Targeting Glutathione Metabolism in cancer

A new therapeutic approach

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To my family

“In fact, it is now conceivable that our children's children will know the term cancer only as a constellation of stars”

Bill Clinton (2000)

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ABSTRACT

Antioxidants have long been considered as potential cancer-preventive agents, though most clinical trials have failed to demonstrate significant benefits from antioxidant supplements. Recent scientific findings, however, have shifted this view by suggesting that pro-oxidant therapies could offer promising anti-cancer strategies. Despite this potential, few pro-oxidant therapies have successfully transitioned into clinical use. Herein, we aimed to determine the impact of antioxidant-targeting therapies on cancer progression, with a focus on pro-oxidative drugs as novel treatments. To explore how antioxidant supplementation affects the initiation and progression of colorectal cancer, we treated a mouse model of hereditary colorectal cancer (ApcMin/+) with N-acetyl cysteine (NAC) and Vitamin E. Our results showed that antioxidant supplementation with NAC, at serum concentrations equivalent to patients given prescription NAC, promoted tumor progression and worsened histological tumor grading. We then examined how nutritional factors affect susceptibility to the glutathione-depleting drug buthionine sulfoximine (BSO) and discovered that amino acid availability is a key factor governing ferroptosis sensitivity, an iron-dependent form of cell death triggered by lipid peroxidation. This sensitization was mediated by the integrated stress response (ISR) pathway, with the transcription factor ATF4 lowering the threshold for lipid peroxidation. To identify novel targets for pro-oxidant therapy, we performed a CRISPR-Cas9 screen to uncover genes that exacerbated the effects of three different pro-oxidant drugs. Notably, we found that activation of the Wnt signaling pathway increased iron uptake via the transferrin receptor, enhancing the effects of BSO treatment. In our final study, we focused on identifying targets of the transcription factor ATF4. We discovered that TRIB3 may represent a novel therapeutic target in combination with BSO treatment. In conclusion, our findings raise concerns about the use of antioxidant

supplements in individuals at risk of developing colorectal cancer. Amino acid restriction presents a promising avenue for further investigation as a therapeutic intervention alongside ferroptosis-inducing drugs. Additionally, Wnt-targeting agents such as CHIR show potential as effective combination therapies with BSO.

Keywords: Glutathione, lipid peroxidation, radical oxygen species, ferroptosis

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SAMMANFATTNING PÅ SVENSKA

Under de senaste decennierna har betydelsen av antioxidanter för tumörutveckling varit föremål för debatt, och nya vetenskapliga rön har utmanat den tidigare vedertagna uppfattningen om antioxidanter som skyddande mot cancer. Reaktiva syreföreningar (ROS) bildas normalt i cellens ämnesomsättning och spelar en viktig roll i normal fysiologi. Men när mängden reaktiva syreföreningar ökar och överstiger cellens antioxidantkapacitet, utsätts cellen för så kallad oxidativ stress. Detta leder till oxidativa skador på flera delar av cellen, inklusive DNA och de fetthöljen som omger våra celler, även kallade cellmembran.Forskning tyder på att läkemedel riktade mot cellens antioxidant system kan vara viktiga nya behandlignar mot framförallt lungcancer.

Argumenten för att rikta in sig på antioxidanter i cancerterapi inkluderar bland annat: 1) randomiserade kliniska studier visar att antioxidant tillskott ökar risken för prostata- och lungcancer, 2) mutationer i gener som kodar för antioxidanter är vanliga vid lungcancer, 3) tumörens miljö är starkt oxidativ på grund av den kärlnybildning som tumörer stimulerar, och 4) cancerceller har ett ökat järnupptag, vilket leder till ökade oxidativa skador.

Syftet med denna avhandling har varit att undersöka hur läkemedel som riktar sig mot antioxidanter kan användas som en ny behandlingsstrategi mot cancer, med särskilt fokus på lungcancer. För att identifiera nya måltavlor som kan kombineras med antioxidantinriktade behandlingar för att hämma tumörtillväxt, använde vi CRISPR-Cas9 screeningteknologi på lungcancerceller i cellkulturer.

I studien, som utgör det tredje delarbetet i denna avhandling, visade vi att aktivering av en viktig signalöverföringsprocess i cancerceller, kallad WNT-signalering, ökar järnupptaget och därmed även känsligheten för hämning av den viktiga antioxidanten glutation. Vi undersökte även hur näringsstatus påverkar lungcancercellernas känslighet för läkemedel som riktar sig mot glutation. Våra resultat visade att aminosyrabrist ökar lungcancercellernas beroende av glutation, vilket i sin tur ökar deras känslighet för en sådan behandling.

Resultaten ökar vår förståelse för tumörbiologi och öppnar bland annat upp för vidare studier kring proteinfattig kost i kombination med glutation riktade terapier.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Zou ZV, Le Gal K, El Zowalaty AE, Pehlivanoglu LE, **Garellick V**, Gul N, Ibrahim MX, Bergh PO, Henricsson M, Wiel C, Akyürek LM, Bergo MO, Sayin VI, Lindahl P. (2021). *Antioxidants Promote Intestinal Tumor Progression in Mice*. Antioxidants. Feb 4;10(2):241. doi: 10.3390/antiox10020241.

II. **Garellick, V.J.**, Gul, N., Horrieh, P., Mustafa, D., Alvarez, S., Dankis, M., Patel A.H., Berndtsson, J., Schwarz, M., Persson, A., Zahirovic, F., Wiel, C., Sayin, V., Lindahl, P. (2024). *Amino acid supply determines susceptibility to ferroptosis-inducing agents in lung cancer cells*. (Manuscript in revision)

III. Gul, N., **Garellick**, **V.J.**, Horrieh, P., Mustafa, D., Karlsson, J., Patel, A.H., Dzanan, J., Ali, K., Alvarez, S., Tuyizere, S., Zahirovic, F., Nilsson, J., Wiel, C., Sayin, V., Lindahl, P. (2024). *By increasing transferrin uptake in cancer cells, WNT-signaling is synthetic lethal with buthionine sulfoximine, a glutathione synthesis inhibitor*. (Manuscript)

IV. **Garellick**, **V.J.**, Berisha, T., Gul, N., Lindahl, P.(2024). *Genomic profiling of the integrated stress response pathway in amino acid deprived lung cancer cells*. (Manuscript)

CONTENTS

ABBREVIATIONS

GSH	Glutathione
BSO	L-Buthionine sulfoximine
PUFA	Polyunsaturated fatty acid
MUFA	Monounsaturated fatty acid
NAC	N-acetylcysteine
NSCLC	non-small cell lung cancer
SCLC	Small-cell lung cancer
CRC	Colorectal cancer
ROS	Reactive oxygen species
ChIP	Chromatin immunoprecipitation
TfR	Transferrin receptor
TrX	Thioredoxin
ISR	Integrated stress response pathway
CRISPR	Clustered regularly interspaced short palindromic repeats
Cas9	CRISPR associated protein 9
EGFR	Epidermal growth factor receptor
mTOR	Mammalian/mechanistic target of rapamycin
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog
TME	Tumor microenvironment

ECM	Extracellular matrix
ATF4	Activating transcription factor 4
GPX4	Glutathione peroxidase 4
IC50	Half maximal inhibitory concentration
FBS	Fetal bovine serum
GCN2	General control nonderepressible 2
RSL3	Ras-selective lethal small molecule 3
TRIB3	Tribbles pseudokinase 3
GO	Gene ontology

DEFINITIONS IN SHORT

Synthetic lethality	The process by which the loss of two genes infers cell death but the loss of either one gene does not affect health.
Genome-wide	Spanning the entire genome, including all coding and non-coding regions, regulatory elements, and other genomic sequences.
Pro-oxidative	A process or agent that promotes the formation of reactive oxygen species (ROS) or other free radicals, leading to oxidative stress.
Homeostasis	The process by which living organisms regulate and maintain a stable internal environment, including both intracellular and extracellular conditions, to ensure proper function and health despite external changes.
Ferroptosis	An iron-dependent regulated cell death pathway triggered by lipid peroxidation
Lipid peroxidation	The addition of a peroxyl (O-O) moiety at specific carbon atoms in polyunsaturated fatty acids forming the reactive lipid hydroperoxides.
GO-term	Gene Ontology (GO) term is a standardized concept used to describe the properties and functions of genes and their products (such as proteins) in a consistent way across different species.

1 INTRODUCTION

This thesis investigates the potential of targeting the glutathione system as a novel therapeutic approach in cancer treatment. It focuses on elucidating the mechanisms by which the glutathione system influences cancer cell adaptation to oxidative stress and lipid peroxidation. A key aspect of this PhD project is the examination of nutritional status, particularly amino acid availability, and its role in maintaining redox homeostasis. The following sections outline key themes explored in this project, e.g. including the role of reactive oxygen species, amino acid metabolism in cancer, ferroptosis and the cellular antioxidant defense mechanisms.

1.1 PATHOPHYSIOLOGY OF COLORECTAL AND LUNG CANCER

Colorectal cancer (CRC) and lung cancer are two of the most prevalent and deadliest cancers worldwide [1]. Both cancers display common features in their pathophysiology, particularly regarding genetic mutations, molecular pathways, and the influence of environmental factors.

1.1.1 COLORECTAL CANCER

CRC stems from cells in the epithelial duct lining the colon or rectum. CRC disease development is a well-defined sequential process of genetic mutational events and epigenetic alterations [2]. A central model of CRC development is the adenoma-carcinoma concept which postulates that most colorectal cancers develops from benign adenomatous polyps (non-cancerous tumors) into malignant carcinomas [3, 4]. Key genetic mutations facilitating malignant transformation, include the activation of oncogenes such as *KRAS* and the inactivation of tumor suppressor genes like *APC* (Adenomatous Polyposis Coli) and *TP53* (tumor protein p53) [5].

The Wnt/β-catenin signaling pathway is crucial in CRC development. Mutations in the APC gene lead to the accumulation of β-catenin, which in turn activates transcription of genes involved in cell proliferation and survival [6]. Additionally, microsatellite instability (MSI), a condition of genetic hypermutability, results from defects in DNA mismatch repair (MMR) genes such as *MLH1, MSH2, MSH6*, and *PMS2* [7, 8]. Approximately 15% of CRC cases exhibit high levels of MSI, which are associated with a distinct molecular phenotype and better prognosis.

Due to better diagnostics and colonoscopy screening programs, the mortality rate of colorectal cancer has declined in recent decades, as precancerous polyps can be effectively detected and removed [9, 10]. Despite this, the average age of diagnosis is decreasing and many patients carries the disease without experiencing symptoms allowing the cancer to progress and spread [11]. Therefore, the development of new medical treatment options is crucial for effectively managing and treating CRC, especially in those patients harboring metastatic lesions [12].

1.1.2 LUNG CANCER

Lung cancer originates from epithelial cells that line various parts of the respiratory tract, including the alveoli and bronchial passages [13, 14]. It is primarily classified into two main types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC which makes up approximately 85% of cases, encompasses adenocarcinoma, squamous cell carcinoma, and large cell carcinoma [15]. In contrast, small cell lung cancer (SCLC), a more aggressive type, accounts for about 15% of cases. Lung adenocarcinoma is the most common form of NSCLC and typically originates in the peripheral or outer regions of the lung. Lung adenocarcinomas often metastasize to the brain, bones, liver, and adrenal glands [16].

Lung cancer remains the deadliest cancer globally, despite the advent of revolutionary immune therapies, primarily because it is often diagnosed at advanced stages and can progress without noticeable symptoms for a long time [17-19]. The development of neoadjuvant therapies, particularly the combination of immunotherapy using PD-L1 inhibitors with chemotherapy, has shown significant effectiveness in patients with resectable NSCLC tumors [20, 21]. However, as the disease progresses and metastasizes to nearby or distant organs, tumors often become unresectable, leaving many patients with palliative care as the only remaining option [22]. Therefore, there is a pressing need for new therapies in the treatment of NSCLC.

Key genetic driver mutations in lung cancer involve the *EGFR* (epidermal growth factor receptor) gene, *ALK* (anaplastic lymphoma kinase) gene rearrangements and *KRAS (*Kirsten rat sarcoma viral oncogene) mutations. These lesions drive oncogenesis in NSCLC by promoting uncontrolled cell growth, proliferation and survival through various signaling pathways, including the PI3K/AKT, RAS/RAF/MEK (**Fig.1**) [23, 24]. Furthermore, approximately 20% of lung adenocarcinoma tumors harbor mutations in *KEAP1* (Kelch-like ECH-associated protein 1), a key tumor suppressor gene. When mutated, *KEAP1* fails to regulate the Nrf2 pathway, leading to the upregulation of antioxidant genes that promote tumor survival and resistance

to oxidative stress [25]. Tobacco smoking is a major risk factor for lung cancer, contributing to DNA damage and mutations in tumor suppressor genes such as *TP53* and *RB1* (retinoblastoma 1) and oncogenes like *KRAS*, especially in SCLC [26, 27].

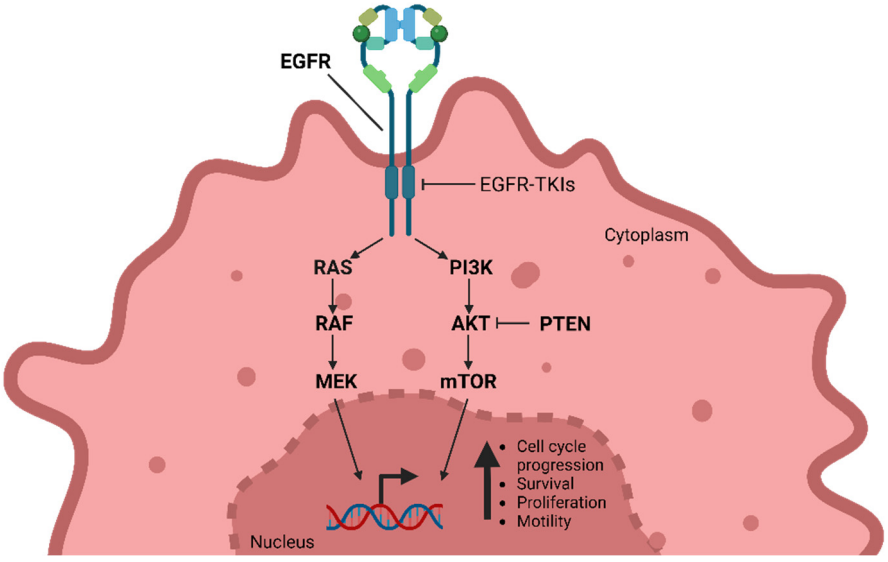

Figure 1 *"EGFR Signaling Pathway and its Role in Cancer Progression".* A simplified overview of the RAS-RAF-MEK-ERK and PI3K-AKT-mTOR signaling cascades, which regulate essential cellular processes such as cell cycle progression, survival, proliferation, and motility. These pathways play crucial roles in the development of cancers, including colorectal and lung cancer.

Despite originating from different tissues and environments, CRC and lung cancer share several similarities in their disease development. In the following section, I will explore these commonalities in the onset of both CRC and lung cancer.

1.1.2.1 SHARED TRAITS IN PATHOPHYSIOLOGY

CRC and lung cancer have many pathophysiological features in common. Molecular events like mutations in *KRAS* and *TP53* plays crucial roles in the development of both cancers. In addition, environmental factors like smoking is contributing to both lung cancer and CRC development. In lung cancer, smoking is a primary risk factor, while in CRC, it is associated with an increased risk of adenoma formation and malignant transformation [28, 29].

Moreover, dysregulated Wnt signaling is a common feature shared by both CRC and lung cancer. The vast majority of colorectal cancer cases exhibit dysregulated Wnt signaling, often due to mutations in the tumor suppressor gene *APC*, a key component of the Wnt destruction complex [30]. These mutations drive malignant transformation in CRC. Although mutations in Wnt pathway genes are rare in lung cancer and insufficient on their own to drive tumorigenesis, Wnt/β-catenin signaling has been shown to enhance cancer progression with studies showing its role in promoting metastasis to distant organs, further contributing to lung cancer's aggressiveness [31].

Another common aspect is the role of chronic inflammation. Autoimmune conditions like inflammatory bowel disease (IBD) significantly increase CRC risk by promoting a pro-carcinogenic environment [32]. Also, prolonged exposure to irritants such as tobacco smoke or air pollutants can cause chronic inflammation, which may lead to lung cancer [33] .

In addition, both CRC and lung cancer show significant genetic heterogeneity by various mutational and epigenetic alterations that influence tumor behavior and treatment response [34, 35].

The tumor microenvironment is a critical factor in both cancers, with interactions between cancer cells and surrounding stromal cells, immune cells, and components of the extracellular matrix playing a key role in tumor progression and metastasis [36, 37].

1.2 WNT SIGNALING: MECHANISMS, REGULATION, AND IMPLICATIONS IN CANCER DEVELOPMENT

In both Paper I and Paper III, Wnt signaling is a central focus. In this section, I will delve deeper into the details of this crucial pathway. Wnt signaling is an evolutionarily conserved pathway that plays a pivotal role in development, particularly in morphogenesis, and is essential for regulating stem cell maintenance. Additionally, it has significant implications in cancer development.

The Wnt family of proteins consists of 19 members, each with a distinct function. Wnt ligands undergo lipidation in the Golgi apparatus, a process essential for their subsequent binding to the Frizzled (FZD) receptors. After lipidation, Wnt ligands are secreted into the extracellular space via vesicle exocytosis. The enzyme Notum can cleave the lipid moiety from the Wnt

ligands, which inhibits their ability to activate signaling. This cleavage regulates Wnt signaling by preventing the Wnt ligands from effectively binding to their primary receptor, FZD [38].

Wnt signaling is a complex pathway that branches into several distinct pathways. In the canonical Wnt signaling pathway (**Fig.2**), the binding of a Wnt ligand to the FZD receptor initiates a cascade that inhibits the destruction complex, leading to the accumulation of the transcription co-activator β-catenin, which then targets a variety of downstream genes.

When Wnt signaling is inactive, a destruction complex forms, consisting of the adaptor protein APC, which binds β-catenin, along with Axin, and the serine/threonine kinases CK1 and GSK3α/β. This complex facilitates the phosphorylation of β-catenin, marking it for polyubiquitination by an E3 ligase, which targets it for proteasomal degradation [38] . However, upon Wnt activation, the destruction complex is sequestered into multivesicular bodies (MVBs), preventing it from interacting with β-catenin. This sequestration allows β-catenin to accumulate and translocate to the nucleus, where it binds

to TCF/LEF transcription factors, activating the transcription of Wnt target genes [39, 40].

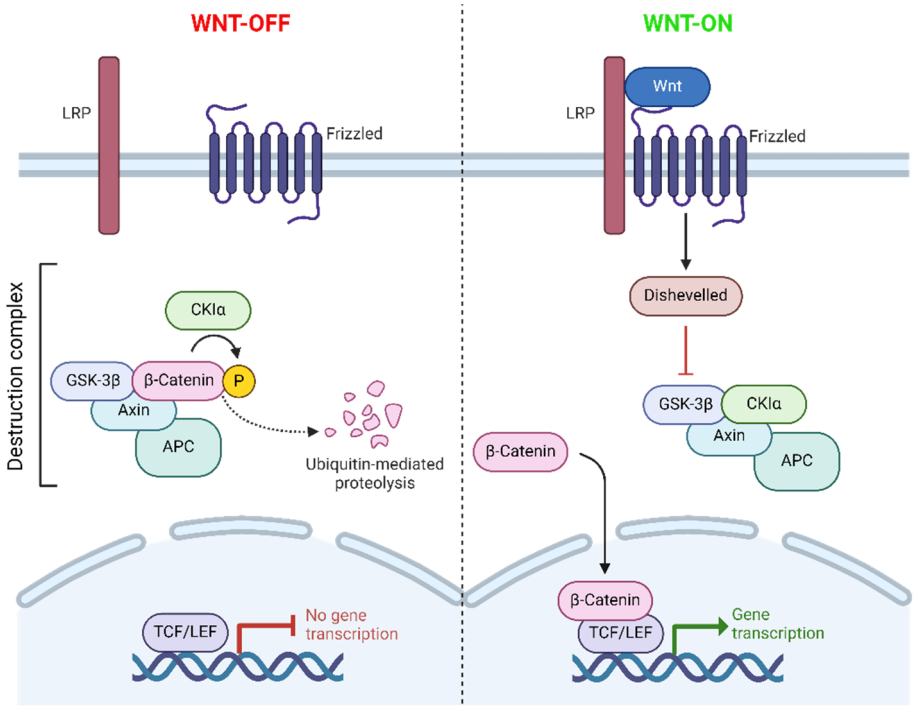

Figure 2 *"Canonical WNT-signaling".* This figure illustrates the two states of the Wnt signaling pathway. On the left, in the "Wnt-Off" state, the absence of Wnt ligand leads to the phosphorylation of β-catenin by the destruction complex (composed of GSK-3β, CKIα, Axin, and APC). Phosphorylated β-catenin is targeted for ubiquitin-mediated proteolysis, preventing gene transcription. On the right, in the "Wnt-On" state, the binding of Wnt to its receptors (Frizzled and LRP) inhibits the destruction complex via Dishevelled, allowing β-catenin to accumulate and translocate into the nucleus, where it associates with TCF/LEF to promote gene transcription

Activation of the Wnt pathway is a critical driver of cancer, as it promotes cell survival, proliferation, and the maintenance of stemness, preventing differentiation, a process that can inhibit tumorigenesis. The frequent APC mutations in CRC highlight the significance of deregulated Wnt signaling in cancer development [41, 42]. There is considerable cross-talk between the Wnt pathway and other pathways involved in cancer progression, such as the EGFR pathway. Wnt ligands can activate EGFR signaling through the Frizzled receptor, while EGFR can activate β-catenin via the receptor tyrosine kinase-PI3K/Akt pathway. The interaction of these pathways enhances invasiveness and therapeutic resistance, contributing to a more aggressive cancer phenotype.

Mutations in both Wnt and EGFR-related genes often coexist, further driving cancer progression [43, 44].

1.3 LIPID METABOLISM IN CANCER

In cancer cells, reprogramming lipid metabolism is a crucial strategy to meet the increased energy demands of carcinogenesis. However, this deregulated lipid metabolism extends beyond mere energy production, impacting vital cellular processes such as proliferation, growth, differentiation, and immune response [45].

Lipids encompass a diverse group of biomolecules, including waxes, sterols, phospholipids, triacyl glycerides, and fatty acids. These molecules serve multiple roles within the cell, such as acting as immunological mediators, substrates for hormone synthesis, energy reservoirs, second messengers in signaling pathways, and as the building blocks of cell membranes [46].

Fatty acids can exist freely within the cell and serve multiple functions. They can be converted into acetyl-CoA via beta-oxidation for energy production, used in lipogenesis and triacyl glyceride biosynthesis, serve as substrates for sterol synthesis, or be incorporated into phospholipids [47].

Fatty acids are categorized as either saturated or unsaturated, with the latter further divided into monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) [48]. While saturated and monounsaturated fatty acids can be obtained from the diet or synthesized through lipogenesis, polyunsaturated fatty acids are primarily acquired from dietary sources. Linoleic acid and alpha-linolenic acid, two essential PUFAs, cannot be synthesized by the body and must be obtained from food [49].

The primary distinction between saturated and polyunsaturated fatty acids lies in the presence of double bonds between carbon atoms in the hydrocarbon tail of unsaturated fatty acids. These double bonds introduce "kinks" in the molecular structure, preventing tight packing of the molecules. This structural feature reduces membrane rigidity and enhances fluidity, which has significant implications for membrane structural integrity, function and cellular processes such as metastasis [50, 51].

Lipogenesis, the *de novo* synthesis of fatty acids from acetyl-CoA, plays a crucial role in tumor development and involves several distinct enzymes. These enzymes are typically expressed only in highly metabolic tissues, such as the liver and musculoskeletal system, as most other tissues obtain fatty acids from the diet [52].

Lipotoxicity refers to the disruption of fatty acid metabolism, specifically the imbalance between saturated and unsaturated fatty acids, which triggers apoptosis and has significant implications in cancer [53]. Desaturation of fatty acids during lipogenesis, the synthesis of fatty acids from acetyl-CoA, plays a critical role in cancer cell stress tolerance. The enzyme stearoyl-CoA desaturase (SCD) is responsible for converting stearic acid into monounsaturated fatty acids. Inhibition of SCD has been associated with lipotoxicity in cancer, and studies show that overexpression of SCD is a negative predictor of survival in various cancers [54].

Deregulated lipid metabolism also plays a pivotal role in the complex interactions between the tumor and the tumor microenvironment (TME), comprising stromal and immune cells. Tumors can export various lipids to manipulate the cells in the TME, altering their metabolism to support tumor growth [55].

The plasma membrane, essential for cellular life, functions as a critical barrier that maintains homeostasis by regulating the uptake and release of biomolecules and adapting to fluctuations in the extracellular environment. It controls the movement of molecules, such as amino acids and sugars, through mechanisms like diffusion, ion channels, pumps, and vesicle-mediated transport [56].

The membrane’s dynamics are regulated by the types of phospholipids, specific fatty acids, and cholesterol distribution. Phospholipids, which consist of a hydrophilic 'head' (phosphate group) and hydrophobic 'tails' (fatty acids), form the bilayer that serves as the membrane's structural foundation. This bilayer, described as a fluid mosaic [57], contains integral proteins, such as ion channels and receptors, that move within the membrane, although their movement is influenced by membrane fluidity. Membrane fluidity regulates receptor function, cellular communication, and motility, processes essential for tumor cell metastasis [58-60].

1.4 THE CRUCIAL ROLE OF AMINO ACIDS IN CANCER METABOLISM AND THERAPEUTIC STRATEGIES

Amino acids play a crucial role in cancer cell metabolism, extending far beyond their function as mere building blocks for protein synthesis. They serve as essential substrates for nucleotide synthesis, contribute to energy production through processes such as the transamination of glutamine to alpha-ketoglutarate, and are vital for maintaining redox homeostasis through their

role in antioxidant production, including glutathione [61]. In addition to being protein building blocks, amino acids play a vital role in cellular signaling pathways, such as mTOR, a key metabolic hub involved in cancer cell metabolism [61].Cancer cells, due to their rapid proliferation and increased exposure to ROS, have an elevated demand for amino acids to maintain redox balance and support the synthesis of biomass, making these molecules essential for their survival and growth [61].

Amino acids are composed of a carboxyl group (-COOH) and an amino group (-NH2), but they differ significantly in their side chains, known as R-groups. These R-groups determine the unique properties and functions of each amino acid, leading to a variety of classes with distinct traits. The twenty-one proteinogenic amino acids, including selenocysteine, which plays a crucial role in redox homeostasis, serve as the building blocks of proteins. These amino acids are divided into two categories: essential, which must be obtained through the diet, and non-essential, which the body can synthesize [48] .

The distinction between essential and non-essential amino acids is crucial for understanding their roles in nutrition and metabolism. Essential amino acids cannot be synthesized by the human body and must be obtained through diet. These include histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine.

Non-essential amino acids are those that the body can synthesize on its own. These include alanine, asparagine, aspartic acid, glutamic acid, serine, and in some cases, arginine, cysteine, glutamine, glycine, proline, and tyrosine, which may become conditionally essential under certain physiological conditions such as stress or illness or during rapid proliferation as in cancer cells [62].

The altered amino acid metabolism in cancer cells has gained significant attention in recent years as a potential therapeutic target. Mutational transformations in cancer cells can lead to auxotrophy, a condition where cancer cells become dependent on certain non-essential amino acids (NEAA) for survival due to mutations in amino acid metabolic enzymes rendering cancer cells incapable of synthesizing NEAA. This dependency, or "amino acid addiction," presents an opportunity to develop treatments that selectively target cancer cells while sparing healthy cells [63-65].

A few drugs that exploit this metabolic vulnerability are already in clinical use. For example, asparaginase is a drug used to target asparagine which leads to its degradation. This treatment is particularly effective against certain types of leukemia, such as acute lymphoblastic leukemia (ALL), that are addicted to asparagine for their survival, while normal cells remain largely unaffected

[66]. In the following subparagraphs I will discuss in more detail some of the most important amino acids in cancer.

Glutamine plays a crucial role in cancer metabolism due to its diverse and essential functions. Often referred to as the "nitrogen donor," glutamine is vital for synthesizing amino acids, nucleotides, and glutathione. In many tumors, the enzyme glutaminase is frequently upregulated, which catalyzes the conversion of glutamine to glutamate, a process known as glutaminolysis. This catabolic pathway supports cellular respiration by converting glutamate into alpha-ketoglutarate, a key intermediate that fuels the tricarboxylic acid (TCA) cycle, thereby sustaining the rapid growth of cancer cells [67-69].

Cysteine, a sulphur-containing amino acid, is vital for maintaining redox balance within cells as it is a precursor for glutathione. Its metabolism is tightly regulated, with cysteine imported into cells via the system xc- antiporter, which exchanges cysteine for glutamate in a 1:1 ratio [70, 71].

Methionine, an essential sulphur-containing amino acid, plays a crucial role in the synthesis of glutathione through the transsulfuration pathway, where it is converted into homocysteine, a key substrate for glutathione production [72]. Research has shown that restricting methionine intake can provide significant health benefits, including enhanced lipolysis and improved insulin sensitivity [73]. Moreover, methionine restriction has emerged as a promising therapeutic strategy in cancer treatment, as many tumors rely on methionine for growth and survival, making them vulnerable to dietary interventions that limit this amino acid [74, 75].

Branched chain amino acids **(**BCAAs in short), including leucine, isoleucine, and valine, are crucial not only for protein synthesis but also for regulating the mTOR signalling pathway, which is critical for cell growth and proliferation. In cancer, elevated levels of BCAA catabolic enzymes are often observed, linking their metabolism directly to tumor growth and progression [76, 77].

Arginine is essential for synthesizing important molecules such as nitric oxide, polyamines, and proline, all of which contribute to tumor biology. In many cancers, arginine metabolism is altered, with enzymes like argininosuccinate synthase 1 (ASS1) being downregulated, making cancer cells more dependent on external sources of arginine. This lack of ASS1 is an example of the aforementioned auxotrophic condition. This dependency has been identified as a potential target for cancer therapy [78].

Serine and glycine are critical components of cancer metabolism, particularly in nucleotide synthesis and one-carbon metabolism. The enzyme phosphoglycerate dehydrogenase (PHGDH), which diverts glycolysis intermediates into the serine biosynthesis pathway, is often overexpressed in cancers, enhancing the production of serine and glycine. These amino acids are crucial for purine synthesis, which is essential for DNA replication and repair, supporting the rapid proliferation of cancer cells [79, 80].

1.4.1 THE INFLUENCE OF AMINO ACID SIGNALING ON CANCER CELL SURVIVAL AND PROLIFERATION

Due to the high proliferation rate of cancer cells, there is an increased demand for anabolic pathways to sustain the accumulation of biomass. Consequently, cancer cells require a greater supply of substrates for biomolecule synthesis, particularly nitrogen donors like the amino acid glutamine, which is essential for nucleotide and protein production. This reprogrammed metabolism is driven by common driver mutations, such as those in *TP53, PTEN, KRAS*, and *c-MYC*. When these mutations co-exist, they lead to hyperactivation of mTOR, which in turn promotes glycolysis, glutamine metabolism, altered lipid metabolism, enhanced protein synthesis, and suppresses autophagy [81-84].

The Integrated Stress Response (ISR) pathway (**Fig.3**) is an evolutionarily conserved mechanism that integrates stress signals from four distinct kinases, converging on the eukaryotic initiation factor 2 alpha (eIF2α), a critical component of the pre-initiation complex and a central regulator of protein synthesis. One of the key kinases in this pathway is General Control Nonderepressible 2 (GCN2), which is particularly relevant in the context of amino acid deprivation. This kinase senses the accumulation of deacetylated tRNAs lacking their corresponding amino acids, an outcome of amino acid deprivation.

When eIF2α is phosphorylated at its serine 51 position by any of these upstream kinases, cap-dependent translation is inhibited, leading to a global reduction in protein synthesis. This reduction helps to conserve resources by slowing down the synthesis of new proteins and salvaging nutrients under stress conditions [85, 86]. This process simultaneously leads to the transcription of specific mRNAs containing a cis-regulatory element known as internal ribosomal entry sites (IRES), which enable the recruitment of the 40S ribosomal subunit without the need for a 5' cap. Two such transcripts are Activating Transcription Factor 4 (ATF4), a key effector in the ISR-pathway and DDIT3/CHOP, a C/EBP family transcription factor involved in regulating both pro-survival and pro-death cellular processes [87].

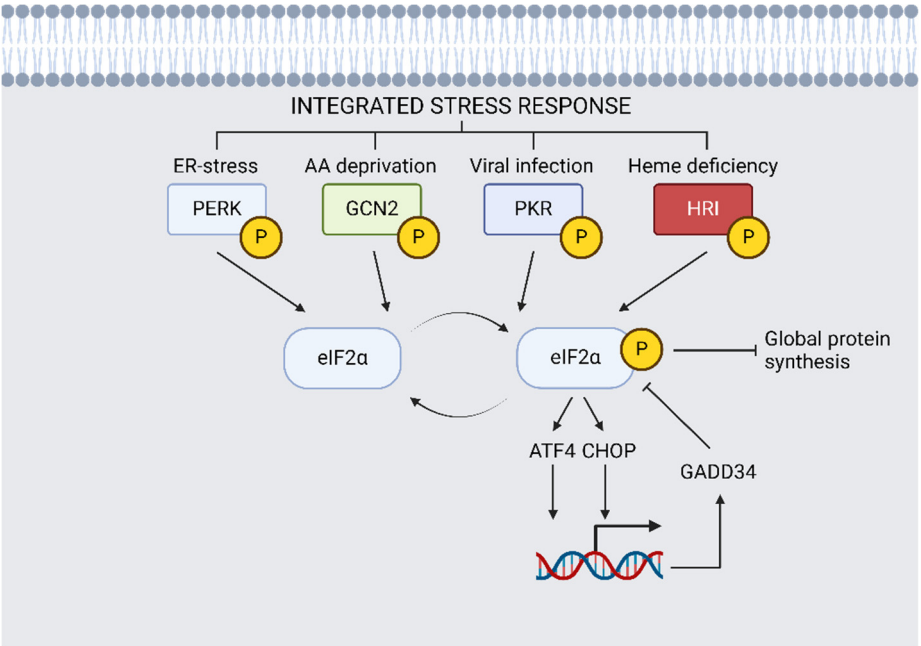

Figure 3 "*An illustration of the integrated stress response pathway".* This schematic illustrates the Integrated Stress Response (ISR) pathway, where various stress signals—such as ER stress (PERK), amino acid (AA) deprivation (GCN2), viral infection (PKR), and heme deficiency (HRI)—activate distinct kinases. These kinases phosphorylate eIF2α, leading to the attenuation of global protein synthesis while selectively inducing the transcription of stress response genes like ATF4 and CHOP. The downstream effects include cellular adaptation to stress or cell death depending on the duration of the stressor. GADD34 acts as a negative feedback control, regulating ISR-activity by dephosporylating eIF2α.

As a result, ISR signaling is considered a double-edged sword. In the acute phase it, helps maintain cellular homeostasis under stress conditions, such as oxidative stress. However, if the stress is prolonged or unresolved, the balance shifts causing the pathway to promote cell death. Notably, studies have shown that ATF4-CHOP transcription is associated with increased oxidative stress and onset of cell death [87].

1.5 IRON HOMEOSTASIS

Iron is a crucial mineral for cell health and is vital for many functions in the body such as oxygen transport, oxidative phosphorylation and DNA synthesis. Iron balance is tightly regulated to maintain homeostasis and preventing iron toxicity by balancing iron absorption, storage and recycling [88].

Iron is primarily absorbed in the duodenum, where enterocytes take it up via the divalent metal transporter 1 (DMT1) of absorptive enterocytes [89]. DMT1 is a H^+ proton pump regulated metal symporter that also transport metals other than Fe^{2+} like Mn^{2+}, Cu^{2+}, Zn^{2+}. However, the major pathway for iron import into cells is through the transferrin receptor [90]. Transferrin, an iron-binding protein, carries ferric iron (Fe^{3+}), and when loaded with ferric iron, known as holotransferrin, it binds to the transferrin receptor (TfR) on the cell surface. The transferrin-receptor complex is then internalized via clathrin-mediated endocytosis (CME), and ferrous iron (Fe^{2+}) is released into the cell [90]. Once inside the cell, iron is either stored by the protein ferritin or remains as free iron in what is known as the labile iron pool. The labile iron pool can be further increased by processes like ferritinophagy, the autophagic degradation of ferritin [91].

Cancer cells harbor a large, labile iron pool due to their increased demand for iron to support tumorigenesis. Iron is primarily imported into cancer cells via TfR, increasing the availability of free iron, which is crucial for various metabolic processes, including DNA synthesis, the formation of iron-sulphur (Fe-S) cluster proteins, and heme synthesis. This elevated iron supply not only fuels cell proliferation and angiogenesis but also contributes to genetic instability, accelerating mutagenesis and promoting cancer progression [92, 93].

However, excessive levels of labile iron can be harmful, as iron can catalyze the production of reactive oxygen species through Fenton reactions, leading to oxidative stress and cellular damage [88]. The Fenton reaction (see below), where ferrous iron (Fe^{2+}) reacts with hydrogen peroxide to produce hydroxyl radicals (•OH), plays a central role in ferroptosis (described more in detail in following paragraphs) a form of iron dependent regulated cell death pathway.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$$

1.6 REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) are a group of highly reactive oxygen compounds primarily arising from the incomplete reduction of oxygen during oxidative phosphorylation in the mitochondria [94]. Other sources of ROS in the cell include peroxisomes, the endoplasmic reticulum, and plasma membrane-associated enzymes like NADPH oxidases (NOXs). ROS encompasses several different molecules like hydrogen peroxide (H_2O_2), superoxide (O_2^-), and the hydroxyl radical (HO·).

Historically, ROS were primarily regarded as toxic byproducts of cellular metabolism and key contributors to disease development. However, increasing evidence shows that ROS also play crucial physiological roles, such as in cellular signaling, proliferation, and apoptosis, making them essential for maintaining cell health [95, 96]. ROS is generated as a response to xenobiotics and cytokines to maintain homeostasis and serves many important functions in the immune system [97-99] .

However, excessive accumulation of ROS can be detrimental to cells, leading to oxidative stress that damages various biomolecules, including lipids, proteins, and DNA [100]. This oxidative stress is linked to the development of a wide range of diseases, such as neurodegenerative disorders and cardiovascular conditions [101, 102].

The dual nature of ROS can be summarized as at moderate levels, they are beneficial, but in excess, they can be harmful. To mitigate oxidative damage, cells have evolved complex antioxidant defense systems. The following paragraph will explore these systems in detail.

1.7 ANTIOXIDANT DEFENSE SYSTEMS

The balance between ROS production and antioxidant defenses is tightly regulated by cellular signaling pathways. Among these, the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is regarded as the primary regulator of the cellular antioxidant response and plays a critical role in maintaining redox homeostasis. Nrf2 drives the expression of a wide array of antioxidant and detoxification genes by binding to antioxidant response elements (AREs) in their promoters.

Under conditions of oxidative stress, Nrf2 is released from its repressor, Kelch-like ECH-associated protein 1 (Keap1). Normally, Keap1 sequesters Nrf2 in the cytoplasm and targets it for proteasomal degradation. However, oxidative modifications to key cysteine residues on Keap1 inhibit this interaction, allowing Nrf2 to escape degradation. Once stabilized, Nrf2 translocate into the nucleus, where it initiates a comprehensive antioxidant response, including the upregulation of multiple defense systems designed to neutralize ROS and restore cellular redox balance [103]. Among these defense systems are various antioxidant enzymes and molecules that work synergistically to protect the cell from oxidative damage.

The cell possesses a robust array of different antioxidant defense systems including both enzymatic and non-enzymatic components. Endogenous

enzymes include superoxide dismutases (SODs), catalases and glutathione peroxidases. These act in a coordinated fashion to reduce ROS and inhibit oxidative stress, the oxidative damage to macromolecules. SODs convert superoxide O_2^- to H_2O_2 that is subsequently converted to water by the enzyme catalase. This conversion is essential because superoxide anions are highly reactive and can contribute to the generation of additional ROS like the hydroxyl radical [104, 105].

Additionally, the cell is equipped with thioredoxins (Trx), which are a crucial component of the enzymatic antioxidant defense system. Trxs are a family of thiol-containing enzymes that catalyze the reduction of disulfide bonds in proteins, thereby regulating protein function and maintaining redox homeostasis. The active site of Trxs contains two redox-active cysteine residues referred to as a CxxC-motif. Through a thiol-disulfide exchange reaction, Trx transfers electrons to oxidized thiol groups on target proteins, reducing them. The oxidized thioredoxins are then regenerated by thioredoxin reductases using electrons from NADPH [106]. Studies have demonstrated a degree of redundancy between the thioredoxin and glutathione systems (which will be discussed in more detail in the following paragraphs). Thioredoxin reductases can reduce oxidized glutathione (GSSG) in the absence of glutathione reductase (GSR) [107], while glutaredoxins can serve as backups when the thioredoxin system is compromised [108]. This cross-functional capability highlights the flexibility of these systems in maintaining cellular redox balance.

Non-enzymatic antioxidants include small molecules like glutathione (GSH), vitamins C and E, and other thiol-containing compounds. Vitamin C (ascorbic acid) and vitamin E (tocopherol) are vital lipid-soluble antioxidants. Vitamin E protects cell membranes from oxidative damage (known as lipid peroxidation), while vitamin C, which is water-soluble, regenerates reduced vitamin E and scavenges a variety of ROS in the aqueous phase. Additionally, the redox cycling between these two vitamins enhances their antioxidant capacity [109].

The long-held notion that antioxidants suppresses cancer has been under substantial scrutiny during the last decades as evidence suggests that their cancer-preventive effects are not well-supported. Clinical trials investigating the use of antioxidant supplements as cancer prevention agents have been generally been unsuccessful [110, 111]. Furthermore, antioxidant agents as adjuvant therapies with chemo- and radiotherapy has shown mixed results [112].

Contradicting the initial assumption, some clinical trials have linked vitamin E supplementation to an increased risk of prostate and lung cancer [26, 113]. Similarly, pre-clinical animal studies have also demonstrated that treatment with the antioxidant N-acetylcysteine (NAC) promotes tumor progression and metastasis in lung and melanoma cancers [114-116]. Additionally, Harris et al. (2015) demonstrated that the GSH and Trx systems work synergistically to promote tumor initiation and progression, and inhibiting both pathways together induced cell death in mouse models xenografted with the MDA-MB-231 breast cancer cell line [117].

To better understand the impact the impact of antioxidant supplementation and explore the potential paradox of antioxidants inhibiting tumor initiation while promoting progression, we conducted a study using a mouse model of familial CRC driven by an APC mutation. In paper I, we specifically tested the effects of the cysteine analog NAC and Vitamin E supplementation, on both tumor initiation and progression.

1.7.1 GLUTATHIONE: SYNTHESIS, FUNCTIONS, AND ITS ROLE IN CANCER

Since the emphasis of this thesis is on glutathione-targeted treatment I will in this paragraph delve deep into glutathione, how it is synthesized and the many functions it has in the cell.

Glutathione is a tripeptide composed of cysteine, glutamate, and glycine, and it is the most abundant intracellular antioxidant, playing a central role in maintaining cellular redox homeostasis. Glutathione is synthesized (**Fig.4**) through a two-step process. The first and rate-limiting step involves the enzyme gamma-glutamyl cysteine ligase (GCLC) catalyzing the joining of glutamate and cysteine to form gamma-glutamyl cysteine. In the second step, glycine is added to gamma-glutamyl cysteine, resulting in the formation of glutathione [118]

Once synthesized, glutathione performs a variety of essential functions in the cell. It is widely recognized as a general detoxifying agent with a broad range of functions, including directly scavenging ROS and acting as a substrate for glutathione peroxidases (GPXs) and glutathione S-transferases (GSTs) in detoxification processes [119].

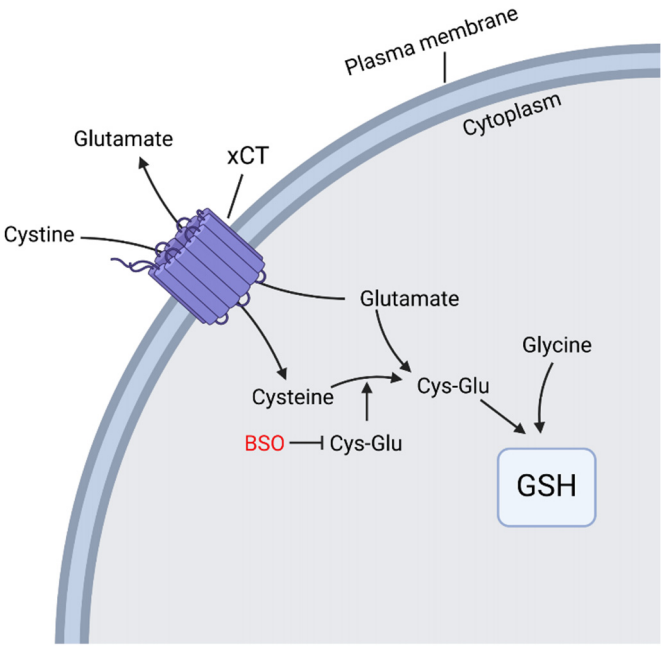

Figure 4 *"Cystine-Glutamate Antiporter (xCT) and Glutathione (GSH) synthesis".* This illustration depicts the role of the xCT transporter in importing cystine (dimer of cysteine) into the cell in exchange for glutamate export. Inside the cytoplasm, cystine is reduced to cysteine, which, along with glutamate and glycine, is used to synthesize glutathione (GSH). The compound BSO (buthionine sulfoximine) is shown inhibiting GSH synthesis by blocking the formation of γ-glutamylcysteine (Cys-Glu), a precursor to GSH. This process is crucial for maintaining cellular redox balance and protection against oxidative stress.

Cancer cells exhibit a strong dependency on glutathione to manage the oxidative stress associated with their high metabolic activity and rapid proliferation [120]. A primary function of glutathione is to serve as an electron donor to GPXs, which in turn reduce hydrogen peroxide and lipid peroxides to water and corresponding alcohols. This results in the conversion of glutathione to its oxidized form, glutathione disulfide (GSSG). GPX4, in particular, plays a crucial role in protecting cell membranes from lipid peroxidation [121]. To sustain cellular antioxidant defenses, the enzyme glutathione reductase regenerates reduced glutathione (GSH) from its oxidized form (GSSG), ensuring the continued antioxidant capacity of cells to combat oxidative stress.

In addition to its antioxidant functions, glutathione also participates in a process known as glutathionylation, in which it forms mixed disulfide bonds

with cysteine residues on proteins. This post-translational modification can alter the function and metabolism of target proteins and plays an important role in regulating the immune response, as well as influencing drug metabolism [122-124]. In particular, glutathionylation promotes chemotherapeutic resistance by stimulating drug efflux mechanisms and protecting cancer cells from oxidative stress [125, 126].

Buthionine-sulfoximine (BSO) is a glutamate analog that irreversibly inhibits the rate-limiting enzyme in glutathione synthesis GCLC, effectively depleting cellular glutathione levels. BSO has been evaluated in phase I clinical trials as part of a combination therapy with the cytotoxic drug melphalan for the treatment of high-risk neuroblastoma [127]. The trial reported acceptable side effects, with some patients achieving mixed responses or stable disease. However, BSO has not been tested as a monotherapy in clinical trials.

Despite glutathione's crucial role in supporting cancer cell proliferation, drug screening studies have revealed that most cancer cell lines exhibit resistance to BSO when used as a single agent [128]. This paradox presents an unresolved gap in our understanding. In Paper II, we investigate the influence of cell nutrition on BSO susceptibility, aiming to provide new insights into the mechanisms underlying this resistance.

1.8 FERROPTOSIS – IRON DEPENDENT CELL DEATH

Cell death plays a crucial role in embryonic development and is implicated in numerous disease conditions. In cancer, a key characteristic is the ability of tumor cells to evade the regulated cell death pathways that normally trigger death in response to specific signals [129]. This ability allows cancer cells to escape the regulatory mechanisms that govern normal cell behavior. Therefore, finding ways to reinitiate cell death in cancer cells is a critical focus in the development of effective anti-cancer therapies.

Ferroptosis is a recently discovered form of regulated cell death characterized by iron-dependent lipid peroxidation (**Fig.5**). Targeting ferroptosis is gaining attention as a promising therapeutic strategy in cancer treatment. This approach exploits distinct metabolic vulnerabilities of cancer cells, particularly their altered lipid metabolism and reliance on iron.

The concept of ferroptosis was first introduced in the seminal paper by Dixon et al. in 2012 in which the term ferroptosis was coined [130]. At the time, ferroptosis was postulated as distinctly differentiated from other regulated cell

death pathways (RCDs) such as apoptosis and necroptosis, both biochemically and morphologically. Ferroptosis, unlike these pathways, is caspase independent and does not exhibit similar morphological changes, such as the swelling and bursting seen in necroptosis or the rupture of the endoplasmic reticulum and formation of apoptotic bodies characteristic of apoptosis [130].

Instead ferroptosis presents itself with formation of shrunken mitochondria accompanied with particular gene expression changes related to lipid and glutathione metabolism and an increased uptake of iron via the transferrin receptor resulting in increased lipid peroxidation [131] .

Lipid peroxidation involves the substitution of specific carbon atoms in the polyunsaturated fatty acid, for an oxyl-group (O-O) causing oxidation of membrane phospholipids. As mentioned previously, eexcess iron catalyzes the formation of highly reactive ROS that causes lipid peroxidation. This lipid peroxidation initiates a chain reaction that, unless countered, leads to cell death via ferroptosis. GPX4, as mentioned previously, is central in resolving these oxidized phospholipids by reducing them to benign lipid alcohols via the electron donating glutathione (**fig. 5**) [132]. RSL3 which inhibits GPX4 or the GCLC inhibitor BSO are classical ferroptosis inducing drugs (**fig. 5**). The identification of erastin, a Xct inhibitor, and RSL3 came from a compound screening for specific synthetic lethal RAS compound, hence the name RAS-selective lethal (RSL) [133, 134].

In addition to elevated iron levels, cancer cells also exhibit deregulated lipid metabolism, resulting in the accumulation of PUFAs, which are the substrates for lipid peroxidation. Acyl-CoA synthetase long-chain family member 4 (ACSL4) and Lysophosphatidylcholine Acyltransferase 3 (LPCAT3) are key enzymes that facilitates the incorporation of PUFAs such as arachidonic acid into phospholipid membranes and plays crucial roles in ferroptosis by providing the substrates for lipid peroxidation [135].

By incorporating MUFAs instead of polyunsaturated fatty acids (PUFAs), cells reduce the substrates available for lipid peroxidation, helping to counteract ferroptosis. This metabolic adaptation, regulated by other members of the ACSL family, represents an important anti-ferroptotic mechanism [136].

Other anti-ferroptotic mechanisms, in addition to the GPX4/GSH system and the regulation of PUFA/MUFA metabolism, involve the NRF2 and mTOR signaling pathways. mTOR signaling increases resistance to ferroptosis by stimulating GPX4 synthesis, while NRF2 exerts multiple roles in ferroptosis suppression. NRF2 regulates the expression of lipoxygenase ALOX5 and

ferritin, thereby modulating lipid peroxidation and iron availability in a context-dependent fashion [137-140].

Several ferroptosis-targeting agents are currently being explored as potential anti-cancer therapies, particularly in combination with traditional chemotherapeutic agents such as the widely used platinum-based drug cisplatin. Combining ferroptosis inducers with established treatments holds promise for enhancing therapeutic efficacy and overcoming drug resistance in cancer [141]. Sorafenib, a multi-kinase inhibitor primarily used to treat advanced renal cell carcinoma and hepatocellular carcinoma, has shown to induce ferroptosis by inhibiting the cystine/glutamate antiporter system Xc^-. This inhibition depletes cellular cysteine and glutathione, thereby promoting ferroptosis [142]. However, research suggests that the response to sorafenib-induced ferroptosis varies among cancer types and resistance mechanisms exist, possibly due to the mutational and metabolic heterogeneity of cancer cells [143].

Pro-oxidant therapies, which target endogenous antioxidants and promote oxidative stress, have shown significant potential as novel anti-cancer treatments [144]. However, these therapies are often linked to severe side effects, highlighting the need for further research to mitigate these adverse outcomes.

In conclusion, the combination of increased iron availability and altered lipid metabolism makes ferroptosis a promising target for cancer therapy. As research into ferroptotic pathways deepens, understanding the metabolic and genetic factors that influence ferroptosis susceptibility will be crucial for developing more effective, targeted therapies. Moreover, the integration of ferroptosis-inducing agents with traditional treatments holds significant

potential for overcoming drug resistance in a wide range of cancers.

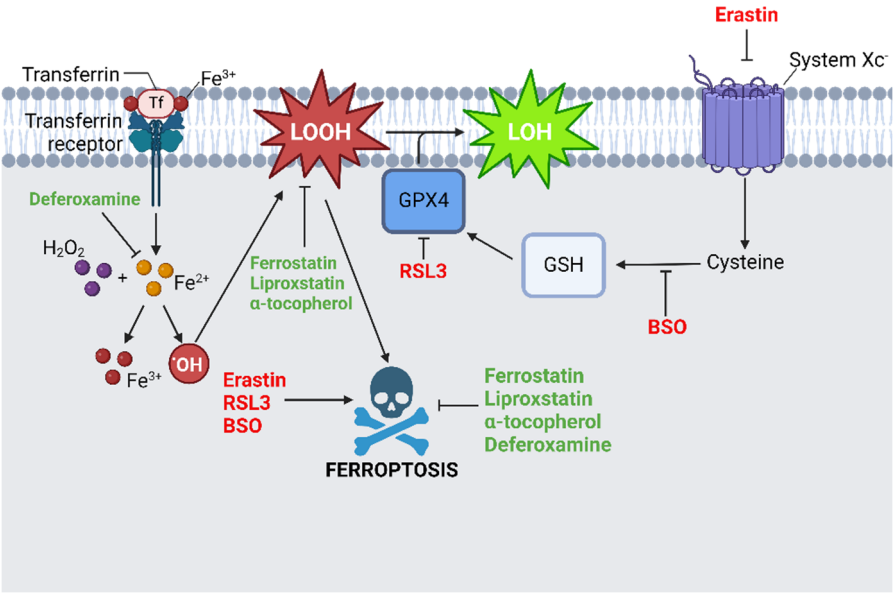

Figure 5 *"Simplified schematic of the ferroptosis pathway".* Mechanisms of ferroptosis: Iron-dependent lipid peroxidation leads to cell death. Key inhibitors (green) of ferroptosis (e.g., Ferrostatin, Liproxstatin, α-tocopherol, Deferoxamine) and inducers (red) (e.g., Erastin, RSL3, BSO) regulate this process through modulating glutathione levels and the activity of GPX4, which reduces lipid hydroperoxides (LOOH) to lipid alcohols (LOH). The accumulation of lipid peroxides (LOOH) ultimately drives ferroptosis in the presence of iron and oxidative stress.

2 AIM

The primary aim of this thesis was to explore the effects of antioxidant-targeting therapies on cancer progression, with a particular focus on pro-oxidative treatments. Specifically, this work investigates the consequences of glutathione depletion and identifies factors that modulate ferroptosis susceptibility, particularly in lung cancer. By addressing how cancer cells respond to oxidative stress and by pinpointing critical vulnerabilities, this research seeks to uncover novel therapeutic strategies to enhance cancer treatment.

Specific aims of the four papers include:

I. To investigate the impact of antioxidant supplements N-acetylcysteine (NAC) and vitamin E on tumor initiation and progression in the $APC^{Min/+}$ mouse model of familial colorectal cancer.
II. To investigate how nutritional factors, influence ferroptosis susceptibility in lung cancer cells.
III. To screen for genetic modifiers that are synthetically lethal with pro-oxidative treatment in lung cancer cells.
IV. To identify direct targets of the ATF4 transcription factor in amino acid deprived lung cancer cells.

3 METHODOLOGICAL CONSIDERATIONS

3.1 CELL CULTURE: THE BACKBONE OF BIOMEDICAL RESEARCH

This PhD project has extensively used commercial 2D cell line models to study cancer, as it is the preferred choice in fundamental biomedical research due to their practicality. These cell lines offer several benefits, including ease of use, simplicity in culture, and scalability, allowing for a broader range of experiments compared to more complex models such as primary cell lines, spheroids, or organoids. For mechanistic studies, *in vitro* models provide a significant advantage, as they offer a more versatile experimental toolkit compared to the often more limited capabilities of complex models, such as screening platforms [145] .

However, these models come with significant limitations. There is an inherent trade-off between feasibility and the biological relevance of the model. Simplified models, such as 2D cell cultures grown in monolayers, do not accurately replicate the complexity of tumors, which have a three-dimensional structure and cellular heterogeneity [145]. Consequently, while 2D cell cultures are convenient, they fail to fully capture the intricate tumor microenvironment, leading to potential discrepancies in experimental outcomes [146, 147]. *In vivo* models on the other hand offer advantages in increased physiological relevance and understanding the metastatic process. Although there has been a considerable development of *in vivo* models during the last decades improving drug development and increasing the relevance and feasibility of *in vivo* models [148], e.g. the use of CRISPR *in vivo* screening platforms [149], they are still limited in gaining mechanistic insight not only due to feasibility but also due to compliance with the 3R's (Replacement, Reduction, and Refinement) making large scale mechanistic studies in animals unethical.

Criticism has been directed at the use of *in vitro* models due to concerns about their relevance to *in vivo* tumor biology. Some studies highlight a significant discrepancy between *in vitro* findings and *in vivo* results, partly due to the inability of *in vitro* models to fully replicate the metabolic phenotypes found in tissues. This is primarily because *in vitro* systems lack the complex cell interactions present in the tissue microenvironment [150]. A particularly troubling statistic is that nearly 90% of drug candidates fail in clinical trials [151], a failure often attributed to the biochemical irrelevance of traditional

cell cultures. This has created a strong incentive within drug discovery to develop more suitable models. Consequently, more complex 3D cell culture models have emerged as a promising solution to bridge the gap between *in vitro* and *in vivo* systems, potentially reducing the reliance on animal testing in line with the principles of the 3Rs [152].

In conclusion, selecting the appropriate model for biomedical tumor research requires balancing feasibility with physiological relevance. There is a growing push to move beyond traditional, simplistic 2D cell cultures in favor of more complex 3D cultures, which offer greater potential for translating laboratory findings into clinical applications.

3.2 DOSE-RESPONSE CURVES

Running dose-response curves has been a crucial method throughout this thesis project, as it is the gold standard for determining drug efficacy under various conditions. In essence, one or more drugs are administered to cell cultures at varying concentrations to establish a dose range. Following treatment, the cells are incubated for a specified period, after which the response is measured using different assays. The raw data is then normalized to untreated controls to establish a baseline. This normalized data is subsequently used in non-linear regression analysis to determine four key parameters: 1) the top asymptote, 2) the bottom asymptote, 3) the Hill slope, which represents the rate of change in response (y) for each unit change in dose (x), and 4) the IC50 value—the concentration at which half of the maximal response is observed. Comparing IC50 values across different experimental conditions provides valuable mechanistic insights into the biological processes underlying specific phenotypes.

However, generating dose-response curves can be challenging due to various sources of variability, some of which may be unnoticed by the operator, and stochastic factors that can introduce differences between experiments. As a result, shifts in IC50 values between experiments are common. One key factor that can significantly impact IC50 values in *in vitro* studies is cell seeding density. This optimization must be tailored for each individual cell line, as it significantly influences drug susceptibility. In our experiments with BSO, we identified seeding density as a crucial factor impacting the results. This observation aligns with reports that low cell density markedly increases ferroptosis susceptibility, mainly through the regulation of the Hippo signaling pathway, a key modulator of ferroptosis [153-155].

3.3 TRANSFECTION WITH INTERFERING RNA

The discovery of RNA interference (RNAi) in the 1990s had a profound effect on molecular biology and earned the inventors a Nobel prize in 2006 [156]. RNAi involves the use of small interfering RNA molecules (siRNAs), typically around 20-25 nucleotides long, which bind to complementary sequences in target mRNA, leading to its degradation and a reduction in protein translation. This process results in transient gene knockdown, making RNAi a valuable tool for studying gene function.

Although the advent of CRISPR has introduced a more precise gene-editing tool, RNAi remains an important technique in molecular biology. One of the advantages of siRNA is its simplicity compared to CRISPR. It can be easily introduced into cells using transfection reagents like Lipofectamine, allowing for effective gene silencing through lipofection. In contrast to CRISPR, where the target cells need to express both the gRNA and Cas9, siRNA can be introduced as a single reagent, streamlining the process and reducing experimental complexity.

However, siRNA has limitations and, in some cases, is not the most appropriate technique to use. One major limitation is that siRNA only achieves a transient knockdown of the target gene, which makes it unsuitable for long-term experiments or establishing stable knock-out clones. Despite this limitation, the transient nature of siRNA can be advantageous in certain contexts, as it mimics the pharmacokinetics of drugs, which require repeated dosing to maintain therapeutic effects.

Another challenge with siRNA is the potential for off-target effects, where the siRNA may bind to unintended mRNA sequences, leading to the silencing of non-target genes. This can result in misleading conclusions about gene function and molecular mechanisms. Although CRISPR-Cas9 also faces the challenge of off-target effects, the guide RNA (gRNA) used in CRISPR systems is typically more specific than siRNAs, which can reduce the likelihood of unintended gene silencing.

3.4 WESTERN BLOTTING

Western blotting, a protein detection analysis, was heavily used throughout this thesis project. This method sometimes referred to as immunoblotting, relies on the use of antibodies to detect specific proteins and involves four main steps: 1) separation of proteins by size using gel electrophoresis, 2) transfer of proteins from a polyacrylamide gel to a membrane 3) antibody incubation and 4) detection using fluorescence or chemiluminescence. Western blotting has

become a standard molecular biology technique and plays a vital part in e.g. cellular signaling studies, however it is associated with some disadvantages. In the following paragraphs I'll discuss the pros and cons of this method.

Since the advent of proteomics, a technique using mass spectrometry, western blotting to some extent have been surpassed by a more advanced method that makes the transition from a reductionist to a holistic perspective possible. Proteomics in contrast to western blotting is a high-throughput method allowing the researcher to study the entire proteome of the cell. In contrast, western blotting is a more laborious, low-throughput method that will not generate as much data per experiment [157].

Another drawback of western blotting is that it is a semi-quantitative method in which the scientist can determine relative protein expression in contrast to absolute levels. In other words, it will generate an estimate of the protein levels rather than a precise determination of the actual protein levels. This is because of different features of the western blotting technique that introduces several sources of variability. This technique relies on the detection of bands on a membrane that will emit a signal (often a chemiluminescent signal) during a stimulus (UV-light during chemiluminescence). The band signal intensity and corresponding protein level, is not always perfectly linear especially at low or high concentrations. Furthermore, for quantitative western blotting, normalization against a housekeeping protein, often referred to as loading control, needs to be done to normalize for unequal loading of the gel [158]. The term dynamic range refers to the interval in which a protein can be reliably quantified as within this range the protein levels is in a linear relationship with signal intensity. Above a threshold level, the signal will get saturated and the protein quantity is no longer increasing linearly with signal intensity. Therefore, the dynamic range of target proteins and reference proteins are an important factor to consider and evaluate when designing a western blot experiment for accurate quantification [159]. Moreover, the transfer efficiency of the proteins from the gel to the membrane is a source of variability that can impact the accuracy of western blotting. In order to control for differences in transfer efficiency it is crucial that the housekeeping protein used for loading normalization is on the same gel as the protein of interest. In our studies we relied heavily on the heat shock protein 90 (HSP90) as a loading control primarily for practical reasons to increase the throughput of each western experiment. It is a constitutively expressed chaperone that can be used for normalization during normal conditions. However, during oxidative stress levels of HSP90 can change thus making it not ideal during these conditions [160]. Although our experimental conditions induced lipid peroxidation and thus oxidative stress, we did not observe alterations of HSP90 levels between the control and experimental conditions.

However, Western blotting have become a standard molecular biology technique because of the many advantages it offers. It is based on the use of antibodies which are characterized by their unique specificity to the epitope to which they bind making this a method with high specificity. In contrast to other techniques like mass-spectroscopy, the use of highly specific antibodies makes it possible to study post-translation modifications of the target protein of interest. It is also a very sensitive method that can detect picograms of protein and in comparison, to proteomics it is very cost-effective. Unlike proteomics which requires many different sets of skills from the operator with complex data analysis, western blotting is more straightforward.

3.5 CRISPR-CAS9 DROPOUT SCREENING

In paper III CRISPR-Cas9 dropout screening was used to identify genes that were synthetic lethal with three different pro-oxidative drugs. As this method was central in this paper and laid the foundation for the following work, I'll be discussing this method in more detail.

In the field of functional genomics, the CRISPR-Cas9 system has emerged as a highly effective and unbiased tool for conducting gene-drug interaction screens to identify genetic modifiers that influence specific phenotypes. This powerful approach enables researchers to interrogate the entire genome in a single experiment, facilitating the discovery of novel genes and potential therapeutic targets that might not be revealed through more traditional methods [161, 162].

A drop-out screen, or negative selection screen, is based on the principle that certain sgRNAs, which target essential genes, are depleted from the mutant cell pool during the screening process when cells are subjected to a selective pressure or experimental challenge. These "dropped-out" sgRNAs correspond to genes that are essential for cell survival under the given conditions. The abundance of each sgRNA in the screened cell pool is compared to the initial mutant cell pool to identify these essential genes. This comparison is typically performed using high-throughput sequencing followed by bioinformatic analysis, which scores each gene based on its depletion, generating a list of candidate genes that can be ranked according to what degree they impact viability. These findings lay the groundwork for further investigation into the molecular mechanisms underlying the phenotype [161, 163].

CRISPR-Cas9 screening generates a vast amount of data and serves as a powerful tool for hypothesis generation. However, once the screening and bioinformatic analysis are completed, the validation phase presents significant challenges. Validating individual gene candidates one at a time is a time-

consuming and labor-intensive process. Despite robust quality control measures, false positives can still occur, leading to the exclusion of many genes from the candidate list. Additionally, establishing knockout clones is a meticulous task; many clones are disqualified because they are either not true knockouts or are heterozygous. Furthermore, unintended effects of the cloning procedure is an issue that can influence the experimental outcomes, potentially making a knockout resistant to the initial conditions under which the candidate gene was identified making it hard to draw the right conclusions [164] .

In our study, we employed CRISPR-Cas9-based screening to uncover new genes that display synthetic lethality when cells are treated with pro-oxidative drugs. Synthetic lethality occurs when the simultaneous loss of function in two genes leads to cell death, whereas the loss of either gene alone is not lethal. This can also occur when the loss of a gene function, combined with the inhibition of a specific protein (e.g., through drug treatment), results in cell death, while the loss of either one independently is non-lethal [165]. Specifically, we identified genes whose disruption sensitizes cells to these oxidative stress-inducing compounds, highlighting potential avenues for targeted cancer therapies that exploit the vulnerability of cancer cells to oxidative damage.

Another drawback with CRISPR-Cas9 is that it is more laborious in comparison with other gene-editing techniques like siRNA. Although sgRNA can be introduced into a cell line using lipofection, in our experience this technique has not worked as good as using lentiviral transduction. Although siRNA can be used for screening purposes, CRISPR is regarded as more reliable and suffers less from off-target effects.

3.6 BATCH EFFECTS OF FETAL BOVINE SERUM ON *IN VITRO* STUDIES

The batch-to-batch variation of fetal bovine serum poses a big challenge in reproducibility of *in vitro* studies and has been a decades long debated issue [166] . Serum contains growth factors, amino acids, cytokines, nucleotides and a range of different components. The supplementation of serum to standard cell culture medium is a prerequisite to support growth of most cell lines in culture.

To enhance the accuracy and reproducibility of *in vitro* studies, the use of a chemically defined, serum-free medium is highly advantageous and desirable from multiple perspectives. It not only standardizes experimental conditions but also aligns with the principles of the 3Rs (Replacement, Refinement, and

Reduction) by reducing the reliance on animal-derived products, thereby benefiting both animal welfare and sustainability. Despite these advantages, many laboratories continue to use Fetal Bovine Serum (FBS) due to the significant challenges associated with serum-free media. These challenges include higher costs, the risk of selecting subpopulations of cells, and the need for extensive optimization for each specific cell line. FBS is a complex mixture containing many components that are not fully characterized, making it difficult to completely replace in cell culture systems.

Traditionally, heat inactivation of serum has been a common practice, primarily to inactivate the complement system, which is crucial for immunological studies, and to reduce variability between experimental conditions [167]. However, there are several arguments against this practice. Heat inactivation is labor-intensive and increases costs, and the process can degrade important serum components, potentially hindering cell growth. As a result, heat inactivation is generally discouraged unless absolutely necessary.

Fetal Bovine Serum (FBS) is the most commonly used serum in cell culture, and it contains lower levels of complement system components compared to calf serum. Additionally, FBS is less likely to contain contaminants, further reducing the need for heat inactivation. Therefore, unless specific experimental conditions require it, avoiding heat inactivation is often recommended to preserve the integrity of the serum and ensure optimal cell culture conditions.

Another way of standardizing conditions is the process of dialysis which is a practice used in many labs to filter away small molecule components like salts, growth factors, cytokines, amino acids and nucleotides. This can be a crucial step in cellular signaling studies and metabolomics. Recently we made attempts to identify new ferroptosis-permissive serum and in our preliminary efforts we discovered that dialysis of serum components up to 10 kDa transformed a non-permissive serum into a permissive one (**Fig. 6**). Although this is only preliminary results the data indicates that the presence of unknown components in regular FBS is protecting against ferroptosis and that dialysis of FBS enhances susceptibility by removing these protective factors.

Previous studies have demonstrated that varying levels of transferrin, a common component in FBS, significantly impact cellular susceptibility to ferroptosis-inducing drugs [70]. Furthermore Vande Voorde et al. (2019) demonstrated that the addition of sodium selenite to a physiologically relevant culture medium (Plasmax™) protected breast cancer cell lines cultured from ferroptosis [168]. This protective effect was observed primarily in low-density cultures, while more confluent cultures showed no signs of growth retardation due to ferroptosis. Selenium which is largely supplied to culture media by FBS,

is a critical element for selenocysteine-containing proteins like GPX4.

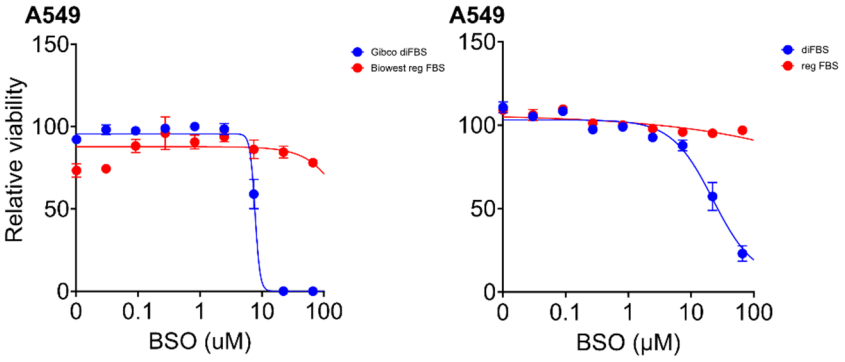

Figure 6 *Dialysis of FBS transforms a non-permissive serum to a ferroptosis permissive serum.* Dose-response curves for A549 cells cultured in F12 medium, supplemented with either regular or dialyzed FBS, are shown. The left panel presents data for two different batches of FBS with dialyzed (blue) and regular (red) FBS, while the right panel compares in-house dialyzed (blue) and non-dialyzed FBS (red) from the same batch. Cells were treated with BSO for 72 hours. Data points represent n=4 replicates, with error bars indicating the standard error of the mean (SEM).

4 RESULTS AND DISCUSSION

4.1 PAPER I: ANTIOXIDANTS PROMOTE INTESTINAL TUMOR PROGRESSION IN MICE

In Paper I, we explored the impact of antioxidant supplementation, specifically NAC and vitamin E, on tumor development in the APCMin/+ mouse model of hereditary colorectal cancer. Our findings revealed that antioxidant supplementation at physiologically relevant serum levels, similar to those obtained after administrating prescription dosages to human subjects, did not influence tumor initiation but did promote tumor progression.

To investigate the effects of antioxidant supplementation on tumorigenesis at different stages, we administered vitamin E through chow and NAC via drinking water during pregnancy and until 12-weeks post-partum and from 12 to 19 weeks of age. While there was no significant increase in the number or distribution of tumors in either cohort, we observed a notable difference in tumor growth. During early tumorigenesis, tumors in the first cohort were significantly larger, whereas in the second cohort (12-19 weeks post-partum), although tumor size was not significantly affected, pathohistological analysis showed that tumors treated with vitamin E/NAC had more advanced lesions, indicating a higher risk of malignant transformation.

Interestingly, when we tested NAC and Vitamin E *in vitro* using the CRC cell lines LoVo and Colo230, both of which carry APC mutations, we observed a different outcome. Despite the reduction in ROS levels, antioxidant treatment did not alter the proliferation rate in either cell line. This suggests that while antioxidants may influence tumor progression *in vivo*, their effects on tumor cells might involve interactions with the tumor microenvironment.

4.1.1 DISCUSSION PAPER I

The widely accepted belief that antioxidants protect against cancer is prevalent, largely due to numerous preclinical studies linking oxidative stress to cancer development [169]. However, clinical trials have failed to show consistent benefits of antioxidants in cancer prevention or treatment, with some studies even suggesting an increased risk of certain cancers with antioxidant use [114-116, 170]. This discrepancy has led to a paradigm shift, challenging the traditional view that antioxidants are universally beneficial for cancer prevention.

One possible explanation for these conflicting results is that while antioxidants may help prevent tumor initiation, they could also promote tumor progression. To investigate how antioxidants, affect both processes in colorectal cancer, we treated the $APC^{Min/+}$ mouse model of familial colorectal cancer with two antioxidants, N-acetylcysteine and Vitamin E. These antioxidants were administered during early development to assess tumor initiation and later to study their effects on tumor progression.

Research suggests that superoxide plays a critical role in driving tumor progression in APC-mutated CRC, potentially by inducing DNA damage and promoting chromosomal instability (CIN) [171]. Increased superoxide dismutase (SOD) activity, which correlates with more aggressive tumor behavior, supports this hypothesis [172, 173].

Wnt signaling in APC-mutated CRC tumors has also been shown to promote superoxide production via NADPH oxidase 1 (Nox1) in a Rac1-dependent manner [174]. This superoxide production leads to the oxidation of nucleoredoxin (NRX), stabilizing β-catenin and driving cell proliferation.

In accord, a study using NAC treatment on conditionally APC-knockout crypt cells in APC^fl/fl Lgr5GFP-CREER RAC1^fl/fl mice, NAC significantly reduced tumor formation [175]. Of note, the dosage used in this study were 5-times the dosage used in our study, potentially explaining the conflict of results.

As a synthetic antioxidant, NAC serves as a precursor for glutathione synthesis and directly scavenges reactive oxygen species [176, 177]. Interestingly, although we observed no effect on tumor initiation, NAC increased tumor volume and grade. Given the previously reported anti-proliferative effects of NAC treatment following APC loss, a reasonable explanation for this paradox may be that NAC exhibits a dose-dependent dual effect on tumor behavior. At lower doses, NAC may support tumor progression by reducing oxidative stress, while at higher doses, it could potentially promote tumor suppression by disrupting the delicate balance of ROS required for cellular homeostasis and proliferation.

In our study, GCMS analysis confirmed that plasma levels of NAC corresponded to the typical prescription doses used in humans. In contrast, the referenced study administered a dosage five times higher [175]. Previous research has shown that the ROS response in cancer cells can vary: RAC-1 stimulates superoxide production, promoting proliferative Wnt signaling, while TIGAR, a regulator of apoptosis, autophagy and inflammation, decreases ROS levels to curtail oxidative stress and to support proliferation

[178, 179]. Also, oncogenic mutations such as $KRAS^{G12D}$, common in both NSCLC and CRC, infers oxidative stress tolerance by inducing NRF2-transcritption, a global antioxidant regulating signaling pathway [180]. This activation strengthens the cell's defense against reactive oxygen species, contributing to the survival and growth of cancer cells in oxidative environments.

Cheung et al. (2013) further demonstrated that in a 3D organoid culture of an APC^fl/fl TIGAR^fl/fl mouse model, NAC rescued organoid growth and reduced lipid damage. However, at higher doses, NAC inhibited growth, likely due to suppressed proliferative ROS signaling. Their study concluded that both too little and too much ROS could impair cell growth, highlighting the importance of ROS balance.

Despite these findings, antioxidant supplementation had no impact on the proliferation of *in vitro* cultured CRC cell lines (LoVo and Colo230), both of which carry APC mutations. While antioxidants reduced overall ROS levels, their lack of effect on tumor cell proliferation suggests that the tumor microenvironment (TME) may mediate antioxidant effects. Further studies using co-culture systems and RNA sequencing could help uncover how antioxidants interact with other cell types within the TME, potentially influencing cancer proliferation during treatment.

Although our study did not resolve whether antioxidant supplementation facilitates the transformation of benign polyps into malignant tumors, our findings suggest a potential link. The complexity of redox signaling, however, warrants caution, as antioxidants can influence both tumor suppression and progression depending on the context and dosage. Further research is needed to clarify the role of redox signaling in malignant transformation.

4.2 PAPER II: AMINO ACID SUPPLY DETERMINES SUSCEPTIBILITY TO FERROPTOSIS-INDUCING AGENTS IN LUNG CANCER CELLS

In Paper II, we investigated how varying nutritional conditions influence the sensitivity of cancer cells to pro-oxidative drugs, with an emphasis on ferroptosis induction through the glutathione inhibitor, BSO. Our findings revealed that the culture medium significantly impacted drug susceptibility. Multiple cell lines derived from different tissues and grown in the nutrient-rich RPMI medium showed resistance to BSO. In contrast, cells cultured in the nutrient-poor F12 medium responded effectively to treatment. Notably, F12-cultured A549 cells exhibited increased sensitivity to several pro-oxidative

drugs, including the GPX4 inhibitor RSL3, the TrxR1 inhibitor Auranofin and the system Xc- inhibitor Erastin. This increased sensitivity was accompanied by significantly higher lipid peroxidation levels, which could be reversed by ferroptosis inhibitors, such as lipid peroxide scavengers and iron chelators, confirming the cells' sensitization to ferroptosis.

Furthermore, when cells were cultured in an amino acid-fortified version of F12 (F12AA), which matched RPMI levels except for cysteine and glutamine, the BSO sensitivity was reversed. Gas chromatography-mass spectrometry (GC-MS) data showed that F12 culture led to depletion of intracellular serine, methionine, leucine, and isoleucine, though levels of GSH substrates like cysteine and glutamine were unaffected. Accordingly, glutathione levels did not differ between cells cultured in F12 and F12AA.

Amino acid scarcity is detected by the key metabolic regulator mTOR, which plays a crucial role in sustaining and promoting cancer cell growth. To assess the potential involvement of activated mTORC1 signaling in F12-cultured cells, we measured the protein levels of mTORC1 downstream substrates.

While basal levels of the mTORC1 substrate 4E-BP1 were higher in F12-cultured cells, no difference in phosphorylation was observed. Additionally, treatment with the mTORC1/2 inhibitor torin1, at concentrations that effectively inhibited downstream substrates S6 and 4E-BP1, had no impact on BSO sensitivity, providing no evidence of mTOR influencing ferroptosis susceptibility during mild amino acid depletion.

Instead, F12 culture activated the integrated stress response (ISR) pathway, a nutrient-sensing mechanism partly triggered by the accumulation of uncharged tRNAs due to amino acid scarcity. This pathway is mediated by the kinase GCN2, which autophosphorylates itself in response to deacetylated tRNA accumulation, subsequently phosphorylating the initiation factor eIF2α. This blocks global protein translation while selectively activating the transcription of proteins crucial for cellular homeostasis.

To determine whether ISR activation increased macroautophagy, a known ISR target, we measured autophagic degradation rates in F12 vs. F12AA-cultured A549 cells. Although basal autophagic flux was higher in F12-cultured cells, treatment with the autophagy inducer torin1 or the autophagy inhibitor 3-methyladenine (3-MA) did not affect BSO sensitivity. Ferritinophagy indicators, such as protein levels of transferrin receptor and ferritin, also showed no differences between F12 and F12AA cultures. Although the lysosomal inhibitors chloroquine and bafilomycin A1 rescued BSO-treated

cells, their effect on ATF4 levels made it difficult to interpret how lysosomotropic agents influence BSO sensitivity. Ultimately, we found no evidence supporting the involvement of macroautophagy in BSO sensitivity.

Knockdown of key components in the ISR pathway, particularly the transcription factor ATF4, protected cells from lipid peroxidation and ferroptosis. Similarly, inhibition of the eIF2α phosphatase GADD34 increased BSO sensitivity. qPCR analysis confirmed that ATF4 knockdown decreased the expression of known downstream targets, such as asparagine synthetase (ASNS) and ChaC Glutathione-Specific Gamma-Glutamylcyclotransferase 1 (CHAC1). CHAC1, which degrades glutathione, is mechanistically linked to ferroptosis susceptibility and could potentially explain the increased sensitivity to ferroptosis downstream of ATF4. However, despite the transcriptional changes, CHAC1 protein levels remained unchanged, and its knockdown did not rescue A549 cells from ferroptosis, suggesting that CHAC1 does not play a significant role in ferroptosis susceptibility in this context.

Additionally, ATF4 knockdown decreased lipid peroxidation, as measured by the lipid peroxidation-sensitive probe BODIPY-C11. This established a causative link between ISR-activation and ferroptosis susceptibility.

Induced cDNA expression of CHOP further heightened sensitivity to BSO in cells cultured in F12 medium, whereas overexpression of ATF4 in cells cultured in RPMI had no effect on BSO susceptibility. This indicates that ATF4 activation alone is insufficient to induce ferroptosis. This also suggests that amino acids may influence ferroptosis through mechanisms beyond ISR activation, or that the inhibition of cap-dependent translation via eIF2α phosphorylation, which is upstream of ATF4, plays a key role. This hypothesis can be tested by knocking down the eIF2α phosphatase, GADD34, in RPMI-cultured cells. Additionally, other components present in RPMI medium may provide protective effects against ferroptosis.

4.2.1 DISCUSSION PAPER II

The discovery of ferroptosis originated from observations that cancer cells rely heavily on cysteine, and that depleting cysteine induced a previously unknown form of cell death, later identified as ferroptosis [130]. Cysteine plays a central role in ferroptosis regulation as a critical substrate for glutathione synthesis. The impact of cell culture medium on ferroptosis susceptibility has been well-documented, with cysteine and glutamate identified as key modulators due to

their roles in GSH production [81]. However, the role of other amino acids in ferroptosis, particularly across different cancer types, remains less explored.

Standard cell culture media, such as RPMI, contain supraphysiological nutrient levels that fail to accurately represent *in vivo* tumor metabolism. This can lead to artefactual phenotypes and distort scientific results [181]. Moreover, compound screenings conducted by the Broad Institute, as part of the extensive data repository known as the Cancer Dependency Map (depmap.org), have been performed in physiologically irrelevant media like RPMI [128]. This raises concerns about the validity of past findings, particularly regarding metabolically targeted drugs like BSO, as most cell lines screened show resistance to BSO. To address this issue, we sought to explore how cell nutrition affects ferroptosis susceptibility, focusing on amino acids beyond cysteine and glutamine, which are already known regulators of ferroptosis.

To isolate the specific impact of amino acids, we used a custom made medium (F12AA) that mirrors the amino acid composition of RPMI but keeping cysteine and glutamine unchanged.

Intriguingly, supplementation with other amino acids in F12AA protected cells from ferroptosis without altering GSH levels. This outcome, though seemingly counterintuitive, is logic since the substrates for GSH synthesis were unchanged. GC-MS analysis of intracellular amino acid levels revealed that essential amino acids such as methionine, leucine, and isoleucine were progressively depleted in cells cultured in the F12 medium. Methionine depletion, in particular, is known to activate the Integrated Stress Response (ISR), a finding consistent with our observations [182].

The mechanisms by which methionine triggers the ISR are complex. While several studies have shown that methionine restriction induces ISR and ATF4 expression through GCN2- and eIF2α-independent pathways [183-185] ,our findings suggest GCN2 phosphorylation in F12-cultured cells. We also detected activation of both PERK and PKR, but their activation levels were consistent across amino acid-deprived and replete conditions. Knockdown of these ISR kinases did not alter the phenotype, suggesting they are not involved in ferroptosis regulation.

Methionine restriction has been reported to have paradoxical effects on ferroptosis. Xue et al. (2023) [186] demonstrated that short term methionine restriction enhanced cystine-deprivation induced ferroptosis, whereas prolonged methionine depletion inhibited it. This effect was attributed to a complex regulatory mechanism involving the GSH-degrading CHAC1. Methionine indirectly supports GSH synthesis by contributing to cysteine

production through the transsulfuration pathway. Previous studies have shown that simultaneous restriction of both cysteine and methionine can accentuate the effects of the GPX4 inhibitor RSL3[187]. In human triple-negative breast cancer cells, erastin-induced cysteine depletion activated the GCN2-p-eIF2α-ATF4 signaling axis, leading to the upregulation of CHAC1. This, in turn, promoted the breakdown of glutathione (GSH) to release cysteine, thereby sensitizing the cells to ferroptosis [188].

In our study, although ATF4 knockdown led to reduced mRNA levels of CHAC1, there were no significant differences in CHAC1 protein levels between cells cultured in F12 and F12AA media. This suggests that CHAC1 may be regulated post-translationally, a finding that aligns with the observations by Xue et al., that prolonged methionine depletion inhibits ferroptosis by preventing CHAC1 protein synthesis. Additionally, the lack of difference in CHAC1 protein levels further supports our GSH measurement results. Also, knockdown of CHAC1 had no effect on BSO toxicity further reinforcing this. Instead it seems as ISR activation increases dependency on GSH without affecting its levels.

In addition to methionine, leucine and isoleucine, were also depleted in F12-cultured cells.Leucine and isoleucine are key amino acids involved in amino acid signaling through the mTOR pathway [189]. In our study, F12AA contained 4 times more leucine and 13 times more isoleucine compared to the F12 medium, which likely explains their significant depletion over time in F12-cultured cells. Interestingly, the mechanism we observed appeared to be independent of mTOR signaling. Although the depletion of leucine and isoleucine can trigger ISR signaling through the accumulation of uncharged tRNAs, thereby amplifying the effect of the ISR.

Overexpression of ATF4 in cells cultured in RPMI did not sensitize lung cancer cells to glutathione deprivation, suggesting that amino acids influence targets beyond ATF4 in mediating ferroptosis susceptibility. Alternatively, it also raises the possibility that inhibition of cap-dependent translation, acting upstream of ATF4, might serve a role that is independent of ATF4. Moreover, it is possible that other factors in RPMI provide protection against ferroptosis.

Interestingly, a 2024 study by Su et al. revealed paradoxical effects of a ketogenic diet, which replaces carbohydrates with fat as the fuel for energy, in a xenograft model using the MDA-MB-231 breast cancer cell line. While mice on the ketogenic diet experienced slowed primary tumor growth, they exhibited increased metastasis. This unexpected outcome was linked to the strong activation of ATF4, which in turn stabilized the pro-metastatic transcription factor BACH1 [190]. Moreover, Ferrer et al. (2023) demonstrated

that in a murine model of cancer cachexia, a ketogenic diet increased tumor sensitivity to ferroptosis but also accelerated the progression of cachexia [191]. These findings highlight the critical role of nutrient sensing and metabolic reprogramming pathways, such as the Integrated Stress Response (ISR), in regulating cancer progression. Exploring the therapeutic potential of dietary interventions and amino acid restriction, particularly by targeting ATF4 and the ISR, could provide novel strategies for cancer treatment.

4.3 PAPER III: BY INCREASING TRANSFERRIN UPTAKE IN CANCER CELLS, WNT-SIGNALING IS SYNTHETIC LETHAL WITH BUTHIONINE SULFOXIMINE, A GLUTATHIONE SYNTHESIS INHIBITOR

In Paper III, our goal was to identify genetic modifiers of oxidative stress to uncover potential new targets for pro-oxidative drug treatments. We conducted an unbiased CRISPR-Cas9 screen in the lung cancer cell line A549, where the entire genome was systematically knocked out in the presence of pro-oxidative drug treatment. This approach allowed us to identify genes that exhibited synthetic lethality under oxidative stress conditions.

Bioinformatic analysis revealed a significant enrichment of negative regulatory genes associated with WNT signalling. To validate this finding, we treated cells with the WNT ligand WNT3A or the GSK3α/β inhibitor CHIR99021 (CHIR) and demonstrated that WNT activation significantly enhanced BSO-induced cytotoxicity. CHIR, a dual inhibitor of the serine/threonine kinase GSK3α/β, a key component of the WNT destruction complex regulating β-catenin levels, played a central role in this process.

To specifically address the role of β-catenin in ferroptosis susceptibility, we generated β-catenin knockout clones using CRISPR-Cas9. Surprisingly, despite the loss of β-catenin, cells remained sensitive to BSO in the presence of CHIR or WNT3A, although the β-catenin knockout clones were less sensitive than wild-type cells. This suggests that while β-catenin influences ferroptosis susceptibility, it is not essential for WNT-mediated ferroptosis induction. Further experiments using doxycycline-inducible gain-of-function β-catenin mutants showed that β-catenin overexpression did not enhance BSO sensitivity, indicating that β-catenin alone is not sufficient to drive ferroptosis susceptibility.

Knockdown of the transferrin receptor (TFRC) effectively rescued cells from BSO-induced cell death. Additionally, transferrin uptake assays with Alexa-labeled transferrin demonstrated that CHIR significantly enhanced clathrin-mediated endocytosis of transferrin, suggesting a direct link between WNT activation and increased iron import, which in turn heightens sensitivity to ferroptosis.

Transcriptomic analysis of WNT-activated, BSO-treated cells revealed deregulation of genes involved in cell junctions and extracellular matrix (ECM) remodelling. We hypothesize that GSK3α/β may regulate cell contacts and ECM dynamics, which could influence redox signalling. Notably, previous studies have shown that cell detachment, a key event during epithelial-to-mesenchymal transition (EMT), elevates ROS levels[192]. The potential link between WNT activation, ECM remodelling, and increased cancer cell invasiveness warrants further investigation.

4.3.1 DISCUSSION PAPER III

In Paper III, we conducted a genome-wide CRISPR-Cas9 screen to identify synthetic lethal genes in combination with pro-oxidative therapies in the *KRAS* and *KEAP1* mutated lung adenocarcinoma cell line A549. This screening was carried out across 14 cell passages using three different pro-oxidative drugs targeting the folate cycle, thioredoxin- and glutathione system. The large-scale approach, combined with high coverage of the screening library, generated a robust dataset of candidate genes influencing the oxidative stress phenotype. The validity of the screening data was underscored by the finding that GCLC emerged as a top candidate in Auranofin-treated samples, while conversely, TxNRD2 was identified as a top candidate in BSO-treated cells. This reciprocal pattern reinforces the robustness of the results. To the best of our knowledge, a screening of this nature of pro-oxidative agents under amino acid constricted conditions had not been previously conducted.

One of the most striking and unexpected findings in our study was the identification of negative regulators of wnt-signaling as potential mediators of BSO-sensitivity. This result is notable since Wnt activation is typically associated with chemotherapeutic resistance and tumor promotion [193]. Recent studies have shown that targeting the Wnt/β-Catenin signaling axis can enhance ferroptosis and lipid peroxidation [194, 195]. For example, Wang et al. (2024) demonstrated that the relatively uncharacterized gene FAM83A, which is highly expressed in malignant lung tissue and linked to poor prognosis, inhibits ferroptosis by activating Wnt signaling in lung squamous cell carcinoma. Similar to the dual GSK3α/β inhibitor CHIR, FAM83A blocks the phosphorylation of GSK3α/β, thereby activating Wnt signaling.

Overexpression of FAM83A was associated with increased cell growth, reduced ROS levels, decreased lipid peroxidation markers such as MDA, and increased GSH synthesis [195]. These results are in conflict with our findings suggesting a pro-ferroptotic role of wnt-activation. Interestingly, Zheng et al. (2020) reported that FAM83A promoted lung cancer progression by altering Hippo pathway signaling, leading to the stabilization of the transcription factor YAP1, a known co-activator with TAZ in inducing ferroptosis. By repressing Hippo signaling, Wnt facilitates YAP activation, which in turn promotes the transcription of several genes involved in ferroptosis, including ACSL4 involved in PUFA metabolism and the transferrin receptor (TfR) [196, 197]. This discrepancy in the implications of wnt-activation in ferroptosis onset warrants further studies.

Our data revealed that CHIR treatment significantly upregulates iron import, as indicated by increased uptake of Alexa-labeled transferrin. CHIR also elevated TfRc mRNA levels; however, this increase did not translate to higher TFRC protein levels, suggesting post-translational regulation. This could be attributed to aconitase's known role in TfRc regulation [198].

Transcriptomic analysis demonstrated that Wnt activation, induced by either CHIR or recombinant WNT3A, led to differential expression of genes related to cell junctions and extracellular matrix components, and that this correlated with increased BSO sensitivity. Notably, previous studies have shown that cell-cell contacts and E-cadherins regulate YAP/TAZ levels via the NF2 pathway [196].

Our findings demonstrate that CHIR treatment synergizes with ferroptosis inducers by enhancing iron import, thereby increasing the sensitivity of lung cancer cells to ferroptosis. This underscores the therapeutic potential of combining Wnt-targeting drugs with ferroptosis inducers in cancer treatment.

4.4 PAPER IV: GENOMIC PROFILING OF THE INTEGRATED STRESS RESPONSE PATHWAY IN AMINO ACID DEPRIVED LUNG CANCER CELLS

In paper IV we aimed at exploring the transcriptomic changes of amino acid deprivation and to identify downstream targets of ATF4 in lung adenocarcinoma cells. We used an integrated RNA-seq and ChIP-seq approach with cells cultured in F12 and F12AA and treated with or without BSO. Transcriptomic analysis identified a set of differentially expressed genes after correcting for multiple testing in F12 vs F12AA cultured cells with and without BSO treatment. Among the upregulated genes in F12 vs F12AA, there was an

enrichment of GO terms associated with biological processes like apoptosis and the PERK-mediated unfolded protein response, though the gene ratio and statistical significance were low. In contrast, downregulated genes showed a strong enrichment for GO terms related to cell attachment and extracellular ECM components. This was also enriched among the BSO treated samples as downregulated. This finding is notable, as cell confluency and attachment via the Hippo signaling and E-cadherin/NF2 pathways, has been linked to ferroptosis. Importantly, similar GO term enrichment was observed in previous RNA-seq data from paper III, reinforcing the idea that low amino acid conditions impact cell adhesion signaling.

During glutathione depletion, upregulated genes showed a significant enrichment in prostaglandin and eicosanoid metabolic pathways. In ATF4 knockdown samples, downregulated genes were predominantly enriched in amino acid metabolic pathways, tRNA acetylation, and ER-stress responses. Notably, upregulated genes during ATF4 knockdown also showed an enrichment in TNF-signaling pathways. In view of the findings done in paper II where we could observe that antagonists of the TNF- and Toll like receptors rescued the cytotoxicity of BSO makes this an intriguing finding.

To identify direct targets of ATF4, we integrated RNA-seq data with ChIP-seq data. The ChIP-seq analysis identified 1089 peaks within 5kb from a known transcription start site (TSS). By cross-referencing this list with the differentially expressed genes from the RNA-seq experiment, we identified 51 genes as direct targets of ATF4. This list included well-known ATF4 targets such as *ASNS* and *CHAC1* [188], with a functional enrichment analysis highlighting amino acid metabolism pathways. Furthermore, the enrichment analysis underscored a robust activation of the integrated stress response (ISR) pathway, identifying it as the most significantly enriched pathway.

4.4.1 DISCUSSION OF PAPER IV

ISR is primarily an adaptive response aimed at maintaining homeostasis during fluctuations in nutrient supply, and it plays a significant role in cancer development. However, the ISR can act as a double-edged sword, as prolonged activation of this pathway can lead to cell death [86] .

Our results mainly indicated an adaptive response orchestrated by ATF4. One limitation of our study could be the timing of sample collection; previous observations have suggested that glutathione depletion does not become toxic until 48 hours post-drug treatment, whereas our study only treated cells for 24 hours. Nevertheless, we sought to explore what precedes the death induction

potentially facilitated by ATF4, which may set the stage for ferroptosis. To identify pro-death genes regulated by ATF4 more effectively, it would be beneficial to analyze samples taken at later time points beyond the initial 24 hours of BSO treatment. This approach could provide a more comprehensive understanding of the death mechanisms downstream of ATF4 during glutathione depletion. Our results from paper II, which show that ATF4 knockdown reduced lipid peroxide levels 24 hours post-transfection, suggest that ATF4 had already initiated a pro-ferroptotic program by this time point.

The integrated analysis identified several known ferroptosis-associated genes, including the glutathione degrading gene *CHAC1*, as well as *TRIB3*, a direct target of ATF4 that represses its activity. Although TRIB3's role in ferroptosis is not well-documented, one study has shown that *TRIB3* inhibits ferroptosis in head and neck squamous cell carcinoma [199]. Furthermore, it has been shown that *TRIB3* cooperates with *DDIT3/CHOP* in ER-stress induced cell death [200]. Thus, the role of TRIB3 in regulating ferroptosis during amino acid depleted conditions warrants further studies.

Among the enriched direct targets of ATF4, were several tRNA synthetases, including CARS, a cysteinyl-tRNA synthetase that has been shown to suppress ferroptosis indirectly by promoting GSH synthesis through the transsulfuration pathway [201]. In their 2016 study, Hayano et al. demonstrated that knocking down CARS in the HT-1080 fibrosarcoma cell line inhibits ferroptosis by triggering the integrated stress response pathway. This is achieved through the phosphorylation of eIF2α, leading to the activation of ATF4. Further research is needed to clarify the role of tRNA synthetases in the process of ferroptosis.

5 CONCLUSION

Overall, this thesis reveals that antioxidant supplementation may enhance malignant transformation in colorectal cancer and that glutathione-targeted therapies can be more effective when combined with amino acid deprivation and/or Wnt activation in lung cancer cells. Specifically, the key findings of this thesis include:

- **Promotion of Tumor Progression by Antioxidants:** Supplementation with NAC or Vitamin E during the early stages of intestinal tumorigenesis accelerated tumor progression, indicating that patients with hereditary APC mutations may face an increased risk of developing colorectal cancer when taking antioxidant supplements
- **Amino Acid Deprivation and Glutathione Dependence:** Amino acid deprivation induces a reliance on glutathione in a panel of human lung cancer cell lines, regulated through the stress-adaptive integrated stress response (ISR) pathway. The ISR effector transcription factor ATF4 lowers the threshold for lipid peroxidation thus sensitizing cancer cells to ferroptosis.
- **WNT Activation and Ferroptosis Sensitivity:** Activation of Wnt signaling upregulates transferrin uptake, thereby increasing the sensitivity of cells to ferroptosis-inducing agents such as the glutathione-targeting BSO.
- **ATF4 and Amino Acid Metabolism:** Amino acid deprivation in lung adenocarcinoma cells leads to a robust initiation of the ISR-response. ATF4 directly regulates genes involved in nutrient stress and amino acid metabolism, which aligns with an enhanced response to glutathione deprivation.

These findings highlight critical interactions between antioxidant supplementation, amino acid metabolism, and therapeutic strategies targeting glutathione, with significant implications for cancer treatment.

6 FUTURE PERSPECTIVES

The findings from this PhD project raise numerous questions that warrant further investigation. In paper II we showed that amino acid depletion increased susceptibility to BSO by activating the integrated stress response pathway lowering the threshold for lipid peroxidation. To support our concept, we conducted an experiment using C57BL/6 mice that were subcutaneously injected with the KP [25] mouse lung cancer cell line ($KRAS^{G12D/+}$, $p53^{-/-}$) in both flanks. The mice were divided into groups and fed either a control diet (20% kcal from protein) or a protein-restricted diet (2.5% kcal from protein) with or without BSO treatment. Protein restriction alone resulted in a strong activation of ATF4 and reduced tumor growth, as evidenced by a lower *ex vivo* tumor weight compared to the control group (**Fig.7**). This growth inhibition was further enhanced by glutathione depletion via BSO treatment although the interaction term determined by Two-way ANOVA was not significant. Notably, BSO treatment alone did not affect tumor growth, consistent with our *in vitro* data.

However, to avoid unacceptable weight loss observed in the mice during combined protein restriction and BSO treatment, BSO was administered intermittently. This intermittent treatment may have caused damage to healthy tissues and is a factor that needs to be considered in a potential clinical scenario and further studied. Also, because of this intermittent treatment GSH measurements at end point was not doable as GSH levels were restored with BSO removal. However, during an *in vivo* experiment for the study in paper III using the same dosage and administration (data not shown), GSH levels were efficiently depleted with 80%.

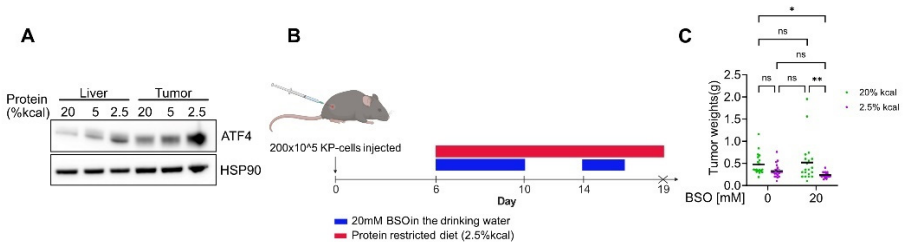

Figure 7 *"Protein restriction slows down tumor growth in vivo and facilitates BSO susceptibility".* A) Western blot analysis of ATF4 in liver and tumor tissues from mice fed either a control diet with 20% kcal from protein or protein-restricted diets containing 5% and 2.5% kcal from protein. HSP90 served as the loading control. B) Schematic illustration of the experimental design of a BSO treatment study in combination with protein restriction. C) Scatter plot showing tumor *ex vivo* weights.

n=20, error bars represents SEM. ns=not significant, **P<0.01, *P<0.05. Difference was tested using Two-way ANOVA with Šídák's multiple comparisons test.

A limitation of this preliminary finding is that the protein-restricted diet does not fully replicate the nutritional composition of F12 medium. While we observed a robust activation of ATF4, it is possible that factors beyond ISR activation are contributing to the growth-inhibitory effects of protein restriction. Additionally, the same diet was used in a study by Wu et al. (2021), where the authors observed an inactivation of mTOR signaling in mice fed a 2.5% kcal protein diet. In our studies, however, we have demonstrated that the ferroptosis-sensitizing effect of ISR is independent of mTOR. Therefore, the *in vivo* findings may indicate a different underlying mechanism, potentially involving crosstalk between the ISR and mTOR pathways [202]. Nevertheless, protein restriction and concomitant BSO treatment warrants further study *in vivo*.

Several improvements of the preliminary *in vivo* experiment could be made for a follow-up study. For instance, an adjusted dosage regimen to maintain consistent BSO treatment in the mice. However, this would require a preliminary pilot study to optimize doses needed for effective GSH depletion. Alternatively, increasing the protein content to 5% kcal might enhance tolerability to BSO treatment, as weight loss was observed in the treated mice. In a follow-up study, biopsies could be collected during BSO treatment to measure GSH levels.

Additionally, the comprehensive collection of tissue samples gathered from both the pilot study on protein restriction tolerability (**Fig.7 A**) and ISR activation, as well as the larger BSO/protein restriction *in vivo* experiment (**Fig.7 B,C**), could provide valuable material for RNA sequencing. This would allow cross-referencing with the data from Paper IV to determine if the transcriptomic changes downstream of ATF4 are consistent across cohorts. Such an analysis would deepen our understanding of the mechanistic aspects of ATF4 signaling and ferroptosis susceptibility *in vivo*, and could potentially inform translational research from bench to bedside. We have also discussed the possibility of staining lung tissues for ATF4 and conducting histological analyses to assess histological scores and metastasis counts, which could reveal whether protein restriction influences these processes.

A more refined *in vivo* diet that closely mimics the F12 culture medium might improve the translation of mechanistic results from *in vitro* studies to an *in vivo* setting. This would also facilitate metabolomic studies *in vivo*, such as trace element experiments to investigate the fate of amino acids. Based on our experience from the *in vivo* study in Paper II, a protein-restricted diet combined

with CHIR and BSO treatment should be considered. Previous attempts to study the *in vivo* response to BSO+CHIR have shown that mice fed a standard chow diet do not respond to BSO treatment, consistent with our *in vitro* findings.

The role of ferroptosis in metastasis remains unclear, with studies showing both anti-metastatic effects through ferroptosis induction and, conversely, pro-metastatic outcomes. This paradox suggests that ferroptosis can either suppress or promote metastasis depending on the context, highlighting the complexity of its influence on cancer progression [203].

For instance, ferroptosis onset, driven by the pro-metastatic transcription factor BACH1, which represses ferroptosis inhibitors like SLC7A11, has been shown to promote metastasis of esophageal squamous cancer cells through lymphatic vessels, associated with a more aggressive cancer development [204]. Mechanistically, this occurs by inhibiting the synthesis of the monounsaturated fatty acid oleic acid, creating a gradient that acts as a chemoattractant, driving migrating cells into the lymphatic system. Interestingly, antioxidant supplementation has been found to stabilize BACH1 in melanoma cells, further promoting metastasis [114].

In parallel, investigating ATF4's role under conditions of amino acid restriction and glutathione deprivation, particularly in the context of lung cancer invasiveness, would be of considerable interest. Metabolism of proline, a non-essential amino acid crucial for maintaining redox balance [205], has been proposed as an attractive anti-cancer target [206] . ATF4 is known to regulate collagen synthesis in cancer-associated fibroblasts (CAFs), which support tumor growth and vascularization [207]. A study by Verginadis et al. (2022) demonstrated that ATF4 promotes the production of glycine and proline, essential for collagen synthesis in the extracellular matrix. In ATF4 knockout mice, impaired angiogenesis and reduced tumor growth were observed in melanoma and pancreatic cancer models, suggesting that ATF4 plays a multifaceted role in tumor progression and metastasis.

To gain deeper understanding, more complex culture systems, like co-cultures or organoids, combined with advanced techniques such as single-cell RNA sequencing or spatial transcriptomics, would offer valuable insights.

Screening the identified direct targets of ATF4 in combination with various pro-oxidative drugs would significantly enhance the findings of this thesis. This approach would allow for the precise identification of effectors downstream of ATF4, potentially uncovering novel targets for combination therapies.

In Paper III, we observed deregulated Wnt signaling during pro-oxidant treatment. To further elucidate the role of Wnt signaling during glutathione depletion, a screening using a library of sgRNAs targeting Wnt-associated genes could provide deeper insights into how Wnt signaling renders cells susceptible to glutathione depletion.

In conclusion, this PhD project uncovers promising avenues for further exploration into the mechanisms of ferroptosis and the role of the integrated stress response (ISR) in cancer therapy. The *in vivo* experiments, focusing on amino acid depletion and BSO treatment, provide support for this approach, though follow-up studies are needed to address limitations such as intermittent BSO dosing and refining the protein-restricted diet to enhance glutathione depletion and model accuracy. Further investigation into the effects of protein restriction and ATF4's role in the tumor microenvironment (TME) and metastasis is also warranted. Incorporating advanced techniques like scRNA-seq and spatial transcriptomics could yield deeper insights into the tumor-TME interplay during nutrient and oxidative stress. Collectively, these findings lay a strong foundation for further development of combination therapies that increase cancer cell susceptibility to ferroptosis.

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