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# ANTIOXIDANTS STIMULATE BACH1-DEPENDENT TUMOR ANGIOGENESIS

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# Antioxidants stimulate BACH1-dependent tumor angiogenesis

Thesis for Doctoral Degree (Ph.D.)

By


## Ting Wang


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*To my beloved family*

# Abstract

Reactive oxygen species (ROS) can inactivate tumor suppressors, activate transcription factors, and stimulate tumor progression, which partly explains the belief that antioxidant supplements should fight cancer. Yet, despite decades of studies, the cancer-fighting potential of antioxidants has never been clearly established. Large clinical trials show that antioxidant supplementation either has no effect or increases cancer risk. Moreover, studies from my host group showed that antioxidants promote malignant melanoma and lung cancer metastasis. Indeed, endogenous and exogenous antioxidants stabilize the transcription factor BTB and CNC homology 1 (BACH1) which increases *GAPDH* and *HK2* gene expression, stimulates aerobic glycolysis, and thus fuels metastasis.

In this thesis I have addressed two questions that arose from the earlier studies. First, as glycolysis is often linked with angiogenesis, I wanted to test the hypothesis that antioxidants through BACH1 can stimulate angiogenesis. Second, our team wanted to study the impact of antioxidant administration on the progression of hematological malignancies, such as B cell lymphoma, where cancer cells can be professional ROS-producing cells, and determine if they respond similarly as solid tumors.

In paper I, we demonstrate that antioxidant-stabilized BACH1 directly controls angiogenesis and glycolysis gene expression in lung cancer spheroids, tumor organoids, and xenograft tumors. Moreover, while HIF1$\alpha$ can control BACH1 expression under both normoxia and hypoxia, BACH1's ability to stimulate angiogenesis gene expression is HIF1$\alpha$-independent. In vivo, we found that antioxidant administration increased BACH1-dependent tumor angiogenesis and renders tumors more sensitive to anti-angiogenesis therapy. Notably, BACH1 expression in lung cancer patients' tumor sections correlates with angiogenesis markers. We conclude that BACH1 is a novel redox and oxygen-sensitive transcription factor.

In paper II, we found that high MYC expression appears to promote proliferation and shield B lymphoma cells from apoptosis at steady-state ROS levels and that compounds such as VitC and NAC in doses that reduce ROS levels induce apoptosis. Injections of VitC and NAC effectively curbed tumor growth from B lymphoma cells with high but not low MYC expression. Consequently, MYC knockdown imparts resistance to VitC and NAC whereas MYC activation makes B cells responsive to these compounds. Mechanistically, VitC and NAC stimulate MYC's binding to EGR1 via Cys117 of MYC, shifting its transcriptional focus from cell cycle to apoptosis gene expression. Our findings establish a redox-regulated mechanism through which MYC sustains proliferation and averts apoptosis, suggesting a potential use of VitC or NAC as therapeutic agents for MYC-driven B cell lymphoma.

Overall, the studies put forward in this thesis provide a refined understanding of the complex role of ROS, oxidative stress, and antioxidants in cancer.

# List of scientific papers

I. **Wang T**, Dong Y, Huang Z, Zhang G, Zhao Y, Yao H, Hu J, Tüksammel E, Cai H, Liang N, Xu X, Yang X, Schmidt S, Qiao X, Schlisio S, Strömblad S, Qian H, Jiang C, Treuter E, Bergo MO. Antioxidants stimulate BACH1-dependent tumor angiogenesis. *J Clin Invest*. 2023 Oct 16;133(20):e169671.

II. Yao H, Chen X, **Wang T**, Kashif M, Qiao X, Tüksammel E, Larsson LG, Okret S, Sayin VI, Qian H, Bergo MO. A MYC-controlled redox switch protects B lymphoma cells from EGR1-dependent apoptosis. *Cell Rep*. 2023 Aug 29;42(8):112961.

# Contents

# List of abbreviations

| | |
|---|---|
| ROS | Reactive oxygen species |
| BACH1 | BTB and CNC homology 1 |
| HIF | Hypoxia-inducible factor |
| NAC | *N*-acetylcysteine |
| VitC | Vitamin C |
| CUT&Tag | Cleavage Under Targets and Tagmentation |
| Redox | Reduction–oxidation |
| $O_2^{\bullet -}$ | Superoxide radical |
| $^{\bullet}OH$ | Hydroxyl radical |
| $ROO^{\bullet}$ | Peroxyl radical |
| $H_2O_2$ | Hydrogen peroxide |
| $^1O_2$ | Singlet molecular oxygen |
| SOD | Superoxide dismutase |
| $GP_X$ | Glutathione peroxidase |
| CAT | Catalase |
| NRF2 | Nuclear factor erythroid 2-related factor 2 |
| KEAP1 | Kelch-like ECH-associated protein 1 |
| ARE | Antioxidant response element |
| NSCLC | Non-small cell lung cancer |
| SCLC | Small cell lung cancer |
| COPD | Chronic obstructive pulmonary disease |
| TKIs | Tyrosine kinase inhibitors |
| GTP/GDP | Guanosine triphosphate/guanosine diphosphate |
| VEGF | Vascular endothelial growth factor |
| EGF | Epidermal growth factor |
| FGF | Fibroblast growth factor |
| Ang | Angiopoietin |
| TIMP | Tissue inhibitors of metalloproteinase |
| PlGF | Placental growth factor |
| NRP | Neuropilin |

| | |
|---|---|
| VEGFR | Vascular endothelial growth factor receptor |
| PI3K | Phosphoinositide 3 kinase |
| mAb | Monoclonal antibody |
| mRCC | Metastatic renal cell carcinoma |
| mCRC | Metastatic colorectal cancer |
| EGFR | Epidermal growth factor receptor |
| HL | Hodgkin Lymphoma |
| NHL | Non-Hodgkin Lymphoma |
| DLBCL | Diffuse Large B-Cell Lymphoma |
| 2D culture | Two-dimensional culture |
| 3D culture | Three-dimensional culture |
| NBT | Nitroblue tetrazolium test |
| IP | Immunoprecipitation |
| ChIP | Chromatin immunoprecipitation |
| RT-qPCR | Reverse Transcription Polymerase Chain Reaction |
| cDNA | Complementary DNA |
| *NSG* | NOD-SCID-gamma |
| US | Ultrasound |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| HK2 | Hexokinase 2 |
| TCGA | The Cancer Genome Atlas |
| GSEAs | Gene set enrichment studies |
| EGR1 | Early growth response protein 1 |
| Igλ | Immunoglobulin λ |
| PHDs | Prolyl-hydroxylases |
| PDGF | Platelet-derived growth factor |
| HGF/SF | Hepatocyte growth factor/scatter factor |
| TGFβ | Transforming growth factor β |
| TNFα | Tumor necrosis factor α |

# Introduction

Lung cancer is one of the leading causes of cancer deaths all around the world. Current traditional treatments offer limited benefit. Therefore, improvement and development of new therapeutic strategies are needed. The lung is a highly vascularized organ and angiogenesis is critical for lung tumor growth and metastasis. Anti-angiogenic drugs targeting tumor vasculature have been developed and approved by Food and Drug Administration in the US in combination with conventional chemotherapy as first or second line treatment in non-small cell lung cancer (NSCLC). Numerous antiangiogenic agents are currently evaluated in therapeutic clinical trials, but their effect is unclear and information on which patients might benefit from this type of therapy is missing.

B lymphocyte cancers, which encompass Hodgkin's lymphoma, non-Hodgkin's forms of diffuse large B cell lymphoma (DLBCL), and Burkitt lymphoma (BL), are responsible for several hundred thousand deaths globally each year. Despite progress in scientific research and improved survival rates, treatment options are often confined to radiation and high-dose chemotherapy. These treatments are linked with toxicity and long-term negative impacts on survivors. Patients with unsatisfactory initial responses to therapy and those who relapse - particularly those with MYC-driven lymphomas - have limited treatment alternatives and a poor prognosis. MYC is challenging to target due to its crucial role in gene regulation in healthy cells. Therefore, identifying vulnerabilities specific to tumor cells and oncogenes, as well as the development of new therapies, is of significant interest.

# 1 Literature review

## 1.1 Antioxidants in cancer treatment

### 1.1.1 Reactive oxygen species (ROS) and oxidative stress

Oxygen, an essential element for life, plays a crucial role in various oxidation–reduction (redox) and enzymatic processes within living organisms. It acts as the ultimate electron acceptor in mitochondria that produces ATP energy (1, 2). However, uncoupled electron flow can generate free radicals, which are highly reactive due to their unpaired electrons, and non-radical oxidizing species (3).

Notably, Reactive Oxygen Species (ROS) encompass all reactive forms of oxygen, which includes both free radicals and non-radical oxidizing species that participate in chain reactions (4, 5). Among these free radicals are oxygen-centered ones such as superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), and peroxyl radical ($ROO^{\bullet}$) (2, 5). Conversely, non-radical oxidizing agents, like hydrogen peroxide ($H_2O_2$) and singlet molecular oxygen ($^1O_2$), are species that can accept electrons but do not have unpaired electrons (5).

ROS originate not only from intracellular sources but also from the 'exposome', a term that refers to cumulative environmental exposure (6). Specifically, the body generates intracellular ROS primarily in the mitochondria during aerobic respiration or as a byproduct of metabolic enzyme activity (7, 8). Environmental factors such as smoke, pollutants, radiation, certain chemicals, drugs, and industrial solvents also contribute to ROS production (5, 9). Thus, both internal and external factors play a significant role in ROS generation.

ROS play a crucial role in the redox-dependent regulation of signaling processes and homeostasis during normal physiological events (2, 5, 10). However, excessive ROS can trigger lipid peroxidation, leading to the accumulation of lipid peroxides and damage to crucial biomolecules such as DNA, RNA, and proteins (11-15). If not effectively neutralized, ROS can stimulate free radical chain reactions that further damage these biomolecules, potentially leading to mutations and diseases such as aging and cancer (11, 16, 17).

Oxidative stress, caused by an imbalance between ROS and antioxidant defenses, can disrupt cellular functions and lead to various pathological conditions (18). This

occurs when ROS overwhelm the organism's antioxidative defenses, leading to oxidative changes in biological macromolecules, accelerated cellular death, and serving as the root cause for many diseases (12, 13, 17, 19, 20).

### 1.1.2 Antioxidants

The levels of ROS are strictly controlled by antioxidant mechanisms, which include both enzymatic and nonenzymatic antioxidant defense components.

Enzymatic antioxidant systems, such as superoxide dismutase (SOD) (21), glutathione peroxidase ($GP_X$) (22), and catalase (CAT) (23) can neutralize an increase in ROS levels. These antioxidant enzymes are activated in response to oxidative stress to reduce the risk of oxidative damage. Similarly, non-enzymatic antioxidant systems, including vitamin C (VitC) (24, 25), *N*-acetylcysteine (NAC) (26-28) and Glutathione (29) , are also capable of eliminating ROS to reduce oxidative stress.

In conclusion, antioxidants—whether they are enzymatic or non-enzymatic—play a crucial role in neutralizing ROS in our bodies. They help us combat free radicals and reduce the risk of diseases associated with oxidative stress.

### 1.1.3 Antioxidants in cancer

Given the significant involvement of oxidative stress in the processes of carcinogenesis and cancer progression (30), the utilization of antioxidants as a potential therapeutic strategy for cancer treatment is highly attractive. Several therapy approaches involving antioxidants have been investigated in both pre-clinical and clinical studies (25, 31, 32).

NRF2 has been recognized as a key regulator of several homeostatic genes that protect cells from cellular oxidative stress (33, 34). This protection is initiated when NRF2 is released from its principal negative regulator, Kelch-like ECH-associated protein 1 (KEAP1), in response to oxidative stress (34). Following this release, NRF2 translocates to the nucleus, where it interacts with the antioxidant response element (ARE) and stimulates the transcription of antioxidant genes (34).

Interestingly, NRF2 plays a dual role in cancer. On one hand, research has shown that mice lacking NRF2 are more prone to chemically induced carcinogenesis, suggesting that NRF2 may act as a tumor suppressor in the early stages of cancer

(35-39). In fact, clinical studies show that some NRF2 activators play a protective role against carcinogenesis and cancer development (40-43). On the other hand, NRF2 has been found to be overexpressed in various cancers, including lung (44, 45), ovarian (46), and breast cancers (47). This overexpression is associated with poor prognosis as it provides cancer cells with survival and growth advantages, as well as resistance to chemo- and radiotherapy (44, 48-50). Given these observations, the inhibition of NRF2 activation is seen as a promising approach for cancer prevention, treatment, and enhancing the effectiveness of drugs. While many NRF2 inhibitors have been discovered and have shown anticancer effects, none have yet produced strong and practicable results (51-54).

At present, NAC is among the most extensively researched antioxidant agents due to its ability to be rapidly absorbed across the anion exchange membrane and deacetylate into cysteine, thereby restoring glutathione (55). Many human disorders, including respiratory diseases, benefit from NAC's therapeutic use because of its ability to reduce levels of cysteine conjugates (56). However, NAC was found to promote melanoma and lung cancer metastasis (57-60).

L-ascorbic acid (vitamin C) and α-tocopherol (vitamin E), the two most common dietary antioxidants, have been studied extensively in cancer. Water-soluble vitamin C is an essential nutrient because it cannot be produced by the human body and instead must be obtained through the diet (61). The oxidized form of vitamin C, known as dehydroascorbic acid, is absorbed by renal epithelial cells from the renal tubules where it performs dual roles as a reductant and an enzyme cofactor (62). High doses of vitamin C have been reported to have encouraging anticancer effectiveness in individuals with advanced cancer (24, 63-65). However, vitamin C's significance in cancer treatment is still debatable, with half of research showing that it has no meaningful effect on cancer incidence and mortality (66-68).

Lipid-soluble vitamin E predominantly localizes to the plasma membrane, where it protects against oxidative stress by neutralising reactive oxygen species (ROS) via interacting with free radicals (69). Clinical trials have revealed a surprising outcome: vitamin E supplementation, rather than providing benefits, either has no impact or even heightens the risk of cancer (70, 71). Adding to this, studies conducted on animals have found that supplementing with vitamin E can accelerate the development of cancer and increase the chances of carcinogenesis (59, 60, 72).

In general, much debate has arisen with regard to antioxidant supplements due to the contentious impact of antioxidants on cancer. Therefore, further experimental research is needed to fully understand the complex function of antioxidants in cancer and to determine how to best use them in cancer prevention and treatment. There should be no additional clinical studies performed in humans using dietary antioxidant supplementation.

## 1.2 Lung Cancer

Lung cancer, a type of cancer characterized by uncontrolled cell growth that begins in the lungs, is one of the leading causes of cancer deaths worldwide (73). According to a report by the World Health Organization from June 2023, lung cancer has the highest mortality rates among both genders, with smoking being the primary cause, accounting for approximately 85% of all cases. A study revealed that in 2020 alone, there were 2.21 million new cases and 1.8 million deaths globally due to lung cancer (74). Alarmingly, this study also forecasts a significant increase by 2050, with new cases expected to double to 3.8 million and deaths anticipated to surge to 3.2 million globally (74). These data highlight the urgent need for effective prevention measures and treatments to combat this deadly disease.

### 1.2.1 Histology

Lung cancer, a complex and heterogeneous disease, can be histologically classified into two main types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (75).

NSCLC, which accounts for approximately 85% of all lung cancer cases, can be further subdivided into non-squamous cell carcinoma (78%), squamous cell carcinoma (18%), and large-cell carcinoma (4%) (76). Among these subtypes, non-squamous cell carcinoma primarily includes adenocarcinoma, the most prevalent subtype of NSCLC (75). Squamous cell carcinoma is characterized by the presence of keratinization or intercellular bridges (77). Large-cell carcinoma is a group of cancers that do not have the features of adenocarcinomas or squamous cell carcinomas (75). Unlike NSCLC, small cell lung cancer (SCLC), which comprises about 15% of all cases, is particularly aggressive (75). Typically diagnosed at an advanced stage, it has a poorer prognosis compared to NSCLC. This highlights the

diversity and complexity of lung cancer and underscores the need for continued research and individualized treatment approaches (**Figure 1**).

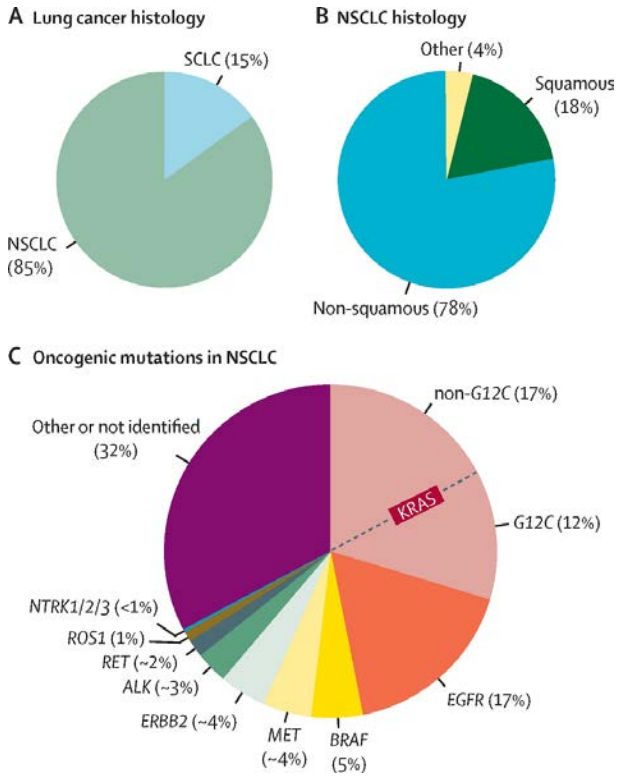


**Figure 1. Lung cancer histology**.



### 1.2.2 Risk factors

There are several risk factors associated with lung cancer initiation and progression, namely, smoking, age, genetic mutations, pulmonary disease, and air pollution. Among these, smoking is the most well-established risk factor, accounting for more than 80% of cases in Western countries (78-80). Squamous cell carcinoma and SCLC have stronger association with smoking than non-squamous cell carcinoma (81, 82). Non-squamous cell carcinoma, which primarily includes adenocarcinoma, is not as strongly linked to smoking but is associated with various environmental exposures (83). For instance, second-hand smoking is a well-known risk factor for lung cancer

(84). Furthermore, exposure to air pollution, particularly fine particulate matter in the air, has also been linked to an increased risk of lung cancer (85, 86).

Additionally, certain lung diseases such as pulmonary fibrosis or chronic obstructive pulmonary disease (COPD) (87, 88), as well as specific genetic mutations or a family history of lung cancer can heighten the risk of lung cancer (89, 90).

It's important to note that while these factors increase the risk, they do not guarantee that an individual will develop lung cancer. Conversely, individuals without these risk factors can still develop lung cancer. Therefore, regular check-ups and leading a healthy lifestyle are crucial.

### 1.2.3 Oncogenic mutations

Surgery, chemotherapy, and radiotherapy have traditionally played a critical role in lung cancer therapy. However, with the advancement of medical science, new therapeutic options have emerged, including immunotherapy and targeted therapy.

Immunotherapy, a novel form of cancer treatment, uses targeted antibodies, vaccines, tumor-infecting viruses, and more to activate the body's own immune system to effectively fight against cancer.

Targeted therapy is a type of cancer treatment that targets specific changes in cancer cells that facilitate their growth, division, and spread. This approach uses drugs to target genes, proteins, or the microenvironment that support cancer growth and survival. Most targeted therapies fall into two categories: monoclonal antibodies and small-molecule drugs (91). Monoclonal antibodies are lab-produced proteins designed to bind to specific targets present on cancer cells. Small-molecule drugs, due to their size, can easily enter cells, making them ideal for targets located inside cells. To develop targeted therapies, it becomes essential to identify specific genetic alterations that drive tumor growth and transformation, known as the drug's "target." Ideally, this target ought to be a protein that is present in cancer cells but absent in healthy ones.

Several critical oncogenic mutations, such as *EGFR, KRAS, MET, BRAF, LKB1, PIK3CA, ROS1, ALK* and *RET*, have been identified as crucial players in the initiation and progression of lung cancer (73) (**Figure 1**). Interestingly, these mutations also serve as attractive targets for therapeutic intervention, paving the way for the development of targeted therapies.

*EGFR* mutations account for 15% and 62% of oncogenic mutations found in NSCLC patients in Europe and Asia, respectively (92, 93). These mutations, which notably occur in exons 18, 19, 20, and 21, have been extensively studied and screened for (94, 95). In light of these findings, a third-generation *EGFR* tyrosine kinase inhibitors (TKIs) – osimertinib has been approved as first-line treatment for NSCLC patients with EGFR mutations (96). This represents a significant step forward in the targeted treatment of this disease.

*KRAS* mutations, which are dominated by single-base missense mutations, have been identified in 25% of patients with NSCLC (97). Remarkably, 98% of these mutations occur at codon 12 (G12), codon 13 (G13), or codon 61 (Q61) (98). These include variants such as G12A, G12C, G12D, G12S, G12R and others (98). Notably, among these mutations, the KRAS G12C mutation is one of the most prevalent in NSCLC (99). Targeting KRAS has been considered challenging for several reasons. First, KRAS has a high affinity for guanosine triphosphate/guanosine diphosphate (GTP/GDP), making it difficult to outcompete these molecules with drugs (100). Second, KRAS lacks distinct drug-binding pockets, which makes it difficult to design drugs that can bind to it effectively (100). Last, even if KRAS itself is inhibited, the activation of major signaling pathways downstream of KRAS, such as the PI3K/AKT and RAF/MAPK pathways, can still drive cancer progression (100, 101). However, recent advancements have led to the successful development of KRAS inhibitors such as sotorasib (AMG510) and adagrasib (MRTX849) (102, 103). Both sotorasib and adagrasib specifically target the KRAS G12C mutation. These drugs work by selectively forming a covalent bond with cysteine 12 in the switch-II pocket of the KRAS-G12C protein and locking the KRAS protein in an inactive state (104). This prevents it from sending signals that promote cell growth and survival, thereby helping to slow down cancer progression (99). Both drugs have been approved to treat people with NSCLC that has the KRAS G12C mutation (100). In addition to G12C, other common KRAS mutations such as G12D and G12V are also being explored as potential targets for therapeutic intervention. For instance, Revolution Medicines' KRAS inhibitors RMC-6236 and RMC-6291 have shown encouraging phase I data in various cancers, including those with common KRAS mutations like G12D and G12V.

*MET* mutations are found in about 4% of patients with NSCLC. Specifically, mutations in exon 14 have been found and are commonly screened for. In terms of treatment,

the FDA has approved two MET inhibitors, capmatinib and tepotinib, for the treatment of NSCLC (105, 106).

*BRAF* mutations, which are identified in approximately 4% of NSCLC samples, predominantly feature V600E mutations, accounting for 50% of the cases (107-109). Clinical trials have shown that *BRAF* inhibitors can have striking initial responses in patients with BRAF V600E-mutant NSCLC. However, even more promising results were observed when BRAF inhibitors were combined with MEK inhibitors, leading to longer median progression-free survival (PFS) (about 10 months) and higher response rates (about 60%) (110-112). In addition, the FDA has approved the combination of binimetinib (Mektovi) and encorafenib (Braftovi) for the treatment of adults with metastatic NSCLC with a BRAF V600E mutation. This represents a significant advancement in the treatment options available for these patients.

In summary, oncogenic mutations have revolutionized NSCLC treatment. Through targeted therapies, we can now offer personalized treatment, thereby enhancing patient outcomes. Even though the complexity and diversity of these mutations present significant challenges, continuous research is essential for enhancing our comprehension and creating more potent therapies.

### 1.3 Tumor Angiogenesis

In 1787, Dr. John Hunter discovered that new blood vessels are required for tissue expansion in adult animals and coined the term ‘‘angiogenesis’’ (from the Greek words “angeion” [vessel] and “genesis” [creation]) to describe newly formed blood vessels (113). The sprouting of new blood vessels from pre-existing vasculature is called angiogenesis (114). The process of angiogenesis includes several steps: proliferation and migration of endothelial cells, degradation of basement membranes, lumen formation and stabilization of newly formed vessels (115, 116).

Under physiological conditions, angiogenesis is only restricted to processes such as reproduction, tissue repair and wound healing (117). However, under pathological conditions, blood vessels are necessary for tumor development not only by providing oxygen and nutrients but also by disposing metabolic waste. In addition, blood vessels provide fundamental routes for cancer cells to metastasize to other organs (118). Interestingly, tumors are limited to a size smaller than 1–2 $mm^3$ and obtain oxygen and nutrients by diffusion from nearby blood vessels (118-120). To grow greater than

2 $mm^3$, tumors need to stimulate angiogenesis to acquire a new blood supply (121, 122) (**Figure 2**). Tumor angiogenesis has been demonstrated to play important roles in tumor growth and metastasis and regarded as a hallmark of tumor progression.

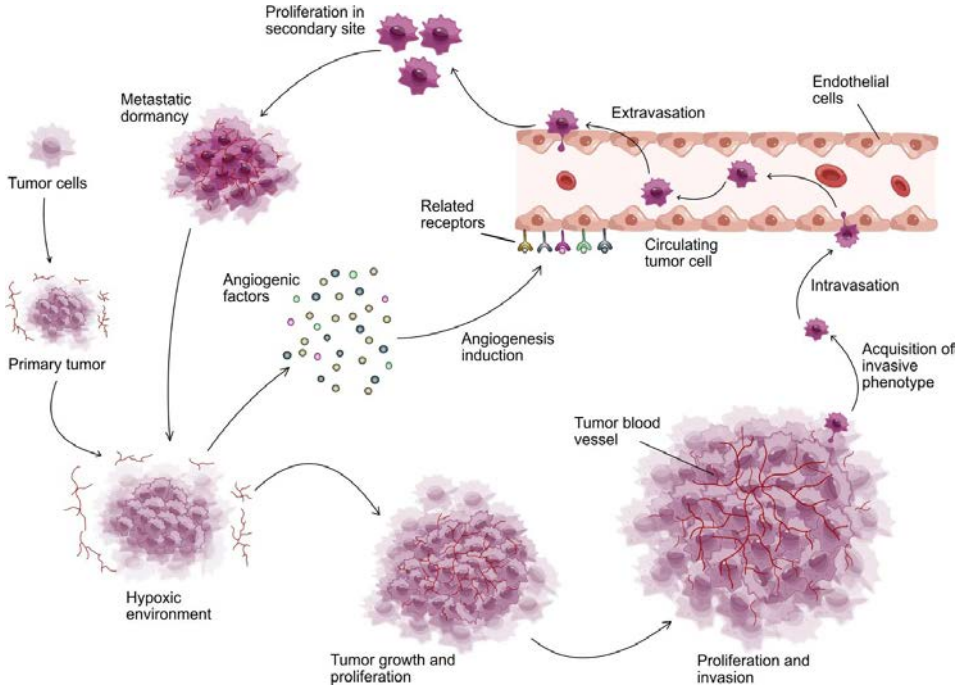


**Figure 2. The progression of the canceration through angiogenesis.**



Angiogenesis is a coordinated process which is regulated by a balance of multiple anti-angiogenic and pro-angiogenic factors secreted by endothelial cells, tumor cells, and other stromal cells (123, 124). The pro-angiogenic growth factors include vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), angiopoietin (Ang) and others. The anti-angiogenic factors include thrombospondin, angiostatin, endostatin and tissue inhibitors of metalloproteinase (TIMP) (125). During the process of tumorigenesis, “the angiogenic switch” occurs, where the balance between pro- and anti-angiogenic factors shifts toward a proangiogenic state (114). This shift results in the predominance of proangiogenic factors, accelerating the formation of vascular networks necessary for tumor progression. “The angiogenic switch” can be triggered by hypoxic signals, the lack of

nutrients, acidosis and inducers of reactive oxygen species (126-128). For example, hypoxia upregulates the transcription of hypoxia inducible factors (e.g., HIF1A, HIF2A), which drive the transcription of proangiogenic factors (e.g., VEGF, FGF) and suppress antiangiogenic factors (e.g., thrombospondin-1, thrombospondin-2), making the tumor microenvironment in a more proangiogenic state to allow tumors to progress (126, 129-132). Compared with normal blood vessels, the newly formed vascular networks are characterized as distorted, dilated, tortuous, and high-permeability vessels. Therefore, tumor vasculature is typically dysfunctional, causing irregular blood flow (114).

### 1.3.1 Main Pro-angiogenic Factors

#### *1.3.1.1 Endothelial Growth Factor (VEGF)*

Among the various pro-angiogenic growth factors, vascular endothelial growth factor (VEGF) and their corresponding receptors stand out as the main drivers of tumors angiogenesis (133). These factors can stimulate endothelial cell proliferation and migration, decrease endothelial cell apoptosis, increase vascular permeability and activate proteases to degrade extra-cellular matrix (134-136).

The human VEGF family comprises VEGFA, VEGFB, VEGFC, VEGFD, placental growth factor (PlGF) and VEGF tyrosine kinase receptors VEGFR1, VEGFR2, VEGFR3. In addition to these tyrosine kinase receptors (RTKs), there are two VEGF co-receptors, namely neuropilin 1 (NRP1) and neuropilin 2 (NRP2) (136, 137).

In this family, VEGFA (VEGF) is the most well-studied proangiogenic factors. VEGFA binds to vascular endothelial growth factor receptors (VEGFR1 and VEGFR2), transducing VEGF mediated angiogenesis signaling pathways (123, 137, 138). VEGFB promotes neuron survival and cardiovascular growth in specific organs such as the heart through angiogenesis (139). VEGFC and VEGFD, mediated by VEGFR3, drive tumor growth and metastasis through lymphangiogenesis. Blocking this pathway disrupts the lymphatic network and triggers lymphatic endothelial cell apoptosis (140, 141). PlGF's role, having both pro- and anti-angiogenic effects, sparks increasing debates (142).

VEGFR is a transmembrane tyrosine kinase receptor, consisting of an extracellular ligand-binding domain, a transmembrane domain and an intercellular tyrosine kinase domain (143, 144). VEGFR1, which serves as a co-receptor for VEGFA, VEGFB, and

PlGF, is the first identified dual-function VEGF receptor (145). In its role as a negative regulator, it inhibits the activation of redundant VEGFA/VEGFR2, manages serum VEGFA levels, and prevents excessive vascular formation. However, when it switches roles and acts as a promoter, it fosters the development and metastasis of cancers including prostate cancer, malignant melanoma and breast cancer (146-148). VEGFR2, a key player in tumor angiogenesis, binds with VEGFA. This binding triggers VEGFR2 dimerization, activates tyrosine kinases, and transduces critical signaling pathways like the phosphoinositide 3 kinase (PI3K)/Akt and Ras-Raf-MEK-ERK pathways (149, 150). These pathways are essential for endothelial cells' growth and survival as well as angiogenesis (151). Moreover, VEGFR2 is overexpressed in various solid tumors, including thyroid cancer, ovarian cancer, and melanoma (152-154). Given these findings, VEGFA/VEGFR2 is a major focus in the research of angiogenic inhibitors. VEGFR3, which is primarily expressed in lymphatic endothelial cells, serves as a mediator for the activation of VEGFC and VEGFD and drives lymphoid proliferation and tumor metastasis (144). The VEGFC, D/VEGFR3 pathway is identified as the main driver of lymphangiogenesis (155). Furthermore, overexpression of VEGFR3 is found in cancers such as cervical, breast, and lung cancer (156-158). Co-receptors NRP1 and NRP2 can form complexes with VEGFR to further enhance affinity of these receptors towards VEGF (159).

#### *1.3.1.2 Angiopoietins (Ang)*

The angiopoietin family includes four ligands (angiopoietins 1–4) (160). Tie is a receptor family that has a high affinity for angiopoietins. Among these receptors, Tie-2 is commonly studied and is known to mediates angiopoietin functions (161). Tie-1, an orphan receptor, can modulate Tie-2 receptor activity (160, 161).

Angiopoietin-1 (Ang-1) mediates vessel remodeling and vascular stabilization (162). It activates the Tie-2 receptor mediated signaling pathway, thereby reducing tumor vessel interstitial pressure and leakage, and preventing tumor metastasis (163). Furthermore, Ang-1 promotes tumor growth by increasing pericyte coverage and matrix deposition, enhancing endothelial cell survival and vascular maturation, and maintaining the integrity of healthy blood vessels (162, 164). Overexpression of Ang-1 intensifies the malignancy of gliomas, breast cancer and NSCLC (165-167).

Ang-2's role can vary. It can either promote or inhibit angiogenesis, depending on VEGF's dynamic concentrations (168). Stimulated by VEGF, Ang-2, through its

competitive interaction with Tie-2 and integrin receptors, stimulates angiogenesis and pericyte shedding, leading to a disruption in vascular stability (168, 169). However, when the concentration of VEGF is low, Ang-2 triggers endothelial cell apoptosis and vascular degeneration, thereby inhibiting tumor growth by cutting off their blood supply (164). Moreover, Ang-2's ability to promote other angiogenic growth factors such as VEGF induces vascular maturation, stabilization, and remodelling (170). Overexpression of Ang-2 encourages vascular proliferation and carcinoma growth, and has correlation with leakiness and delicateness of tumor vessels and poor prognosis (171).

#### *1.3.1.3 Fibroblast Growth Factor (FGF)*

The fibroblast growth factors (FGFs), which are heparin-binding proangiogenic factors, include 18 ligands and 4 tyrosine kinase receptors (FGFR1-4) (172). Among them, FGF1, an acidic fibroblast growth factor, stimulates endothelial cell proliferation and differentiation (173). FGF2, a basic fibroblast growth factor, stands out as the FGF family's most potent pro-angiogenic factor. It propels angiogenesis-related processes such as endothelial cell invasion, migration, and plasminogen activator production (174, 175). Overexpression of FGF2, prevalent in leukemia, bladder cancer and lung cancer, correlates with cancer metastasis and poor prognosis (175, 176). When FGF binds to FGFRs, it triggers FGF-related signaling pathways such as PI3k/Akt and Ras/Raf-MAPK pathways. These pathways control angiogenesis, cell differentiation and growth (177-179).

Moreover, FGF2 can stimulate VEGF secretion and enhance the effects of VEGF in vivo (180) and in vitro (181, 182). Overexpression of FGF and FGFR in various cancers stimulates the release of other proangiogenic factors, leading to enhanced angiogenesis (183-185). Furthermore, it is suggested that targeting both FGF and VEGF pathways simultaneously may be more effective in suppressing tumor angiogenesis than targeting either pathway alone (183).

#### *1.3.1.4 Other pro-angiogenic factors*

There are other types of pro-angiogenic growth factors. For example, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor β (TGF β), hepatocyte growth factor/scatter factor (HGF/SF) and tumor necrosis factor α (TNF α) (186).

## 1.4 Tumor Angiogenesis in NSCLC

In NSCLC, overexpression of VEGFA has been reported to correlate with poor overall survival (187-191). Similarly, high VEGFR1 expression is associated with a low survival rate (151). Additionally, high VEGFR2 expression closely associates with poor prognosis (192). Patients with advanced-stage lung cancer have been observed to have increased serum or plasma VEGF levels (189). Furthermore, NSCLC patients with higher VEGF levels had poor clinical treatment outcomes (192).

FGF2 and its receptors are overexpressed in NSCLC (193-195). It has been observed that high serum FGF2 levels correlate with a poorer prognosis in NSCLC (195, 196), although some studies present conflicting findings (197). There is a lack of correlation between FGF2 expression and overall survival (195). Moreover, the serum FGF2 level does not differ significantly among NSCLC patients in different clinical stages. However, higher serum FGF2 levels in NSCLC patients have been associated with better clinical outcomes (198). Frequent FGFR1 amplification is demonstrated and confirmed in squamous cell lung cancer (199).

Recent studies have shown that Ang-1, Ang-2 and Tie2 are overexpressed in NSCLC (163, 196), suggesting that regulating Ang-1 and/or Ang-2 expression could be a potential therapeutic method for targeting tumor angiogenesis. The epidermal growth factor receptor, matrix metalloproteinases, angiopoietin-2 and some other angiogenic factors have also been investigated and studied in NSCLC. However, some of these factors were demonstrated to correlate negatively with poor prognosis in some studies (196, 197).

## 1.5 Anti-Angiogenic Therapy in NSCLC

In 1971, Judah Folkman proposed that tumor growth is strongly influenced by angiogenesis and postulated that anti-angiogenic therapy could be considered as a pan-cancer therapeutic strategy, aiming at targeting tumor angiogenesis to ‘starve cancer cells to death’ (123). Since then, several drugs against tumor angiogenesis regulators have been developed and approved for the treatment of specific cancer types, either alone or in combination with traditional treatment (200).

### 1.5.1 Anti-VEGF Agents

One classical class of anti-angiogenic agents are the ones specifically targeting angiogenic growth factors and their receptors. It includes anti-VEGF monoclonal antibody (mAb); anti-VEGFR monoclonal antibody (mAb); tyrosine kinase inhibitors (TKIs); Decoy VEGF-trap receptor (**Figure 3**).

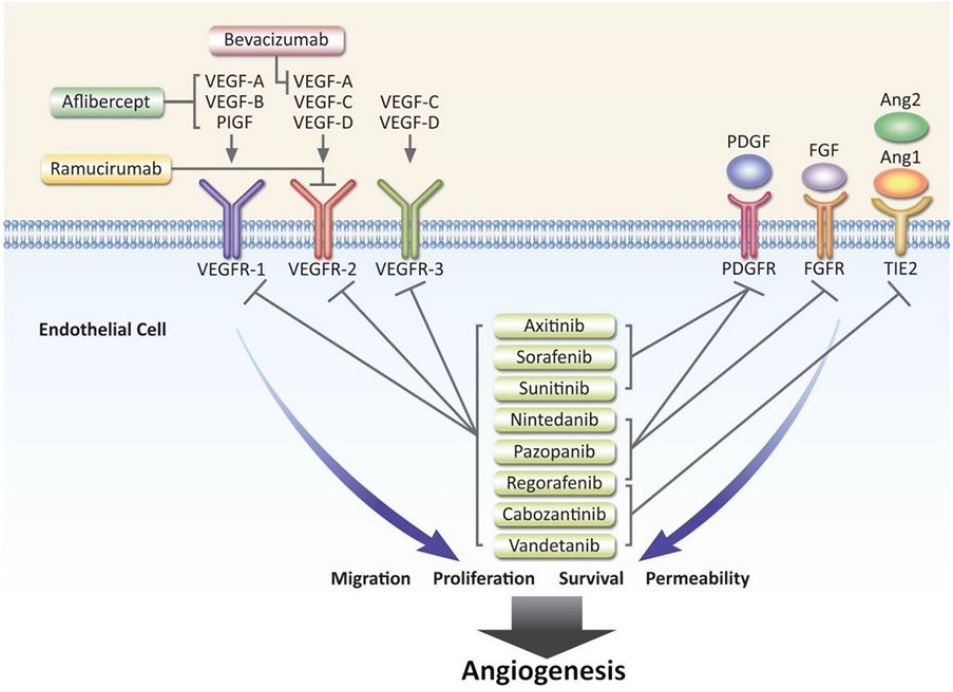


**Figure 3. Angiogenesis signaling and targets of inhibition in approved antiangiogenic agents.**



#### *1.5.1.1 Anti-VEGF monoclonal antibody (mAb)*

Ferrara and his colleagues demonstrated that an antibody neutralizing one of the main pro-angiogenic factors VEGFA could reduce tumor vascular density and growth in an in vivo study performed in 1993 (201). This work laid foundation for further development of antibody targeting VEGFA as the antiangiogenic therapy. Bevacizumab (Avastin®) is a humanized monoclonal antibody targeting vascular endothelial growth factor A (VEGF-A) to prevent VEGF/VEGFR interaction (202, 203). This antibody, approved by the FDA, is utilized alongside chemotherapy to treat

advanced recurrent metastatic glioblastoma, non-small cell lung cancer (NSCLC), metastatic renal cell carcinoma (mRCC) and metastatic colorectal cancer (mCRC) (203). The FDA approved it as a first-line treatment for patients with recurrent or metastatic non-squamous NSCLC, when used in combination with paclitaxel and carboplatin in 2006 (204).

#### *1.5.1.2 Anti-VEGFR monoclonal antibody (mAb)*

Ramucirumab (Cyramza®), a humanized monoclonal antibody, targets VEGFR2's extracellular domain and disrupts the binding of VEGF to VEGFR2 (205). It has been approved by FDA for the treatment of patients with advanced NSCLC, gastric cancer and colon cancer in combination with chemotherapy. In 2014, it has been approved by FDA to patients with advanced NSCLC combined with docetaxel as a second-line therapy (206). In 2020, ramucirumab, when used with erlotinib, received FDA approval as a first-line treatment for metastatic NSCLC patients with deletions in exon 19 or mutations in exon 21 (L858R) of the epidermal growth factor receptor (EGFR) (207).

#### *1.5.1.3 Tyrosine Kinase Inhibitors (TKIs)*

Tyrosine kinase inhibitors (TKIs) are a group of small molecule compounds which bind to the kinase domain of angiogenic receptors, inhibit the activation of these receptors, and disturb the associated signaling pathways (208, 209). Certain TKIs, such as Sorafenib, Axitinib, and Sunitinib, actively target not only VEGFR but also FMS-like tyrosine kinase-3 (FLT-3) and PDGFR (210). These multikinase inhibitors work by concurrently blocking signaling pathways mediated by VEGFR and PDGFR. Pazopanib, on the other hand, inhibits multiple pathways, including those mediated by VEGFR, FGFR, and PDGFR (211-213).

In 2014, docetaxel was approved for use in combination with the multitargeting VEGFR, FGFR, and PDGFR antibody nintedanib for European patients with recurrent or advanced NSCLC who had received initial chemotherapy (183). For advanced NSCLC patients with EGFR mutations, several TKIs, including EGFR TKIs like Osimertinib, are preferred treatment options (214). Simultaneously inhibiting several kinases has potential advantages over single kinase inhibition. However, the disadvantage is the kinase inhibitors have certain levels of toxicity resulting from off-target effects.

#### *1.5.1.4 Decoy VEGF-trap Receptor (aflibercept)*

Aflibercept, a humanized monoclonal antibody, is commonly known as a “VEGF trap”. It serves as a soluble decoy receptor, binding to circulating VEGFs more effectively than the body’s own receptors. This action prevents VEGF from interacting with its receptors, thereby decreasing VEGF’s activity (215). Although it demonstrates a potent effect in patients with metastatic colorectal cancer, it does not show the same efficacy in patients with NSCLC (216).

### 1.5.2 Multi-agent treatments

In addition to VEGF-mediated signaling pathways, alternative VEGF-independent pathways have been identified that can also promote tumor angiogenesis (217). These include several well-established proangiogenic pathways such as PDGF/PDGFR, ANG/TIE, FGF/FGFR, and the hepatocyte growth factor (HGF)/MET signaling pathways (**Figure 3**).

The concept of multi-targeting antiangiogenic treatments, which disrupt multiple signaling pathways simultaneously, is gaining traction as a strategy to enhance antitumor efficacy and combat drug resistance. For example, both in vivo and in vitro data have shown that simultaneous targeting of the VEGF and FGF pathways can inhibit the proliferation and migration of endothelial cells (218). Furthermore, higher FGF levels have been reported in patients with recurrent glioblastoma or pancreatic neuroendocrine tumors resistant to bevacizumab treatment, providing evidence that tumor angiogenesis also relies on VEGF independent signaling pathway (219, 220). Preclinical studies have demonstrated that dual inhibition of VEGF/Ang2 can suppress tumor angiogenesis and development. Clinical trials using vanucizumab, a humanized VEGF/Ang2 bi-specific monoclonal antibody, are currently underway and have shown promising results so far (221-223).

Current studies have also explored the combination of antiangiogenic agents with traditional cancer therapies. For example, in 2016, the combination of bevacizumab with the EGFR inhibitor erlotinib was approved as first-line treatment for European patients with EGFR-mutant NSCLC (224). A study published in 2021 further demonstrated that combining erlotinib with the VEGF antibody bevacizumab could improve progression-free survival (225).

## 1.6 Challenges of Antiangiogenic Agents

### 1.6.1 Anti-angiogenic drug resistance

Bevacizumab, when used with conventional chemotherapy, slightly enhances overall survival in NSCLC (226). However, similar to chemotherapy and radiotherapy, resistance to antiangiogenic therapy remains a significant limitation due to its limited efficacy (139).

At the treatment's initial stage, current anti-angiogenic drugs show promising results. However, resistance to these drugs emerges after long-term use. The mechanisms behind this resistance include: (1) The compensatory activation of other pro-angiogenic factors and signaling pathways. (2) Tumor cells adapting to their environment and inducing hypoxia-inducible factors, leading to the upregulation of pro-angiogenic gene expression (131, 132). Inhibitors of HIF1A or HIF2A may alleviate this resistance (227, 228). (3) Pro-angiogenic factors stimulating the recruitment of endothelial progenitor cells and myeloid cells to the tumor microenvironment. Under certain cytokines' influence, myeloid cells differentiate into tumor-associated macrophages that secrete more pro-angiogenic factors (229-231). (4) Tumor cells themselves instead of endothelial cells can form vascular-like structures, a phenomenon known as vasculogenic mimicry, which is unresponsive to anti-angiogenic therapy as these drugs are designed to target only the pro-angiogenic factors or their corresponding receptors in endothelial cells (232-234). (5) Gene mutations in tumors, the type of tumor, the development stage of the tumor, differentiation of cancer stem cells (235), autophagy of tumor cells, the patient's medication history and other factors (**Figure 4**).

### 1.6.2 Lack of predictive biomarkers

Despite numerous efforts by different groups, no valid predictive biomarkers currently exist to determine which cancer patients might respond best to antiangiogenic therapies. The challenge in identifying potential biomarkers lies in the complexity of the angiogenic signaling pathways. Their multiple overlaps and interactions make it difficult to eliminate an angiogenic stimulus. While some potential biomarkers, such as levels of circulating VEGFA, have been identified from post hoc analyses of clinical trial data, confirming their predictive value across multiple studies has proven difficult (236, 237).

Currently, several types of biomarkers are under investigation. These include soluble angiogenic ligands like VEGFA in circulation, genes that upregulate the expression of angiogenic factors, angiogenic receptors identified by immunohistochemical staining in human cancer tissues, and physiological parameters such as hypertension (238, 239).

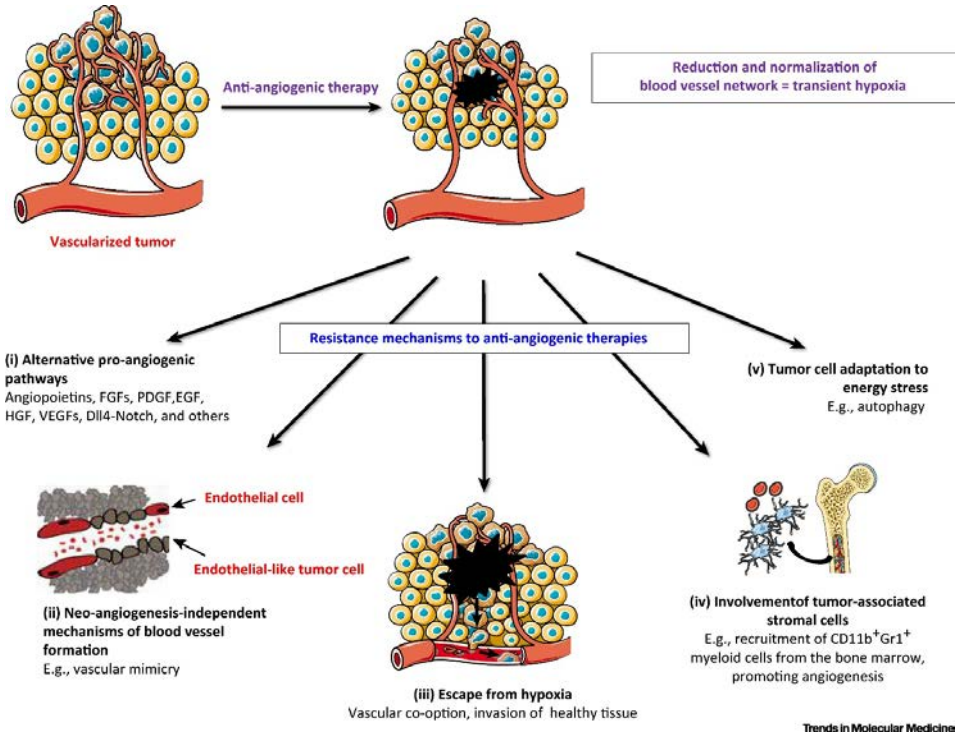


**Figure 4. Tumor Resistance Mechanisms to Anti-Angiogenic Therapies.**



### 1.6.3 Concerns about anti-angiogenic therapy

Combining these drugs with other treatments like radiotherapy and chemotherapy presents additional challenges. The choice of cytotoxic drug to pair with anti-angiogenic drugs for the best clinical outcome remains unclear. The ideal timing for administering anti-angiogenic drugs, whether before or after standard cancer therapy, is yet to be determined. Furthermore, establishing the optimal dosages of anti-angiogenic drugs poses a significant challenge.

## 1.7 B-cell lymphoma

In the Western world, each year sees about 20 new lymphoma cases per 100,000 individuals (240). Surprisingly, despite the similar distribution of T and B cells in the human body, around 95% of these lymphomas originate from B-cells, with the rest being T-cell malignancies (241).

Lymphomas are broadly categorized into two categories: Hodgkin Lymphoma (HL) and Non-Hodgkin Lymphoma (NHL) (242). HL, which is less common, typically originates in a type of B cell found in the bone marrow and is considered one of the most curable forms of cancer (243). The treatment options for HL can include chemotherapy, immunotherapy, and stem cell transplantation. On the other hand, NHL is the most prevalent form of lymphoma, accounting for about 90% of cases (244). It usually develops in older adults and encompasses a diverse group of 60 lymphomas, with B-cell lymphoma and T-cell lymphoma being the two major subgroups (245). Among these, Diffuse Large B-Cell Lymphoma (DLBCL) and Burkitt Lymphoma are main types of non-Hodgkin lymphoma that originate from B cells (244). Notably, DLBCL is the most common type of NHL. Treatments for NHL can include chemotherapy, targeted therapy, radiation therapy, immunotherapy, and stem cell transplantation.

In the 2016 WHO classification, the previously unclassifiable category of B-cell lymphoma, with features intermediate between DLBCL and Burkitt lymphoma, was removed (246). It was replaced by two new categories: High-grade B-cell lymphoma, not otherwise specified, and High-grade B-cell lymphoma with translocations of MYC and BCL2 and/or BCL6 (246).

The MYC gene on chromosome 8 (8q24) encodes the c-MYC oncogenic protein (247). This transcription factor regulates genes involved in various cellular processes such as cell cycle progression, DNA damage repair, protein synthesis, metabolism, and stress response (247, 248). MYC activation can occur due to chromosomal translocation, mutations in regulatory or promoter regions, or copy number amplification. Normally, MYC induces apoptosis via the TP53 pathway (249). However, cancer cells with MYC translocations and TP53 mutations can evade this process (249). Approximately 10% of DLBCL cases exhibit MYC translocation, which

leads to aggressive clinical behavior (250). Additionally, Burkitt lymphoma is characterized by the activation of the MYC gene (251).

Targeting MYC, a transcription factor involved in many cellular processes, can be an efficient therapeutic strategy in cancer. However, MYC is considered an undruggable target due to its lack of a druggable binding pocket (252). Furthermore, inhibiting MYC can lead to significant side effects, as it plays a crucial role in normal cell function. Despite these challenges, researchers are exploring various strategies to overcome them.

# 2 Research aims

Researchers have tested the hypothesis that antioxidant compounds could be utilized for both cancer prevention and treatment. However, the majority of clinical trials and preclinical studies have concluded that the strategy either has no effect or increases cancer risks (59, 70, 71, 253). The effect of antioxidants on lung cancer and B cell lymphoma, as well as underlying mechanisms, is of interest to us.

In paper I, my aim was to uncover mechanisms involved in the acceleration of lung cancer metastasis by antioxidants; to find novel proteins and mechanisms that control tumor angiogenesis, along with identifying biomarkers for tumors that are more likely to respond to anti-angiogenic therapy.

In paper II, we aimed to investigate the impact and mechanisms of antioxidants on the progression of B cell lymphoma.

# 3 Materials and methods

A detailed description of all experimental methods used in the thesis are provided in the the constituent papers. The goal of this section is to discuss the advantages and disadvantages of key methods.

## 3.1 Cell lines

A cell line is a permanently established cell culture that will proliferate indefinitely given appropriate fresh medium and space. This group of cells, formed from the subculture of primary culture consisting of a pure culture of cells, is an invaluable tool in fields such as cancer research, drug development, and genetics. Cell lines are generally homogeneous and can be used by any number of laboratories, thereby promoting consistency in research data and the ability to reproduce findings. They are relatively easy to maintain and manipulate compared to whole organisms, making them a convenient model for studying biological processes. Moreover, the use of cell lines can be more cost-effective than using whole organisms, especially for high-throughput screening. Once established, cell lines provide an unlimited supply of material without the ethical or legal issues associated with the use of animal and human tissue. Genetic manipulation is much easier in cell lines than in whole organisms, allowing for the study of specific genes and their functions (254).

However, while cell lines have many advantages, they also have several disadvantages (255). First, cell lines can undergo genetic drift over time, which may cause them to lose the characteristics of the original tissue. This can lead to inaccurate research results. Second, cell lines can be easily contaminated by other cells or microorganisms, which can affect the accuracy of experimental results. Moreover, the use of human cell lines, especially when the cells are derived from embryos or fetuses, brings up ethical concerns. Last, primary cell lines have a limited lifespan and can only divide a certain number of times before they stop proliferating. All these disadvantages need to be carefully considered when choosing to use cell lines in research.

In this thesis, several human lung cancer cell lines were utilized, including A549 (CRL-7909, ATCC), H838 (CRL-5844, ATCC), ZFN-generated *HIF1A* knockout and control A549 cells (CLLS-1014, Sigma-Aldrich), CRISPR-CAS9-generated *BACH1*-knockout and control A549 cells, as well as CRISPR-SAM-generated *BACH1*-

overexpressing and control A549 cells (72). Additionally, various human B cell lines were employed such as IM9 (CCL-159, ATCC), BL2 (ACC-625, DSMZ), L428, LY1, LY3, Karpas-422, DB, Daudi, Ramos (from Dr. S. Okret, Karolinska Institutet), and P493-6 with doxycycline-off-induced MYC expression. Furthermore, human T-ALL cell lines were also used in the study. These include Jurkat (gift from Dr. Y. Bryceson, Karolinska Institutet) and TALL1 (ACC 521, DSMZ). All the cell lines tested negative for mycoplasma and were cultured under specific conditions. Some were cultured in a normoxic environment with 21% O2 while others were cultured in a hypoxic environment with 1% O2. Both environments maintained a constant temperature of 37ºC with 5% CO2.

## 3.2 Spheroids and mouse lung tumor organoids culture

Organoids, tiny self-organized three-dimensional tissue cultures, are derived from stem cells as well as primary human and mouse tissues. These cultures can replicate much of an organ's complexity or express selected aspects, such as producing only certain types of cells. A key feature of organoids is their ability to self-renew and self-organize into complex three-dimensional (3D) structures, which enables them to mimic the general tissue structure found in vivo (256). Organoids provide a completely different new research model for medical research, including histopathology, drug development and screening, and precision medicine. Compared to conventional two-dimensional (2D) cell cultures, organoids are better at representing cellular environments found in vivo. This includes not only interactions between cells but also between cells and the matrix. However, despite their advantages, organoids still face some challenges. For instance, the effect of the basement membrane gel on cellular behavior is unclear – it's uncertain whether it supports the natural differentiation of the cell or reprograms growth in an undetermined way. The use of organoids as a tumor model is a topic of ongoing debate, primarily due to the unclear influence of the basement membrane gel. Another challenge is that the basement membrane gel has batch-to-batch differences, which can affect experimental reproducibility. Furthermore, as of now, organoids lack vascular and immune cells. This means there is a strict limit on how big they can grow without cell death. Therefore, while organoids hold great promise for advancing medical research, these challenges need to be addressed for their full potential to be realized.

In our study, human cancer cells were trypsinized, counted and mixed with Matrigel (356231, Corning). Domes of 25 µl cell/Matrigel suspension was pipetted into wells of a pre-warmed 48-well plate which were allowed to solidify for 10 min at 37ºC. Mouse lung tumor tissue was dissected into ~1 mm3 fragments with sterile scissors and incubated at 37ºC for 1 h in Eppendorf tubes with 1 ml digestion medium (Advanced DMEM/F-12, 10% FBS, Glutamine, HEPES, and penicillin/streptomycin (ThermoFisher) supplemented with Collagenase type IV (100 mg, Sigma-Aldrich) and Dispase II (20 mg, ThermoFisher)). The cells were mixed with Matrigel. Domes of 25 µl cell/Matrigel suspension was pipetted into wells of a pre-warmed 24-well plate which were allowed to solidify for 10 min at 37ºC.

Prewarmed growth medium (same as digestion medium without Collagenase and Dispase) supplemented with 10 µM RHO Kinase inhibitor (Rocki, Y-27632, Sigma-Aldrich), Insulin-Transferrin-Selenium Supplement (Sigma-Aldrich), and TGFβR inhibitor (A83-01, ThermoFisher) was added to the wells and the plate was incubated at 37ºC.

### 3.3 ROS measurements

ROS measurements, which are used to detect the level of oxidative stress in cells, have been used in the field of cancer research as an indicator to predict the efficacy of anticancer drugs. There are various tools available for ROS detection, which can be broadly divided into two main categories.

The first category, Direct Methods, measures ROS directly. These methods allow for real-time detection of ROS, providing immediate insights into the dynamic changes of ROS in cells. Techniques in this category include chemiluminescence, a method that uses light emission produced during a chemical reaction as a result of the formation of an excited state that then decays to a lower energy state. Other techniques include the nitroblue tetrazolium assay, flow cytometry, electron spin resonance, and the xylenol orange-based assay (257).

The second category is Indirect Methods. Due to the highly reactive and unstable nature of ROS, they are difficult to measure directly. However, the end products of ROS are stable and easier to measure, which is why indirect methods are used. These methods measure the oxidized products of ROS, providing a more reliable and feasible way to assess ROS activity (257).

Both categories of tools, which use different indicators to measure ROS levels, can provide valuable information about cellular health and function. However, there are some challenges associated with ROS Measurement. First, measuring ROS can be technically challenging due to their reactivity and short lifespan. The techniques used need to be highly sensitive and specific, and the timing of the measurement is crucial. Second, there is considerable data variability across laboratories. This variability is compounded by factors such as pH, temperature, and the presence of other reactive species, all of which can influence the results. Consequently, this can make it challenging to draw general conclusions from the data.

In our study, we measured ROS using the ROS-Glo-$H_2O_2$ assay (G8820, Promega), which is a type of direct ROS measurement. This homogeneous, fast, and sensitive bioluminescent assay measures the level of hydrogen peroxide ($H_2O_2$), a reactive oxygen species (ROS), directly in cell culture or in defined enzyme reactions. Specifically, a derivatized luciferin substrate is incubated with the sample and reacts directly with $H_2O_2$ to generate a luciferin precursor. In addition to this, we also utilized the GSH/GSSG-Glo Assay (V6611, Promega), an indirect ROS measurement method. This luminescence-based system detects and quantifies total glutathione (GSH + GSSG), GSSG, and the ratio of GSH to GSSG in cultured cells. By measuring the oxidized products of ROS, it provides a more reliable and feasible way to assess ROS activity. Finally, the fluorescence was recorded with a Synergy multimode reader from BioTek. This comprehensive approach allowed us to effectively study the role and impact of ROS in our research.

### 3.4 Cleavage Under Targets and Tagmentation (CUT&Tag) and Chromatin immunoprecipitation (ChIP) qPCR

CUT&Tag and ChIP-seq are both techniques used to investigate interactions between proteins and DNA. Specifically, CUT&Tag, a molecular biology method, is designed to identify DNA binding sites for a protein of interest. This process begins with live permeabilized cells or isolated nuclei as the starting material (258). Uniquely, CUT&Tag can selectively target antibody-bound chromatin in intact nuclei or cells without the need for cross-linking, fragmentation, or immunoprecipitation (IP). CUT&Tag provides higher resolution and lower background signal than ChIP-seq, allowing for more precise mapping of protein-DNA interactions at a reduced price. However, CUT&Tag has its own set of limitations. For instance, while it can generate

robust data for certain targets like histone post-translational modifications, its reliability may be lower for others like chromatin-associated proteins.

ChIP-qPCR is a technique commonly used in studies that focus on specific genes and potential regulatory regions across differing experimental conditions. ChIP-seq uses an antibody to enrich or "pull-down" targets from a large pool of fragmented chromatin (259). qPCR enables real-time, truly quantitative DNA analysis by analyzing fluorescent signal intensities proportional to the amount of amplicon. However, ChIP-qPCR also has its drawbacks. The main disadvantage is that it requires well-designed primers and probes, as well as optimization of each primer/probe set. Additionally, it requires millions of cells and involves several technically challenging steps that require optimization. Furthermore, it requires high sequencing depths and can be time-consuming.

While ChIP-seq has been a reliable method for chromatin mapping for many years, CUT&Tag provides a new approach with several advantages, especially when dealing with low cell numbers and specialized applications.

In Paper I, we used CUT&Tag to assess the genome-wide chromatin enrichment of BACH1, HIF1α and H3K27ac in A549 3D spheroids. CUT&Tag was performed on $10^5$ cells from 3D spheroid cultures using digitonin (Sigma, D5628) for cell permeabilization and concanavalin A–coated magnetic beads (Bangs Laboratories, BP531) for immobilization. Two biological replicates were used for all experiments. Primary antibodies were H3K27ac (Abcam, ab4729), BACH1 (R&D, AF5776), and HIF1α (Novus Biologicals, NB100-134); secondary antibodies were anti-goat (Sigma, SAB3700280) and anti-rabbit (EpiCypher, 13-0047). Samples were incubated with pAG-Tn5 (EpiCypher, 15-1117) for 1 h. After tagmentation, the cleaved DNA was extracted using the DNA Clean & Concentrator-5 Kit (Zymo Research, D4013). IDT primers (Illumina, 20027213) and PCR enzyme mix (NEB, M0541S) were used for library preparation, and AMPure bead (Beckman Coulter, A63881) was used for PCR cleanup. DNA concentration was measured by Qubit (Invitrogen, Q32851). Library samples were sequenced on NextSeq 2000 (PE100) platform (BEA, Karolinska Institutet) using pair-ended output.

In Paper II, we used ChIP-qPCR assays to reveal whether VitC and NAC increased MYC occupancy on BAK1, NOXA, BAX, and CDKN2A promoters and reduced MYC

occupancy on CDC25A and CCNG1 promoters. Cells were incubated with 500 μM NAC or 250 μM VitC for 16 h and double crosslinked with 2 mM disuccinimidyl glutarate (DSG) for 30 min and 1% formaldehyde for 10 min. Nuclei were isolated and sonicated for 65 min (in 30 s ON–30 s OFF cycles) using the Bioruptor Pico (Diagenode). For chromatin immunoprecipitation, anti-cMYC (10 μg, 9402S, Cell signaling) and control rabbit IgG (10 μg, ab171870, Abcam) antibodies were conjugated with Protein A Dynabeads (Invitrogen), added to the nuclear lysates, and incubated overnight at 4°C. The formaldehyde cross-links were reversed and the immunoprecipitated DNA was purified using the QIAquick PCR purification kit (Qiagen).

## 3.5 Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR is a technique that is used to detect and quantify RNA. In this process, total RNA or mRNA is initially transcribed into complementary DNA (cDNA) (260). This cDNA then serves as the template for the quantitative PCR or real-time PCR reaction, also known as qPCR. The amount of amplification product is measured in each PCR cycle using fluorescence. One of the key advantages of RT-PCR is its speed and high reproducibility. Additionally, it generates a stable cDNA pool during the process, which can be stored for long periods and used for multiple reactions, thereby making it a versatile tool for various applications. Another significant benefit of RT-PCR is its ability to detect genetic material in real time, leading to highly sensitive results. However, like any other technique, RT-PCR has its disadvantages. Its high sensitivity can sometimes be a double-edged sword as it not only allows for the detection of low amounts of target nucleic acid but also makes the technique more prone to contamination, potentially leading to false positives if not handled with utmost care.

In our study, we used RT-PCR as a tool to measure and analyze the expression of specific genes. To begin with, RNA was isolated using the RNeasy Plus Mini kit (74136, Qiagen). Following this, cDNA was synthesized with the iScript cDNA synthesis kit (170-889, Bio-Rad). Once we had our cDNA, we proceeded to analyze gene expression. This was done using the SYBR Green Master Mix (KCQS00, Sigma-Aldrich) on a CFX384 Real-Time System (Biorad). For this process, we used predesigned KiCqStart SYBR-Green Primers, all of which were sourced from Sigma-Aldrich.

## 3.6 Western Blotting

Western blotting, also known as protein immunoblotting, is a technique widely used in molecular biology and immunogenetics. Its primary function is to detect specific proteins in a sample of tissue homogenate or extract (261). The steps involved in Western blotting are as follows: 1. Sample Preparation. 2. Gel Electrophoresis: This is a commonly used method for separating proteins based on size, shape, and/or charge. Differently sized, shaped, and charged molecules in the sample move through the matrix at different velocities. 3. Transfer: On completion of gel electrophoresis, the proteins are transferred from the gel onto a membrane made of nitrocellulose or PVDF (polyvinylidene fluoride). 4. Blocking: The membrane is then blocked to prevent nonspecific binding of antibodies to the surface. 5. Primary Antibody Incubation: The membrane is incubated with a primary antibody that is specific to the target protein. 6. Secondary Antibody Incubation: After washing off the unbound primary antibody, the membrane is incubated with a secondary antibody that recognizes the primary antibody. 7. Detection: The secondary antibody is usually tagged with an enzyme or fluorophore that allows visualization of the target protein. 8. Imaging and Analysis: The final step involves capturing an image of the Western blot and analyzing the results.

One of the key advantages of western blotting is its ability to detect as little as 0.1 nanograms of protein in a sample, thereby making it an effective early diagnostic tool. Furthermore, the specificity of the antibody-antigen interaction allows the process to selectively detect a target protein even in a mixture of many different proteins. However, western blotting does have its limitations. For instance, a false-positive can occur when an antibody reacts with a non-intended protein. Similarly, a false-negative can result if larger proteins are not given sufficient time to transfer properly to the membrane. Additionally, the cost of Western blotting can be quite high due to the large individual expenditures for tagged antibodies, skilled analysts, and laboratory equipment.

In our study, we used Western blotting as a technique to detect the presence of a specific protein in a given sample. Initially, cells were lysed in Laemmli buffer, which was supplemented with β-mercaptoethanol. Following this, equal amounts of proteins were resolved on either 4–20% or 10% Mini-PROTEAN TGX Stain-Free gels (456-8036, Bio-Rad). These proteins were then electro-transferred onto nitrocellulose membranes (0.2 μm, 1704158; Bio-Rad). Subsequently, the membranes were

blocked with 5% milk in TBST to prevent non-specific binding. After blocking, the membranes were incubated with primary antibodies overnight and then with secondary antibodies for 1 hour at room temperature. For the detection of protein bands, we used the Western ECL substrate (1705061, Bio-Rad) with the ChemiDoc Touch Imaging system (1708370, Bio-Rad). Finally, band densities were quantified using Image Lab Software. This comprehensive process allowed us to effectively detect and analyze specific proteins in our samples.

### 3.7 Animal experiments

Animal experimentation involves using non-human animals to control variables that influence behavior or biological systems (262). This method is particularly useful for understanding disease mechanisms at cellular and molecular levels. This method is crucial for developing effective treatments. There are several reasons why animal experiment is used in scientific research. First, there is a significant biological similarity between animals and humans. For instance, mice share more than 98% of their DNA with us (263). Second, animals are susceptible to many of the same health problems as humans – such as cancer, diabetes, and heart disease. Third, animals have a shorter life cycle than humans. This allows animal models to be studied throughout their whole life span and across several generations. It's important to note that a staggering 95% of all animals necessary for biomedical research in the United States are rodents – specifically rats and mice that are especially bred for laboratory use.

The disadvantages of animal experiments are manifold (262). First, there is the issue of cruelty. Animal testing can involve procedures that cause significant distress and suffering to the animals. In some cases, animals may die during the experiment or be killed after their use, and others may lose their limbs, eyesight, hearing, or muscle coordination. Second, the results obtained from animal experiments can be unreliable. Animals do not naturally get many of the diseases that humans do, such as major types of heart disease, many types of cancer, HIV, Parkinson’s disease or schizophrenia. Third, there are ethical issues associated with animal experiments. Some argue that making animals suffer for any reason is morally wrong. Fourth, animal experiments can be expensive due to costs associated with housing, feeding, and caring for animals, as well as regulatory compliance. Fifth, the lack of direct applicability to humans. While animals and humans share a significant amount of genetic material, there are still many differences between us. These differences can

make it difficult to apply the results of animal experiments to humans. For instance, a drug that works well in mice may not work in humans due to differences in metabolism, immune system, and many other factors. This is why many drugs fail in clinical trials despite promising results in animal experiments. This highlights the complexity and unpredictability of biological systems across different species.

In conclusion, while there is a shared long-term goal of reducing animal use in scientific research and eventually stopping it, not all research questions can be answered using only animal-free methods at present. Therefore, for the foreseeable future, there remains a crucial need for animal models to understand health and disease and to develop medicines.

### 3.7.1 Mice

In our study, Animal experiments were approved by the Research Animal Ethics Committees in Gothenburg and Linköping, Sweden.

In paper I, *Kras2*$^{LSL/+}$ mice were on a C57BL/6-129/Sv mixed genetic background (72); controls were always littermates. A low dose of *Cre*adenovirus ($5 \times 10^5$ pfu, University of Iowa, Iowa City, IA) were administered intranasally to 6–7-week-old male and female mice. For xenograft experiments, NOD-SCID-gamma mice (*NSG*; NOD.Cg-*Prkdc*$^{scid}$*Il2rg*$^{tm1Wjl}$/SzJ, from Charles River) were transplanted subcutaneously with $5 \times 10^5$ *BACH1* knockout, *BACH1* overexpression, and *BACH1* knockout *BACH1* overexpression A549 cells; when tumors were detected (i.e., reached 1–3 mm in size), the mice were injected intraperitoneally with DC101 (40 mg/kg, BE0060, Bio X Cell) 3 times per week; control mice were injected with PBS. Tumor volume was measured three or five times per week with an electronic caliper and calculated as width$^2$ × length × ½; tumors were weighed at the endpoint.

In paper II, *λMYC* mice were on a C57BL/6–129/Sv mixed genetic background. *λMYC* mice were injected intraperitoneally with NAC (1 g/kg) and VitC (3 g/kg) 5 times per week after inguinal lymph node tumors reached 100 mm$^3$ in size; control mice were injected with PBS. In some experiments, antioxidants were orally administered to *λMYC* mice; littermate mice at weaning were randomly selected to receive NAC (1 g/L), VitC (4 g/L), or no drug in the drinking water. The water was changed weekly; the mice were killed at the endpoint defined as when they became listless because of primary tumor burden or when primary tumors ulcerated. For xenograft experiments,

NOD-SCID-gamma mice (*NSG*; NOD.Cg-*Prkdc*$^{scid}$*Il2rg*$^{tm1Wjl}$/SzJ) were subcutaneously transplanted with $10^7$ IM9, Ramos, and LY1 cells; and Ramos-sh*MYC* and LY1-sh*MYC* cells. One week (Ramos), two weeks (LY1), and three weeks (IM9) following cell transplantation, NAC (800 mg/kg and 1 g/kg), VitC (1.5, 2, and 3 g/kg), or PBS was injected 5 times per week. Tumor volume was measured three or five times per week with an electronic caliper and calculated as width$^2$ × length × ½; tumors were weighed at the endpoint.

### 3.7.2 High-frequency ultrasound imaging

High-Frequency Ultrasound Imaging, a safe and cost-effective imaging technology, utilizes high-frequency sound waves to visualize and characterize various types of tissues (264, 265). This method provides higher resolution images, making it a powerful tool in medical imaging. One of the key advantages is that ultrasound images are captured in real-time, enabling visualization of the movement of the body's internal organs as well as blood flowing through the blood vessels. Moreover, it is a non-invasive technique used to visualize subcutaneous body structures including muscles, joints, vessels, and internal organs. However, this technique does have its limitations. For instance, the use of high-frequency probes can lead to reduced penetration of acoustic waves in the tissue due to scattering and absorption. Consequently, this results in insufficient visualization of deeper structures. Furthermore, high-frequency waves are more attenuated than lower frequency waves for a given distance; thus, they are primarily suitable for imaging superficial structures. Lastly, the technique requires skilled operators for accurate interpretation of the images, adding to its complexity. Despite these challenges, High-Frequency Ultrasound Imaging remains a critical tool in the field of medical imaging.

In our study, we used this method for assessing tumor vascularity. *NSG* mice were subcutaneously transplanted with 5 × $10^5$ *BACH1* knockout and control A549 cells and received NAC (1 g/l) or VitC (3.47 g/l) in the drinking water, or VitE (DL-α-tocopheryl acetate) in the chow (Lantmännen) at a dose of 0.5 g/kg chow (61.5 mg/kg body weight), calculated from an observed daily food intake. Ultrasound (US) imaging of tumors was performed on a Vevo LAZR-X Imaging Station (VisualSonics, Inc., Toronto, Canada) using a high-frequency ultrasound probe MX250 (15–30 MHz, 75 µm image axial resolution). Mice were anesthetized with 1.5% isoflurane and medical air flow of 2 l/min during the imaging process; hair over the imaged area was removed

using a depilatory cream; and US gel (Parker Laboratories) was applied over the region of interest. Tumor size quantification was performed using 18 MHz B-mode. 3D images were acquired via a 3D acquisition motor scanned along the vertical axis. 3D volumetric quantification was performed be integrating multiple two-dimensional US images. Nonlinear contrast imaging was acquired at 18 MHz frequency, 10% power, 30 dB contrast gain, and 20/sec frame rate—immediately after an intravenous bolus injection of 50 µl non-targeting microbubbles ($2 \times 10^9$/ml, VevoMicroMarker Contrast Agent, VisualSonics). Tumor perfusion/vascularity (peak enhancement) was quantified with VevoCQ Software (VisualSonics).

# 4 Results

## 4.1 PAPER I: Antioxidants stimulate BACH1-dependent tumor angiogenesis

The redox-sensitive transcription factor BTB and CNC homology 1 (BACH1) binds antioxidant response elements and inhibits heme oxygenase 1 transcription (266). Subsequent studies have demonstrated that the antioxidants VitE, NAC, or activating NRF2 reduce oxidative stress, reactive oxygen species (ROS) and heme levels in lung cancer cells, thereby stabilizing BACH1 and increasing transcription of pro-metastatic genes like glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hexokinase 2 (HK2). Interestingly, antioxidants promote aerobic glycolysis to stimulate lung cancer metastasis via BACH1 (72). It is also known that lung tumor growth requires angiogenesis. Furthermore, angiogenesis and glycolysis are intertwined with tumor progression (267). In light of these findings, this study investigated how angiogenesis affects antioxidant-induced tumor metastasis.

In line with previous research (72), the administration of VitC, NAC, and Trolox increase BACH1 mRNA and protein levels. Remarkably, this effect was observed not only in tumor organoids derived from mice with $KRAS^{G12D}$- induced lung cancer and 3D cultured human lung cancer cell lines A549 and H838, but also in xenograft tumors from *NSG* mice that were subcutaneously injected with A549 cells. Additionally, the compounds functioned as antioxidants, reducing $H_2O_2$ levels and increasing GSH/GSSG ratios. Furthermore, these antioxidants also upregulated the expression of angiogenesis and glycolysis genes, such as vascular endothelial growth factors (VEGFs), VEGF receptors, and PFKFB2. Moreover, an increase in protein levels was also noted for two selected genes, VEGFR2 and NRP2.

In our study, we utilized CRISPR/CAS9 techniques to manipulate BACH1 expression in A549 cells and examine its effects on angiogenesis and glycolysis gene and protein expression (72). Interestingly, we found that most evaluated angiogenesis genes and VEGFR2 and NRP2 protein levels were higher in cells with high *BACH1* expression than in cells with low *BACH1*. Moreover, both antioxidants and BACH1 modification altered angiogenesis gene expression in a similar manner. Furthermore, *BACH1* knockout cells had considerably lower antioxidant-induced increases in angiogenesis gene expression and VEGFR2 protein levels compared to BACH1 wildtype cells. This evidence suggests that BACH1 mediates the effects of

antioxidants on angiogenesis-related gene expression. Consistent with this, we observed similar results when examining glycolysis gene expression.

Following our initial experiments, we used Cleavage Under Targets and Tagmentation (CUT&Tag) to investigate H3K27ac-marking of transcriptionally active enhancers and promoters, as well as BACH1 genome-wide chromatin binding. We discovered that BACH1 binds predominantly to promoter regions near transcriptional start sites, in addition to potential enhancer elements located within intergenic regions and introns. Interestingly, *BACH1* knockout decreased H3K27ac levels across the genome, as well as at the enhancers and promoters of glycolysis and angiogenesis genes. This suggests that BACH1 directly activates these regulatory regions. Further investigations revealed that BACH1 deletion led to a reduction in both basal and VitC-induced expression of a wide variety of angiogenesis and glycolysis genes.

The stabilization of HIF1α under hypoxic conditions promotes angiogenesis and glycolysis gene expression (267). Given this, we investigated whether the upregulation of HIF1α gene or protein levels is associated with antioxidant-induced gene expression in angiogenesis and glycolysis under normoxia. Notably, VitC, NAC, and trolox increased *HIF1A* gene expression in A549 spheroids but not *HIF2A*. In addition, the antioxidants dose-dependently increased HIF1α protein levels in lung tumor organoids, A549 and H838 spheroids, but had little effect on HIF2α levels. It is also known that hypoxia induces BACH1 gene expression and protein levels (268, 269). Considering this, we examined this modulation's mechanism using the prolyl hydroxylase inhibitors FG0041 and DMOG (270). Remarkably, our findings revealed that both drugs significantly elevated the levels of BACH1 protein under normoxic conditions.

Under normoxia, *HIF1A* overexpression in A549 spheroids increased BACH1 gene and protein levels. Conversely, *HIF1A* knockout A549 spheroids had considerably lower basal BACH1 protein levels. Moreover, these *HIF1A* knockout cells were unable to increase BACH1 in response to antioxidants. As observed in previous studies, hypoxia increased BACH1 levels (268, 269); similarly, hypoxia also elevated BACH1 levels in *HIF1A* knockout cells. Interestingly, both DMOG and FG0041 enhanced BACH1 protein in *HIF1A* knockout cells. Reintroduction of exogenous HIF1A into *HIF1A* knockout cells normalized both basal and NAC-induced BACH1.

From these observations, our study concluded that under normoxia, HIF1α regulates BACH1 levels and mediates the increase in BACH1 levels induced by antioxidants. It is important to note that HIF1α is also necessary for the increase in BACH1 gene expression and protein levels under hypoxia. Interestingly, both hypoxia and hypoxia-mimetic drugs increased BACH1 protein levels in *HIF1A* knockout cells, indicating that the regulation of BACH1 is independent of HIF1α and dependent on prolyl hydroxylase.

CUT&Tag studies had unveiled that *HIF1A* knockout cells display lower genome-wide BACH1 chromatin occupancy, a finding supported the observed reduction in BACH1 expression in these cells. Interestingly, the overexpression of *BACH1* in *HIF1A* knockout cells triggered an increase in gene expression related to angiogenesis and glycolysis. This observation implied that BACH1 might have the capacity to regulate these processes independently of HIF1α. Moreover, CUT&Tag experiments conducted on hypoxic cells had revealed an increase in HIF1α chromatin occupancy. This increase was not limited to the genome at large but was also noticeable at specific gene loci, including BACH1. Taken together, these findings suggested that BACH1, targeted by HIF1α as a transcription factor, might also promote the expression of genes involved in angiogenesis and glycolysis even in the absence of HIF1α.

The Cancer Genome Atlas (TCGA) data revealed a link between BACH1 expression and various angiogenesis genes in lung cancers. Similar patterns were found in breast and kidney cancer cohorts, reinforcing this link. Further immunohistochemical examination of tumor sections from KRAS-mutant NSCLC patients showed correlations between BACH1-VEGFA and BACH1-VEGFR2.

To explore the potential role of antioxidant-mediated BACH1 activation in tumor angiogenesis, we administered VitC, NAC, and VitE to *BACH1* wild type and *BACH1* knockout tumor-bearing *NSG* mice. Using ultrasonic imaging, we discovered that VitC and NAC increased tumor vascularity, an effect that disappeared when *BACH1* was knocked out. Interestingly, VitE produced similar results. Building on this, we postulated that increased BACH1 expression could enhance the tumor's response to anti-angiogenic therapy. To test this theory, we injected anti-VEGFR2 antibodies (DC101) into NSG mice with xenograft tumors, which had varied BACH1 states: overexpressed, knocked out, or re-expressed. Our findings were compelling. Tumors

overexpressing *BACH1* stopped growing in mice treated with DC101 after an initial growth period, unlike in the control group given saline. While DC101 did cause a delay, it did not significantly slow the growth of *BACH1* knockout tumors. Notably, re-expression of *BACH1* in the knockout cells restored their sensitivity to DC101. These results highlight the complex role of BACH1 in tumor angiogenesis and response to therapy.

## 4.2 PAPER II: A MYC-controlled redox switch protects B lymphoma cells from EGR1-dependent apoptosis

Lymphomas, which include Hodgkin's and non-Hodgkin's types, are a diverse group of blood cancers. Among non-Hodgkin lymphomas, the majority are B-cell lymphomas (271). The most common type of B-cell lymphoma is Diffuse Large B Cell Lymphoma (DLBCL). Currently, many clinical trials are investigating the use of high-dose Vitamin C as an anticancer treatment, including for B cell lymphoma (24, 272-274). However, the impact of antioxidants that reduce ROS levels on the growth of B cell lymphoma is still unclear.

To decipher the effects of antioxidants on the progression of B cell lymphoma, a study was conducted using nine different B cell lines. These lines were obtained from patients diagnosed with various types of lymphomas, such as Hodgkin's lymphoma (L428), diffuse large B cell lymphoma (Karpas-422, DB, LY1, LY3), and Burkitt lymphoma (Daudi, BL2, Ramos). The control cell line used was IM9, a B lymphoblast cell line transformed by the Epstein Barr virus. Upon treatment with VitC and NAC, it was observed that the viability of six out of the nine cell lines was decreased. However, the remaining three cell lines appeared to be largely unaffected. Notably, when sensitive cell lines were incubated with a combination of VitC and NAC, an additive effect was observed, suggesting a potential synergistic interaction between these two antioxidants. Consistent with their antioxidant properties, VitC and NAC lowered overall ROS levels with $H_2O_2$ analyses, 2',7'-dichlorofluorescein (DCF) probes, and GSS/GSH ratios.

The study discovered that high-dose intraperitoneal administration of VitC or NAC reduced tumor growth curves and endpoint weight in *NSG* mice, which had been subcutaneously injected with the sensitive cell lines LY1 and Ramos. Furthermore,

it was observed that concurrent administration of lower doses of VitC and NAC effectively halted tumor progression. However, it's noteworthy that VitC and NAC did not influence the growth of IM9-derived xenograft tumors, consistent with the in vitro findings.

To elucidate the anticancer mechanisms of VitC and NAC, RNA-seq was performed on treated LY1 and Ramos cell lines. The HOMER analysis revealed an enrichment of MYC-binding motif genes among the differentially regulated genes. Concurrently, Gene Ontology studies identified a collection of MYC-related genes in the protein interaction dataset. This enrichment of MYC-target genes was further validated by gene set enrichment studies (GSEAs). The antioxidant treatment interestingly led to a dual effect: a decrease in cell proliferation and an increase in cell death. Consequently, genes associated with the apoptosis pathway were enriched, while those related to the G2M checkpoint and E2F targets were depleted. More specifically, there was a decrease in MYC-dependent cell-cycle genes, while a corresponding increase in apoptosis-related genes.

The selected B cell lines were further analyzed for their origin and mutations. MYC-activating genetic changes were identified in six responsive cell lines. Western blotting revealed that these cell lines expressed ten times more basal MYC than the three non-responsive cell lines. Notably, in these sensitive B lymphoma cell lines, antioxidants did not affect MYC or phosphorylated MYC. To evaluate the hypothesis that MYC contributes to antioxidant-induced apoptosis, three experiments were conducted. First, preincubation with I-BET762, a chemical that inhibits MYC expression, or exposure to lentiviruses with short hairpin (sh) RNAs that knockdown *MYC* expression, diminished the impact of VitC and NAC. Second, neither VitC nor NAC inhibited tumor growth in mice injected with sh*MYC*-transfected cells, and similarly, the combination of VitC and NAC also did not affect tumor growth in mice transplanted with sh*MYC*-transfected cells. Lastly, VitC and NAC reduced cell numbers in human B cells expressing tetracycline-off-inducible MYC, but not when MYC was off (275). VitC and NAC induced apoptosis and inhibited cell-cycle progression, as confirmed by fluorescence-activated cell sorting (FACS) of propidium iodide and Annexin V-labeled cells and western blots using antibodies to cleaved caspase-3 and NOXA. Interestingly, VitC and NAC did not increase NOXA in sh*MYC*-transfected cells, which supports the role of MYC in apoptosis. Moreover, the inhibition of apoptosis also inhibited NAC-induced cell death in lymphoma cells.

MYC-regulated transcription, which is pivotal in actively dividing cancer cells, enhances cell-cycle progression and reduces apoptosis (276, 277). Co-factors like CDKN2A (ARF) and early growth response protein 1 (EGR1) can modulate MYC transcription, thereby suppressing cell-cycle progression and triggering apoptosis. (276-279). To understand the role of ARF and EGR1 in MYC-mediated apoptosis, particularly after VitC and NAC administration, we employed quantitative polymerase chain reaction (qPCR) as a tool to measure their expression levels. The results showed a dose-dependent upregulation of both genes in LY1 and Ramos cells. Conversely, the expression levels in IM9 cells remained unchanged, indicating a different response in this cell type. In lentivirus-shRNA experiments, it was observed that *EGR1* reduction offered some protection against VitC and NAC-induced apoptosis, a phenomenon not seen when *ARF* was knocked down. On the contrary, *EGR1* overexpression not only increased VitC and NAC-induced apoptosis but also increased the expression of apoptotic genes. Moreover, downregulating *EGR1* effectively inhibited the upregulation of apoptosis-related genes and the downregulation of most cell-cycle genes.

In our study, we examined the EGR1-MYC relationship and its subsequent effects. Utilizing immunoprecipitation (IP)-western blot techniques, we discovered that VitC and NAC increased the EGR1-MYC interaction in Ramos and LY1 cells by two to three-fold. Following this, we performed ChIP-qPCR tests. These tests revealed that VitC and NAC increased MYC's occupancy on the promoters of BAK1, NOXA, CDKN2A and BAX, while concurrently decreasing its occupancy on the promoters of CDC25A and CCNG1. Interestingly, downregulating *EGR1* not only amplified the effect of VitC and NAC in enhancing MYC's occupancy on cell cycle gene promoters, but also prevented VitC and NAC from increasing MYC's occupancy on apoptotic gene promoters.

Protein redox sensitivity is often mediated by cysteine residues. In this investigation, we specifically targeted four conserved cysteines located at positions 117, 171, 300, and 342 on the MYC protein (280, 281). Our primary objective was to determine whether any of these cysteines interact with EGR1. To achieve this, we inserted plasmids encoding four cysteine-to-serine MYC mutants and wild-type (WT) MYC into HEK293 cells, which are known to express little MYC. Subsequently, we conducted a Co-immunoprecipitation (CoIP) analysis. The results of this analysis revealed a considerably reduced interaction between MYC and EGR1 in all mutants,

with the C117S mutant showing a particularly notable reduction. Furthermore, we found that transduction of IM9 cells, which have low MYC levels and resist NAC-induced apoptosis, with WT-MYC enhanced their sensitivity to VitC and NAC. However, transduction with MYC-C117S did not have the same effect.

Two studies were conducted using *λMYC* mice, which are characterized by a mutant human MYC gene under a rebuilt immunoglobulin λ (Igλ) locus and are prone to B cell lymphoma (282). In the first study, tumor-bearing mice were randomly selected and injected intraperitoneally with either VitC/NAC or a vehicle. It was observed that the injections of VitC/NAC slowed down tumor growth and improved survival. In the second study, VitC and NAC were added to the drinking water of *λMYC* mice from weaning until the endpoint, leading to a significant increase in survival. Therefore, both studies underscore the potential benefits of VitC and NAC in enhancing survival rates in *λMYC* mice.

# 5 Discussion

Paper I

In this study, we discovered that BACH1, a redox- and oxygen-sensitive transcription factor, governs tumor angiogenesis and their responsiveness to antiangiogenic treatment. Interestingly, we found that both hypoxia and antioxidants activate BACH1 in lung cancer cells through two distinct processes: transcriptional and post-translational. On one hand, HIF1α controls BACH1 transcription. Remarkably, both *HIF1A* wild type and knockout cells exhibit BACH1 protein accumulation when exposed to prolyl hydroxylation inhibitors. This observation suggests that reduced prolyl hydroxylation-dependent degradation may be responsible for the posttranslational stabilization of BACH1 under hypoxia, independent of HIF1α. On the other hand, under reducing conditions, such as those encountered after antioxidant administration, BACH1 stabilization is mediated through reduced heme-dependent degradation (72, 283, 284).

Antioxidants were found to increase HIF1α gene and protein levels, which is essential for the subsequent increase in BACH1 gene and protein levels. This discovery has led to the hypothesis that endogenous NRF2-driven, dietary and pharmaceutical antioxidants could accelerate lung tumor development and metastasis via HIF1α and BACH1-mediated angiogenesis and glycolysis activation (59, 72, 253).

The discovery that antioxidants enhance HIF1α levels was indeed surprising, particularly as VitC has been previously shown to decrease HIF1α levels in cancer cells (285). This unexpected rise in HIF1α levels, induced by ROS-lowering antioxidants, was intriguing given that ROS production from mitochondria under hypoxia is known to increase HIF1α levels by inhibiting hydroxylation-dependent degradation (286, 287). However, this could be attributed to the transient nature of mitochondrial ROS production under hypoxia, which lasts only a few hours (288), while the present study evaluated the effects over a span of 7 days. Additionally, the study found that antioxidants upregulate *HIF1A* gene expression, which could potentially explain the increase in protein levels, an action thought to be unrelated to posttranslational hydroxylation.

BACH1, a protein that has been found to promote lung tumor angiogenesis, is associated with the expression of angiogenesis genes and proteins in human lung tumor samples. Interestingly, treatment targeting VEGFR2, a key player in angiogenesis, has been shown to decrease the growth of tumors with high BACH1 levels. However, this effect was not observed in tumors with low BACH1 levels. Consequently, this implies that BACH1 could potentially act as a biomarker for predicting the responsiveness to anti-angiogenic treatments. In essence, patients with high BACH1 levels might benefit more from anti-VEGFR2 treatment. This discovery could pave the way for more personalized cancer treatment strategies and ultimately lead to improved patient outcomes.

Paper II

In this study, we uncovered that VitC and NAC, which lower ROS, can inhibit MYC's ability to sustain proliferation and prevent apoptosis in B lymphoma cells. Additionally, these antioxidants were found to reduce tumor growth and increase survival in both xenograft and endogenous B cell lymphoma models. Interestingly, these antioxidants showed a preference for inducing apoptosis in high-MYC human B lymphoma cells. Based on these findings, we suggest the potential incorporation of these antioxidants in future therapeutic strategies for MYC-driven B cell lymphoma.

Our study revealed that VitC and NAC induced apoptosis when MYC expression was high. However, when MYC expression was blocked using shRNAs or i-BET762, the apoptosis-inducing ability of VitC and NAC was consistently reduced and sometimes even eliminated. Given that MYC is a driving oncogene, the ability of its inhibition to restore the effects of VitC and NAC varied. Notably, when MYC was suppressed with shRNAs, VitC and NAC were found to increase tumor growth, a finding consistent with other cancer models (57, 59, 60, 72, 253). These findings suggest that MYC plays a crucial role in determining whether VitC and NAC act as pro- or anti-tumorigenic agents in B lymphoma cells.

In our additional mechanistic investigations, we found that VitC and NAC enhance the binding of MYC to EGR1. This interaction leads to a change in MYC promoter occupancy and gene expression, effectively converting cell-cycle genes into apoptotic genes, thereby activating apoptosis. Simultaneously, we observed a dose-dependent increase in EGR1 expression, suggesting the existence of a feedforward

activation loop. Unlike EGR1, ARF failed to trigger apoptosis. Although ARF does increase the recruitment of MYC to EGR1, our data supports the occurrence of ARF-independent MYC-EGR1-driven apoptosis (278, 289). The impact of EGR1 knockdown research might be understated due to the low knockdown efficiency of *EGR1*-shRNAs. Remarkably, when *EGR1* was overexpressed, VitC and NAC dramatically increased the expression of apoptotic genes. Collectively, these findings propose that VitC and NAC potentially recruit EGR1 to regulate MYC transcription.

Despite the fact that antioxidants have been observed to increase tumor metastasis in preclinical studies (59, 72, 253), clinical investigations tell a different story. In particular, high doses of VitC have been shown to reduce tumor growth (274, 290), a beneficial effect that is attributed to VitC's pro-oxidant activities. Interestingly, this concept is further supported by the observation that NAC, a well-known antioxidant, decreases VitC's ability to kill colorectal cancer cells with KRAS and BRAF mutations (64).

In this study, it was found that both VitC and NAC reduced ROS levels and triggered apoptosis, with the combination of the two having an additive effect. Interestingly, T cells expressing the MYC oncogene exhibited similar responses as B lymphoma cells. However, neuroblastoma cells expressing C- and N-MYC did not show the same response. This suggests that safe redox-related chemicals, when administered at doses that lower ROS, can induce apoptosis specifically in cancer cells that express certain oncogenes. Given their role as ROS-producing cells, T and B cells might be particularly sensitive to VitC/NAC therapy, potentially through a MYC-dependent mechanism. This could provide an explanation as to why other wild-type and solid tumor cell types have not demonstrated a similar cytotoxic effect.

The study demonstrated that both VitC and NAC effectively slowed tumor growth and enhanced survival in mice, regardless of whether they had a fully functional immune system. These findings reduce the possibility that these compounds indirectly reduced tumor growth by triggering antitumor immunity. Instead, it's more likely that their effects were specifically targeted at the specific tumor cells.

# 6 Conclusions

Paper I

- Antioxidants increase BACH1 and HIF1α mRNA and protein levels.
- Antioxidants significantly increase the expression of angiogenesis and glycolysis genes.
- BACH1 plays a role in mediating the effects of antioxidants on the expression of angiogenesis and glycolysis-related genes.
- BACH1 controls expression of angiogenesis and glycolysis genes.
- BACH1 is post-translationally prolyl-hydroxylated by the HIF prolyl-hydroxylases (PHDs).
- HIF1α maintains the basal levels of BACH1 and mediates antioxidant-induced increases in BACH1 levels.
- BACH1 is a HIF1α-targeted transcription factor, but it can promote HIF1α-independent expression of genes involved in angiogenesis.
- The expression of BACH1 in lung, breast and renal cancer samples has been found to be correlated with the expression of a wide variety of angiogenesis genes.
- BACH1 expression is correlated with angiogenesis protein expression in human KRAS-mutant NSCLC samples.
- BACH1 enhances the sensitivity of tumors to anti-angiogenic therapy.
- BACH1 is a redox- and oxygen-sensitive transcription factor and could serve as a potential biomarker for predicting a more favorable response to anti-angiogenic therapy.

Paper II

- VitC /NAC trigger MYC-EGR1-dependent apoptosis in high-MYC-expressing B lymphoma cells.
- NAC/VitC-induced apoptosis is associated with reduced levels of oxidative stress.
- Reduction of conserved MYC cysteine residues stimulates EGR1 binding.
- EGR1 stimulates MYC recruitment from proliferative to apoptotic gene promoters

Our research has unveiled the dual effects of antioxidants in two different types of cancer, namely lung cancer and B cell lymphoma.

In the case of lung cancer, we have found that antioxidants can stimulate BACH1 dependent tumor angiogenesis, providing additional evidence that antioxidants stimulate tumor metastasis. Hence, we strongly advise lung cancer patients, especially those with high BACH1 expression, to avoid taking supplementary antioxidants and to consider undergoing anti-angiogenesis treatment.

Conversely, when it comes to B cell lymphoma, our research indicates a contrasting effect. We found that antioxidants can inhibit the progression of the disease. This is particularly relevant for patients with high MYC expression. Therefore, this suggests that antioxidant treatment could potentially be beneficial for these patients.

In conclusion, our research underscores the importance of personalized medicine and highlights the need for careful consideration when recommending antioxidant supplements to cancer patients. It's crucial to consider the type of cancer and the specific genetic expressions involved before making any recommendations, although in most instances the safest course of action seems to be to avoid supplements and focus on a balanced diet that includes fruits and vegetables.

# 7 Points of perspective

Paper I

Our research findings initially indicate that BACH1 promotes angiogenesis in lung cancer, and it positively correlates with angiogenesis-related genes and proteins in lung cancer patients. As a result, it is possible that BACH1 could serve as a potential biomarker for predicting a more favorable response to anti-angiogenic therapy. Drawing on these findings, we recommend that patients with lung tumors expressing high levels of BACH1 or NRF2 could be considered to undergo anti-angiogenic therapy, as this could potentially improve their treatment outcomes. Nonetheless, the utility of this biomarker in a clinical setting should be determined by future studies. Intriguingly, our work suggests these investigations may expand beyond lung cancer. In particular, the expression of angiogenesis-related genes in breast and kidney cancer was also found to correlate with BACH1.

Paper II

While our findings could not rule out that VitC and NAC may interact with other proteins/pathways to indirectly promote MYC/EGR1 binding, it seems clear that MYC-driven B lymphoma cancer cells are vulnerable to antioxidants. This vulnerability raises the possibility that this idea could be tested in human clinical trials. Interestingly, in a proof-of-principle experiment, we found that the survival rate improved moderately but statistically significantly when VitC or NAC was added to drinking water of the mice. However, injections of VitC or NAC was more effective than oral dosing for B cell lymphoma therapy. Considering this, one potential treatment option could be to use traditional lymphoma treatments before and after administering VitC or NAC. Alternatively, VitC or NAC could be alternated with R-CHOP. However, it is important to note that it is unlikely that concurrently administering R-CHOP chemotherapy drugs like cyclophosphamide and doxorubicin with VitC/NAC would work, as these chemotherapeutic drugs' capacity to increase ROS levels may counteract the effects of VitC/NAC or vice versa.

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