resistance of cancer cells to classic neoadjuvant therapies? This review is an attempt to answer this question. The first part will discuss the mechanisms which lead to ferroptosis,

while the second part will introduce the concept of using ferroptosis in the treatment of breast cancer.

## Cysteine metabolism

Although the concept of ferroptosis was suggested by Stockwell in 2012, (Dixon et al., 2012), this pioneering work on disruption to the cysteine metabolism, which is crucial to the process of ferroptosis, was started in the 1950s by Harry Eagle. He showed that depriving cells of cysteine resulted in their death (Eagle 1955), while endogenous cysteine synthesis inhibited this process of self-destruction (Eagle et al., 1961). These observations indicated that cysteine, as the main substrate for the biosynthesis of reduced glutathione, is an important factor in maintaining the level of intracellular oxidation potential and its presence inhibits ferroptosis. Due to the key role of glutathione in cellular metabolism, its biosynthesis from cysteine has been evolutionarily secured by several pathways. The source of cysteine may be cystine collected from the environment surrounding the cell via the  $x_c^-$  cystine/Glu antiporter system (Fig. 1). This is a transmembrane protein complex, consisting of the subunits Solute Carrier Family 7 Member 11 (SLC7A11) and Solute Carrier Family 3 Member 2 (SLC3A2), which replace Glutamate (Glu) with cystine at a ratio of 1:1. (Lin et al., 2020, Bannai & Kitamura 1980). In this mechanism, the availability of Glu, which is formed from glutamine, is important. The glutamine released from tissues is carried into the cell by means of the Solute Carrier Family 1 Member 5 (SLC1A5) transporter and then converted to Glu by means of glutaminase 1 (GLS1) and glutaminase 2 (GLS2). During the deamination reaction, Glu forms  $\alpha$ -ketoglutarate ( $\alpha$ -KG), which is then degraded in the mitochondrial tricarboxylic acid (TCA) cycle. This reaction is catalysed by the aminotransferase Glutamic-Oxaloacetic Transaminase 1 (GOT1). It has been proven that the knockdown of GLS2, which inhibits the glutamine degradation pathway (Gao et al., 2019), as well as the inhibition of GOT1 by amino-oxyacetate (AOA) (Villar et al., 2015) may secondarily inhibit ferroptosis. It should be added



here that an important factor influencing ferroptosis is the ratio of intra- and extracellular Glu concentrations. When the extracellular concentration of Glu is abnormally high, the uniform exchange of Glu and cystine is disrupted, which indirectly affects the amount of available cysteine and ultimately leads to ferroptosis due to the accumulation of oxidised lipids (Lin et al., 2020). The second source of cysteine is the transsulfuration pathway, a multi-step sequence of reactions in which cysteine is formed from methionine via s-adenosylmethionine, s-adenosylhomocysteine, homocysteine and cystathione. A third possible source of cysteine is the blockage of cysteinyl-tRNA biosynthesis by inhibiting cysteinyl-tRNA synthetase (CARS) or a knockout of the gene encoding it. This situation causes the inhibition of protein synthesis, but then increases the amount of free intracellular cysteine. The inhibition of CARS additionally induces the transsulfuration pathway, thus inhibiting ferroptosis induced by cysteine deficiency (Hayano et al., 2016).

#### The Role of Glutathione (GSH) and Glutathione peroxidase 4 (GPX4)

GSH and GPX4 are part of the  $X_c^-$ -GSH-GPX4 pathway responsible for the inhibition of ferroptosis. This pathway inhibits lipid oxidation, i.e. the formation of phospholipid hydroperoxides (PLOOH) - a form of reactive oxygen species (ROS) that destroys the cell. Formed from cysteine, GSH is an intracellular antioxidant buffer that maintains redox balance with the help of GPX4. The reduction of PLOOH and cholesterol to the corresponding alcohols by GPX4 requires the catalytic selenocysteine residues of GPX4 and two electrons mainly supplied by GSH, but also by other low molecular weight thiols or even protein thiols (Maiorino et al., 2017). In other words, GPX4 is responsible for the reduction of PLOOH to lipid alcohols (PLOH) (Lv et al., 2019). The substrates are two GSH molecules, which are the reducers of PLOOH to PLOH (Ursini et al., 1982, Ursini et al., 1985). In addition to PLOH, this reaction produces oxidised glutathione (GSSG). This form of glutathione must be reduced so that glutathione can be reused in the hydroperoxide reduction reaction. The reduction of

glutathione occurs with the participation of glutathione-disulfide reductase (GR) and nicotinamide adenine dinucleotide phosphate + hydrogen (NADPH+H<sup>+</sup>). Since GPX4 is the main PLOOH neutralising enzyme, its regulation is crucial to ferroptosis. Through the regulation of GPX4, ferroptosis is intensified by erastin and ras-selective lethal small molecule 3 (RSL3). Erastin indirectly inactivates GPX4 by inhibiting cystine import, thereby depriving the cells of cysteine, an essential cellular antioxidant and building block of GSH. Additionally, RLS3 is a direct GPX4 inhibitor. As a consequence of the action of both of these inhibitors, an accumulation of PLOOH occurs, which can cause rapid and irreparable damage to the plasma membrane, leading to the death of the cell. GPX can also be regulated by the MVA pathway. Selenocysteine-tRNA (Sec-tRNA) uses a direct product of the MVA pathway, isopentenyl pyrophosphate (IPP), to promote GPX4 maturation (Warner & Berry, 2000, Ingold et al., 2018). It is worth adding that statins, inhibiting 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGCR), reduce the regulation of the MVA pathway and the production of IPP, which may intensify ferroptosis. (Viswanathan et al., 2017).

### Lipid Peroxidation Metabolism

While it is known that the level of unsaturation of lipid bilayers is a key determinant of ferroptosis, it is still unclear how the lipid peroxidation process is initiated. It is known that it proceeds in two different ways - non-enzymatic and enzymatic. Failure to control the lipid peroxidation process chain leads to the rise of innumerable by-products, the breakdown of membrane integrity, and ultimately disruption to the membranes of organelles and cells. Thus, membranes with a high of polyunsaturated-fatty-acid-containing phospholipids (PUFA-PL), as seen in neurons, are particularly prone to peroxidation. Non-enzymatic peroxidation is a spontaneous process – an oxygen-dependent free radical chain reaction. It is divided into three stages: initiation, propagation and termination (Frank, 1950). The initiation process is associated with the generation of a free radical, formed in the Fenton reaction with the

participation of iron (Lai & Piette, 1978). Next the bis allylic hydrogen atom is removed between the two carbon-to-carbon double bonds in the polyunsaturated acyl molecules. At the next stage, acyl molecules react with molecular oxygen, resulting in the formation of a phospholipid peroxy radical (PLOO<sup>•</sup>). (Conrad & Pratt 2019, Bateman, 1954). This PLOO<sup>•</sup> removes hydrogen from another polyunsaturated-fatty-acid (PUFA), turning itself into PLOOH. With the participation of GPX4, PLOOH is converted into PLOH. If this does not happen, PLOO<sup>•</sup> and PLOOH react with further PUFA molecules (Davies & Guo, 2014). In the presence of oxygen, another hydrogen atom is removed from PUFA and further PLOOH molecules, which are pathognomonic for ferroptosis, are formed. However, enzymatically controlled lipid peroxidation seems to be of greater importance for ferroptosis. At least some enzymes are involved in regulating this process. Research on enzymatic lipid peroxidation has mainly focused on lipoxygenase (LOX), acyl-CoA synthetase long-chain family member 4 (ACLS4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) (Yang et al., 2016, Doll et al., 2017, Golej et al., 2011). LOX is iron-containing non-heme dioxygenase which catalyses the dioxygenation of free and esterified PUFA. Where there is redox equilibrium, LOX products (PLOOH) are reduced by GPX4, with the participation of glutathione. Thus, in a sense, both of these enzymes collaborate with each other – LOX increases ferroptosis, while GPX4 inhibits it. These observations were confirmed in studies of the pharmacological inhibition of LOX. Both the administration of LOX inhibitors such as baicalein as well as the knockout of the LOX coding gene inhibited ferroptosis (Li et al., 1997, van Leyen et al., 2006, Jin et al., 2008). Subsequently, Yang et al. performed pharmacological inhibition of LOX under conditions of glutathione depletion in the cell (Yang et al., 2016). It became apparent that the inhibition of LOX stops ferroptosis even in the presence of glutathione deficiency. Thereby it inhibits the cysteine dependent pathway of ferroptosis induced by erastin and other regulating factors of the Xc<sup>-</sup> cistine/Glu antiporter system. Interestingly,

however, the simultaneous knockout of the LOX and GPX4 genes does not prevent ferroptosis in mouse fibroblasts. (Friedmann et al., 2014). It therefore appears, that there must be alternative mechanisms compensating for the lack of LOX activity and/or that the "LOX specific" inhibitors used exert non-specific activity on the radical-trapping antioxidants (RTA) (Zilka et al., 2017). Research has confirmed the thesis which challenges the importance of the role of LOX in ferroptosis (Shah et al., 2018). Moreover, the complete inhibition of all LOX isoenzymes does not prevent ferroptosis caused by ferroptosis activator RSL3, probably due to the fact that treatment with erastin activates LOXs (Yang et al., 2016).

The formation of PUFA-PL – a substrate for LOX – is preceded by the action of LPCAT3, although studies indicate that the earlier stage, i.e. the biosynthesis of polyunsaturated fatty acyl-coenzyme A (PUFA-CoA) catalysed by ACLS4, is more important in the regulation of ferroptosis (Doll et al., 2017). The main substrates for ACLS4 are arachidonic acid (AA) and adrenic acid (AdA), which are thioesterified to give membrane phospholipids. (Golej et al., 2011). As in the case of LOX, inhibition of ACLS4 e.g. by thiazolidinediones or knockout of the gene encoding ACLS4 leads to inhibition of ferroptosis even with GPX4 knockout (Doll et al., 2017). In the case of ACLS4 and LPCAT3 deficiency, an alternative pathway for PLOOH formation could be the SCD1 pathway. Substrates for LOX could then be Oleate (C18: 1) and Palmitooleate (C16:1) (Fig. 1). However, this is not the case. Research indicates that the exogenous supplementation of monounsaturated fatty acids (MUFAs), SCD1-mediated cellular MUFAs production, and an ACSL3-dependent enrichment of membranes with MUFAs reduce the susceptibility of cells to ferroptosis (Magtanong et al., 2019, Yang et al., 2016, Tesfay et al., 2019).

### The Role of Iron in Ferroptosis

Iron is a biochemically active element of such cofactors as: heme, iron-sulphur centres [Fe-S], and 1- or 2-atom iron centres, which, in turn, determine the activities and functions of many

proteins necessary for the proper course of key biological processes. Proteins containing iron in their active centres participate in the transportation of electrons in the respiratory chain, the transportation and storage of oxygen, the regulation of gene expression as well as in the synthesis of DNA, microRNA and collagen. The regulation of iron metabolism is controlled by numerous genes, whose expression products are involved in maintaining the iron's homeostasis. An example of such molecules are iron regulatory proteins (IRPs) (Volz, 2008). By regulating the expression of transferrin receptor (TFRC) and ferritin heavy chain 1 etc., IRP1 and IRP2 stabilise the cell's labile iron pools (LIP) composed of  $\text{Fe}^{2+}$ . (Lin et al., 2020). One of the disruptions to iron metabolism is this ferroptosis. Ferroptotic cell death correlates with an increase in iron stores in the cell, which ultimately leads to the oxidation of cell membrane fats. As mentioned earlier, lipid peroxidation takes place during enzymatic and non-enzymatic Fenton reaction. The latter is considered to be the key mechanism of ferroptosis (Jiang et al., 2021, Conrad & Pratt, 2019). The Fenton reaction, as well as the action of LOX, are regulated, inter alia, by the pool of intracellular iron. The main regulators of this pool, and thus of ferroptosis, are transferrin and TFRC (Gao et al., 2015a). Increasing TFRC activity to enrich the cellular iron pool can lead to ferroptosis (Yang & Stockwell, 2008, Lin et al., 2020). In the cell, iron binds to ferritin and is deposited in this form. The release of iron from the deposits occurs in the lysosomes. The process is controlled in three stages – phagocytosis of ferritin bound to iron, the reduction of iron and its release into the cytoplasm. The key factor regulating phagocytosis of ferritin is nuclear receptor coactivator 4 (NCOA4). The combination of ferritin with NCOA4 forms autophagosomes which are then internalised into lysosomes. Studies have shown that reducing the content of NCOA4 in the cytoplasm inhibits the internalisation of ferritin into lysosomes and reduces susceptibility to ferroptosis (Mancias et al., 2014). The second stage, i.e. the reduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ , takes place with the participation of metalloredutase six-transmembrane epithelial antigen of

prostate 3 (STEAP3) (Zhang et al., 2012). STEAP3 activity has been proven to be dependent on fanconi anaemia complementation group D2 (FANCD2), a nuclear protein involved in DNA damage repair, which protects against ferroptosis-mediated injury in bone marrow stromal cells (BMSCs) (Song et al., 2016). Knockdown of the FANCD2 gene inhibits STEAP3 expression and releases  $\text{Fe}^{2+}$  (Song et al., 2016) and thus reduces the cytoplasmic LIP pool and susceptibility to ferroptosis. The third stage, the release of  $\text{Fe}^{2+}$  into the cytoplasm occurs with the participation of the divalent metal transporter 1 (DMT1). Many other factors, such as iron responsive element-binding protein 2 (IREB2) (Dixon et al., 2012), autophagy-related protein 13 (ATG13) (Gao et al., 2016), heme oxygenase-1 (HMOX1) (Kwon et al., 2015), heat shock protein beta-1 (HSPB1) (Sun et al., 2015), and ferroportin (Fpn) (Li et al., 2018) etc. also participate in the cytoplasmic metabolism of iron.

#### Glutamine, Mitochondria and Ferroptosis

Mitochondria are involved in the implementation of various types of regulated cell death, such as extrinsic and intrinsic apoptosis and autophagy, thus playing a major role in tissue homeostasis (Bebber et al., 2020, Mattson et al., 2008, Xie et al., 2018). They are essential for most normal cell types due to their role in generating adenosine triphosphate (ATP) via the mitochondrial oxidative phosphorylation (OXPHOS) (Gao et al., 2015b, Gao et al., 2019). The involvement of mitochondria in ferroptosis thus emphasises its metabolic nature. Characteristic mitochondrial changes in ferroptosis include rupture of the mitochondria, reduction of mitochondrial membrane density, and reduction of mitochondrial ridges (Lin et al., 2020). It has been shown, for example, that the experimental induction of ferroptosis by pharmacological inhibition of  $\text{Xc}^-$  causes mitochondrial fragmentation, production of mitochondrial ROS, the loss of mitochondrial membrane potential (MMP), and depletion of ATP (Zhou et al., 2019, Neitemeier et al., 2017, Xie et al., 2016, Yagoda et al., 2007, Yuan et al., 2016). The inhibition of OXPHOS or mitochondrial loss produced by mitophagia impedes

the ferroptosis either induced by erastin or by the lack of cystine. The metabolism of glutamine and, especially, its conversion to Glu are crucial for cystine starvation-induced ferroptosis and the mitochondrial Krebs cycle. In other words, without glutamine, erastin-induced ferroptosis, in the absence of cysteine, is also inhibited. (Gao et al., 2015b). One of the factors regulating glutamine decomposition is amino-oxyacetate (AOA) (Wu et al., 2021), an inhibitor of GOT 1, an enzyme which mainly takes part, inter alia, in the biosynthesis of keto acids, principally  $\alpha$ -ketoglutaric acid, which is necessary in the mitochondrial process of ATP production. The action of AOA inhibits ferroptosis, but it can be restored by the supplementation of  $\alpha$ -KG (Luo et al., 2018, Gao et al., 2015b, Jennis et al., 2016). Research indicates that further mitochondrial metabolites of  $\alpha$ -KG such as succinate (suc), fumarate (fum) and malate (mal) could modulate cysteine-deprivation induced ferroptosis (Gao et al., 2016). It is believed that the electron transport assisted by the glutaminolysis-TCA cycle may facilitate lipid peroxidation by generating ROS which are produced as an inevitable byproduct of mitochondrial respiration. Sensitivity to ferroptosis may also be determined by the modulation of adenosine monophosphate-activated protein kinase (AMPK) activity by the glutaminolysis-TCA cycle and the associated production of ATP in the electron transport chain (Ma et al., 2020). The AMPK, activated in this mechanism, phosphorylates and thus inhibits acetyl-CoA 1 (ACC1) and 2 (ACC2) carboxylase, which leads to a reduced conversion of acetyl-CoA to malonyl-CoA necessary for PUFA biosynthesis (Lee et al., 2020). It is possible that this mechanism reduces the level of lipid peroxidation and slows down ferroptosis (Wu et al., 2021, Li et al., 2020[a], Lee et al., 2020). It seems, however, that endogenous biosynthesis can be replaced by the transport of fatty acids (FAs) from the tumour microenvironment (TME), hence the influence on the inhibition pathway of ACC1 and ACC2 may be of less importance in the regulation of ferroptosis. Additionally, AMPK activation also triggers another mechanism. Due to the AMPK phosphorylation, there is

upregulation of sterol regulatory element-binding protein 1 (SREBP1) and the downstream CoA desaturase-1 (SCD1) and this results in a decrease in the amount of MUFAs which inhibit ferroptosis. (Zhao et al., 2020).

## Breast Cancer

Breast cancer is a disease that is molecularly and clinically extremely heterogeneous. Unlike normal tissue, cancer cells develop under conditions of metabolic and oxidative stress. This situation may be favourable or unfavourable to their growth, depending on whether they are exposed to moderate or excessive levels of stress and the internal mechanisms of both antioxidant protection and the stress defences of the tumour. (Ackerman & Simon, 2014, Harris, 2002, Reczek & Chandel, 2017, Manda et al., 2015). Changes in oxygen and nutrient levels in a hypoxic TME may limit tumour growth, but can also alter the tumour phenotype and provoke conversion to more malignant tumour types (Sanna, 1994). The TME is key to the growth of the tumour. In the breast cancer TME, the role of adipocytes has received great attention due to their proximity to a developing breast tumour. Clinical trials clearly indicate an increased invasiveness of breast cancer leading to worse treatment outcomes and prognosis (Dirat et al., 2011, Wang et al., 2012). Adipocytes influence the growth of cancer cells through two main mechanisms. Firstly, adipocytes secrete adipocytokines (adipocyte-secreted growth factors and cytokines) which activate growth-promoting signalling pathways in tumour cells and stimulate various oncogenic processes. Interleukin-6 (IL-6) and leptin secreted by adipocytes induce and regulate epithelial-mesenchymal transition (EMT) in cancer cells (Gyamfi et al., 2018, Gyamfi et al., 2019, Lee et al., 2015). The secreted leptin activates pathways which increase stem cell renewal and also increase drug resistance (Ayob & Ramasamy, 2018). Secondly, adipocytes release metabolites and biomolecules that remodel the metabolism of cancer cells to increase tumour growth. It should be added that adipocytes in close/direct contact with tumour cells are commonly called cancer-associated adipocytes



(CAAs) and have different characteristics from adipocytes not associated with tumour cells. The adipose tissue infiltrated by breast cancer has a substantially reduced lipid droplet (LD) size. Importantly, CAAs have a greatly increased expression of IL-6 and leptin. The CAAs release increased amounts of IL-6 and leptin which are important factors in boosting the growth of tumours (Zaoui et al., 2019, Balaban et al., 2017, Giordano et al., 2016, He et al., 2018). This proximity of the adipocytes to the cancer cells means the breast cancer cells can parasitise their energy substrates and obtain metabolites including lactate, glutamine, and FAs that are restricted in the environment of the growing tumour (Dias et al., 2019, De Berardinis et al., 2008, Wang & Li, 2019). The metabolites thus obtained support metabolic needs and provide a selective advantage for cancer survival and progression in a difficult microenvironment. The reprogramming of energy metabolism is therefore a key hallmark of tumour growth (Hanahan & Coussens, 2012, Dias et al., 2019). Systemic therapies have improved overall survival for many cancers. However, relapses are the most common problem among many primary cancer treatments (Mahvi et al., 2018). Small tumours remaining after chemotherapy or targeted therapies can survive under conditions of constant change between hypoxia and re-oxygenation in the TME during treatment and later cause a relapse and/or the development of aggressive tumours (Cardenas-Navia et al., 2008). Uncontrolled proliferation overloads the existing vessels and causes the tumour to become hypoxic (Brown & Giaccia, 1998), and neoadjuvant therapies may exacerbate tumour hypoxia, which may drive drug resistance (Samanta et al., 2014, McIntre & Harris, 2015). Alternating hypoxia and re-oxygenation lead to chronic oxidative stress destabilising the cell's redox potential (Semenza, 2017). As a consequence, this affects genomic instability, changes in tumour metabolism, and the progression of malignancy (Brown & Wilson, 2004, Zhang et al., 2015, Li et al., 2015), and ultimately resistance to neoadjuvant therapies.

It should be clearly emphasised that ferroptosis is only one of several pathways through which tumour cells may be killed therapeutically. Unfortunately, this same process may be induced, in parallel, in normal cells, which may be the basis for adverse effects. So, the question arises: how to increase or induce ferroptosis in cancer cells during cancer treatment without causing ferroptosis in normal cells and how to suppress the repair mechanisms in cancer cells that inhibit ferroptosis. The starting point for the answer to these questions should be the identification of the factors which promote ferroptosis. The key compounds involved in ferroptosis are FAs. A number of macro - and micromolecules, involved in the transport and biosynthesis pathways of FAs, are involved in their metabolism, biosynthesis and penetration into cells (Table 1).

#### The SCD1 - FABP4 - CD36 Axis

The SCD1 pathway and FAs membrane transport pathway deserve special attention. These pathways disrupt the metabolism in the specific microenvironment of the tumour and this disruption is crucial in inhibiting the tumour regrowth process. This applies, inter alia, to the biosynthesis of FAs in which SCD1 participates. According to many authors, *de novo* biosynthesis of FAs (Sounni et al., 2014, Iwamoto et al., 2018) is emerging as a key driver of cancer malignancy dependent on the hypoxic and metabolic stress induced by antiangiogenic treatment. Expression of SCD1 [EC 1.14.19.1] has been detected in recurrent human breast cancer samples and has been correlated with a worse prognosis in patients with different types of cancer (Luis et al., 2021). SCD1, also called  $\Delta^9$ -desaturase, is an endoplasmic reticulum-related enzyme that catalyses the formation of MUFAs from *de novo* synthesised or food-supplied FAs. SCD is a four-transmembrane domain protein composed of 359 amino acids with a mass of 37 kDa (Kucharski & Kaczor, 2014, Paton & Ntambi, 2009). In humans, there are two homologs of the gene encoding SCD. One is on chromosome 10 (SCD1) and the other is on chromosome 17 (SCD5). Both isoforms differ in structure and size of the

transcripts (Zhang et al., 1999). SCD1 catalyses the reaction of introducing a first double bond, at the *cis* position between carbon atoms 9 and 10, into Saturated Fatty Acids (SFA) such as palmitic acid (C16: 0) and stearic acid (C18:0). The MUFAs, such as palmitoleic acid (POA; 16:1) and oleic acid (C18:1), resulting from this reaction competitively disturb the activity of PUFAs, thus inhibiting ferroptosis (Yang et al., 2016, Megtanong et al., 2019, Tesfay et al., 2019). PUFAs, unlike MUFAs, are a preferential substrate for lipid peroxidation. The inhibitory effect on ferroptosis is due to the fact that MUFAs do not have bis-allylic carbon atoms and are therefore much less prone to peroxidation. The bis-allylic position seems to be crucial to the progress of the ferroptosis process. Research has shown that deuterated at the bis-allylic position PUFAs (D-PUFAs) reduce oxidative stress and protect against ferroptosis caused by erastin or RSL3 (Yang et al., 2016). This mechanism suggests that PUFA-induced ferroptosis may depend on the structure of these FAs. The regulation of ferroptosis in cancer via SCD1 also involves the lactate-activated hydroxycarboxylic acid receptor 1/monocarboxylate transporter 1 (HCAR1/ MCT1) system. Lactate-rich cancer cells show increased resistance to ferroptotic damage induced by commonly used ferroptosis inducers such as RSL3 and erastin. The uptake of extracellular lactate by MCT1 may promote ATP production as  $L$ -Lactate has the ability to support ATP production via the Krebs cycle (Martinez-Reyes and Chandel, 2017). On the other hand, blocking HCAR1/MCT1 by gene knockdown or the use of MCT1 inhibitor AZD3965 slows ATP biosynthesis which results in increasing the AMP:ATP ratio (Fig. 2). A change in the energy status of a cell may initiate phosphorylation of AMPK which leads to inhibition of the expression of sterol regulatory element-binding protein 1 (SREBP1) (Zhao et al., 2020, Bertolio et al., 2019) and the eventual repression of SCD1. The inhibition of SCD1 results in a decrease in MUFA amount, a decrease in tumour cell resistance to ferroptosis and an increase in the accumulation of cytotoxic lipid ROS in the cytoplasmic membrane. The

intracellular concentration of MUFAs is therefore one of the key factors in the regulation of ferroptosis. Studies have shown that the administration of SCD1 products such as palmitooleate (C16:1) and oleate (C18:1) with simultaneous inhibition of HCAR1/MCT1 reduces the susceptibility of cells to ferroptosis (Zhao et al., 2020). The partial restoration of cancer cells proves that other non-ferroptotic mechanisms of cell regulation, such as apoptosis, may be involved (Zhao et al., 2020, Tesfay et al., 2019, Li et al., 2017, Ma et al., 2017). An example is the regulation of AMPK/ mammalian target of rapamycin (mTOR) pathway by sodium-glucose co-transporter-2 (SGLT-2) (Zhou et al., 2020). SGLT-2 is the primary co-transporter involved in renal glucose reabsorption (Cowie & Fisher, 2020). It belongs to the sodium/glucose cotransporter-1 family (SLC5A) and is responsible for creating a  $\text{Na}^+$  gradient in cells located in the proximal tubule which drives glucose transport from the renal tubules into the cells. Physiologically, SGLT-2 is found only in the kidneys. Pathologically, SGLT-2 expression has been observed in, inter alia, breast cancer (Zhou et al., 2020). Studies have shown that blocking SGLT-2 with specific inhibitors stops the cellular cycle of breast cancer cells at the G1/G0 phase and induces apoptosis. Canagliflozin and dapagliflozin, hypoglycaemic and cardioprotective drugs, have been used as SGLT-2 inhibitors. It has been observed that SGLT-2 inhibitors cause a decrease in glucose uptake and an increase in AMPK phosphorylation, i.e. its activation. A further effect of this is the inhibition of p70S6K phosphorylation (p-p70S6K) which means mTOR inhibition (Zhou et al., 2020). Canagliflozin and dapagliflozin reduce ATP production, inhibit oxidative phosphorylation, and decrease the intracellular concentration of ATP in breast cancer cells. This results in a large increase in the AMP:ATP ratio and thus the activation of AMPK, which is a sensor of cellular energy and nutrition levels in eukaryotic cells. This signalling pathway leading from the activation of AMPK to the inhibition of mTOR is believed to be, at least partially, responsible for inhibiting the proliferation, arresting the cellular cycle at the G1/G0

phase and inducing apoptosis in breast cancer cells by canagliflozin and dapagliflozin (Zhou et al., 2020). The activation of AMPK inhibits protein synthesis and cell proliferation. Zakikhani et al (Zakikhani et al., 2012) also showed that another diabetes medication, metformin, inhibits the proliferation of breast cancer cells by activating AMPK, resulting in an inhibition of mTOR signalling. Thus, the mTOR signalling axis has emerged as a promising area in breast cancer therapy (Kennedy et al., 2020). The activation of AMPK also influences the activity of SCD1 as previously described. Thus, the action of canagliflozin and dapagliflozin must inhibit the biosynthesis of MUFAs, which directly connects the SGLT-2 inhibition pathway with ferroptosis. The MUFAs, a product of this reaction, inhibit ferroptosis by competitively interfering with the activity of PUFAs. In contrast to MUFAs, PUFAs are preferential substrates for lipid peroxidation. Thus, the inhibition of SCD1 induced by SGLT-2 connects the AMPK activation and SREBP1 inhibition pathway to ferroptosis. SCD1 inhibition causes diminished production of MUFAs, resulting in a surplus of PUFAs and increased lipid peroxidation. This, then, causes the ferroptosis to intensify.

In addition to the previously discussed lactate, acyl-CoA synthetase long chain family member 4 (ACSL4) is also involved in the process of regulating ferroptosis. Lactate can inhibit ACSL4 via other pathways (Brown et al., 2017, Pucino, et al., 2019; Xie et al., 2020) and reduce the production of oxidisable PUFAs (Doll et al., 2017; Li et al., 2019). Relative changes in SCD1 and ACSL4 levels after lactate entry into the cell suggest that lactate-induced rearrangement in the biosynthesis of MUFAs and PUFAs may act synergistically – enhancing ferroptosis resistance in cancer cells. That is, lactate may protect tumour cells from ferroptotic cell death through highly regulated mechanisms.

Another important macromolecule involved in cancer ferroptosis is Fatty Acid Binding Protein 4 (FABP4). FABPs are intracellular lipid chaperones - a group of molecules that coordinate lipid responses in cells (Hauerland & Spener, 2004, Chmurzyńska, 2006, Makowski

& Hotamisligil, 2005). FABPs are abundantly expressed 14–15 kDa proteins that reversibly bind hydrophobic ligands, such as saturated and unsaturated long-chain fatty acids, eicosanoids and other lipids, with high affinity (Coe & Bernlohr, 1998, Zimmerman & Veerkamp, 2002). In addition to the intracellular transport of FAs, FABP4 is responsible for the organisation of LD in the cell, which, in the case of cancer cells, is important in the process of sensitising them to ferroptosis. Studies have shown that FABP4 derived from adipocytes or endothelial cells present in TME can promote the uptake of PUFAs necessary for tumour regrowth and stimulate the formation of LDs in cancer cells, which is essential for cell survival under hypoxic conditions (Luis et al., 2021). The administration of FABP4 inhibitors caused a dose-dependent increase in ROS production in cells and inhibited clinical recurrence of breast cancer. However, the best efficacy was when SCD1 and FABP4 inhibitors were administered simultaneously. The inhibition of SCD1 led to a halt in FA desaturation and thus the pool of FAs, necessary for the formation of PLOOHs, was increased, which, in turn, increased ferroptosis. Moreover, the inhibition of FABP4 decreased the transport of FAs to the tumour cells and inhibited LD formation in response to hypoxia.

Fatty acid translocase/CD36 plays an important role in the SCD1-FATP4 axis, which regulates FA metabolism in tumour cells and TME. CD36 is a key membrane glycoprotein involved in importing adipocyte-released fatty acid into breast cancer cells (Febbraio et al., 2001, Pepino et al., 2014, Liang et al., 2018). The primary functions of CD36 include FA uptake, cell adhesion, and regulation of inflammatory processes. In the adipocyte-breast cancer cell interaction, CD36 plays a role in reprogramming the metabolism with a shift towards increased fatty acid oxidation (Wang & Li, 2019). Thus, CD36 may be a unique FA receptor and regulate various oncogenic processes. In patients with breast cancer, the expression of CD36 is increased, with a simultaneous increase in the expression of lipid transport receptors (LDL) and FABP4. (Gyamfi, 2021). An increased expression of CD36 and

FABP4 worsens the prognosis in breast cancer. An appropriate expression of CD36 and FABP4 maintains homeostasis at the rate of FAs import and metabolism, ensuring that imported FAs are efficiently transported to subcellular locations (mitochondria and peroxisomes). This collaboration between the two transport proteins is probably as a result of the presence of similar amino acid sequences creating possible motives for the interaction between CD36 and FABP4 as well as other proteins. Presumably, CD36 acts as a transmembrane FA importer. In contrast, FABP4 interacts with CD36 to transport FAs to various cell locations. In all likelihood, these interactions are responsible for the reprogramming of the metabolism, which induces the drug resistance seen in breast cancer cells cultured with adipocytes. (Iwamoto et al., 2018, Bochet et al., 2011). Studies have shown that the chemical inhibition of CD36 and FABP4 induces apoptosis in breast cancer cells. The use of CD36 and FABP4 inhibitors, sulfosuccinimidyl oleate (SSO) and BMS309403 respectively, greatly decreased the lifespan of breast cancer cells and also notably reduced the proliferative, migratory and invasive abilities of breast cancer cells (Gyamfi, 2021).

#### Acyl starvation

More than 100 years of cancer research have provided an enormous amount of information on the role of FAs in tumorigenesis (Koundouros & Poulogiannis, 2020). It is known that due to their exceptional growth dynamics, cancer cells consume large amounts of FAs for building and energy purposes, thus maintaining a high proliferation rate especially under metabolic stress conditions associated with alternating hypoxia and reoxygenation. The source of FAs in cancer cells is de novo synthesis from nutrients such as glucose or glutamine and exogenous uptake from the surrounding microenvironment. The above-mentioned macromolecules, such as SCD1, CD36, FATP or FABP and many others, participate in these processes, especially in the uptake from the surrounding environment. Appropriate regulation of their expression or

activation/inhibition promotes tumour growth. The spread of breast cancer metastases is also stimulated by the presence of adipocytes located in periglandular regions. Adipose tissue functions as an endocrine system which secretes growth factors and cytokines as well as releasing FAs after lipolysis (Nieman et al., 2013). It also serves as a reservoir of FAs and other fats for the cancer cells, which is why people with obesity have a higher propensity to develop cancer (De Pergola & Silvestris, 2013). Adipocytes activate the endogenous lipolysis of triglycerides to produce free FAs, which can then be secreted and taken up by FABP4-overexpressed metastatic cells (Nieman et al., 2011). Thus, adipose cells perpetuate a network of reciprocal interactions and function as active mediators of endocrine and paracrine signalling. Adipokines, secreted by adipocytes, stimulate cancer cells to release exosomes containing pro-lipolytic factors such as miRNA-144 and miRNA-126, which in turn promote lipolysis in adjacent adipocytes by activating AMPK signalling and inducing autophagy (Lengyel et al. 2018, Wu et al. 2019). Ultimately, increased lipolysis and the release of free FAs fundamentally change the metabolic dependence of the migrating cancer cells, shifting their reliance onto exogenous lipid uptake and  $\beta$ -oxidation for energy (Wu et al. 2019, Balaban et al. 2017). The main effect of this increased uptake of exogenous FAs is their subsequent storage in LDs, which sequester excess FAs as TAGs and sterol ester (Olzmann & Carvalho, 2019). Consequently, the accumulation of LDs in cancer cells is not only used to maintain lipid homeostasis and prevent lipotoxicity, but also provides valuable ATP and NADPH during metabolic stress (Olzmann & Carvalho, 2019, Petan et al. 2018). This is mainly due to the  $\beta$ -oxidation of stored lipids, which leads to the production of acetyl-CoA. The acetyl-CoA produced in each round of  $\beta$ -oxidation can then enter the tricarboxylic acid (TCA) cycle to produce, in turn, NADH and FADH<sub>2</sub> for the electron transport chain. Thus, the  $\beta$ -oxidation of FAs contained in LDs provides a sufficient amount of ATP to drive the metastatic cascade and generate NADPH, which is essential for anabolic metabolism and



detoxification of reactive oxygen species (ROS) (Pascual et al. 2017, Samovski et al. 2015, Lu, 2009, Mitsuishi et al. 2012). This is particularly important in the case of hypoxic cells, which have an increased FAs uptake and LD accumulation following the action of the cellular oxygen sensing and hypoxia-inducible factor (HIF)-1 $\alpha$  dependent expression of FABP3 and FABP7 (Bensaad et al. 2014). Thus, the proliferation capacity of cancer cells as well as their metastasis is closely related to FAs metabolism not only in the cancer cells but also in adjacent tissues, especially adipose tissue. Studies show that dysregulation of lipid metabolism in TME contributes to a reduction in or successful treatment of metastasis (Koundouros & Poulogiannis, 2020). However, cancer cells have the ability to bypass the major pathways by activating other pathways which allow them to synthesise or take up FAs from the environment. It is therefore more effective in the fight against cancer to intervene at multiple levels in the FAs metabolism of cancer cells, such as supporting neoadjuvant therapies with induced ferroptosis. This leads to a blockade of FAs biosynthesis, their abnormal metabolism, PLOO $\cdot$  production, disruption of FAs transport into the cell and the loss of proper biochemical communication between cancer cells and the TME, especially, adipocytes. The result is a lack of FAs needed to maintain the proper rate of tumour growth. Apart from energy and general metabolic issues, FAs are necessary for the construction of cell membranes, so any deficiency and, particularly, incorrect proportions constitute a key obstacle in uncontrolled proliferation of cancer cells. The deficiencies in question concern short-, medium-, and long-chain saturated, monounsaturated, polyunsaturated, n-3, n-6 acids etc. and lead to FAs starvation. However, due to the fact that those FAs present in cells, especially in cell membranes, occur in combination with other compounds such as glycerol to form mono-, di- and triacylglycerols, or are part of phospholipids, sphingolipids etc. a more precise term is "acyl starvation" (Fig.3). It can therefore be assumed that "acyl starvation" is a consequence of, amongst other things, ferroptosis. Ferroptosis disturbs the delicate balance in

the correct proportion and amount of FAs by generating useless PLOO', which additionally cause heavy metabolic stress on a cancer cell and lead to its death. Studies show that inducing ferroptosis in tandem with neoadjuvant therapy gives much better results in cancer treatment than neoadjuvant therapy alone because it reduces the cells' invasiveness and inhibits the spread of metastases to distant tissues.

### Breast Cancer and Other Cancers

Tumours differ in location, tissue of origin, metabolism, etc. However, they all consist of rapidly dividing cells. They require building materials to maintain their rate of proliferation. The main component of their cell membranes are fatty acids and especially their acyl derivatives such as phospholipids and triacylglycerols. Ferroptosis and the subsequent acyl starvation, affect the supply of these acyl derivatives in the overall metabolic process, but, above all, affect the rate of construction of the cell membranes. This may be of particular importance in tissues with intensive FA metabolism, such as the mammary gland.

Despite, a relatively good understanding of ferroptosis, it is still not known how cells die. There are already quite a few publications that present the results of studies supporting neoadjuvant therapies with induced ferroptosis. However, the vast majority of these are in vitro studies. Ferroptosis has been studied in several types of cancer. These include hepatocellular, gastric, ovarian, pancreatic and colorectal carcinomas among others. Of particular interest, however, seem to be the results of studies on ferroptosis in association with FABP4 and SCD1 inhibition in breast cancer cells. There, drastically reduced tumour recurrence (Luis et al., 2021) was observed as well as the inhibition of tumour growth. The interference of FABP4, SCD1 and CD36, to a very large extent, disrupts the normal metabolism of fatty acids. This leads to the development of their defective forms such as PLOO', as well as abnormal ratios and quantities of FAs. Uncontrolled peroxidation of PUFA-PLs, is one of the mechanisms explaining the inhibition of tumour growth. Peroxidised

phospholipids cause membrane damage and pore formation, thereby compromising membrane integrity. This is especially true for phospholipids which have two chains of PUFAs, and which are particularly prone to ferroptosis leading to acyl starvation and membrane damage. However, it should be added that at least some of these described mechanisms inhibit tumour growth in other tissues. For example, it is known that Canagliflozin downregulates SCD1 in hepatocellular carcinoma cells, resulting in the inhibition of fatty acid synthesis and the proliferation of these cells (Nakano et al., 2020). Downregulation of SCD1 is also known to inhibit prostate cancer cell proliferation by suppressing the production of MUFAs (Fritz et al., 2010).

## Conclusion

Ferroptosis is a form of cell death driven by iron-dependent lipid peroxidation. Its complicated mechanism is gradually becoming better understood, but many aspects still remain unclear, such as the role of cell organelles in the development of ferroptosis. The use of ferroptosis in the treatment of cancer, and especially in supporting classic neoadjuvant therapies, gives hope for effective anti-cancer therapies (Pizon et al., 2022). Cancers, adaptively, create mechanisms that inhibit lipid oxidation, which lead to disease relapse. This adaptation is driven by alternating hypoxia and reoxygenation as well as the specific structure and metabolism of TME, which all support tumour growth. A major role in both the development of cancer and in the process of ferroptosis is played by FAs. These FAs play both an energetic and a formative role (cell membranes) in tumour development. In the process of ferroptosis, they are oxidised, causing cell damage. Therefore, the regulation of the FAs metabolism in cancer cells seems to be a promising therapeutic direction. In this study, we have focused mainly on the SCD1-FABP4-CD36 axis, because both cell line studies and clinical trials show the effectiveness of therapies acting on these biochemical pathways (Table 2). The inhibition of SCD1 causes an increase in the amount of long chain PUFAs, which can

further be used for the formation of PLOOH. The inhibition of FATP4 and CD36 in cancer cells and TME causes dysregulation of both the transport of FAs in TME and the intracellular transport of FAs and also inhibits the formation of LDs in tumour cells. FATP4 and CD36 are also involved in the apoptosis of cancer cells through other mechanisms such as the SGLT-2 cotransporter and the AMPK/mTOR pathway. Ferroptosis reduces cell resistance to classic neoadjuvant therapies by rearranging the metabolism of FAs, which are in great demand in the rapidly growing tumour cells. Limiting the consumption and biosynthesis of the relevant FAs in tumour cells results in a deficiency of FAs and an inhibition of the tumour progression. We suggest this mechanism should be named “acyl starvation”. The tumour’s dependency on lipids and its sensitivity to FAs oxidation by ferroptosis are the starting points for designing new treatment strategies to prevent tumour recurrence. Cancer cells should be the sole target for ferroptosis-inducing therapies. It is also important to monitor any possible adaptation processes of cancers to ferroptosis.

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#### Authorship contribution

Participated in research design: Rafał Bobiński

Conducted experiments: Rafał Bobiński, Monika Pizon

Contributed new reagents or analytic tools: Rafał Bobiński, Anna Pielesz

Performed data analysis: Rafał Bobiński, Mieczysław Dutka, Wioletta Waksmańska

Wrote or contributed to the writing of the manuscript: Rafał Bobiński, Mieczysław Dutka

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## Captions

### Figure 1

Processes leading to ferroptosis and mechanisms of its regulation, taking into consideration key pathways such as the mevalonate pathway, transsulfuration pathway, SCD1 pathway,

lipid peroxidation pathway and fatty acid membrane pathway. The figure also shows the role of lysosomes and mitochondria in the process of apoptosis.

## Figure 2

The participation of the SCD1, FABP4, CD36 and the HCAR1 / MCT1 pathway in the regulation of ferroptosis and other processes contributing to the death of tumour cells. Blocking HCAR1 / MCT1 slows ATP biosynthesis, resulting in an increase in the AMP/ATP ratio. This initiates the phosphorylation of AMPK, which affects the inhibition of SREBP1 and ultimately the repression of SCD1. AMPK activity is also regulated by SGLT2 present in cancer cells. When this cotransporter is inhibited, it activates AMPK. This activation represses SCD1 and, independently, blocks the mTOR pathway leading to the activation of apoptosis. The inhibition of FABP4 and CD36 causes a reduction in the amount of lipid droplets in cancer cells and an increase in ROS. In addition, this inhibition intensifies acyl starvation and, secondarily, damages cell membrane phospholipids. These mechanisms, on the one hand, reduce the number of PUFAs available to tumours, and on the other hand, intensify ferroptosis and the formation of PLOOHs from the remaining available pool of PUFAs.

## Figure 3

In breast cancer cells, there is an increased synthesis and uptake from the environment of FAs necessary for energy and building purposes. Blocking this synthesis and transport from the TME leads to a reduction in the number of available FAs for metabolic purposes, and particularly, to the construction of cell membranes. Regardless of the overall FA deficiency, when fat metabolism inhibitors are administered, a change in the reciprocal ratios of different types of FA is observed, which may promote, for example, ferroptosis. An example of such a mechanism may be the inhibition of SCD1, as a result of which the number of FAs



susceptible to oxidation increases. Disruption of the physiological ratios of the different types of fatty acids present in TAG and phospholipids and the formation of their damaged forms leads to a loss of cell membrane fluidity and, ultimately, to its disintegration. Most of the FA of cell membranes is incorporated into derivatives such as TAG or phospholipids, therefore the mechanisms presented in his figure are called "acyl starvation".

Molecule	Regulation	Promote/inhibit ferroptosis	reference
SCD1	[inhibition] Desaturation of saturated fatty acids - competitive formation of monounsaturated fatty acids	promote	[Luis 2021]
CD 36	[upregulation] increase in the import of fatty acids into cells	promote	[Pizon 2018]
Transferrin receptor	[upregulation] Increase in unstable iron pool	promote	[Yang & Stockwell, 2008]
FABP	[inhibition] creation of lipid droplets in cells	promote	
Heat shock protein $\beta$ -1	[knockdown] increase in iron uptake	promote	[Sun 2015]
FANCD2	[knockdown] increase in iron uptake	promote	[Song 2016]
Ferritin heavy chain 1	[knockdown] reduction in the content of iron-binding proteins in the cell	promote	[Yang & Stockwell 2008]
Heme oxygenase	[upregulation] enhancement of heme catabolism and iron release	promote	[Kwon 2015]
SGLT2	[inhibition] increase in AMPK phosphorylation	promote	[Zhou 2020]
Erastin/sorafenib	[increase in concentration] blockage of system $x_c^-$	promote	[Lin 2020]
Iron response element-binding protein2	[knockdown] regulation of RNA translation responsible for iron consumption	inhibit	[Dixon 2012]
NCOA4	[knockdown] slowdown in the degradation of ferritin in the process of autophagy	inhibit	[Mancias 2014]
ferroportin	[upregulation] increase in transport of iron outside the cell	inhibit	[Zhao 2018]
Autophagy related protein 3	[knockdown slowdown in the degradation of ferritin in the process of autophagy	inhibit	[Gao 2016]

Table 1. Selected molecules involved in the regulation of ferroptosis.

Table 2. Molecules involved in the promotion of apoptosis in breast cancer cells.

<b>Breast cancer</b>	<b>Therapy</b>	<b>Type of evidence</b>	<b>Reference</b>
	Erastin	Cell culture	Li et al. 2020b
	Siramesine	Cell culture	Ma et al. 2016
	Lapatinib	Cell culture	Ma et al. 2016, Yang, et al. 2020
	Sulfasalazine	Cell culture	Yu et. al. 2019
	Sorafenib	Cell culture	Luis et al. 2021
	SCD 1 Inhibitor	Cell culture	Luis et al. 2021
	FABP 4 inhibitor	Cell culture	Luis et al. 2021
	CD36 inhibitor	Cell culture	Liang et al. 2018
	SGLT2 inhibitor	Cell culture	Zhou et al. 2020

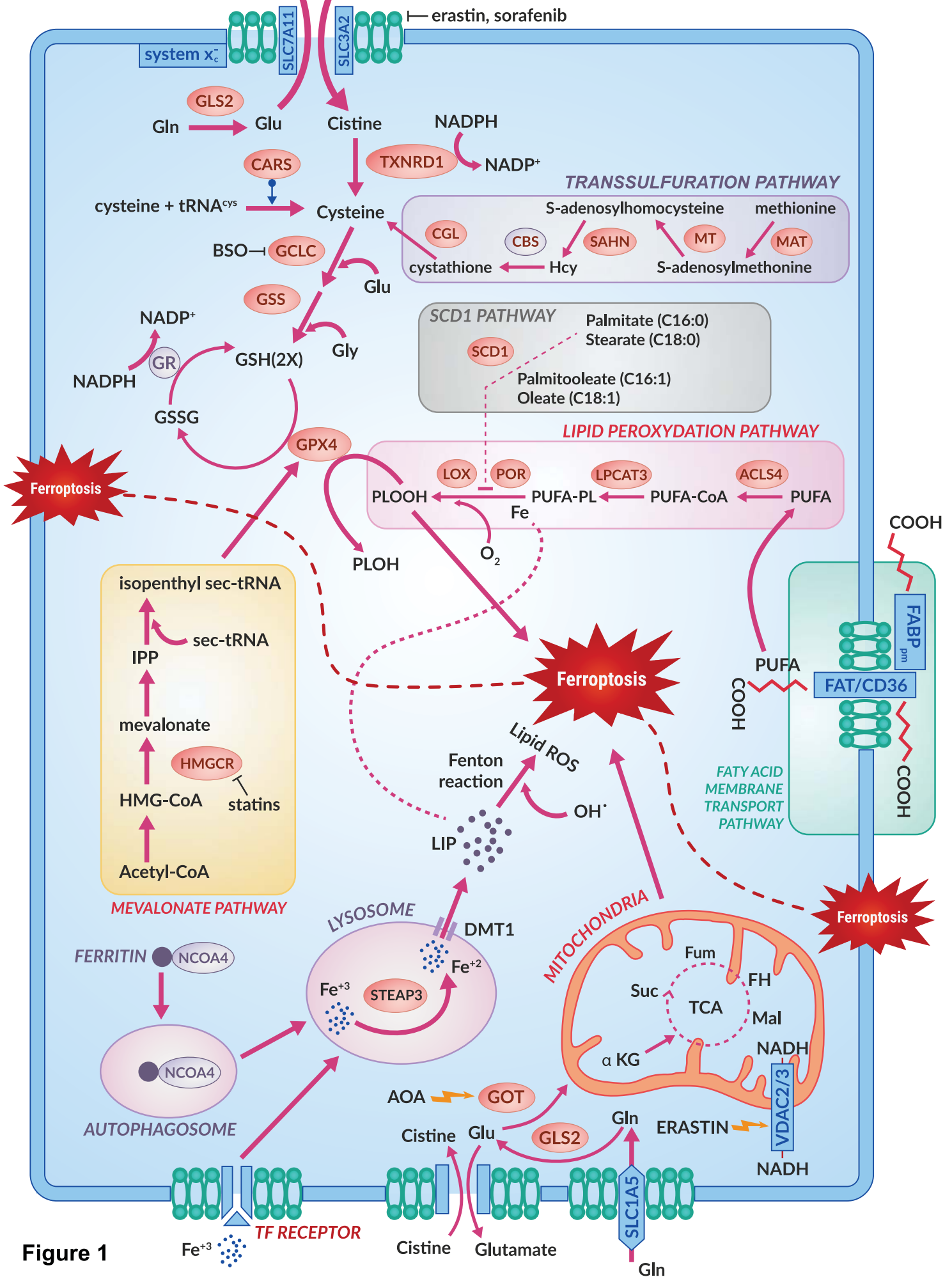


Figure 1

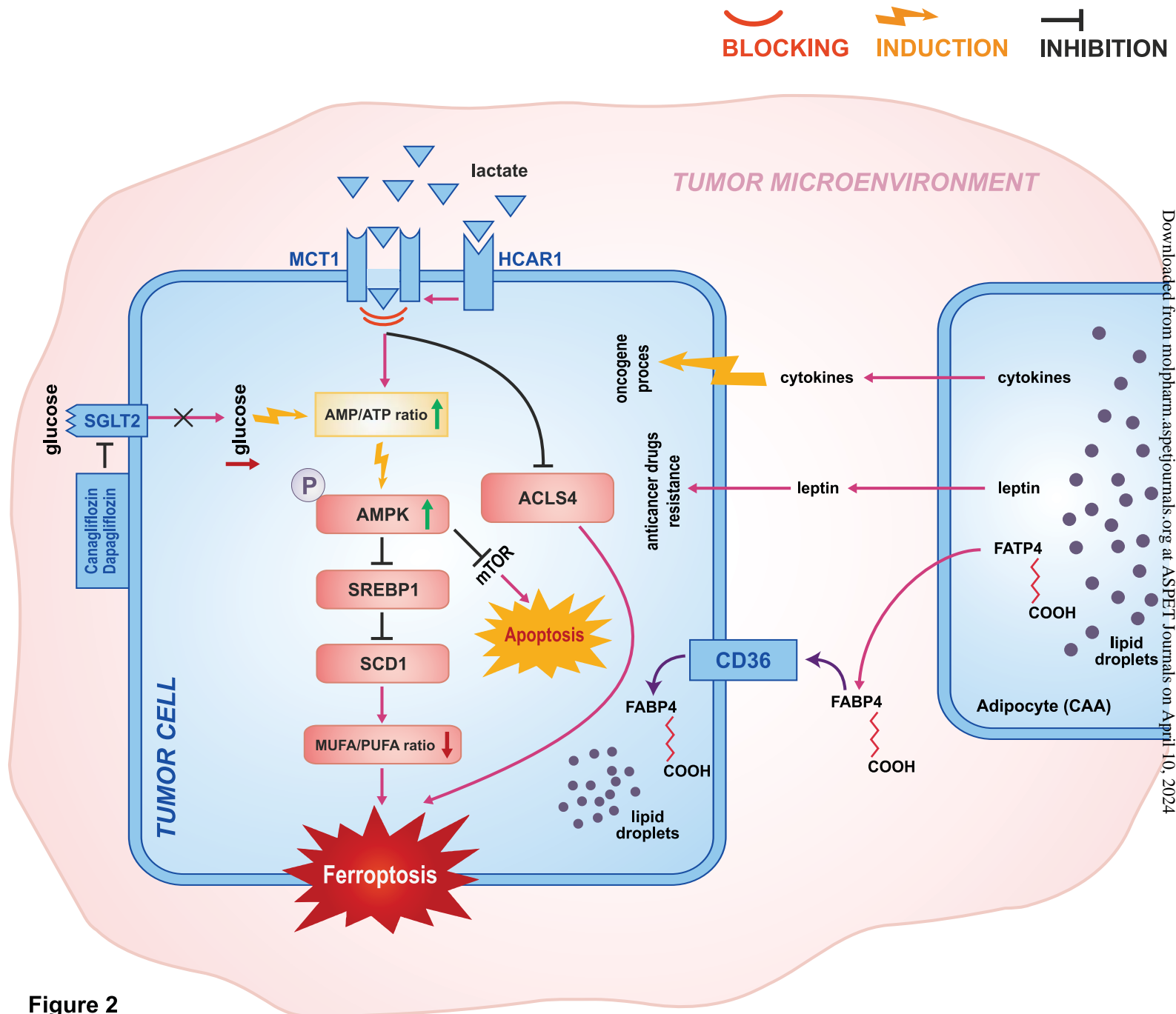
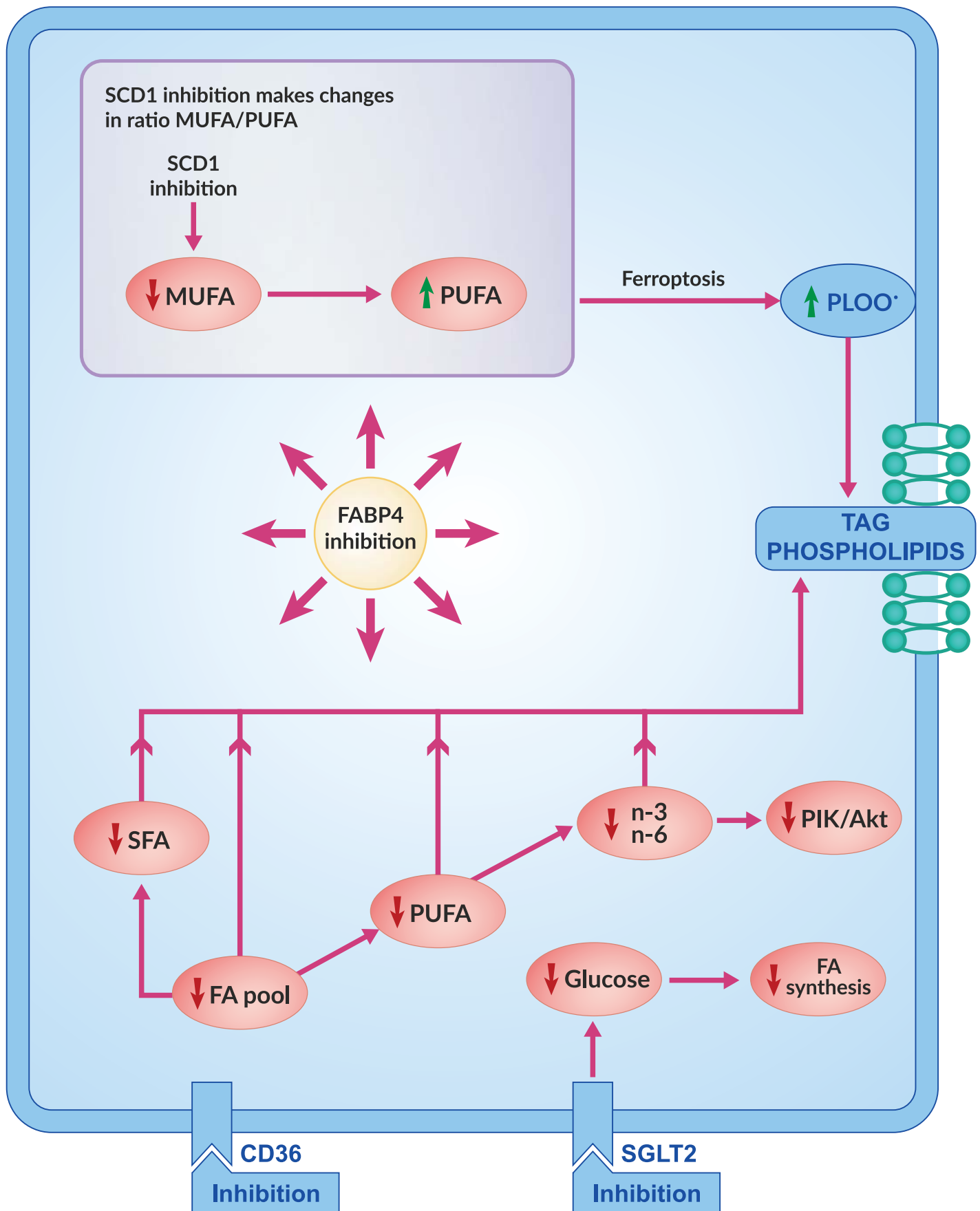


Figure 2

## ACYL STARVATION



**Figure 3**