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Remodeling Anaplastic Thyroid Cancer's Aggressive Profile and Metabolic Signature by Natural Alkaloid Berberine

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Remodeling Anaplastic Thyroid Cancer's Aggressive Profile and Metabolic Signature by Natural Alkaloid Berberine

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List of Abbreviations

AKT: Ak strain transforming
AMP: adenosine monophosphate
AMPK: adenosine monophosphate protein kinase
ATC: anaplastic thyroid carcinoma/cancer
ATP: adenosine triphosphate
Bax: Bcl-2 Associated X-protein
BBR: berberine
Bcl-2: B-cell lymphoma 2
BMI: body mass index
BRAF: V-Raf murine sarcoma viral oncogene homolog B
BSA: bovine serum albumin
β-catenin: cadherin-associated protein beta 1
CAF: cancer-associated fibroblast
cAMP: cyclic adenosine monophosphate
CBC: complete blood count
CCL: C-C motif chemokine
CM: conditioned media
CO_2: carbon dioxide
CT: computed tomography
CTNNB1: catenin Beta 1
CXCL: chemokine (C-X-C motif) ligand
DEG: differentially expressed gene
DMSO: dimethyl sulfoxide
DRAM: damage-regulated autophagy modulator
DTC: differentiated thyroid carcinoma/cancer
EGFR: epidermal growth factor receptor
EIF1AX: eukaryotic translation initiation factor 1A X-linked
ELISA: enzyme-linked immunosorbent assay
EMT: epithelial-to-mesenchymal transition
ERK: extracellular signal-regulated kinase
ETE: extrathyroidal extension
FDG-PET: fludeoxyglucose-18 positron emission tomography
FNA: fine needle aspiration
FNMTC: familial non-medullary thyroid carcinoma/cancer
FTC: follicular thyroid carcinoma/cancer
FVPTC: follicular-variant papillary thyroid carcinoma/cancer
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
G-CSF: granulocyte-colony stimulating factor
GEO: Gene Expression Omnibus
GM-CSF: granulocyte macrophage colony-stimulating factor
GOLPH3: Golgi phosphoprotein 3

GRO-α: growth-related oncogene alpha
GTP: guanosine-5'-triphosphate
HCC: Hürthle cell carcinoma/cancer
HIF-1α: hypoxia inducible factor 1 subunit alpha
HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A
HO-1: hemeoxygenase-1
IAP: inhibitor of apoptosis
ICAM-1: intracellular adhesion molecule 1
IDO: indoleamine-2,3-dioxygenase
IFN-γ: interferon-gamma
IL: interleukin
IP-10: interferon-gamma inducible protein
IPA: Ingenuity Pathway Analysis
JAK: Janus tyrosine kinase
JNKC: c-Jun NH2-terminal kinase peptide control
LDL: low density lipoprotein
LKB1: Liver Kinase B1
LN: lymph node
LPS: lipopolysaccharide
mAb: monoclonal antibody
MACIS: metastasis, age, completeness of resection, invasion, and size
MAPK: mitogen-activated protein kinase
Mcl-1: Myeloid cell leukemia-1
MCP: monocyte chemoattractant protein
M-CSF: macrophage colony-stimulating factor
MCT: monocarboxylate transporter
MED12: mediator of RNA polymerase II transcription subunit 12 homolog
MEK: mitogen-activated protein kinase kinase
MIG: membrane immunoglobulin
MIP-1: macrophage inflammatory protein-1
MKK4: mitogen-activated protein kinase 4
mRNA: messenger ribonucleic acid
MTC: medullary thyroid carcinoma/cancer
mtDAMPS: mitochondrial danger-associated molecular patterns
mtDNA: mitochondrial deoxyribonucleic acid
mTOR: mammalian target of rapamycin
NADPH: nicotinamide adenine dinucleotide phosphate
NADH: nicotinamide adenine dinucleotide + hydrogen
NCBI: National Center for Biotechnology Information
NF-κB: nuclear factor kappa B
NIFTP: noninvasive follicular thyroid neoplasm with papillary-like nuclear features
Nrf2: nuclear factor erythroid 2-related factor 2
OS: overall survival
OXPHOS: oxidative phosphorylation

p53: tumor protein p53
PAX8: paired-box gene 8
PBS: phosphate buffered saline
PDGF-BB: platelet-derived growth factor-BB
PDTC: poorly differentiated thyroid carcinoma/cancer
PGC-1α: peroxisome proliferator-activated receptor-gamma coactivator
PI3K: phosphatidylinositol 3-kinase
PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PIK3CB: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta
PIK3CD: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta
PPARγ: peroxisome proliferator-activated receptor gamma
PTC: papillary thyroid carcinoma/cancer
PTEN: phosphatase and tensin homolog
Raf: rapidly accelerated fibrosarcoma
RANTES: regulated upon activation, normal T cell expressed and secreted
Ras: rat sarcoma virus
RBM10: RNA-binding motif 10
RET/PTC: rearranged during transfection / papillary thyroid carcinoma
RIPA: radioimmunoprecipitation assay
RNA Seq: ribonucleic acid sequencing
ROS: reactive oxygen species
RPMI-1640: Roswell Park Memorial Institute 1640
rpS6: ribosomal protein S6
RT: radiation therapy
SAPK: stress-activated protein kinase
SDS-PAGE: sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SIRT: sirtuin
STAT3: signal transducer and activator of transcription 3
STING: stimulator of interferon response cGAMP interactor 1
T3: triiodothyronine
T4: thyroxine
TAM: tumor-associated macrophage
TBST: tris-buffered saline with 0.1% Tween® 20 detergent
TC: thyroid carcinoma/cancer
TERT: telomerase reverse transcriptase
Tg: thyroglobulin
TGF: transforming growth factor
TH: thyroid hormone
TIMP-2: tissue inhibitor of metalloproteinases 2
TME: tumor microenvironment
TNF-RI: tumor necrosis factor receptor 1
TNF-RII: tumor necrosis factor receptor 2
TNF-α: tumor necrosis factor alpha
TNM: tumor, node, metastasis

TOMM20: translocase to the outer mitochondrial membrane 20
TP53: tumor protein p53 gene
TPA: O-tetradecanoylphorbol-13-acetate
TR: thyroid hormone receptor
TRH: thyrotropin releasing hormone
TSH: thyroid stimulating hormone
UTR: untranslated region
V600E: valine to glutamic acid
VEGF: vascular endothelial growth factor

Abstract

Anaplastic thyroid cancer is a rare, fatal cancer with a five-year survival of 4%. Universally diagnosed at stage IV, anaplastic thyroid cancer is characterized by its lack of differentiation, rapid proliferative rate, highly inflammatory tumor microenvironment, and metabolic dysregulation. Refractory to all established therapies, anaplastic thyroid cancer requires a novel therapeutic approach that targets all of these drivers of anaplastic thyroid cancer carcinogenesis. We propose natural alkaloid berberine as a therapeutic with multitarget efficacy to alter mitochondrial metabolism and reprogram anaplastic thyroid cancer's aggressive phenotype. Our *in vitro* model uses monocyte cell line U937, anaplastic thyroid cancer cell lines T238 and SW1736, and immortalized normal thyroid cell line Nthy-ori-3-1. Validation of *in vitro* findings via RNA Sequencing was conducted by Genewiz from Azenta and Qiagen's Ingenuity Pathway Analysis was used for *in silico* modeling.

In targeting the aggressiveness of anaplastic thyroid disease, berberine selectively slowed proliferation by 80% in anaplastic thyroid cancer cells from 48 to 72 hours while sparing normal cells. Berberine reduced migratory capacity by 33% in T238 cells and 51% in SW1736 cells after 24 hours. Berberine reduced both migration and invasion by 30% in T238. These observations were substantiated by Western blot analysis – berberine selectively decreased phosphorylation of MEK, ERK, and ribosomal protein S6, crucial downstream regulators of the pro-proliferative and pro-survival pathways in anaplastic thyroid cancer cells. Further, berberine specifically modulated cancer-associated metabolism as observed through an increase in AMPKα phosphorylation, a major rate-limiting protein in cancer-induced dysregulation with an anti-tumor effect.

Modeling the anaplastic thyroid cancer tumor microenvironment, U937 cells were activated and polarized into a proinflammatory macrophage phenotype. Following berberine treatment at the activation/polarization stages, 19 soluble inflammatory mediators were significantly downregulated in the conditioned media compared to controls. U937 cells polarized using anaplastic thyroid cancer-conditioned media pre-treated with berberine also showed decreased IFN-γ and TNF-α secretion.

Validation of *in vitro* findings via RNA Sequencing revealed more than 400 significantly differentially expressed genes involved in mitochondrial metabolism, glycometabolism, sirtuin signaling, apoptosis, and proliferation. Following a comprehensive analysis, we identified significant downregulation of 22 of 37 total mitochondrially encoded genes and 13 of 13 mitochondrially encoded protein-coding genes comprising the oxidative phosphorylation complexes, illuminating a clear link between berberine treatment and altered mitochondrial metabolism in anaplastic thyroid cancer. Additionally, protein expression of significantly downregulated mitochondrial genes identified via RNA Sequencing was validated via Western blot, demonstrating decreased mitochondrially-encoded protein expression related to oxidative phosphorylation.

This work reveals a novel role for berberine as an inhibitor of mitochondrial metabolism that can be used to reprogram the aggressive nature of anaplastic thyroid cancer and open the door for promising combination therapy in treating fatal anaplastic thyroid cancer.

I. Introduction

1. Thyroid Characteristics

1A. The Thyroid Gland

The thyroid gland is a butterfly-shaped endocrine organ situated anteriorly in the neck between the C5 and T1 vertebrae **(Figure 1)** (Benvenga et al., 2018; Beynon & Pinneri, 2016). It is the first endocrine gland to develop in humans, originating from the thyroid diverticulum in the median ventral wall of the pharynx (Benvenga et al., 2018). Primitive thyroid tissue begins hollow but becomes solid throughout embryonic development (Benvenga et al., 2018). The external carotid and subclavian arteries highly vascularize the thyroid gland (Al-Azzawi & Takahashi, 2021; Benvenga et al., 2018). It is divided into left and right lobes (4 cm x 2 cm x 2-3 cm) and the smaller, central isthmus (2 cm x 2 cm x 2-6 mm) connects the two lobes (Benvenga et al., 2018; Beynon & Pinneri, 2016). Approximately 30-50% of the population has an additional pyramidal lobe extending upward, usually to the left, from either lobe or the superior portion of the isthmus (Al-Azzawi & Takahashi, 2021; Benvenga et al., 2018). The thyroid gland weighs 13-20 grams and is, on average, 5 grams heavier in males than females (Al-Azzawi & Takahashi, 2021; Benvenga et al., 2018).

The thyroid gland is encapsulated by layers of deep cervical fascia firmly adhering to the gland and projecting into the thyroid to form septae; these septae subsequently divide the thyroid gland into lobes and lobules (Benvenga et al., 2018; Beynon & Pinneri, 2016; Nilsson & Fagman, 2017). Histologically, these lobules each contain 20-40 spherical follicles that are heterogeneous in size, and surrounded by a rich capillary network (Benvenga et al., 2018;

Nilsson & Fagman, 2017; Young, 1968). Each secretory follicle is lined with a layer of epithelial cells that surround the acidophilic colloid-containing lumen, categorized as thyroid follicular cells or thyrocytes **(Figure 1)** (Benvenga et al., 2018; Beynon & Pinneri, 2016; Young, 1968). Thyroid follicles are surrounded by the stroma – loose connective tissue where vascularization, enervation, lymphatics, and diverse immune cells, including fibroblasts, macrophages, and mast cells are all present (Benvenga et al., 2018; Rosa et al., 2022; Young, 1968).

At the cellular level, epithelial cells are the major cell type within the thyroid parenchyma. Primarily this consists of follicular cells (thyrocytes), which are responsible for thyroid hormone synthesis, but a smaller portion of a distinct epithelial cell subtype, parafollicular cells or C-cells, are also present exclusively in the basement membrane of the follicle (Beynon & Pinneri, 2016; Kameda, 2016; Young, 1968). C-cells only constitute about 0.1% of thyroid epithelial cells but are essential for calcitonin synthesis, storage, and secretion, which are essential for maintaining calcium levels in the blood (Benvenga et al., 2018; Kameda, 2016; Nilsson & Fagman, 2017; Young, 1968).

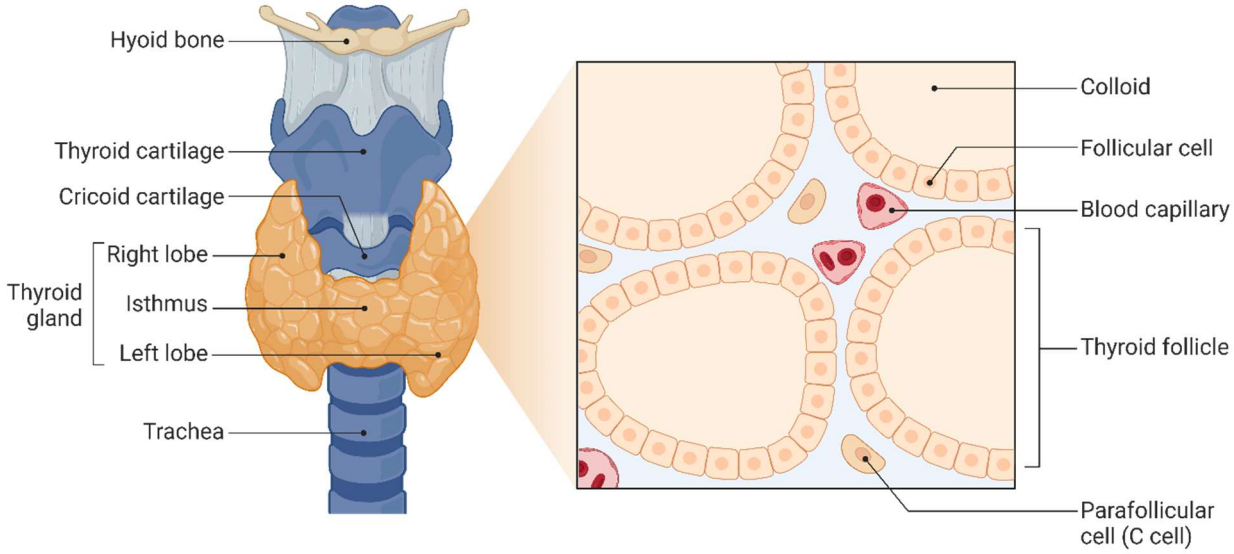


Figure 1. Thyroid gland anatomy and histology. Figure adapted from BioRender.com.

1B. Thyroid Hormones and Function

The thyroid produces three hormones essential for normal growth, development, and metabolism: thyroxine (T4), triiodothyronine (T3), and calcitonin **(Figure 2)** (Warner & Mittag, 2012; Yan-Yun Liu et al., 2020). Thyroid hormone (TH), comprised of thyroxine and triiodothyronine, is a multi-component peptide-derived hormone synthesized and released by the thyroid gland to act on nuclear hormone receptors and through non-genomic pathways (Yan-Yun Liu et al., 2020). Stimulation via thyroid stimulating hormone, iodine availability, and deiodinase activity all impact thyroid hormone synthesis and metabolism (Benvenga et al., 2018). T4 is the main product of the thyroid gland and is converted via an enzymatic reaction catalyzed by type 1 or type 2 5'-deiodinases to the active hormone T3 (Hoermann et al., 2015; Russo et al., 2021; Teixeira et al., 2020). For thyroid hormone synthesis to occur, iodine must be transported into the thyroid gland through a sodium iodide

symporter as it is required for T3 and T4 synthesis (Yan-Yun Liu et al., 2020). T4 and T3 can be subsequently inactivated by type 3 5'-deiodinase (Hoermann et al., 2015; Russo et al., 2021; Teixeira et al., 2020).

Early in development, thyroid hormone plays a critical role in normal growth and brain and sensory development (Yan-Yun Liu et al., 2020). In adults, thyroid hormone has a crucial function in regulating metabolism, and influences proper function of the heart, skeletal muscle, liver, bone, and brain (Yan-Yun Liu et al., 2020). In bone, thyroid hormones are essential for bone growth, development, and bone mass maintenance– hyperthyroidism increases osteoporosis and bone fracture risk as excess thyroid hormone accelerates bone turnover through osteoclast function (Lademann et al., 2020). In the brain, thyroid hormone augments adrenergic signaling of neurotransmitters (Warner & Mittag, 2012; Yan-Yun Liu et al., 2020). Thyroid hormone also influences the signaling of nutrient receptors, particularly peroxisome proliferator-activated receptor alpha, and gamma, fatty acid oxidation, regulation of energy homeostasis, insulin sensitization and glucose metabolism, and liver X receptor involved in lipid homeostasis (Mullur et al., 2014; Yan-Yun Liu et al., 2020).

In the canonical pathway, T4 and T3 reach these target cells through specialized membrane transporters, monocarboxylate transporter 8 (MCT8), MCT10, and organic anion-transporting polypeptide 1C1, wherein T3 can bind to nuclear thyroid hormone receptors (TR) (subtypes include α1, β1, and β2 TRs) and regulate transcription of target genes (Hoermann et al., 2015; Teixeira et al., 2020; Warner & Mittag, 2012). Non-classical pathways of thyroid hormone signaling may be mediated through binding of mitochondrial or cytoplasmic TRs, or activation of intracellular signaling via binding to nonspecific membrane

proteins (Teixeira et al., 2020). These non-canonical signaling pathways are reported to have a particularly influential role in the cardiometabolic effects of thyroid hormone signaling, including metabolic rate, energy expenditure, and exogenous heart rate control, and appear to utilize activation of the phosphatidylinositol 3-kinase (PI3K) signaling cascade to carry out these functions (Mullur et al., 2014; Teixeira et al., 2020; Warner & Mittag, 2012).

Importantly, tissue responsiveness to TH varies across age and sex, and influences changes in energy expenditure and body weight (Teixeira et al., 2020). Overproduction of thyroid hormone can lead to hyperthyroidism, while underproduction of thyroid hormone can lead to hypothyroidism – the two major functional thyroid diseases (Y. Wang et al., 2023; Yan-Yun Liu et al., 2020). Hypothyroidism prevalence is roughly 8-15% with increased incidence with age, reaching roughly 20% in the elderly population (Teixeira et al., 2020). TH has thermogenic effects – hyperthyroid patients have increased heat production and develop heat intolerance, whereas hypothyroid patients produce less heat and are cold intolerant (Teixeira et al., 2020; Y. Wang et al., 2023). These diseases of thyroid hormone over- and underproduction can have major influence on obesity, cardiac diseases, fertility, hyperlipidemia, and fibrosis, amongst others (Y. Wang et al., 2023; Yan-Yun Liu et al., 2020). Genetic defects can also impact normal thyroid hormone signaling, though this is rare (Yan-Yun Liu et al., 2020).

As thyroid hormone levels are crucial for homeostatic regulation, understanding their regulation is also essential. Dynamic interplay between the thyroid and pituitary glands and their secreted hormones are essential for homeostatic equilibrium **(Figure 2)** (Bianco et al., 2019; Hoermann et al., 2015). The pituitary gland secretes thyrotropin, commonly called

thyroid stimulating hormone (TSH), which acts on T4 and T3 in situation, regulatory negative-feedback loop (Beynon & Pinneri, 2016; Bianco et al., 2019; Hoermann et al., 2015). TSH is often used as an indirect indicator of both thyroid function and dysfunction, as well as thyroid hormone homeostasis (Hoermann et al., 2015). Secretion of TSH is subject to circadian regulation (Hadlow et al., 2013; Hoermann et al., 2015). There is a logarithmic relationship between TSH and free T4 levels that is impacted by thyroid status (Hadlow et al., 2013; Hoermann et al., 2015). Negative thyroid hormone feedback mitigates thyroid hormone overproduction and hyperthyroidism, while thyrotropin releasing hormone (TRH) protects against underproduction of thyroid hormone and hypothyroidism by stimulating pituitary TSH secretion, which can in turn stimulate thyroid hormone production (Bianco et al., 2019; Hoermann et al., 2015).

In addition to T4 and T3, calcitonin is an essential hormone synthesized by the thyroid gland. Calcitonin, also referred to as thyrocalcitonin, is secreted by the neuroendocrine parafollicular or C-cells. Calcitonin is a hypocalcemic hormone that functions as a natural antagonist to the hypercalcemic parathyroid hormone (Felsenfeld & Levine, 2015; Nilsson & Fagman, 2017). Parathyroid hormone increases calcium levels in the blood through increasing calcium absorption from digested food and bone resorption via osteoclast activity (Feng et al., 2015). Thus, calcitonin regulates blood calcium levels (Felsenfeld & Levine, 2015; Nilsson & Fagman, 2017). C-cells respond to extracellular calcium levels through activation of calcium-sensing receptors, which in turn leads to the release of stored calcitonin from the dense-core granules (Kameda, 2016; Nilsson & Fagman, 2017).

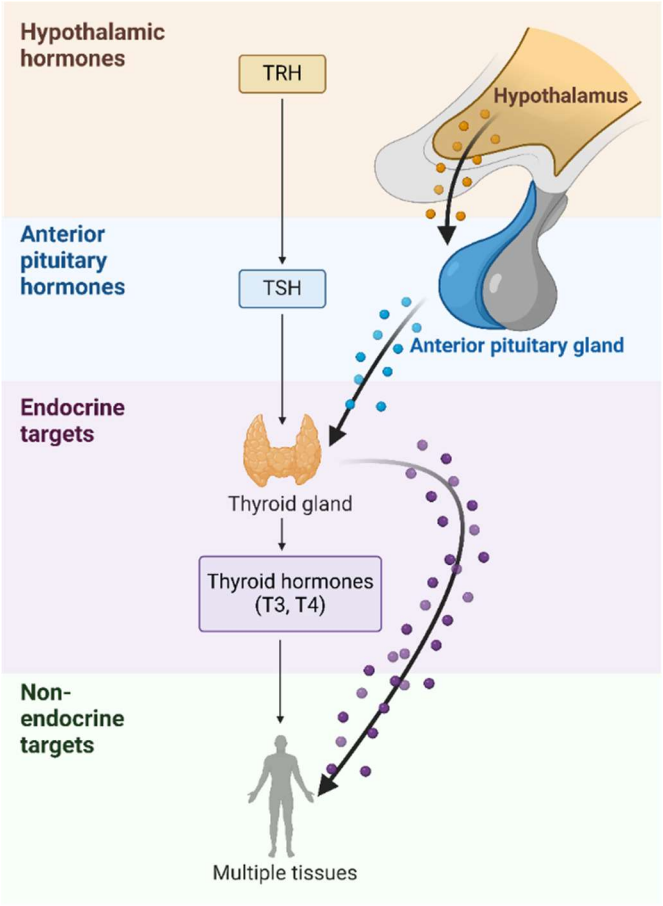


Figure 2. Hypothalamus-pituitary-thyroid axis. Dynamic interplay between the hypothalamus region of the brain, the pituitary gland, and the thyroid gland. Figure adapted from BioRender.com.

1C. Thyroid Cancer

Epidemiology and Incidence

Thyroid cancer (TC) is the most common endocrine malignancy, accounting for greater than 90% of all endocrine malignancies and 3.8% of all cancers (Drozd et al., 2020; Kitahara & Schneider, 2022; Malaguarnera et al., 2020; Nikiforov & Nikiforova, 2011; Zhai et al., 2021). Its incidence both in the United States and internationally has steadily risen over the last four decades (Alhejaily et al., 2023; Drozd et al., 2020; Kitahara & Schneider, 2022; Li et al., 2020; O'Connell et al., 2021). It currently has the highest increase in incidence rate of any tumor in

the United States (Drozd et al., 2020). Thyroid cancer is the 13th most common cancer diagnosis and 6th most common among women, with even higher incidence in women under 50 (Kitahara & Schneider, 2022; Malaguarnera et al., 2020; Tuttle et al., 2010). In young individuals, 15-24 years, thyroid carcinomas comprise 7.5-10% of all diagnosed cancer cases (Alamoudi et al., 2011; Malaguarnera et al., 2020; Tuttle et al., 2010). This accounts for approximately 44,000 new cancer diagnoses per year, as of 2022 (Kitahara & Schneider, 2022). Generally, these increases in incidence have been partially attributed to increased screening using diagnostic imaging such as thyroid ultrasonography, more sensitive diagnostic tools, and occasionally overdiagnosis (Drozd et al., 2020; Kitahara & Schneider, 2022; Xie et al., 2016; Zhai et al., 2021). Overdiagnosis in this context is the detection or confirmation of disease that would have otherwise not been diagnosed even by time of death if the testing was not conducted (Li et al., 2020; Pizzato et al., 2022; Zhai et al., 2021). However, increased incidence of more advanced thyroid cancers and more aggressive thyroid tumors with higher mortality imply that etiological and environmental factors are also contributing to the rising disease incidence (Kitahara & Schneider, 2022; Pizzato et al., 2022).

Risk Factors

Thyroid cancer incidence is impacted by modifiable and non-modifiable risk factors **(Table 1)**. Non-modifiable risk factors for thyroid cancer include gender and age and differ by histological subtype **(Figure 3)** (Kitahara & Schneider, 2022; Rahbari et al., 2010; Zhai et al., 2021). Disease etiology may also be impacted by race, genetic susceptibility factors, and reproductive and hormonal factors (Bogović Crnčić, 2020; Drozd et al., 2020; Kitahara & Schneider, 2022). Medullary thyroid cancer specifically seems to have a strong genetic basis

(Bogović Crnčić, 2020; Kitahara & Schneider, 2022). Geographic location also impacts incidence, particularly cold climate (Kitahara & Schneider, 2022; Lehrer & Rosenzweig, 2014). Modifiable risk factors include childhood exposure to ionizing radiation, obesity and metabolic syndromes, presence of other benign thyroid pathologies, iodine and other dietary factors, nitrites, use of endocrine-disrupting chemicals, environmental contaminants, mercury in the thyroid gland, and recently, cell phone usage when certain genetic variants are present (Drozd et al., 2020; Kitahara & Schneider, 2022; Luo et al., 2020; Pamphlett et al., 2021; Shah, 2015; Zhai et al., 2021).

By sex, thyroid cancer incidence is roughly three times higher in women than in men (Kitahara & Schneider, 2022; Rahbari et al., 2010). Age and histological subtype also factor into this incidence rate (Malaguarnera et al., 2020). The average age of thyroid cancer incidences peaks ten years earlier in women than men, around 55 years in women and 65 years in men (Kitahara & Schneider, 2022; Rahbari et al., 2010). Thyroid cancer incidence occurs commonly from adolescence through middle age, and declines after the aforementioned peak into older age (Kitahara & Schneider, 2022; Malaguarnera et al., 2020; Rahbari et al., 2010). Increased incidence in females may be associated with more active humoral and cellular immune responses generating increased inflammation in tandem with higher likelihood of other autoimmune and inflammatory thyroid pathologies, such as Grave's Disease and Hashimoto's Thyroiditis (O'Connell et al., 2021). Although thyroid cancer is less common in males, men tend to experience more aggressive disease; both of these phenomena may possibly be attributed to higher levels of androgens and increased average age at time of diagnosis (O'Connell et al., 2021; Tuttle et al., 2010). At present, one in 55 women and one in 149 men

in the United States are anticipated to receive a thyroid cancer diagnosis, demonstrating the high prevalence of this disease (Kitahara & Schneider, 2022).

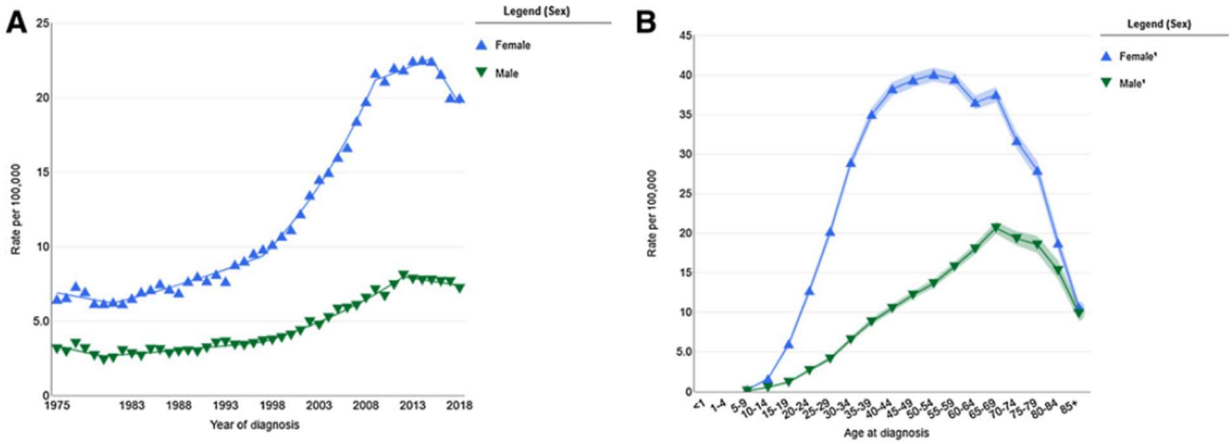


Figure 3. "Thyroid cancer incidence in U.S. men and women, by calendar year at diagnosis (SEER-9, 1975–2018) (A) and age at diagnosis (SEER-21, 2014–2018) (B) (all races). Data source and graph production: SEER, Bethesda, MD. Estimates based on <16 cases are suppressed." Figure and Legend Taken From: Kitahara and Schneider, 2022.

Geographical differences in thyroid cancer incidence are particularly pronounced in women (Kitahara & Schneider, 2022). Generally, highest incidence occurs in wealthier nations, including Austria, Canada, Croatia, France, Israel, Italy, the Republic of Korea, and the United States (Kitahara & Schneider, 2022; Zhai et al., 2021). These discrepancies in incidence by geographical location have minimal bearing on mortality, and so this disparity is thought to be attributed to the overdiagnosis epidemic that has followed more rigid screening practices and better technology (Kitahara & Schneider, 2022; Li et al., 2020; Zhai et al., 2021). This is seen most readily in South Korea where national screening initiatives led to vast increases in detection (Drozd et al., 2020; Li et al., 2020). Interestingly, incidence is also higher in many

island nations, including Cabo Verde, Cyprus, French Polynesia, New Caledonia, and Puerto Rico (Kitahara & Schneider, 2022; Zhai et al., 2021). These differences are often attributed to socioeconomic disparities including access to diagnostic screening and patient care (Kitahara & Schneider, 2022). Further still, across the United States, living in a colder climate, such as Alaska, increases thyroid cancer risk two-fold compared with a warmer climate state (Lehrer & Rosenzweig, 2014). With overall climate fluctuations increasing, these cold-related differences in incidence could impact mortality in the future, particularly in vulnerable populations, such as the elderly (Lehrer & Rosenzweig, 2014).

Childhood exposure to ionizing radiation is the most established modifiable risk factor for thyroid cancer. Although less well-studied, obesity has emerged as an influential risk factor in thyroid cancer (Bogović Crnčić, 2020; Kitahara & Schneider, 2022). The mechanisms behind this trend require further investigation. Endocrine-disrupting chemicals have also emerged recently as an important factor influencing thyroid dysfunction and thyroid cancer development (Bogović Crnčić, 2020; Kitahara & Schneider, 2022). Further evidence has demonstrated that metabolic disorders related to insulin resistance are associated with increased TC risk through a hyperinsulinemia mechanism or by impacting other known TC risk factors, such as elevating TSH, inducing chronic autoimmune thyroiditis, or creating an iron deficiency (Malaguarnera et al., 2020).

In anaplastic thyroid cancer (ATC), specifically, history of goiter or prior or co-existing differentiated or medullary thyroid carcinoma may contribute to likelihood of developing ATC (Smallridge & Copland, 2010). Approximately 25% of ATC patients had prior history of goiter and an additional 10% had familial history of goiter (Taccaliti et al., 2012). Areas where goiters

are more endemic have higher ATC risk (Taccaliti et al., 2012). Additionally, a case-control study (n=126 patients) using benign goiter surgery patients vs. ATC patients found ATC patients to more commonly have lower overall educational status, other past or present malignancies, later age at first menstrual cycle, earlier age at first pregnancy, and blood group B (Smallridge & Copland, 2010). Although risk factors present in some cases, many patients present with no previous history of thyroid disease or carcinoma and additional risk factors impacting initiation and progression remain unknown (Smallridge & Copland, 2010).

HIGH RISK	Radiation exposure (head and neck region) Chromosomal (genetic) alterations Hereditary Conditions • Hereditary MTC • Non syndromic FNMTC • Syndromic FNMTC
LOW RISK	Thyroid imaging with iodine 131 Iodine deficiency High thyroid stimulating hormone (TSH) level Autoimmunity Thyroid nodule/s Environmental pollutants Lifestyle and diet High BMI
UNCLEAR	Estrogen

MTC-Medullary thyroid cancer; FNMTC-Familial non-medullary thyroid cancer; BMI-body mass index

Table 1. "Stratification of risk factors for thyroid carcinoma." Table and Legend Taken from: Tatjana Bogović Crnčić, 2020.

Prognosis and Survival

Overall survival (OS) amongst thyroid cancer patients is high, as the greatest incidence is of slow-growing, localized papillary or follicular thyroid tumors, however, thyroid cancer incidence and prognosis varies dramatically by histological subtype (Kitahara & Schneider, 2022). As most thyroid cancer cases are from the less aggressive forms, thyroid cancer mortality is low compared with overall incidence (0.5 deaths per 100,000 cases annually) (Kitahara & Schneider, 2022), and survival rate is 98.6% overall. In the United States, five-year survival rate is impacted by localization of the disease; 99% for localized disease, 98.3% for regional, and 54.9% for metastatic disease (Kitahara & Schneider, 2022). However, rare, aggressive subtypes of the disease, such as anaplastic thyroid cancer, have a nearly 100% mortality rate (Keutgen et al., 2015). ATC is exceedingly rare, accounting for 1% of all thyroid cancer cases, but 30-50% of thyroid cancer fatalities (Keutgen et al., 2015; Kitahara & Schneider, 2022).

Detection and Standard of Care Therapy

Thyroid nodules and tumors are screened for during routine physical exams by palpation. Thyroid nodules are also frequently detected by routine imaging, such as MRI, for different complaint (Shah, 2015). They can be further detected by thyroid ultrasonography and fine need aspiration (Drozd et al., 2020). This often leads to overdiagnosis or unnecessary procedures. Historically, nodules that meet certain size and structural criteria are biopsied via fine needle aspiration (FNA) biopsy to confirm whether they are benign or malignant (Kitahara & Schneider, 2022; Shah, 2015). In the last sixteen years, the American Thyroid

Association has stopped recommending biopsies of small nodules (Kitahara & Schneider, 2022).

Differentiated thyroid tumors are managed effectively via ipsilateral lobectomy or total thyroidectomy, when warranted (Kitahara & Schneider, 2022; Tuttle et al., 2010). More advanced cases may pair radioactive iodine therapy with surgery to eradicate any remaining thyroid cells (Kitahara & Schneider, 2022). Prognosis is good in these cases. Additionally, levothyroxine is often used to maintain low TSH levels in patients with differentiated thyroid carcinoma because TSH is a trophic hormone that can stimulate growth of any cells derived from the thyroid follicular epithelium, including thyroid cancer cells (Tuttle et al., 2010). Maintenance of TSH levels below 0.1 mU/L are desired in patients with known carcinoma, while patients with years of progression-free survival want to maintain TSH levels within normal reference ranges (Tuttle et al., 2010). This course of action is primarily recommended in younger patients, as there are less severe side effects, including cardiac arrythmias, but monitoring of sufficient calcium and vitamin D intake must be done concurrently (Tuttle et al., 2010). External beam radiation therapy and chemotherapy are not as commonly used in these cancers. Many differentiated thyroid cancer cases can be fully curable (Coca-Pelaz et al., 2023).

Anaplastic thyroid cancer and other poorly differentiated thyroid cancers do not respond to standard-of-care treatments (Tong et al., 2012). In these cases, more experimental therapeutic options and palliative care strategies have been employed (Tong et al., 2012). Novel therapies aiming to target undifferentiated and poorly differentiated thyroid cancers

include personalized medicine and kinase inhibitors, however, there has been minimal success (Kitahara & Schneider, 2022).

1D. Histological Subtypes of Thyroid Cancer

Thyroid cancer subtypes include differentiated thyroid cancer, medullary thyroid cancer, and undifferentiated or poorly differentiated thyroid cancers **(Figure 4, 5)** (Alhejaily et al., 2023; Beynon & Pinneri, 2016; Hu et al., 2021; Macerola et al., 2021; Tuttle et al., 2010). Pathologically differentiated thyroid tumor deriving from follicular cells include papillary (PTC), follicular (FTC), and Hürthle cell carcinoma (HCC) (Alhejaily et al., 2023; Hu et al., 2021; Macerola et al., 2021). This represents the majority of thyroid cancer cases and five-year survival is greater than 95% (Alhejaily et al., 2023). Medullary thyroid carcinoma (MTC) is a C cell-derived thyroid carcinoma (Alhejaily et al., 2023; Hu et al., 2021; Kameda, 2016). Undifferentiated thyroid cancers also derive from the follicular cells and include anaplastic thyroid cancer, where the follicular cell morphology and biological activity of the follicular cells are lost (Alhejaily et al., 2023; Hu et al., 2021; Hu et al., 2019). This biological activity includes loss of iodine uptake capability and inability to synthesize thyroglobulin (Alhejaily et al., 2023). These tumors have histological patterns that include giant-cell, spindle-cell, and squamoid-cell tumors (Alhejaily et al., 2023). Commonalities amongst subtypes include higher incidence in women, and approximately 60% of PTCs and 25% of ATCs harboring a V-Raf murine sarcoma viral oncogene homolog B valine to glutamic acid mutation (BRAFV600E), which likely serves as one initiating factor in tumorigenesis (Lang et al., 2023; McFadden et al., 2014).

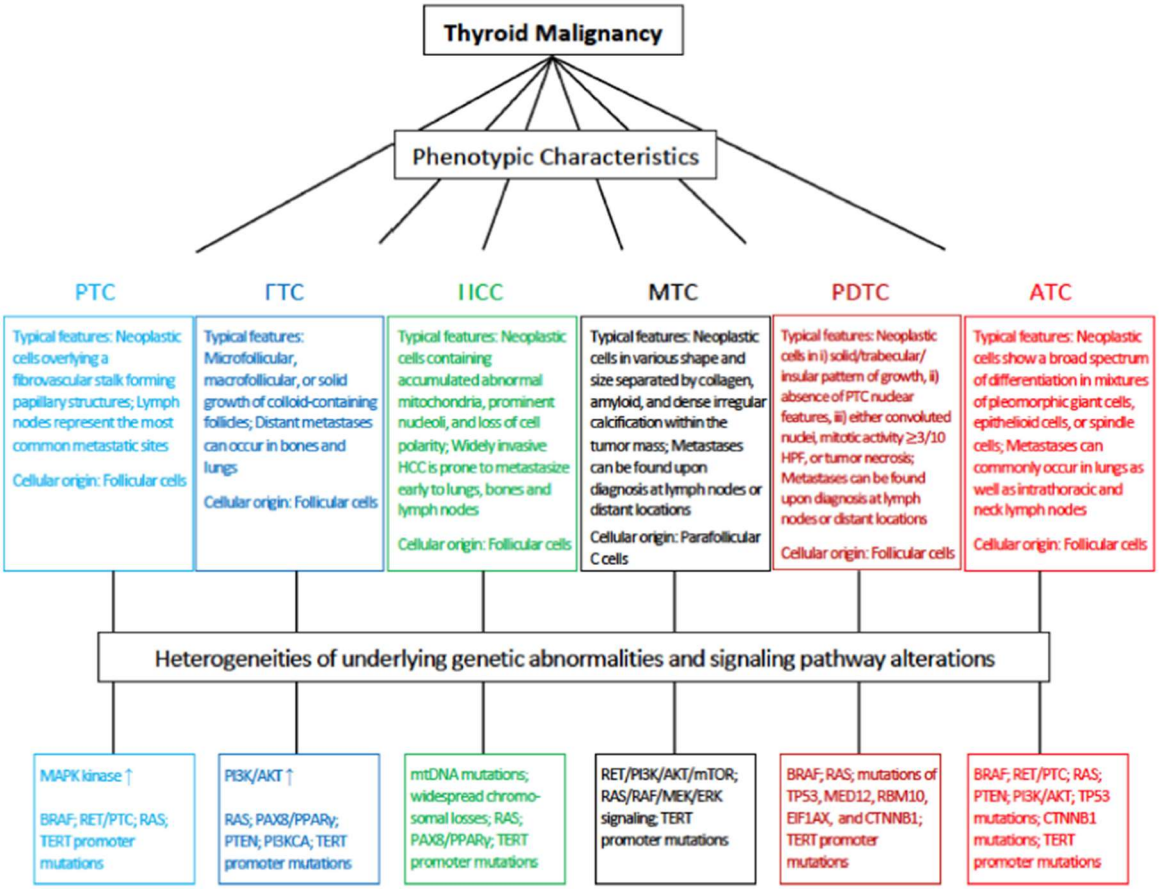


Figure 4. "Overview of the phenotypic characteristics and underlying mechanisms of thyroid carcinomas broken down by histological subtype. PTC: papillary thyroid carcinoma; FTC: follicular thyroid carcinoma; HCC: Hürthle cell carcinoma; MTC: medullary thyroid carcinoma; PDTC: poorly differentiated thyroid carcinoma; ATC: anaplastic thyroid carcinoma." Figure and Legend Taken From: Hu *et al.,* 2021.

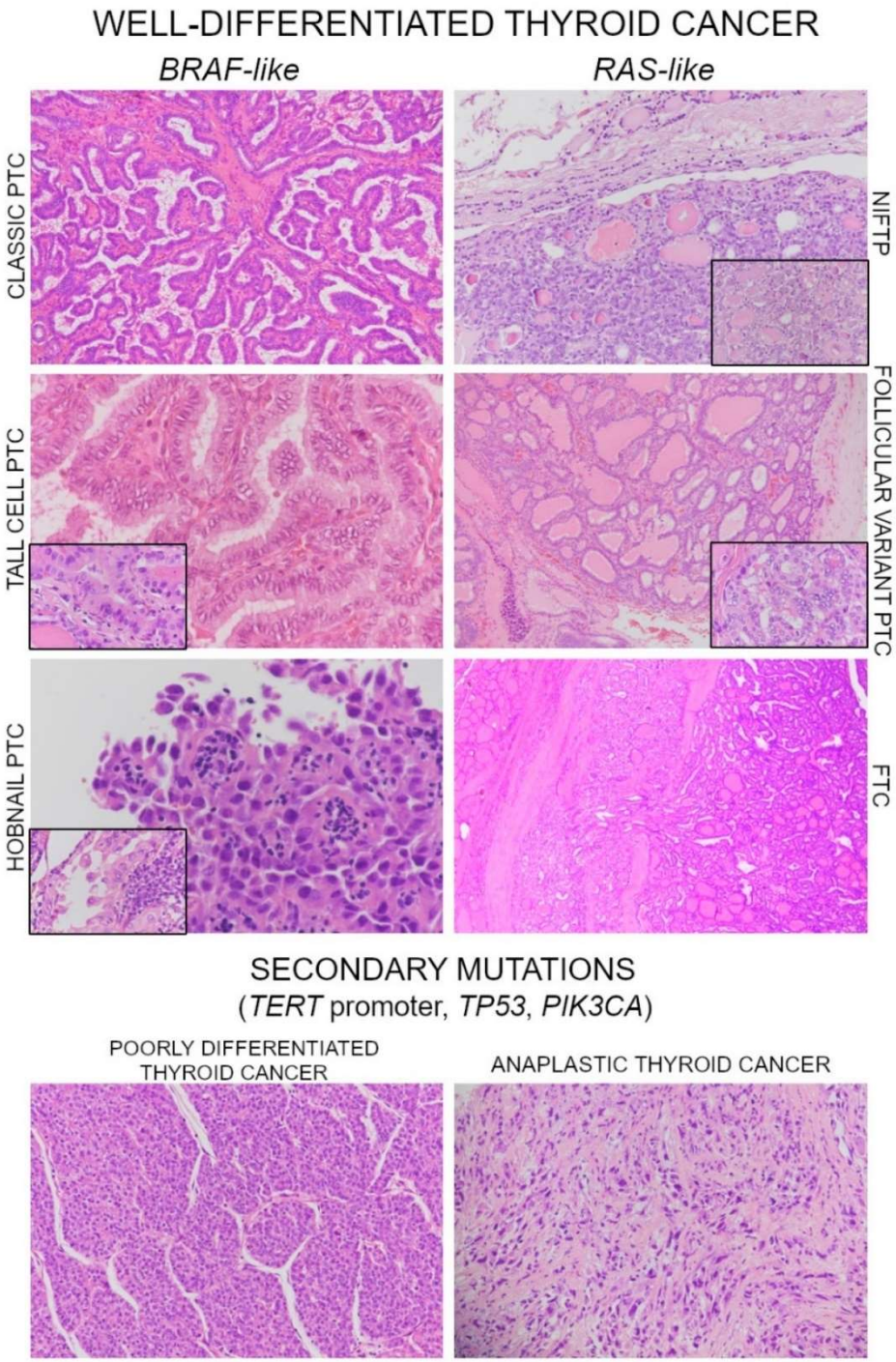


Figure 5. "Histology of well-differentiated and poorly differentiated thyroid cancer subtypes." Figure and Legend Taken From: Macerola *et al.,* 2021.

Differentiated Thyroid Carcinoma

Most thyroid cancers originate in the follicular cells of the thyroid gland (Hu et al., 2019; Macerola et al., 2021; Shah, 2015). Cancers deriving from follicular cells are primarily differentiated thyroid carcinomas (DTC), including papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) (Shah, 2015). Some cases of DTC do not present with exclusively a papillary or follicular pattern, but rather are heterogeneous and have presence of both histologic types – these are described as mixed papillary and follicular carcinomas, but diagnosis generally goes with the predominant histological pattern **(Figure 5)** (Hu et al., 2019; Paniza et al., 2019; Shah, 2015). For example, one such subtype includes follicular-variant papillary carcinoma (FVPTC), which has follicular architecture but papillary cytology (Paniza et al., 2019; Tuttle et al., 2010). When the capsule remains intact, the prognosis for FVPTC remains the same as encapsulated PTC (Paniza et al., 2019; Tuttle et al., 2010). These histological subsets can influence survival rates. PTC with a well-defined capsule is one of the most favorable prognostic indicators, and this is seen in 10% of PTC tumors (Hirabayashi & Lindsay, 1961; Sherman, 2003; Tuttle et al., 2010). Prognosis worsens when tall-cell papillary variants are observed (25% 10-year mortality), columnar variants are present which grow much more rapidly, or diffuse sclerosing variants infiltrate the entire thyroid gland (Tuttle et al., 2010). In contrast, FTC tends to be more aggressive than PTC, although still often a minimally invasive tumor (Tuttle et al., 2010). It is typically a solitary encapsulated tumor with microfollicular histology and frequent follicular cell invasion through the tumor capsule and blood vessels (Tuttle et al., 2010). Capsular invasion can range from only slight penetration of the capsule to full invasion. Vascular invasion in FTC is a worse prognostic indicator than

capsular invasion alone (Goldstein et al., 2000; Tuttle et al., 2010). Up to 80% of FTCs with extensive vascular invasion metastasize and worsen prognosis markedly (Goldstein et al., 2000; Tuttle et al., 2010).

DTC has a significantly younger incidence than most cancers. Historically, age at onset of diagnosis follows a bell-shaped curve with incidence from 20-50 years of age, however, a rise in cases from 40-60 years has been seen in recent years (Shah, 2015). Consistent with the overdiagnosis epidemic discussed above, these increased diagnoses may be attributable to incidental findings found during unrelated imaging studies, with as many as 10% of people receiving autopsy at death from other causes in the United States having the appearance of undiagnosed thyroid cancers – often termed *incidentalomas* (Shah, 2015; Tuttle et al., 2010). Many DTCs present with no symptoms at time of diagnosis, and roughly 50% of malignant thyroid nodules are found during routine physical examination (Tuttle et al., 2010). When symptoms, such as difficulty swallowing, do begin to present, they are usually resultant from invasion of adjacent structures or progression to lateral lymph nodes of the neck (Shah, 2015). Typically, these cancers have a favorable prognosis, as described above, and respond favorably to treatment. At least 85% of thyroid cancer cases are DTCs (Shah, 2015). One school of thought hypothesizes that approximately 10-15% of differentiated carcinomas can follow a tumor progression model that leads from papillary or follicular thyroid cancer to tall-cell or insular carcinoma, then poorly differentiated thyroid carcinoma (PDTC), and finally ATC through mutagenesis (Ibrahimpasic et al., 2019; Shah, 2015; Smallridge & Copland, 2010). Still others find these diseases to all be distinct and to arise from their own initiating factors. Tumor, node, metastasis (TNM) staging criteria categorize PTC and FTC based on tumor size,

lymph node metastases, degree of extrathyroidal extension (ETE) and capsular invasion, and metastasis (Mao et al., 2020) **(Figure 6)**. Greater than 80% of newly diagnosed thyroid tumors are less than 2 cm in diameter, indicating early stage and excellent prognosis (Mao et al., 2020; Shah, 2015).

Additionally, the Mayo Clinic has developed its own multivariate analysis that stratifies differentiated thyroid cancer cases based on metastasis, age, completeness of resection, invasion, and size (MACIS) (Shah, 2015). DTC prognosis, particularly in terms of likelihood or recurrence and more aggressive disease post-recurrence, is largely related to the severity of these factors. Distant metastases are a strong predictor of poorer patient outcomes (Shah, 2015). Interestingly, invasion to local lymph nodes does not negatively impact survival. Younger patients (<45 years) have better prognosis than older patients, with a 10-year survival rate of 99% in younger patients and as low as 25% in patients >70 years (Shah, 2015). Overall, papillary thyroid carcinoma and follicular thyroid cancer, have five-year survival rates of 93% and 76%, respectively (Tuttle et al., 2010). The efficacy of thyroidectomy or ipsilateral lobectomy to fully remove the tumor has strong prognostic value (Tuttle et al., 2010; Wu et al., 2021). Extrathyroidal extension is highly correlated with patient outcomes – major extension into adjacent structures, including the recurrent laryngeal nerve, the trachea, the larynx, or the esophagus contributes to worse outcomes than minor extension outside of the thyroid gland has (Shah, 2015; Wu et al., 2021). Lastly, increasing tumor size leads to increased risk of local recurrence and can negatively impact overall survival (Grant, 2015; Shah, 2015). BRAF mutation status can also impact prognosis and therapeutic approach.

Approximately 30% of DTC patients have tumor recurrences; 66% of recurrences occur within the first decade following initial therapy, however recurrences can still appear several decades after initial diagnosis (Grant, 2015; Tuttle et al., 2010). As these patients tend to be young at time of initial diagnosis, these recurrences often still occur early in life. Central neck recurrences are most frequently seen in the cervical lymph nodes (74%), thyroid remnant (20%), and trachea or muscle (6%) (Tufano et al., 2015; Tuttle et al., 2010). Although not frequently fatal, recurrences have a worse prognosis than the primary diagnosis, and 8% of individuals with local recurrences develop fatal disease (Tufano et al., 2015; Tuttle et al., 2010). When distant metastases present with recurrence in approximately 21% of cases, 50% of these patients die of cancer. Common metastases are seen in the lungs (63%), bones, and brain (Tuttle et al., 2010).

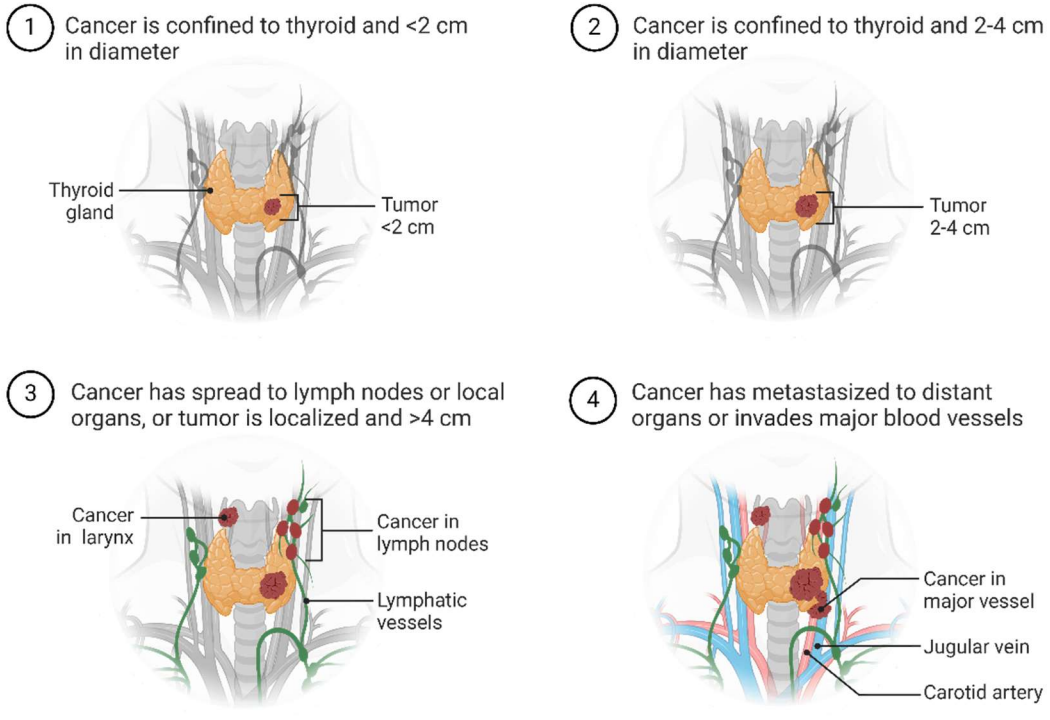


Figure 6. Staging of papillary and follicular thyroid carcinomas. Figure adapted from BioRender.com.

Poorly Differentiated and Undifferentiated Thyroid Carcinoma

Compared to the more common and highly treatable differentiated subtypes, poorly differentiated thyroid carcinoma, and undifferentiated anaplastic thyroid cancer together only make up less than 10% of thyroid cancer cases (O'Neill & Shaha, 2013; Shah, 2015). These cancers have significantly worse prognosis and require more extensive interventions, both surgically and non-surgically, if surgery is even possible (O'Neill & Shaha, 2013; Shah, 2015). Due to the loss of defined thyroid follicular structure, these cancers are refractory to radioactive iodine therapy, as well as other conventional therapies (Aashiq et al., 2019). With the worst associated prognosis, ATC comprises only 2% of all thyroid cancer cases, and its disease-specific mortality is 100% (Jannin et al., 2022; O'Neill & Shaha, 2013; Shah, 2015; Smallridge & Copland, 2010; Taccaliti et al., 2012).

Patients with PDTC and ATC usually present with symptoms that may include a palpable, visual mass in the neck surrounding the thyroid gland on either side, a lump elsewhere in the neck representing invasion to a local lymph node, pressure in the neck and throat, difficulty swallowing, a feeling of choking, airway obstruction from tumors invading the trachea and compromising the airway, and hoarseness due to vocal cord paralysis from invasion of the recurrent laryngeal nerve (Ibrahimpasic et al., 2019; Lowe et al., 2014; Shah, 2015).

Distinguishing between PDTC, ATC, other aggressive primary thyroid malignancies, and other poorly differentiated carcinomas metastatic to the thyroid through core or surgical biopsy can be difficult (Ibrahimpasic et al., 2019; Tuttle et al., 2010). Additional differentiating diagnostic procedures include evaluating complete blood cell count, serum calcium, and TSH; computed tomography (CT) scan of the neck to determine primary tumor size and local

invasion into nearby structures; CT of the head, chest, abdomen, and pelvis to establish extent of distant metastases; bone scans and fludeoxyglucose-18 positron emission tomography (FDG-PET) scans to observed bone metastases and bone density (Tuttle et al., 2010).

Anaplastic Thyroid Cancer

Anaplastic thyroid cancer is an exceedingly rare form of thyroid cancer, accounting for only 1-4% of all thyroid cancer cases, yet it accounts for 30-50% of mortality due to thyroid malignancies – the most of any subtype (Keutgen et al., 2015; Lang et al., 2023). Survival following diagnosis with these well-differentiated thyroid cancer histotypes is in stark contrast to that of undifferentiated ATC, which has a one year-survival rate of 17%, total five year-survival rate of 8%, five year-survival of 3% when metastatic, and a median overall survival of only six months (Tuttle et al., 2010; Venkatesh et al., 1990). Mean survival is up to 8 months when disease is still confined to the neck at time of diagnosis, and drops to 3 months if the disease has extended beyond the neck (Tuttle et al., 2010; Venkatesh et al., 1990). This disease is universally fatal (Keutgen et al., 2015; Lang et al., 2023). Compared to the relatively young age of diagnosis of TCs overall, ATC has a mean age at diagnosis of 71 years with less than 10% of patients under 50 years (Tuttle et al., 2010; Zivaljevic et al., 2014). Similar to other histological subtypes, 60-70% of cases are seen in women (Tuttle et al., 2010). Dissimilar to trends in TC incidence overall, the incidence of ATC has fortunately decreased in the past decades (Tuttle et al., 2010). Studies in Germany from 1965-1997 found a dramatic decrease in incidence from 35% to 7%, and attributed this marked decline to more aggressive management of DTC and iodized salt for goiter prevention (Smallridge & Copland, 2010). A

similar trend was observed in Dublin, Ireland from 1970-1999 (24.3% to 9.8%), and this decline was attributed to an increase in dietary iodine (Smallridge & Copland, 2010).

Fundamental features distinguishing ATC from other TC subtypes include loss of differentiation, local invasion, metastasis, and rapid lethality **(Figure 7)** (McFadden et al., 2014).

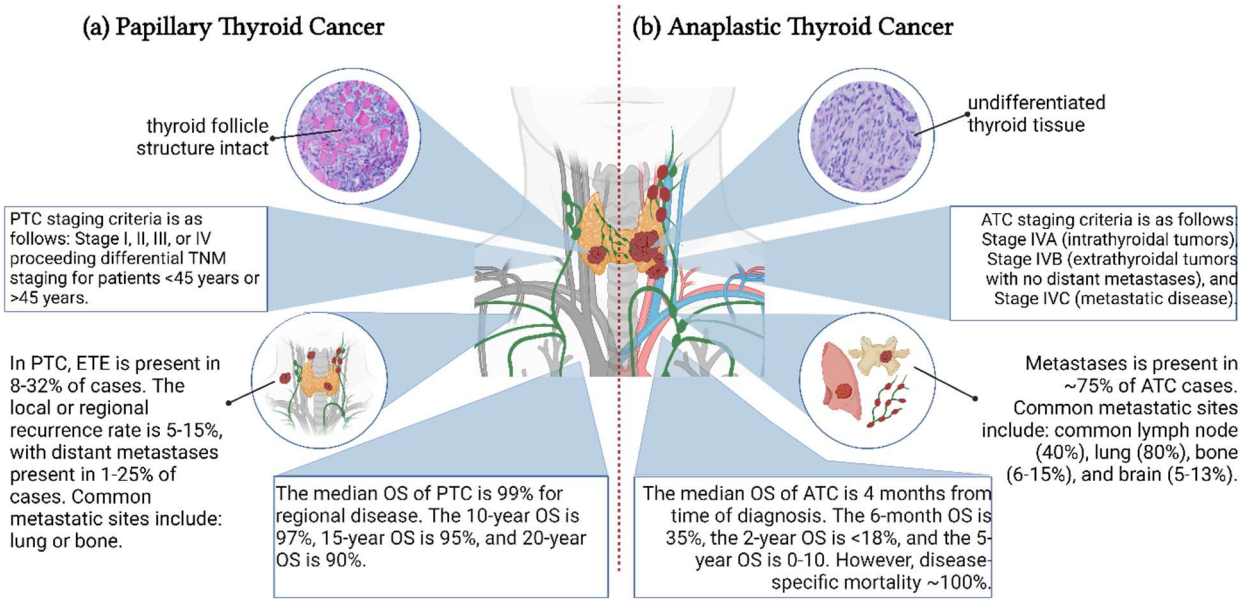


Figure 7. Papillary vs. anaplastic thyroid cancer. (A) PTC and (B) ATC defined by differentiation status, staging criteria, extrathyroidal extension, local invasion, metastasis, and overall survival. Figure created on BioRender.com (*pending publication*)

ATC has complex molecular dysregulation, with many over- and under-expressed proteins contributing to its regulation of critical cellular processes, including transcription, cell signaling, mitosis, cell cycle progression, proliferation, apoptosis, adhesion, and tumor growth (Smallridge & Copland, 2010). Additionally, loss of follicular cell characteristics, such as iodine uptake and thyroglobulin synthesis, contribute to its poor prognosis and lack of effective therapeutic intervention (Lang et al., 2023). Early intervention is essential for better

outcomes as its rapid growth and proliferation quickly lead to significant airway compromise and distant metastasis.

2. Anaplastic Thyroid Cancer

2A. Anaplastic Thyroid Cancer Staging and Criteria

ATC tumors lose the clear thyroid follicle structure through undifferentiation. These undifferentiated tumors are not only the most aggressive TC subtype with a disease-specific mortality of 100%, but they are also one of the most lethal solid malignancies overall (Lang et al., 2023; McFadden et al., 2014). Over 90% of these patients present with extensive local invasion, while distant metastases are found in 15-50% of patients already at time of diagnosis (Lang et al., 2023; Tuttle et al., 2010). Squamoid, spindle cell, and giant cell morphological patterns may be observed upon histological analysis, however, they all present similarly clinically with little to no influence on prognosis (Taccaliti et al., 2012). Reflecting its inferior prognosis, ATC is always diagnosed as a stage four disease, subcategorized as stage IVA, IVB, or IVC (Tuttle et al., 2010), with loss of thyroid-like characteristics through de-differentiation, invasion of major blood vessels, extensive airway obstruction, and distant metastases already likely (Lang et al., 2023; Taccaliti et al., 2012). These upper airway obstructions lead to suffocation in 50% of ATC patients, despite frequent tracheostomy, which is responsible for their swift death (Tuttle et al., 2010). Local ATC presentation includes a rapidly growing anterior neck mass, dysphagia (40%), hoarseness and vocal changes (40%), and stridor (24%) (Keutgen et al., 2015; Taccaliti et al., 2012). Regionally, symptoms include palpable lymph node masses (54%) and neck pain (26%) (Taccaliti et al.,

2012). Systemically, patients experience symptoms of weight loss, anorexia, and shortness of breath due to pulmonary metastases (Keutgen et al., 2015). Length of survival in ATC cases can be attributed to a series of factors, including age, tumor size, extent surgery is possible for resection, dose of radiotherapy received, absence of distant metastases at time of diagnosis, and co-existence of differentiated thyroid cancer (Smallridge & Copland, 2010; Taccaliti et al., 2012).

Stage IVA includes intrathyroidal tumors and is rare. Stage IVA tumors are the only ATC tumors where surgical resection is a therapeutic option. Stage IVB includes extrathyroidal tumors without distant metastases present. These tumors are not surgically resectable (Tuttle et al., 2010). Stage IVC has distant metastases present. In ATC, most common distant metastatic sites include the lungs and pleura (observed in 90% of patients with metastases), bone (present in 5-15% of patients with metastases), and brain (found in 5% of patients with metastases) (Lang et al., 2023; Tuttle et al., 2010). Bone metastases are usually lytic in nature (Tuttle et al., 2010). More rarely, ATC may metastasize to the skin, liver, kidneys, pancreas, heart, and adrenal glands (Besic & Gazic, 2013; Tuttle et al., 2010).

2B. Genesis and Etiology of ATC

Genesis of ATC can occur via multiple distinct mechanisms. Few gene mutations have currently been identified to be associated with ATC, but the commonly found mutations have been seen to be associated with distinct stages of initiation and progression (Smallridge et al., 2009). Some genetic alterations commonly observed in DTC, such as rearranged during transfection/papillary thyroid carcinoma (RET/PTC) rearrangements in childhood and

radiation-induced PTCs and paired box gene 8 (PAX8)/peroxisome proliferator-activated receptor gamma (PPARγ) fusions in FTC, are not found in ATC (Smallridge et al., 2009). Early mutations are comparable in incidence across DTC subtypes and ATC, such as rat sarcoma virus (RAS) and BRAF (Lang et al., 2023; Smallridge et al., 2009). Approximately 50% of ATCs develop as a result of one or more de-differentiating steps progressing from a prior or coexistent differentiated TC (McFadden et al., 2014; Poolakkil et al., 2021; Smallridge et al., 2009; Smallridge & Copland, 2010; Taccaliti et al., 2012; Tuttle et al., 2010). These late mutations include those in the tumor protein p53 gene (TP53), cadherin-associated protein beta 1 (β-catenin), and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), and contribute most significantly to aggressiveness in ATC (McFadden et al., 2014; Smallridge et al., 2009). A major step in this transformation is the loss of tumor protein p53 (p53) tumor suppressor function (McFadden et al., 2014; Tuttle et al., 2010). TP53 mutations serve a major role in dedifferentiation, and also impact growth and angiogenesis (Poolakkil et al., 2021; Taccaliti et al., 2012). Analysis of human TC patient samples has revealed a highly protective role of p53 against tumor progression, and presence of the TP53 mutation is detected at highest frequencies in ATC (Johnson et al., 2015), decreasing frequency in PDTC, and almost never present in PTC (McFadden et al., 2014). Some studies indicate the loss of p53 is required for ATC progression (McFadden et al., 2014). Other precipitating events and mechanisms leading to anaplastic transformation need to be further studied (Tuttle et al., 2010). In addition to TP53, other common mutations in ATC include H-, N-, or K-RAS, BRAFV600E, PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta (PIK3CB), and phosphatase and tensin homolog (PTEN) (Johnson et al., 2015).

These mutations impact signal transduction along the pro-proliferative RAS/rapidly accelerated fibrosarcoma (RAF)/mitogen-activated protein kinase kinase (MEK)/ extracellular signal-regulated kinase (ERK) cascade and the pro-survival PI3K/Ak strain transforming (AKT)/PTEN cascade (Johnson et al., 2015). Constitutive activation of these signal transduction networks allows for the replicative immortality and rapid proliferation observed in ATC. There appears to be a combined role of BRAF mutation and loss of p53 cooperating to facilitate ATC progression, however, additional events, including epigenetic regulation, are largely required for ATC conversion (Acuña-Ruiz et al., 2023; McFadden et al., 2014).

2C. Anaplastic Thyroid Cancer Signaling

Normal Thyroid Signaling

Thyroid stimulating hormone activates the cyclic adenosine monophosphate (cAMP) signaling cascade, and has long been considered the most essential signaling pathway in the thyroid for regular thyroid functionality (Benvenga et al., 2018). In addition to activation of cAMP signaling, calcium signaling within the thyroid gland also serves a vital role (Asghar et al., 2021). Calcium signaling is physiologically fundamental for normal cellular functioning, regulating vast cellular processes: muscular contractions, cellular proliferation, mitochondrial function, and regulation of membrane potential in cells (Asghar et al., 2021). Many of these pathways controlled by calcium signaling also serve an important role in cancer progression when dysregulated.

Thyroid hormone influences adiposity. Adiposity gain and loss depends on the balance between energy expenditure and energy intake, which receive cues from the sympathetic

nervous system, the endocrine system, and the hypothalamus (for satiety control) (Teixeira et al., 2020). Thyroid hormone and TSH regulate metabolic rate, which ultimately influences adiposity (Teixeira et al., 2020). TH increases oxygen consumption in tissues, directly impacting adenosine triphosphate (ATP) utilization and accelerating anabolic and catabolic processes involved in macronutrient catabolism, including fatty acid oxidation and lipolysis (Teixeira et al., 2020). However, this is a bi-directional interaction, as adiposity acts on thyroid function and, possibly, structure via production of hormones, cytokines, and other regulatory compounds influencing thyroid function (Teixeira et al., 2020).

The normal function of the thyroid and its hormone production and signaling is intimately intertwined with general metabolism and mitochondrial metabolism, all of which can become dysregulated within ATC (Mullur et al., 2014).

Mitochondrial Metabolism in Cancer

Under normal physiological conditions, 90% of the cellular energy required for biological functioning is produced via oxidative phosphorylation (OXPHOS) in the inner mitochondrial membrane (Lee et al., 2015). Mitochondria also serve an essential role in regulating steroid hormone and porphyrin synthesis, the urea cycle, lipid metabolism, and interconversion of amino acids; additionally, mitochondria are central in induction of apoptosis, cell proliferation, and cellular calcium homeostasis (Lee et al., 2015). As these processes are essential in both the thyroid gland normally, and in carcinogenesis, the mitochondria appear to have a vital role in energy metabolism in both the normal thyroid gland and thyroid carcinomas (Lee et al., 2015).

In 1923, Otto Warburg discovered a phenomenon called the Warburg effect where he found carcinoma cells in culture to preferentially use anaerobic glycolysis to generate ATP with a byproduct of lactate regardless of oxygen availability, rather than OXPHOS, which is a more energy efficient pathway that generates more ATP per glucose molecule than glycolysis (Liberti & Locasale, 2016; Gill et al., 2016; Warburg O, 1956). This seemingly counterintuitive method of energy production in tumor cells was wildly accepted for decades under the assumption that this switch to glycolysis could confer a growth and survival advantage for these cells (Liberti & Locasale, 2016; Gill et al., 2016; Warburg, 1956). Currently, it is understood that although relevant under some physiological conditions, this effect is an oversimplification of the energy production that occurs in all heterogenous tumor cells (Weljie & Jirik, 2011). This advancement in knowledge has established a clear role for a "reverse Warburg effect" as well (Gill et al., 2016). In the reverse Warburg effect, aerobic glycolysis occurs in cancer-associated fibroblasts (CAFs) in the tumor microenvironment (TME), rather than in the tumor cells themselves (Gill et al., 2016). This produces high-energy metabolites, lactate and pyruvate, that become present in the TME and are utilized by cancer cells at the leading edge of growth nearby these glycolytic CAFs (Gill et al., 2016). These metabolites are eventually transferred to epithelial cells with high levels of inflammation and reactive oxygen species (ROS) serving as second messengers (Gill et al., 2016). This shift towards aerobic glycolysis in the CAFs is coupled with increased mitochondrial metabolism in the tumor cells utilizing these metabolites to produce more ATP, allowing for promotion of tumor progression (Gill et al., 2016).

Further understanding of tumor metabolism has now led to the concept of multi-compartment metabolism, including two- and three-compartment models, and metabolic coupling in the tumor microenvironment (Frades et al., 2021; Gill et al., 2016; Salem et al., 2012). In the two-compartment system of tumor metabolism, tumor cells secrete hydrogen peroxide, which induces oxidative stress in CAFs and stromal cells (Gill et al., 2016; Salem et al., 2012). The CAFs increase their production of ROS as a response to the oxidative stress, which induces aerobic glycolysis and autophagy and resultant production of intermediate catabolites (lactate, glutamine, and ketone bodies) (Degenhardt et al., 2006; Gill et al., 2016; Salem et al., 2012). In turn, these catabolites circulating in the tumor microenvironment (TME) are taken up into the tumor cells and stimulate OXPHOS within the tumor cells themselves (Gill et al., 2016; Salem et al., 2012). This metabolic coupling of glycolysis in some cells and OXPHOS in others promotes proliferation and apoptotic resistance (Degenhardt et al., 2006; Gill et al., 2016; Salem et al., 2012) **(Figure 8).**

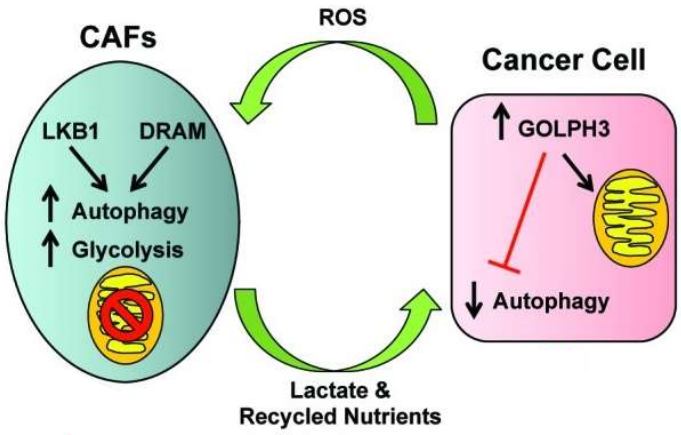


Figure 8. "Metabolic coupling mechanism in cancer. Two-compartment tumor metabolism: autophagy in the tumor microenvironment and oxidative mitochondrial metabolism (OXPHOS) in cancer cells." Figure and Legend Taken from: Salem *et al.,* 2012.

This paradigm of shuttling high-yield metabolites from stromal fibroblasts to fuel cancer cell growth and metastasis has resulted in increased research of various transport proteins (Gill et al., 2016). An important step in this multi-compartment system is that the intermediate catabolites, particularly lactate, actually enter the tumor cells, thus rendering lactate transporters an essential effector of this system (Gill et al., 2016). Monocarboxylate transporters 1 and 4 (MCT1, MCT4), are high-affinity and low-affinity transporters of lactate, respectively (Gill et al. 2016; Sheng et al., 2023). MCT1 allows lactate influx into tumor cells and MCT4 enables lactate efflux from CAFs (Payen et al., 2020; Gill et al., 2016; Sheng et al., 2023). Lactate is further transported when inside the tumor cell to the mitochondria via a translocase to the outer mitochondrial membrane (TOMM20), which ultimately allows for increased ATP production via OXPHOS (Gill et al., 2016; Sotgia et al., 2013). As such, TOMM20 and MCT1 are both OXPHOS markers and MCT4 is a marker of glycolytic metabolism and oxidative stress, but they have also been observed to have prognostic significance in tumors, with MCT4 expression associated with poor outcomes and higher tumor stage in some cancers, including ATC (Payen et al., 2020; Gill et al., 2016; Sheng et al., 2023).

Metabolic Regulation in Anaplastic Thyroid Cancer

Dysregulated cellular metabolism has been heralded as one of the "hallmarks of cancer," which emphasizes its crucial role in oncogenesis with ATC cells having particularly high bioenergetic requirements to maintain such rapid cellular growth (Hirschey et al., 2015; Lee et al., 2015; Gill et al., 2016). In cancer, generally, clinical progression is largely determined by control of cellular energy metabolism by oncogenes and tumor-associated factors (Hirschey et al., 2015; Lee et al., 2015). Metabolic dysregulation in ATC and its TME and their

promotion of carcinogenesis had been understudied for decades, however, recently a clearer picture of the role of a multicompartment metabolism model in thyrocyte metabolism in ATC has been proposed (Gill et al., 2016). ATC has demonstrated a particular metabolic signature, distinct from mitochondrial metabolism observed under normal physiological conditions or in other thyroid pathologies or carcinomas (Johnson et al., 2015).

Key signal transduction pathways that regulate mitochondrial metabolism are frequently altered in ATC, supported by the ATC mutational signature, including commonly mutated TP53, BRAFV600E, PTEN, and PIK3CA (Johnson et al., 2015). These mutations constitutively activate the mitogen-activated protein kinase (MAPK) and PI3K/AKT signal transduction pathways, which have established roles as metabolic modulators (Johnson et al., 2015). Overaction of RAS drives increased mitochondrial metabolism through the TCA cycle, and, coupled with loss of wild type p53, increases mitochondrial metabolism, and specifically, oxidative phosphorylation (Johnson et al., 2015).

TP53 mutations also induce expression of the most common monocarboxylate transporter, MCT1 (Johnson et al., 2015; Payen et al., 2020; Sheng et al., 2023). MCT1 is commonly expressed in fast-twitch muscle fibers, myocardiocytes, and hepatocytes – a common feature of all these cells types are high rates of oxidative phosphorylation facilitated by MCT1 (Johnson et al., 2015). MCT1 takes up monocarboxylates to be incorporated in the mitochondrial tricarboxylic acid cycle (TCA) (Johnson et al., 2015). Expression of MCT1 is under control of both TP53 and MYC, and disruption of its function increases intracellular lactate and pyruvate concentrations. This subsequently reduces glucose transport and levels of ATP, nicotinamide adenine dinucleotide phosphate (NADPH), and glutathione resulting in

increased hydrogen peroxide levels, mitochondrial damage, and apoptosis (Johnson et al., 2015). With very low TP53 expression in ATC, MCT1 expression is high and leads to these disruptions and increased OXPHOS as its primary metabolic driver.

Importantly, high MCT1 expression has been found in many human cancer types compared to non-cancerous cells, and are associated with a worse prognosis in renal cell and non-small cell lung cancers (Johnson et al., 2015; Payen et al., 2020; Sheng et al., 2023). Johnson et al. (2015) validated high MCT1 and TOMM20 expression in human ATC compared to PTC and noncancerous thyroid tissue, with this high expression indicating that disturbed cellular metabolism is a key clinical feature in ATC (Johnson et al., 2015). Importantly, MCT1 expression in PTC cells was rarely high, evidencing the stark clinical phenotypic differences between ATC and PTC (Johnson et al., 2015; Gill et al., 2016). Further, this high MCT1 expression in ATC evidences the high rate of OXPHOS occurring in the anaplastic thyroid cancer cells, comparable to rates observed in other metabolically active cell types that express MCT1 (Johnson et al., 2015; Gill et al., 2016). TOMM20, a key component of the mitochondrial outer membrane protein import system for nuclear encoded subunits of OXPHOS, is also associated with high OXPHOS when highly expressed (Johnson et al., 2015; Sotgia et al., 2013). Of note, ATC xenografts have also shown high uptake of 13C-pyruvate within the tumors, likely mediated by MCT1, which is the main importer of pyruvate into cells (Johnson et al., 2015). In short, genetic alterations in TP53 in ATC induce increased MCT1 expression in ATC cells, which allows for import of pyruvate and lactate, thus fueling mitochondrial respiration via oxidative phosphorylation, and ultimately conferring a significant growth advantage for these ATC cells (Johnson et al., 2015). ATC is one of the fast-

growing cancers, which is an overarching problem in its fatal prognosis. Overall, ATC's high growth kinetics, supported by increased OXPHOS taking place within ATC cells, is a key driver in ATC proliferation and mortality.

2D. Inflammation in Anaplastic Thyroid Cancer

Solid tumors often manipulate their surrounding tumor microenvironment composed of tumor cells, adjacent epithelial cells, stromal cells, immune cells, and cellular matrix components to meet the metabolic requirements required to fuel the demands of the tumor's high proliferative, survival, and invasive needs (Gill et al., 2016). These TME components release cytokines and chemokines, which give subsequent feedback to induce and support required tumorigenic signaling and metabolic needs (Gelfo et al., 2020; Gill et al., 2016). This interplay plays a role in essential tumorigenic signal transduction in numerous cancers via nuclear factor kappa B (NF-κβ), hypoxia inducible factor 1 subunit alpha (HIF-1α), vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), p53, and PI3K/AKT related to gene expression, hypoxia, angiogenesis, cell cycle control, apoptosis, and proliferation, amongst other mechanisms (Gill et al., 2016). Nearly one fifth of all cancer diagnoses in humans arise from pre-existing chronic inflammation, or have inflammation as a crucial player in the malignant phenotype (Gelfo et al., 2020). Within the TME, at least 20% of all tumors exhibit persistent low-level inflammation, with chronic inflammation in the body also being implicated in increased susceptibility to oncogenesis (Gill et al., 2016). In the cancer context, chronic inflammation has been well-defined as a driver of tumor formation and progression, however, in some tumors induction of inflammation-induced adaptive

immune responses is also utilized in a therapeutic fashion (Marchi et al., 2023). A more complete understanding of the role of inflammation in anaplastic thyroid cancer still remains to be fully elucidated, however, increasing evidence points to inflammation as a crucial mediator of thyroid cancer induction and progression and is associated with a poorer prognosis.

The relationship between thyroid cancer establishment, progression, and inflammation is indissolubly intertwined (Laoui et al., 2014; Mantovani A et al., 2008). This inflammation is mediated by cell populations within the TME and secretory factors, such as pro-inflammatory cytokines, chemokines, and extracellular vesicles (Gelfo et al., 2020). The crosstalk between the tumor cells and these immune cells and their interacting molecules has a pro-tumorigenic role. It is possible that the highly inflamed microenvironment is primarily involved in ATC's lack of durable response to therapy and that targeting this inflammation concurrently with ATC's other aggressive pathologies and aberrant signaling will allow for a more robust anti-tumor response.

There are intrinsic and extrinsic pathways that regulate inflammatory cell presence and cytokine production by cell populations in the TME and by cancer cells themselves (Gill et al., 2016). The intrinsic pathway is activated by cellular transformation caused by the inactivation of tumor-suppressor genes and/or the activation of oncogenes (Gill et al., 2016). This induces oncogenic signal transduction, which produces inflammatory molecules that recruit further inflammatory mediators into the TME (Gill et al., 2016). In the extrinsic pathway, chronic inflammation or inflammatory infection predate carcinogenesis (Gill et al., 2016). This has relevance in the context of numerous inflammatory thyroid conditions pre-dating cancer

development in the thyroid. Regardless of the initiating factor, both pathways have downstream activation of transcription factors that produce inflammatory cytokines and chemokines that further recruit and activate various leukocyte and lymphocyte populations into the TME, which further induces inflammation (Gill et al., 2016). In thyroid cancer, both intrinsic and extrinsic pathways of activation have been implicated, interestingly, with intrinsic pathways activation more commonly induced by the frequent genetic alterations in PTC (RET/PTC, HRAS, and BRAF) and extrinsic pathway activation more common in more invasive TC models (Gill et al., 2016). With intrinsic pathway induction, these genetic alterations, such as BRAF, activate transcription of proinflammatory molecules, such as VEGF-A, chemokine (C-X-C motif) ligand 1 (CXCL1)/ growth-related oncogene alpha (GRO-α, CXCL10)/ interferon-gamma inducible protein 10 (IP-10), and CXCL8/interleukin-8 (IL-8) in a RAS/RAF/MAPK-dependent manner (Gill et al., 2016). These inflammatory mediators can then support cancer cell growth and survival in an autocrine and/or paracrine fashion (Gill et al., 2016). The extrinsic pathways involves immune cell populations present at the tumor stroma and invasive front of the thyroid tumor cells, with high regulatory T cell, immunoregulatory natural killer cell, and tumor-associated macrophage density correlating with the increased thyroid cancer aggressiveness seen in ATC (Gill et al., 2016). Additionally, mast cells, key players in inflammatory responses in the body, have recently been found to contribute significantly to epithelial-to-mesenchymal transition (EMT) in TC cells via mast cell production of inflammatory mediators, such as CXCL1 and CXCL10, that stimulate cellular proliferation, and CXCL8, which induces EMT (Visciano et al., 2015). Importantly, mast cell infiltration correlated with ETE and invasiveness in PTC and ATC (Visciano et al., 2015).

2E. Current Therapies in Anaplastic Thyroid Cancer

All established therapies are insufficient in treating ATC, and, unfortunately overall survival rates have shown no improvement over the last 60 years (Smallridge et al., 2009; Smallridge & Copland, 2010). Surgical resection, which is highly effective in eliminating papillary thyroid tumors, is rarely viable in ATC. While differentiated thyroid tumors have the ability to concentrate iodine, express TSH receptors, and produce thyroglobulin (Tg), ATC tumors can no longer uptake iodine properly due to their loss of differentiation (Tuttle et al., 2010). Radioactive iodine therapy, another standard of care for differentiated tumors, does not work in ATC. Radioiodide imaging cannot be used in ATC either (Lee et al., 2015; Tuttle et al., 2010). Other therapies, such as multimodal treatments utilizing external beam radiation and chemotherapy, have some efficacy, but are ultimately insufficient in halting ATC's rigorous spread. More aggressive radiotherapy regimens appear to reduce locoregional recurrences, but have little improvement on median survival (Smallridge & Copland, 2010). External beam radiation can, however, be used palliatively to help prevent asphyxiation and have some local tumor control (Keutgen et al., 2015; Tuttle et al., 2010). Similarly, paclitaxel and doxorubicin may have some palliative benefit (Tuttle et al., 2010) **(Figure 9).**

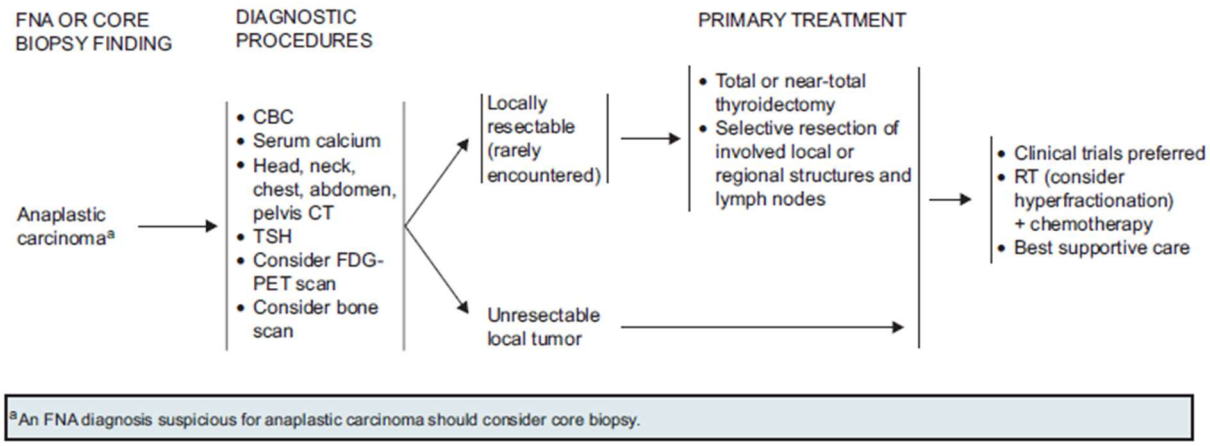


Figure 9. "Current diagnostic and treatment workflow in ATC from the National Comprehensive Cancer Network Thyroid Carcinoma Clinical Practice Guidelines in Oncology." Figure and Legend Taken From: Tuttle *et al.,* 2010.

Although durable responses and improved overall survival were noted in BRAFV600E mutated ATC patients in a clinical trial using a combination of a BRAF inhibitor (dabrafenib) with a MEK inhibitor (trametinib), this recently FDA-approved combination had significant adverse effects and dose interruptions or discontinuations in many patients (Smallridge et al., 2009; Subbiah et al., 2022). There has been some promise in *in vivo* studies using a combination of MEK and ERK inhibitors in patients with a BRAFV600E mutation, however significant continued exploration needs to be done (McFadden et al., 2014). Personalized medicine approaches are also being explored but have had limited efficacy to date. There are currently 48 active (not recruiting), open and recruiting, and not yet recruiting clinical trials looking at combination therapies in ATC and/or PDTC (clinicaltrials.gov). From the work of Lee *et al.* (2015), it is also proposed that new biomarkers and therapeutic targets could be discovered by elucidating the molecular nature of the metabolic remodeling towards increased OXPHOS in ATC compared to more treatable TCs (Lee et al., 2015). This new

approach to biomarker identification and therapy is supported by KS Gill *et al.*, who hypothesize that better understanding the metabolic phenotype of tumor cells and associated stromal cells in ATC can influence discovery of biomarkers to reveal subclinical cancer cases and therapeutic interventions that manipulated dysregulated tumor metabolism to halt tumorigenesis and eradicate tumor cells (Gill et al., 2016).

To date, no new, effective therapeutic approaches have been illuminated. In part, the low incidence of this disease and lack of easy access to tissue samples has hindered additional progress in this area (McFadden et al., 2014). Newer, systemic therapies are being explored, but effective combinations to treat ATC are still lacking (Smallridge & Copland, 2010). As ATC is not amenable to any established therapies, there is an urgent need for novel treatment strategies that could control the rapid progression of this universally fatal disease (Keutgen et al., 2015). As ATC progresses so rapidly, there is a combined need for better early detection methods via biomarkers or advanced screening procedures and therapeutics that can target the primary tumor and its likely metastases systemically.

2F. Therapeutic Needs in Anaplastic Thyroid Cancer

In anaplastic thyroid cancer, there are three major determinants that stand out as controllers of the aggressiveness and fatality of this disease – rapid, uncontrollable proliferation, chronic inflammation, and extreme metabolic dysregulation **(Figure 10)**. Chronic inflammation in the thyroid often precedes ATC establishment, creating a niche in which these cells can swiftly proliferate and invade the local area, presenting significant discomfort for the patient, and ultimately death by asphyxiation in many cases (Ferrari et al., 2019; Guarino et al., 2010;

Liotti et al., 2012; Visciano et al., 2015). The complex network of cell signal transduction pathways dysregulated in ATC allows for this immortalized and rapid growth, and also induced sustained metabolic activity which feeds these rapidly growing and dividing cells. Taken together, with these components working in tandem, current therapies do not have enough time to have an impact. An effective novel therapeutic for the treatment of ATC should target at least one of these critical factors.

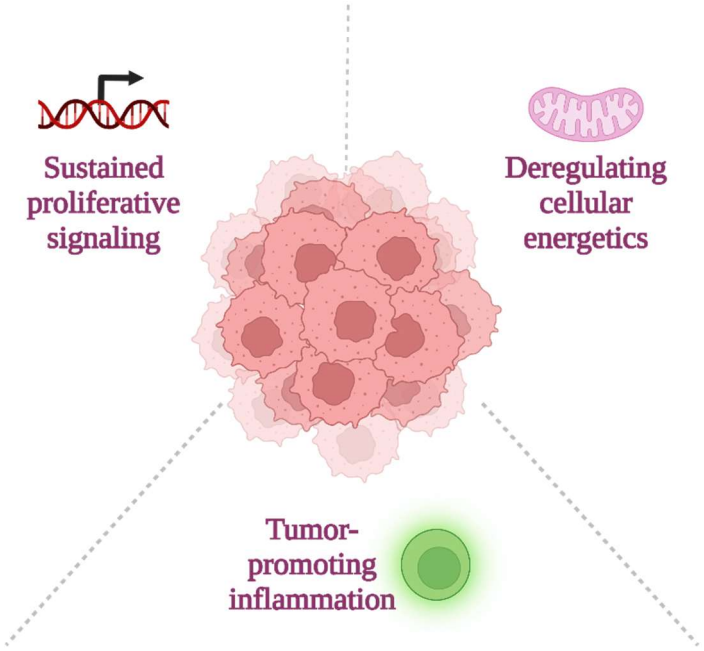


Figure 10. Major hallmarks of ATC aggressiveness to be targeted by novel therapy. Figure created on BioRender.com

To date, combination therapy with dabrafenib and trametinib has been approved in ATC patients with BRAFV600E mutation and inoperable metastatic disease (Yuan & Guo, 2023). The goal of these drugs is to target serine/threonine kinases BRAF and MEK1/2, respectively, along the RAS-RAF-MEK-ERK (MAPK) signaling pathway to inhibit tumor cell proliferation induced by constitutive activation of this pathway **(Figure 11)** (Yuan & Guo, 2023). Although

fairly safe and tolerable with promising short-term outcomes, long-term survival with this combination remains largely unchanged (Yuan & Guo, 2023). Additionally, this therapeutic approach is only appropriate in patients with the BRAFV600E mutation, which only represents approximately a quarter of all ATC cases in the population.

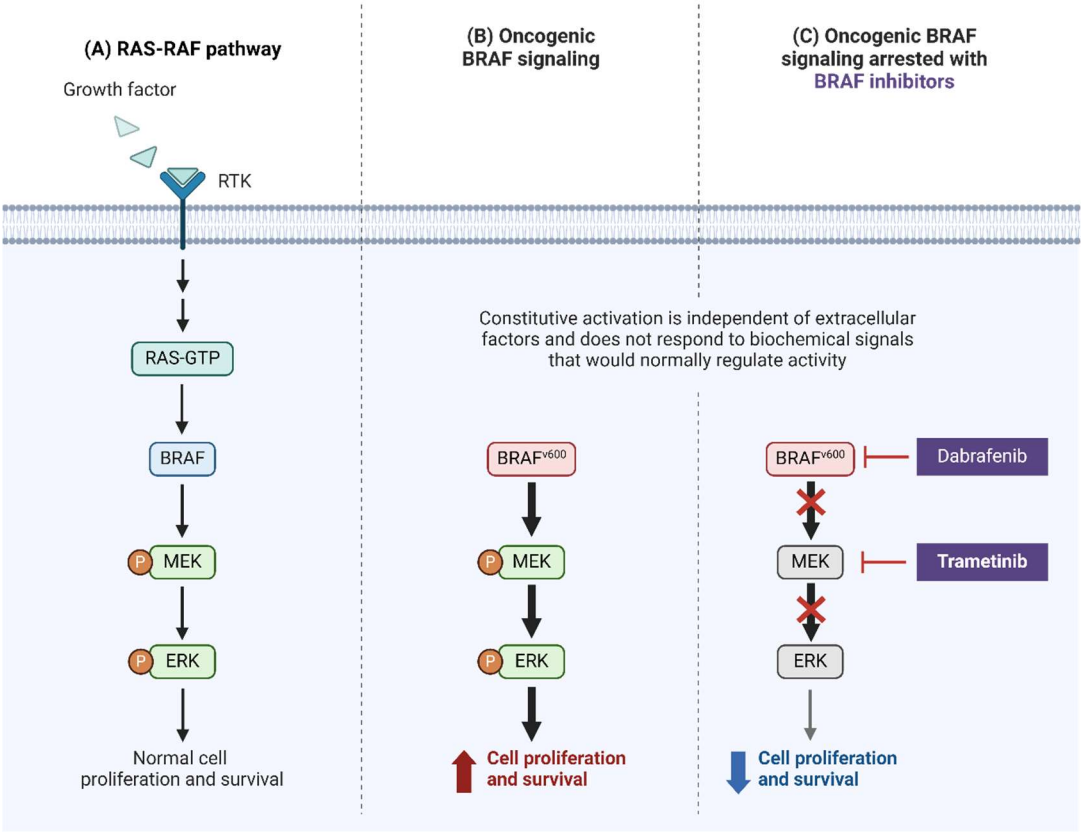


Figure 11. RAS-RAF signaling. (A) Normal MAPK signaling, **(B)** Constitutive activation of MAPK signaling due to BRAFV600E mutation, and **(C)** Targets of dabrafenib and trametinib combination therapy for cancer therapy. Figured adapted from BioRender.com.

Sustained over-activation of PI3K/AKT/mammalian target of rapamycin (mTOR) signaling leads to increased cellular metabolism in ATC, which perpetuates cell growth and proliferation and cellular immortality (Harris et al., 2019; Yuan & Guo, 2023). Inhibitors of

mTOR have been recently subjected to evaluation in treatment of ATC in clinical trials for their ability to target all three of the major determinants mentioned earlier– sustained proliferation, dysregulated metabolism, and inflammation. This seems like a promising direction for ATC treatment, however, there have been major drawbacks in the use of everolimus, a type of mTOR inhibitor, in clinical trials (Harris et al., 2019). One trial by Lim *et al.*, which included six ATC patients, found a median progression-free survival of ten weeks (Lim et al., 2022; Yuan & Guo, 2023). Another phase II clinical trial included seven ATC patients. The medication was fairly well-tolerated, however, it can have serious adverse effects, including notable skin toxicity and respiratory reactions (Yuan & Guo, 2023). Further, it seemed to only have success as palliative care, with more than half of the patients showing tumor progression within three months of the study (Yuan & Guo, 2023). Additionally, one patient who experienced significant tumor regression in the study developed a somatic nonsense mutation that reactivated the mTOR pathway, revealing a possible drug resistance mechanism that can develop early when receiving this treatment (Yuan & Guo, 2023).

While the pathways targeted by these drugs seem promising in treating the drivers of the ATC phenotype, the sustained rewiring of these signals has been insufficient in halting the spread of ATC. There remains a significant unmet clinical need for a systemic, well-tolerated mediator of tumor initiation and progression that can be harnessed to ameliorate the proliferation, chronic inflammation, and metabolic dysregulation of fatal anaplastic thyroid cancer and subsequently provide better outcome for patients beyond palliative care. A therapeutic that fits these requirements needs to have a multi-factorial impact on the tumor cells. Due to the aggressive and fast-growing nature of ATC, a drug with a single-target effect

may be insufficient in treating the disease beyond a palliative approach. Additionally, therapy will need to target regulators of inter-connected cellular pathways that control proliferation, survival, metabolic regulation, and inflammatory feedback simultaneously. Further, all current established therapies have extensive side effects or toxicities that are not well-tolerated, so an approach that can selectively target dysregulation in tumor cells, while leaving non-cancer cells unharmed would be imperative. Historically, natural compounds and biological agents and therapies used for millennia in traditional medicine systems have been repurposed in modern Western medicine with great value (Atanasov et al., 2015). Repurposing such an agent could have tremendous efficacy in improving the prognosis of anaplastic thyroid cancer.

3. Berberine

3A. Historical Perspective

Berberine (BBR) is an isoquinolone alkaloid that is the active ingredient isolated from the root and rhizome of coptis chinesis and hydrastis canadensis species to form a bitter, vibrant yellow powder (Hu et al., 2021; Kumar et al., 2015; Lv et al., 2012; Y. Wang et al., 2020; Yeung et al., 2020). BBR is a phytochemical compound that has a rich history in holistic medicine, including Traditional Chinese Medicine, Ayurveda, Egyptian, Native American, and Iranian medicine for over 3000 years (Hu et al., 2019; Kumar et al., 2015). Historically, BBR was used to inhibit toxins and bacteria and protect the intestinal epithelial layer from injury, with its original use as an anti-diarrheal therapy (Kumar et al., 2015). From this role, its potent anti-

inflammatory and antimicrobial properties, including antibacterial, antiviral, antifungal, anti-protozoan, and anti-helminth functions, became clear (Fang et al., 2022; Lv et al., 2012; Yin et al., 2012; Zhang et al., 2020). BBR is still used consistently in China as an over-the-counter anti-diarrheal medicine, and has been approved as an anti-diabetic agent in China since 1988 with efficacy similar to that of metformin and sulphonureas (Yin et al., 2012).

The chemical formula of isolated berberine is $C_{20}H_{18}NO_4$ **(Figure 12)** (Guamán Ortiz et al., 2014; Zhang et al., 2020). BBR's molar mass is 336.36122 g/mol (Hu et al., 2019). Berberine is often used in a formulation with other active ingredients in a multi-botanical mixture, rather than being extracted from the plant for cost or time purposes (Yin et al., 2012). However, many chemical forms of berberine now exist as well, including berberine hydrochloride, berberine sulfate, berberine citrate, and berberine phosphate, among which berberine hydrochloride is the most commonly used form (Kumar et al., 2015; Yin et al., 2012). When berberine is isolated for use, it is poorly but slowly soluble in water, very slightly soluble in ethanol, slightly soluble in methanol, and is also poorly lipid soluble (Kumar et al., 2015; Zhang et al., 2020). Due to these contributing factors, the oral bioavailability of BBR is poor with absorption of less than 5% of orally administered BBR through the intestinal wall (Hu et al., 2019). Different strategies are in development to improve BBR's bioavailability, including nanoparticle formulations, utilization of more absorbable forms of berberine (dihydroberberine), co-administration with pro-biotics, and co-treatment with glycoprotein inhibitors to enhance absorption (Farooqi et al., 2019; Hu et al., 2019; Yang et al., 2023). Despite its poor oral bioavailability and low presence in the blood, BBR's high tissue distribution and ability to penetrate the blood-brain-barrier both contribute to its

pharmacological effects (Kumar et al., 2015). Organ concentration of both berberine and its bioactive metabolites is high compared to concentration in the blood following oral administration, and this organ distribution takes place rapidly to the liver, followed by the kidneys, muscles, lungs, brain, heart, pancreas, and, to a lesser extent, fat (Kamrani Rad et al., 2017; Kumar et al., 2015). This distribution remains relatively stable for 48 hours (Kumar et al., 2015). Orally administered BBR is metabolized in the liver and excreted in the urine, bile, and feces, with a total recovery rate of approximately 23%, mostly through the feces, after 48 hours (Kamrani Rad et al., 2017; Kumar et al., 2015).

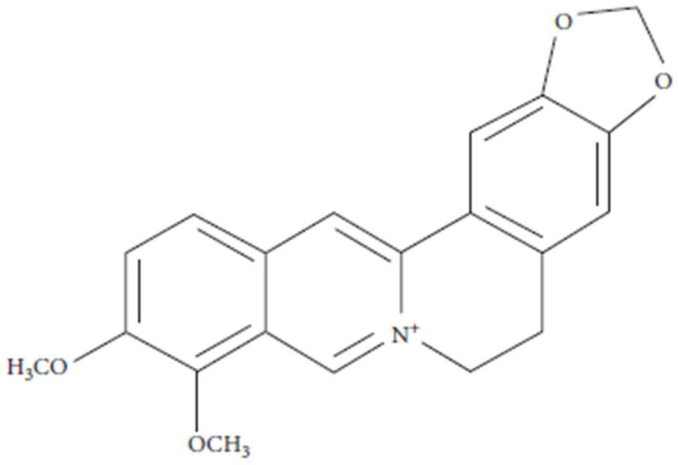


Figure 12. Chemical structure of berberine. Figure Taken From: Hu *et al.,* 2019.

Since its initial usage, BBR has displayed a vast spectrum of beneficial pharmacological activities, including anti-inflammatory, antimicrobial, antihypertensive, antioxidant, antidepressant, antidiabetic, hepatoprotective, nephroprotective, sedative, antiemetic, antinociceptive, anticholinergic, and anti-cancer (Hu et al., 2019; Kumar et al., 2015; Lv et al., 2012; Wang et al., 2020; Yeung et al., 2020.; Zhang et al., 2020). Currently, BBR's vast pharmacological actions are being evaluated in diabetes, cardiovascular disease, digestive

disorders, neurological disorder, allergy, and tumors (Fang et al., 2022; Hu et al., 2019; Tong et al., 2012; Yin et al., 2012). Further clinical trials are needed to better understand the *in vitro* and *in vivo* actions BBR has been observed to exert in cancer and serious neurological disorders, such as Alzheimer's disease and Parkinson's disease (Kumar et al., 2015).

Natural products, such as BBR, are attractive agents for study due to their high potency and low associated side effects (Guamán Ortiz et al., 2014; Kumar et al., 2015). Additionally, it is low cost, low toxicity, and exhibits diverse properties (Guamán Ortiz et al., 2014; Hu et al., 2019). These extensive pharmacological actions of BBR have been studied in animal models and clinical trials, however, the exact mechanism of action of BBR remains unclear (Fang et al., 2022).

Some pathways that have been implicated in inducing BBR's anti-inflammatory and antioxidant therapeutic effects include AMP-activated protein kinase (AMPK) signaling, MAPK signaling, PI3K/AKT/mTOR signaling, Janus tyrosine kinase 2 (JAK)/signal transducer and activator of transcription 3 (STAT3) signaling, and nuclear factor erythroid 2-related factor 2 (Nrf2)/hemeoxygenase-1 (HO-1) signaling – all of which are also important signaling pathways that are dysregulated in cancer (Hu et al., 2019).

3B. Targets of Berberine

Inflammation

From ancient medicinal texts, berberine has been known for its anti-infection and anti-inflammatory properties however, recently, this role as an anti-inflammatory agent has been investigated in different pathologies and drug-induced inflammatory side effects (Yin et al., 2012). These roles are seen in immune cell constituents throughout the body and various cell

types that impact distinct processes in disparate body systems to return to homeostasis. For example, thioacetamide treatment for hepatic fibrosis of the liver causes significant inflammation as a side effect – BBR inhibits this drug-induced inflammation (Wang et al., 2020). In a Zebrafish model, BBR treatment was able to suppress seizure-like behavior via downregulation of crucial soluble inflammatory mediators – tumor necrosis factor alpha (TNF-α), IL-1β and IL-6 (Wang et al., 2020).

BBR has been shown to induce Nrf2 activation in inflammatory macrophages in an AMPK-dependent fashion to inhibit inflammation (Wang et al., 2020). In metabolic disease, BBR sreduces inflammation induced by oxidized low-density lipoprotein again through regulation of the AMPK/mTOR signaling pathway (Wang et al., 2020). BBR also applies its anti-inflammatory actions in skeletal progenitor cells by activating one essential pathway and inhibiting another (Wang et al., 2020). Activation of AMPKα/Sirtuin 1 (SIRT1)/Peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α) is essential to this mechanism controlling inflammation and also interweaves with BBR's metabolic impacts, as PGC-1α is a key regulator of cellular energy metabolism in the mitochondria. Inhibition of mitogen-activated protein kinase 4 (MKK4)-stress-activated protein kinase (SAPK)/c-Jun NH2-terminal kinase peptide control (JNKC)-JUN also allows for reduction of inflammation (Wang et al., 2020). Additionally, in an adjuvant arthritis model in mice, representative of rheumatoid arthritis in humans, BBR alleviated joint destruction and infiltration of inflammatory cells through regulation of AMPK/NF-κB signaling (Wang et al., 2020). Consistent amongst these processes in which BBR inhibits inflammation in significantly different pathologies is the

ability to regulate signaling pathways, notably AMPK signaling, and cytokine production and release (Wang et al., 2020).

Metabolism

BBR's role in metabolic diseases as an insulin-independent hypoglycemic agent is demonstrated via its anti-dyslipidemia and anti-obesity activities, as well as its regulation of blood glucose levels (Yeung et al., 2020; Yin et al., 2012). In animal models, delivery of berberine also impacts its function (Yeung et al., 2020). Intraperitoneal injection improved glucose tolerance in mice, while oral administration reduced plasma triglycerides and insulin action in rats and reduced blood cholesterol levels in hamsters – both routes of administration equally reduced body weight (Yeung et al., 2020). BBR lowers lipid levels by interacting with the 3'UTR (untranslated region) of the low density lipoprotein (LDL) receptor to improve LDL receptor messenger RNA (mRNA) stability and by competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (Wang et al., 2020). BBR can also alleviate nonalcoholic fatty liver through activation of sirtuin 3 (SIRT3) *in vivo* (Wang et al., 2020).

Unlike other pathologies, mechanisms by which BBR exerts its hypoglycemic effects have been extensively studied (Wang et al., 2020). AMPK activation remains a key player. BBR improves insulin action through mitochondrial inhibition and subsequent activation of AMPK (Wang et al., 2020). This mechanism may have potential in other disease processes that also experience dysregulated mitochondrial metabolism, such as tumorigenesis. Additionally, this control of energy expenditure also contributes to the berberine-related weight control of diabetic rats (Yeung et al., 2020). The well-known antioxidant and anti-inflammatory

contributions of berberine also play a significant role in BBR's action in diabetic animals through AMPK, MAPK, Nrf2, and NF-κB (Yeung et al., 2020).

A summary of hyperlipidemia and dyslipidemia clinical trials using berberine treatment demonstrated BBR's efficacy as replacement therapy for patients who are intolerant to statins (Yeung et al., 2020). Statins also come with a host of side effects, while BBR is much more tolerable for patients. Short-term, BBR also significantly reduced fasting plasma glucose and glycated hemoglobin levels relative to controls, however, this treatment lost efficacy when given for over 90 days (Yeung et al., 2020).

Cancer

To date, there have been *in vivo* and *in vitro* studies of the pharmacological effects of BBR treatment in various cancer models as a cancer-preventative agent as well as treatment against established tumors (Guamán Ortiz et al., 2014; Zhang et al., 2020). These studies have shown that BBR can block proliferation, induce cell cycle arrest, promote apoptosis, activate autophagy, and hinder invasion and metastasis by suppressing angiogenesis and EMT (Guamán Ortiz et al., 2014; Wang et al., 2020; Xu et al., 2019). BBR may inhibit cell proliferation by interacting with microRNAs. BBR has also demonstrated an ability to regulate intracellular oxidative stress and exert its anti-inflammatory and anti-oxidant properties to regulate the TME (Wang et al., 2020; Xu et al., 2019). Recent evidence has also shown that BBR improves the efficacy and safety of chemoradiotherapies, particularly doxorubicin (Tong et al., 2012). Many of these properties have been showcased in high-risk cancer types, including lung, breast, prostate, colorectal, and gastric cancers, *in vitro* and *in vivo*, with positive pre-clinical results seen in breast and lung cancers specifically (Xu et al., 2019).

Although numerous pathways have been implicated to be regulated by BBR treatment in cancer, exact mechanisms and targets behind these functional changes are less clearly elucidated.

In a comprehensive review conducted by Xu *et al.* of 26 *in vivo* studies evaluating the dose response effect of BBR in ten different tumor models, almost all studies showed a significantly decreasing trend in tumor volume and tumor weight with increasing concentration of BBR given **(Figure 13)** (Xu et al., 2019). Mechanistically, how BBR is reducing tumor volume in these tumor models is not known.

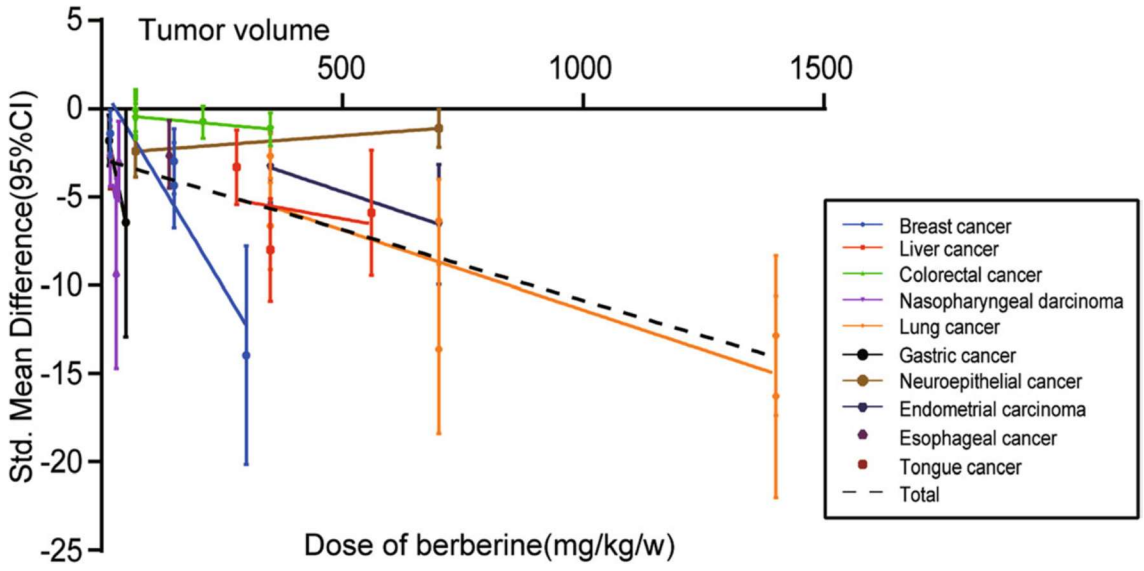


Figure 13. "Dose response effect of BBR on tumor volume following a comprehensive review of 26 studies of various cancer types in animal models conducted from 2000 to 2018." Figure and Legend Taken from: Xu *et al.*, 2019.

Unregulated progression through the cell cycle is a feature common amongst many cancer types, particularly those with loss of p53 function. Depending on dose, BBR can induce cell

cycle arrest at the G0/G1 phase, G1 phase, S, or G2/M phase (Zhang et al., 2020). Arrest at G0/G1 by BBR is often coupled with activation of cyclins D1, D2, E, Cdk2, Cdk4, and Cdk6, thus suppressing cellular proliferation following cell cycle arrest (Guamán Ortiz et al., 2014). Arrest at G1 was observed in tandem with decreased cyclin B1 at low BBR concentration in lung cancer and melanoma cell models (Guamán Ortiz et al., 2014). Ren *et al.* found cell cycle arrest in the S and G2/M phases following lower concentration (20-40 μM) BBR treatment and later arrest in the G2/M phase following higher BBR concentration (60-100 μM) in a melanoma model (Ren et al., 2020). In different *in vitro* cell models of breast cancer (T47D and MCF-7), BBR induced G2/M arrest in T47D but G0/G1 arrest in MCF-7, with all other variables, such as dose, constant (Zhang et al., 2020). These differences in cell cycle arrest appear to be a function of both concentration and cell type, as well as p53 status (Guamán Ortiz et al., 2014).

Regulation of cell cycle and control of apoptosis is largely regulated by key tumor suppressor, p53 (Zhang et al., 2020). An initiating step in tumorigenesis in many cancers, including ATC, is a mutation in p53, which subsequently removes the ability to recognize cellular damage, halt the cell cycle, and induce apoptosis (Zhang et al., 2020). This apoptotic induction occurs through p53 inhibition of anti-apoptotic B cell lymphoma (Bcl-2) protein by pro-apoptotic Bcl-2 Associated X-protein (Bax) (Guamán Ortiz et al., 2014; Zhang et al., 2020). BBR may upregulate p53 expression by different mechanisms – post-transcriptional repression of MDM2, an inhibitor of p53, or increased expression of miR-23a enhancing G2/M cell cycle arrest (Zhang et al., 2020). Interestingly, a study of berberine treatment in two breast cancer cell lines with different p53 mutational status, MCF-7 (wild-type TP53) and MDA-MB-231

(mutant TP53) demonstrates that BBR exerts different effects on p53 expression (Zhang et al., 2020). Berberine was able to recover expression levels of p53 in MCF-7 cells that were depleted of p53, however, expression of p53 did not revert back to functionality in MDA-MB-231 with the TP53 mutation (Zhang et al., 2020). In cells with p53 mutation, BBR cannot directly affect expression levels, but BBR can still induce p53-independent G2/M cell cycle arrest (Guamán Ortiz et al., 2014; Zhang et al., 2020).

Myeloid cell leukemia 1 (Mcl-1) is an anti-apoptotic protein of the Bcl-2 family that inhibits apoptosis via suppressing interactions with pro-apoptotic proteins Bim, Bak, and Bid (Guamán Ortiz et al., 2014). In many cancer types, Mcl-1 becomes constitutively active via oxidative stress pathways and cytokine and growth factor signals, leading to promotion of cell growth, survival, and angiogenesis via transcriptional upregulation of STAT3 (Guamán Ortiz et al., 2014). BBR has been reported to suppress constitutive STAT3 activation in oral cancer, renal cancer, and nasopharyngeal carcinoma through downregulation of Mcl-1 (Guamán Ortiz et al., 2014). This shows an additional mechanism by which BBR can inhibit cellular survival and induce apoptosis (Guamán Ortiz et al., 2014).

Still, despite some progress *in vitro* and *in vivo,* little clinical guidance exists for treatment of cancer patients with BBR (Xu et al., 2019). Further, research in ATC explicitly is very limited. Although carcinogenic properties regulated by BBR have been demonstrated in other cancer types *in vitro* and *in vivo,* full understanding of the mechanisms leading to these changes needs further work. However, an essential player in the actions displayed by BBR *in vitro* and *in vivo* amongst diverse disease processes and cancer appears to be AMPK. Additionally, as inflammatory immune cell activation and the release of soluble inflammatory mediators

propagate carcinogenesis in ATC, BBR may serve as a valuable agent to modulate the tumor immune landscape.

II. Specific Aims

Hypothesis – Berberine is a systemic mediator of inflammation, as well as tumor initiation and progression, that can be harnessed to ameliorate the aggressiveness of fatal anaplastic thyroid cancer through metabolic reprogramming.

To test this hypothesis, we focused on defining the aggressive phenotype of anaplastic thyroid cancer *in vitro* using ATC cell lines. We assessed these elements following berberine treatment of ATC cells themselves and also evaluated the role of berberine in modulating the abundant inflammation produced by cells and cellular mediators that would be present in the tumor microenvironment. To identify a mechanism of action by which berberine controls the aggressiveness and inflammatory components of ATC, we evaluated how berberine alters mitochondrial metabolism in the ATC cells and what downstream consequences it has for the tumor.

The specific aims of this study are as follows:

Specific Aims –

Aim 1: Berberine alleviates the aggressiveness of the anaplastic thyroid cancer phenotype via control of proliferation, survival, invasion, intrinsic migratory capacity, and motility.

a. *In vitro* evaluation of berberine treatment on the aggressive phenotypes of anaplastic thyroid cancer
b. *In vitro* evaluation of expression level and activation of important cell signaling pathways in anaplastic thyroid cancer by Western blot

Aim 2: Berberine reduces the burden of soluble and cellular mediators of inflammation commonly found in the anaplastic thyroid cancer tumor microenvironment.

a. Panning of National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) human anaplastic thyroid cancer tissue data for upregulation of inflammatory elements
b. *In vitro* evaluation of soluble inflammatory cytokines and chemokines released from activated macrophages treated with berberine in conditioned media
c. *In vitro* evaluation of soluble inflammatory cytokines and chemokines released from activated macrophages in conditioned media after polarization with berberine-treated anaplastic thyroid cancer-derived conditioned media

Aim 3: Berberine induces metabolic changes in ATC that alter the tumor's energetics.

a. Comprehensive RNA Sequencing (RNA Seq) analysis of differentially expressed genes (DEGs) in berberine-treated anaplastic thyroid cancer (T238) cells compared to vehicle control
b. Evaluation of the expression of mitochondrial-encoded genes at the transcript and protein level
c. *In silico* analysis of major metabolic pathways impacted by berberine treatment in anaplastic thyroid cancer
d. Analysis of superoxide production from anaplastic thyroid cancer cells following berberine treatment

III. Materials and Methods

Cell Lines and Cell Culture: Nthy-ori-3-1 is a human follicular epithelial cell line derived from normal thyroid and immortalized by SV40 large T gene (Sigma Aldrich Inc.; catalog number 90011609). T238 and SW1736 are anaplastic thyroid cancer cell lines and were obtained from Dr. Rebecca Schweppe at the University of Colorado Cancer Center. All cells were tested for mycoplasma. Cells were cultured using aseptic technique in a biological safety cabinet (NuAire) using Roswell Park Memorial Institute 1640 (RPMI-1640) media with L-Glutamine and phenol red supplemented with 10% fetal bovine serum for all thyroid cell lines; T238, SW1736, and Nthy-ori-3-1. Cells were grown at 37°C in a humidified tissue culture incubator with 5% carbon dioxide (CO_2) atmosphere. U937 is a cell line with monocyte morphology derived from the pleural effusion of a 37-year-old white, male patient with histiocytic lymphoma (ATCC; catalog number CRL-1593.2). These cells were obtained from Dr. Raj Kishore at the Lewis Katz School of Medicine of Temple University.

	Type	$BRAF^{V600E}$	$CDKN2A^{L63R}$	$PIK3CA^{E542K}$	$TERT^{C228T}$	TP53	$TSHR^{I486F}$
T238	Anaplastic thyroid carcinoma	Homozygous	Homozygous	Heterozygous	Heterozygous; in promoter	Homozygous (S183X)	Wild type
SW1736	Anaplastic thyroid carcinoma	Heterozygous	Wild type	Wild type	Heterozygous; in promoter	Homozygous (Q192X)	Heterozygous
Nthy-ori-3-1	Immortalized, normal thyroid epithelial	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type

Table 2. List of mutations present in cell lines used in this study.

Treatment: Pure berberine, without any additional components, was used for all experiments. Purity was confirmed in Dr. Xiu-Min Li's laboratory. Treatment conditions were 24 hours of 100 µM BBR or an equal volume of dimethyl sulfoxide (DMSO) as vehicle control in complete media (10% fetal bovine serum in RPMI 1640 with L-Glutamine and phenol red) in 37°C in a humidified tissue culture incubator with 5% CO_2 atmosphere, unless otherwise described.

Activation and Polarization of U937 Cells and Conditioned Media Collection: Five million U937 monocytes were seeded and activated to macrophages by incubation with 200 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) in RPMI-1640 supplemented media for 48 hours in the presence or absence of 100 µM BBR. After 48 hours, the media was discarded, and the activated cells were washed twice with phenol red-free RPMI-1640 for five minutes. For M1 polarization, cells were incubated with 10 pg/mL of lipopolysaccharide (LPS; Sigma Aldrich Inc.) and 20 ng/mL of recombinant human interferon-gamma (IFN-γ; R&D Systems, MN, USA) in the presence or absence of 100 µM BBR for 24 hours. Cells were washed twice with phenol red-free RPMI-1640 for five minutes and then incubated for a further 48 hours in FBS-free phenol red-free RPMI-1640 media. The supernatant was collected and centrifuged at 1500 rpm for five minutes to remove cellular debris, and the remaining supernatant served as activated macrophage-conditioned media of an M1 phenotype, depending on polarization.

Collection of ATC Conditioned Media: ATC cells, SW1736 and T238, and immortalized, normal thyroid epithelial cells, Nthy-ori-3-1, were grown to approximately 60-70% confluency. Cells were treated with 100 µM BBR or equal volume of DMSO vehicle control

for 24 hours. After 24 hours, cells were washed twice with phenol red-free RPMI-1640 for five minutes and then incubated for a further 48 hours in FBS-free phenol red-free RPMI-1640 media. The supernatant was collected and centrifuged at 1500 rpm for five minutes to remove cellular debris, and the remaining supernatant served as ATC or normal thyroid conditioned media.

Polarization of U937 Cells with ATC Conditioned Media: Five million U937 monocytes were seeded and activated to macrophages by incubation with 200 nM TPA alone in RPMI-1640 supplemented media for 48 hours. After 48 hours, the media was discarded, and the activated cells were washed twice with phenol red-free RPMI-1640 for five minutes. Cells were polarized with conditioned media collected from SW1736, T238, or Nthy-ori-3-1, as described above. Cells were washed twice with phenol red-free RPMI-1640 for five minutes and then incubated for a further 48 hours in 5 mL of FBS-free phenol red-free RPMI-1640 media. The supernatant was collected and centrifuged at 1500 rpm for five minutes to remove cellular debris, and the remaining supernatant served as activated macrophage-conditioned media polarized by ATC cells.

Inflammation Array: The RayBio C3 Human Inflammation Antibody Array (RayBiotech, Norcross, GA, AAH-INF-3-8) was used to evaluate inflammatory cytokines and chemokines present in activated macrophage conditioned media according to manufacturer's instructions (n=3 biological replicates; n=2 technical replicates per membrane). The test membrane includes positive and negative controls. The 40 targets include: Eotaxin-1 (C-C

motif chemokine 11; CCL11), Eotaxin-2 (CCL24), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), intracellular adhesion molecule 1 (ICAM-1/CD54), IFN-γ, I-309 (CCL1), interleukin-1-alpha (IL-1-α), interleukin-1-beta (IL-1-β), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-6 receptor (IL-6R), interleukin-7 (IL-7), interleukin-8 (IL-8 / chemokine (C-X-C motif) ligand 8; CXCL8), interleukin-10 (IL-10), interleukin-11 (IL-11), interleukin-12 p40 (IL-12 p40), interleukin-12 p70 (IL-12 p70), interleukin-13 (IL-13), interleukin-15 (IL-15), interleukin-16 (IL-16), interleukin-17A (IL-17A), interferon gamma inducible protein-10 (IP-10/CXCL10), monocyte chemoattractant protein-1 (MCP-1/CCL2), MCP-2 (CCL8), macrophage colony-stimulating factor (M-CSF), membrane immunoglobulin (MIG/CXCL9), macrophage inflammatory protein-1-alpha (MIP-1-α/CCL3), MIP-1-β (CCL4), MIP-1-δ (CCL15), regulated upon activation, normal T cell expressed and secreted (RANTES/CCL5), transforming growth factor-beta-1 (TGF-β1), tumor necrosis factor-alpha (TNF-α), TNF-β, tumor necrosis factor-receptor 1 (TNF-RI), TNF-RII, platelet-derived growth factor-BB (PDGF-BB), tissue inhibitor of metalloproteinases 2 (TIMP-2).

IFN-γ Enzyme-Linked Immunosorbent Assay (ELISA): BD OptEIA™ Human IFN-γ ELISA kit (Becton Dickinson, Franklin Lakes, NJ, USA; catalog number: 550612) was followed according to manufacturer's instructions. Conditioned media from BBR-treated and control M1 U937 macrophages was used (n=6) for one experiment and activated macrophage-conditioned media polarized by ATC cells (with or without BBR treatment) (n=6) was used for another independent experiment. Mann Whitney U test was used to compared conditions.

TNF-α ELISA: TNF-α human uncoated ELISA kit (Invitrogen, Carlsbad, CA, USA; catalog number: 88734688) was followed according to manufacturer's instructions. Conditioned media from BBR-treated and control M1 U937 macrophages was used (n=6) for one experiment and activated macrophage-conditioned media polarized by ATC cells (with or without BBR treatment) (n=6) was used for another independent experiment. Mann Whitney U test was used to compared conditions.

Proliferation Assay: Cell proliferation rates were assessed using the *Trypan Blue Exclusion Test of Cell Viability* over a distinct time course. Anaplastic thyroid cancer cells, T238 and SW136, and immortalized, normal thyroid epithelial cells, Nthy-ori-3-1, were seeded in 6-well cell culture plates at a density of 25,000 cells per 9.6cm^2 well, each with a total complete medium, cell suspension, and treatment volume of 1 mL at the time of seeding. Treatment conditions were 100 µM BBR (n=3) or an equal volume of DMSO vehicle control (n=3). Every 24 hours for three consecutive days, 250 µL of 0.25% Trypsin, 2.21 mM ethylenediamine tetraacetic acid, 1X [-] sodium bicarbonate was added to each well and was incubated at 37°C for 2 minutes and then neutralized with 750 µL of complete media. Each well was washed and complete media was added to ensure all adhered cells were lifted from the well, and 200 µL of cell suspension was collected. From the collected 200 µL cell suspension, 10 µL was removed and resuspended in 10 µL of 0.4% Trypan Blue solution, rendering a dilution factor of 2. 10 µL was loaded onto a Hemocytometer and placed under a Fisher Micromaster microscope at 10X. Cells were counted in all four quadrants and averaged to determine the total cell count using the following formula: $\bar{x}$ * dilution factor (2) * 10^4. After three

consecutive days of cell counting, growth rate was calculated using the following equation: $\mu = \frac{\ln ({}^{Nt}/_{N0})}{\Delta t}\ x\ 24h$ where μ = growth rate; Δt = hours of growth [h]; N_0 = number of cells seeded; N_t = number of cells harvested. The growth rate for BBR-treated cell lines was compared to controls using a Mann-Whitney U test.

Cell Death Detection ELISA: Cell Death Detection ELISA (Roche, Basel, Switzerland) was followed according to the manufacturer's instructions to allow for the specific determination of mono- and oligonucleosomes in the cytoplasmic fractions of cell lysates representative of induction of apoptosis. 5 x 10^4 cells (SW1736, Nthy-ori-3-1) were seeded per well and treated with 100 μM BBR or an equal volume of vehicle control (DMSO) for 18 hours (n=6). Exponentially growing cells without any treatment were used as a negative control, as a certain number of dead cells are always found under cell culture conditions and they have the ability to impact the absorbance value. The differences in production of cytoplasmic histone-associated DNA fragments for BBR-treated and vehicle control cells was compared using a Mann-Whitney U test. The enrichment factor was calculated using the following equation: $enrichment\ factor = \frac{mU\ of\ sample\ (dying/dead\ cells)}{mU\ of\ the\ corresponding\ control\ (viable\ cells)}$ where mU = absorbance [10^{-3}].

Scratch Wound Assay: Anaplastic thyroid cancer cells, T238 and SW136, were seeded in 12-well cell culture plates at a density of 100,000 cells per 3.6cm^2 well, each with a total complete medium and cell suspension volume of 500 μL. Once cells reached 100%

confluency in each respective well, complete media was replaced with sterile 1X phosphate-buffered saline (PBS), and a wound field was introduced through the center of each well using a p10 pipette tip. Cells were washed with 250 µL 1XPBS post-scratch, and then 500 µL complete media with 100 µM BBR or equal volume DMSO was added per well. Each wound field was imaged at marked {X,Y} coordinates in each well using a Nikon Eclipse Ti microscope at 10X magnification under bright field setting. Wound fields were again measured at marked {X,Y} coordinates at the following time points proceeding scratch induction: 18 hours, 24 hours, 48 hours, and 72 hours. Percent wound field healing was calculated using the following formula: $[T_0(\mu m) - T_X(\mu m)/ T_0(\mu m) * 100]$, where *x* represents the measurements taken at each distinct time point after wound field introduction. Percent healed calculations for BBR-treated cell lines were compared to vehicle controls using a Mann-Whitney U Test.

Transwell Invasion and Migration Assay: Cellular invasion and migration were determined using Matrigel Invasion Chambers (Corning, catalog number 354483, Corning, NY, USA), according to manufacturer's instructions. Control inserts were used to measure migration, while inserts coated with Matrigel were used to measure invasion. Briefly, T238 cells were treated with 100 µM BBR or equivalent volume of vehicle control (DMSO) for 24 hours, following which, cells were passaged and seeded into the chambers. After incubating at 37°C for 24 hours, the cells that invaded through the bottom towards the chemoattractant (complete RPMI media with FBS) were fixed, imaged using the Nikon Eclipse Ti microscope and counted in n=5 locations per well for n=3 wells per condition. Percent reduction in invasion or migration following berberine treatment was calculated using the following

formula: $Percent\ reduction = \frac{(Number\ of\ untreated\ cells\ migrated\ or\ invaded - num\ \ \ of\ BBR\ treated\ cells\ migrated\ or\ invaded}{Number\ of\ untreated\ cells\ migrated\ or\ invaded}$.

Percent invasion and migration for BBR-treated cell lines were compared to vehicle controls using a Mann-Whitney U Test. Percent invasion was calculated using the following formula: $Percent\ invasion = \frac{mean\ number\ of\ cells\ invaded}{mean\ number\ of\ cells\ migrated}$ x 100%. This was used to calculate invasion index using the following formula: $Invasion\ index = \frac{percent\ invasion\ treated\ cells}{percent\ invasion\ control\ cells}\ x\ 100.$

Western Blot: Anaplastic thyroid cancer cells, T238 and SW1736, or immortalized normal thyroid cells, Nthy-ori-3-1, were seeded in 75 cm^2 culture flasks and treated at 65-75% confluence with 100 µM BBR or equal volume DMSO for 24 hours. Cells were harvested by scraping in 1XPBS, centrifuged at 1,200 rpm for 10 minutes, washed in 1XPBS, and pellets were aspirated completely to dryness. Cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer with HALT protease phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA, USA), and incubated on ice for one hour with intermittent rigorous vortex. Lysed cells were centrifuged at 14,000 rpm for 30 minutes at 4°C. The supernatant containing protein was saved, absorbance was read at 595 nm using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA, USA) on the Thermo Scientific BioMate 3S, and the concentration of protein was calculated. Protein samples were prepared to 15 µg total protein in 15 µL total volume with 1:10 beta-mercaptoethanol in Bio-Rad 4X Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA, USA). Samples were boiled for 10 minutes and separated by 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis. They were transferred onto a polyvinylidene fluoride membrane and blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween 20

detergent (TBST) buffer for phosphorylated proteins or 5% milk in TBST buffer for non-phosphorylated proteins for one hour at room temperature with gentle rocking. Membranes were gently rinsed with TBST and incubated in primary antibody in 2% BSA in TBST overnight at 4°C with gentle rocking. Membranes were then washed three times for 5 minutes each with TBST and incubated with corresponding horseradish peroxidase-conjugated secondary antibodies in TBST for two hours. After four 15-minute washes with TBST, membranes were developed with Thermo Scientific SuperSignal West Pico PLUS Chemiluminescent Substrate (Waltham, MA, USA) and imaged on the Biostep Celvin S chemiluminescence imaging machine (Next Advance, Inc., Troy, NY, USA). Western blots were analyzed using ImageJ Software (National Institutes of Health, Bethesda, MD, USA).

Antibody	Manufacturer	Catalog Number	Dilution
Phospho-MEK1/2 (Ser217/221) Rabbit mAb	Cell Signaling Technology	9154S	1:1000
MEK1/2 Rabbit mAb	Cell Signaling Technology	9126S	1:1000
Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) Rabbit mAb	Cell Signaling Technology	4376S	1:1000
P44/42 MAPK (ERK1/2) Rabbit mAb	Cell Signaling Technology	4695S	1:1000
Phospho-S6 ribosomal protein (Ser235/236) Rabbit mAb	Cell Signaling Technology	2211S	1:1000
S6 ribosomal protein Rabbit mAb	Cell Signaling Technology	2217S	1:1000
Phospho-AMPKα (Thr172) Rabbit mAb	Cell Signaling Technology	2535S	1:1000

AMPKα Rabbit mAb	Cell Signaling Technology	2532S	1:1000
GAPDH Rabbit mAb	Cell Signaling Technology	5174S	1:2000
MitoNTS OXPHOS Complex 1 (39 kDa subunit)	Molecular Probes	A21344	1:500
B-actin Rabbit mAb	Cell Signaling Technology	4967S	1:1000

Table 3. List of primary and secondary antibodies used in Western blots.

RNA Isolation: Total RNA was extracted from pelleted anaplastic thyroid cancer cells (T238 and SW1736) and immortalized normal thyroid cells (Nthy-ori-3-1) following 24-hour treatment (conditions described above) and purified using a Quick-RNA™ MiniPrep Kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions. RNA concentration and purity were measured with NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA).

RNA Sequencing: Isolated RNA from T238 treated with 100 µM or equal volume of DMSO vehicle control was sent to GENEWIZ from Azenta Life Sciences (South Plainfield, NJ, USA) for standard RNA Seq. Sequencing results were returned as read counts with log-fold-change, p-value, and adjusted p-value given for each significantly differentially expressed gene (DEG). Read counts were also provided for all genes, whether or not they were differentially expressed. Principal Component Analysis was performed via linear dimensionality reduction to demonstrate the data's maximum variance. A volcano plot was created to show genes downregulated in BBR treated T238 cells compared to vehicle control

and genes upregulated in BBR treated T238 cells compared to vehicle control. Biclustering of significant DEGs into a heat map demonstrated changes in differential expression. Further data analysis, gene ontology analysis, and processing was conducted in excel and using Qiagen's Ingenuity Pathway Analysis Software (Hilden, Germany).

MitoSox Red™ Mitochondrial Superoxide Indicator Assay: SW1736, T238, and Nthy-ori-3-1 cells were seeded at 6,000 cells per well in a 96-well plate. After 24 hours, cells were treated with 100 µM BBR treated or equivalent volume vehicle control (DMSO) for 24 hours at 37°C in a humified atmosphere with 5% CO_2. After 24 hours, cells were washed twice with 1XPBS and MitoSox Red™ Staining was conducted as per manufacturer's instructions (Invitrogen, Carlsbad, CA, USA; catalog numbers: M36007 and M36008). Briefly, 100 µL MitoSox Red™ reagent working solution (500 nm) was added per well of the 96-well plate. Cells were incubated for 30 minutes at 37°C and 5% CO_2 protected from light. After 30 minutes, cells were washed gently three times with warm Hank's balanced salt solution buffer (Sigma Aldrich). Cells were imaged using the Nikon Eclipse Ti Microscope at 40X magnification within two hours of staining.

IV. Results

Specific Aim 1 – Berberine alleviates aggressiveness of the ATC phenotype via control of proliferation, survival, invasion, intrinsic migratory capacity, and motility.

Experimental Design –

Two human anaplastic thyroid cancer cell lines (T238, SW1736) were used for these *in vitro* experiments and one immortalized normal thyroid follicular epithelial cell line (Nthy-ori-3-1). The goal was to evaluate how berberine can selectively alter the hallmark dysregulation that makes ATC one of the most aggressive cancer types. Cells were treated and harvested at the log phase of their growth for all experiments. Subsequent *in vitro* analyses included Trypan Blue Exclusion Assay to evaluate proliferation, Cell Death Detection ELISA to evaluate apoptotic induction, Scratch Wound Assay to evaluate motility, Transwell Migration and Invasion Assay to evaluate migration and invasion, and Western blots to evaluate signal transduction targets.

Rationale –

Anaplastic thyroid cancer harbors hallmark features that drive its rapid lethality, including loss of differentiation, rapid proliferation, local invasion, and early distant metastasis (McFadden et al., 2014). ATC has a markedly high proliferation rate, even compared to other aggressive cancers (Espinosa et al., 2007; Jannin et al., 2022). Impacting both proliferation and differentiation, constitutive activation of BRAF signaling induces malignant transformation and aggressive tumor behavior (Espinosa et al., 2007). BRAFV600E mutation

activating this signaling pathway is present in more than 25% of ATC cases (Lang et al., 2023) and is a common feature amongst the ATC cell lines used in these studies. Additionally, a p53 mutation is present in greater than 70% of ATC cases, while less invasive thyroid cancer subtypes only possess this mutation less than 10% of the time (Espinosa et al., 2007; McFadden et al., 2014). While BRAF mutation is seen as a common initiating somatic event in all thyroid cancer subsets, loss of p53 function is hypothesized to be important in the progression of ATC, specifically (McFadden et al., 2014). As these driver mutations are present in both of the ATC cell lines used in our studies, an important first step in understanding if berberine can control ATC progression is to evaluate its impact on proliferation.

In ATC, the presence of inhibitor of apoptosis (IAP) proteins, such as survivin (present in 89% of ATCs compared to 14% of DTCs), prevent apoptotic induction in the tumor cells and create more resistant tumors (Smallridge et al., 2009; Ito et al., 2003). Proapoptotic protein, such as Bcl-2, expression has an inverse correlation with differentiation status in ATC (Smallridge et al., 2009). Additionally, TP53 mutation present in most ATCs hinders induction of apoptosis in tumor cells (McFadden et al., 2014; Taccaliti et al., 2012). As such, induction of apoptosis of ATC cells is essential for a treatment to be able to clear the disease.

Another major determinant of ATC's aggressiveness is its early propensity to migrate beyond the thyroid gland and invade local and distant tissue (Smallridge & Copland, 2010; Taccaliti et al., 2012). Invasion is the first step to metastasis in patients. Followed by aberrant cellular migration, which is an inherent feature of cancer progression (Gotsulyak et al., 2014).

Although the overarching mechanisms regulating invasion and migration in ATC remain unclear, invasion and migration in ATC are, at least in part, regulated by phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta (PIK3CD) overexpression (PI3K/AKT/mTOR signaling), overexpression of transforming growth factor beta 1 (TGF-β1), and dysregulated microRNA expression (Bu et al., 2017; Sun et al., 2017). Previous studies have found increased PI3K/AKT signaling and TGF-β1 overexpression to promote cell survival, induce EMT, migration, and invasion of many cancers, including ATC (Bu et al., 2017; Sun et al., 2017). As such, the changes in cellular motility, migration, and invasion induced by BBR in anaplastic thyroid cancer cells is a major task in evidencing its control over ATC aggressiveness.

Results –

To evaluate the effect of BBR treatment on proliferation, actively proliferating ATC cells (T238, SW1736) were counted over time using a trypan blue exclusion assay **(Figure 14)**. In T238, BBR significantly reduced proliferation compared to vehicle controls, as evidenced by significantly fewer viable cells over time **(14A)** compared to untreated cells. Growth rate in the BBR-treated T238 cells was 0.16 (doubling time = 104 hours) compared to 0.68 in the vehicle control (doubling time = 24.5 hours), showing a 76% decrease in growth rate following BBR treatment. Similarly, in SW1736, BBR nearly halted proliferation compared to vehicle controls **(14B)**. Growth rate in the BBR-treated SW1736 cells was 0.06 (doubling time = 277.3 hours) compared to 0.59 in the vehicle control (doubling time = 28.2 hours). These demonstrate a significantly slower growth rate following treatment, which validates BBR's ability to restrict growth in highly proliferative ATC cells. Importantly, BBR did not influence proliferation in normal thyroid epithelial cells **(14C)** with growth rate in the BBR-treated

Nthy-ori-3-1 cells 0.34 (doubling time = 48.9 hours) compared to 0.36 in the vehicle control (doubling time = 46.2 hours). This demonstrated BBR's selective toxicity for ATC cells specifically, compared to normal thyroid epithelial cells, which would be advantageous for BBR's development as an anti-cancer drug. ATC's rapid proliferation is a primary factor in its rapid spread, minimal overall survival, and lack of response to conventional therapies, making BBR's ability to curtail growth prognostically impactful.

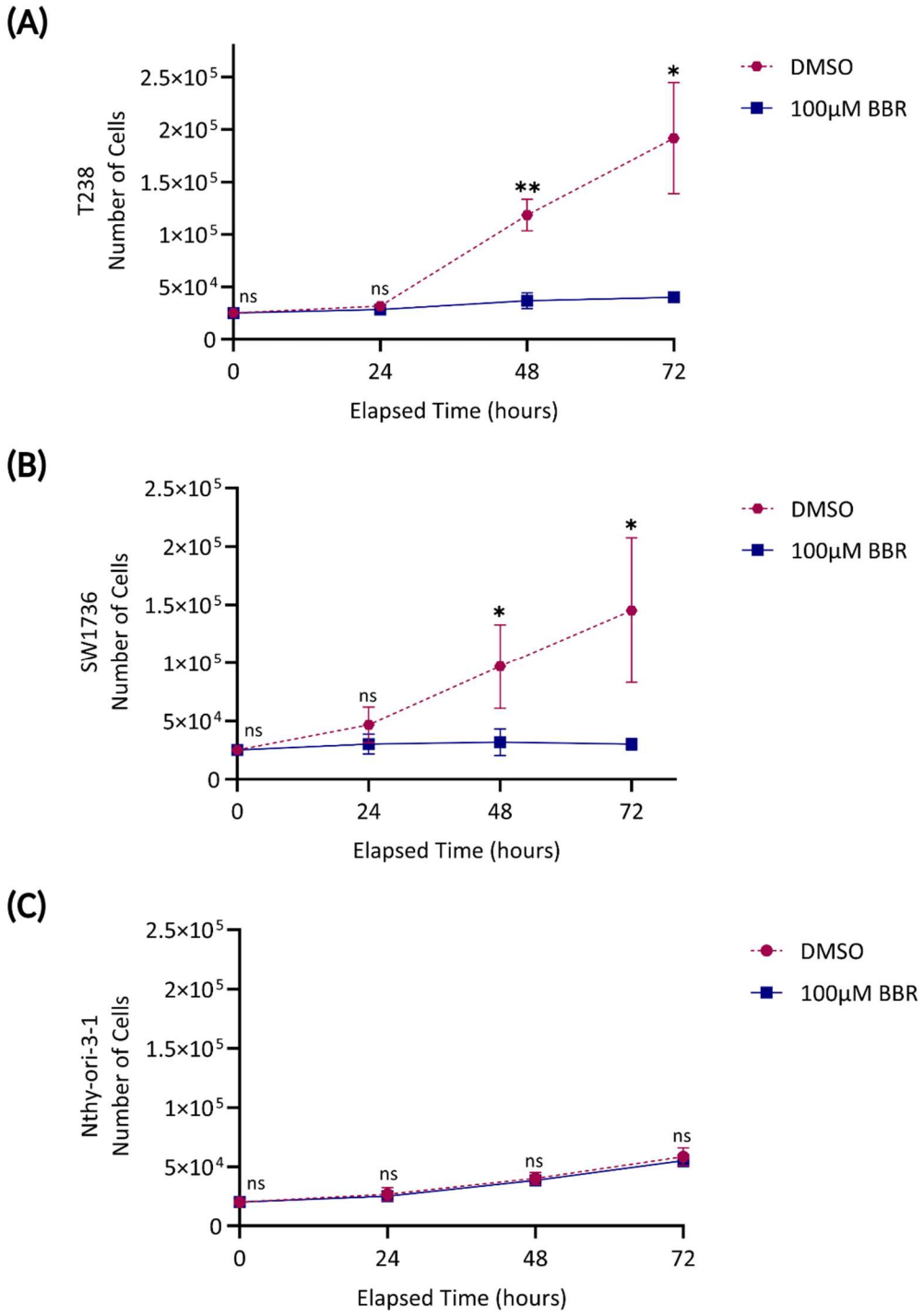


Figure 14. Berberine slows proliferation specifically in anaplastic thyroid cancer cells. Measured via trypan blue exclusion assay, 100 µM BBR treatment slows proliferation in anaplastic thyroid cancer cells **(A)** T238 and **(B)** SW1736 compared to vehicle control (DMSO). Berberine does not significantly impact proliferation in **(C)** Nthy-ori-3-1 immortalized, normal thyroid cells. A Mann-Whitney U Test was used for n=3 independent experiments per time point. *p<0.05, **p<0.01, ***p<0.001

A cell death detection ELISA was performed for detection of mono- and oligonucleosomes in the cytoplasmic fractions of cell lysates representative of induction of apoptosis. Induction of apoptosis is essential for killing and clearing ATC cells. 100 µM BBR treatment significantly induced apoptosis after 12-hours in SW1736 cells compared to vehicle control-treated cells (69% increase) **(15A)** and enriched the number of dead and dying cells produced **(15B)**, while Nthy-ori-3-1 had no significant induction of apoptotic fragments or enrichment of apoptosis **(15A,B)**.

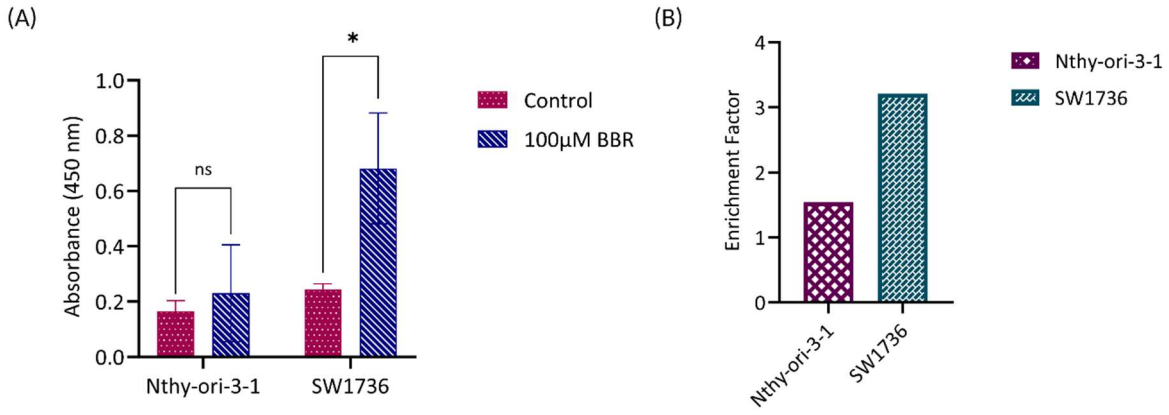


Figure 15. Berberine induces apoptosis in anaplastic thyroid cancer cells. Measured via cell death detection ELISA, 100 µM BBR treatment significantly induces apoptosis in anaplastic thyroid cancer cells **(A)** SW1736 compared to vehicle control (DMSO), but not in Nthy-ori-3-1, as demonstrated by increased absorbance. **(B)** The number of dead and dying cells is enriched compared to viable cells in SW1736 cells treated with BBR compared with control. A Mann-Whitney U Test was used for n=3 independent experiments per time point. *p<0.05, **p<0.01, ***p<0.001

A scratch wound assay was performed to estimate cell motility and migration dynamics in monolayer culture. The movement dynamics and cellular morphology were microscopically monitored in real-time over various time points as the cells migrated from the intact periphery back into the cell-free zone. Treatment with 100 µM BBR delayed wound healing

by 23% in T238 **(16A,C)** and by 49% in SW1736 **(16B,D)** after 18 hours, 33% in T238 **(16A,C)** and by 51% in SW1736 **(16B,D)** after 24 hours, 24% in T238 **(16A,C)** and by 47% in SW1736 **(16B,D)** after 48 hours, and 24% in T238 **(16A,C)** and by 59% in SW1736 **(16B,D)** after 72 hours. This demonstrates how BBR treatment lessens the aggressiveness of ATC *in vitro.* By reducing its intrinsic migratory capacity, BBR may remodel ATC to resemble largely treatable forms of thyroid cancer more closely. This assay is usually most effective in gauging motility before the 24-hour mark. Post 24 hours, it starts to become difficult to distinguish if the changes are directly related to cell motility, or if altered proliferation and cell survival play an important role as well (Gotsulyak et al., 2014). We can see in our two earlier time points (18 and 24 hours) before the cells reach their doubling time that BBR treatment is delaying wound healing and lessening the migratory capacity of these cells. In the later time points we are qualitatively observing again the slowed proliferation in these cells following BBR treatment.

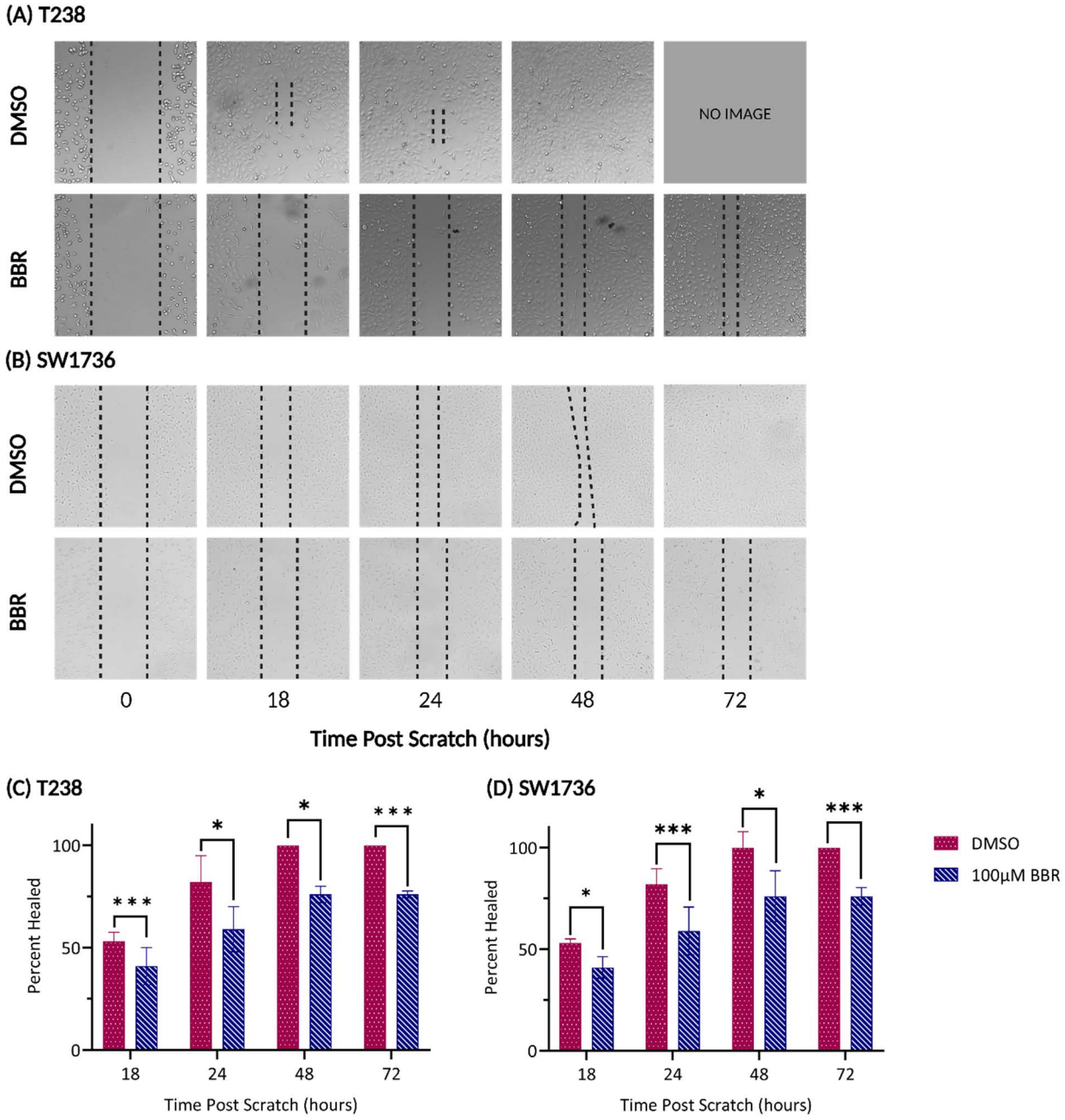


Figure 16. BBR combats aggressive ATC phenotype observed through delayed wound healing. Treatment with 100 µM BBR significantly slows wound healing compared to vehicle control (DMSO) in **(A,C)** T238 and **(B,D)** SW1736 anaplastic cells for 72 hours *in vitro*. Representative images of scratches at 0-, 24-, 48-, and 72-hours post-scratch in T238 **(A)** and SW1736 **(B)** anaplastic thyroid cancer cells following vehicle control (DMSO) or 100µM BBR treatment. There is no representative image 72 hours post-scratch for T238 vehicle control because all cells were overconfluent and floating. BBR delayed healing post-scratch for up to 72 hours in **(A)** T238 and **(B)** SW1736. A Mann-Whitney U Test was used for n=3 independent experiments per time point with n=5 measurements per condition. *p<0.05, **p<0.01, ***p<0.001

Invasion and migration were evaluated by cellular migration towards a chemoattractant through Boyden chambers with and without the presence of Matrigel on the insert, respectively. 100 µM BBR treatment decreased migration by 39% in T238 **(Figure 17A)**. BBR treatment also decreased invasion by 51% in T238 **(17A),** demonstrating its ability to degrade an extracellular matrix layer. The invasion index was also significantly reduced in T238 (21%) **(17B).** Representative images of cells migrating and invading can be observed in **Figure 17C**, and this qualitative picture demonstrates distinctly less purple staining of cells that were able to cross the insert. Importantly, the ability of BBR treatment to decrease invasion and migration in this ATC cell line could be essential in an *in vivo* system to control the early and advanced metastatic spread of this disease.

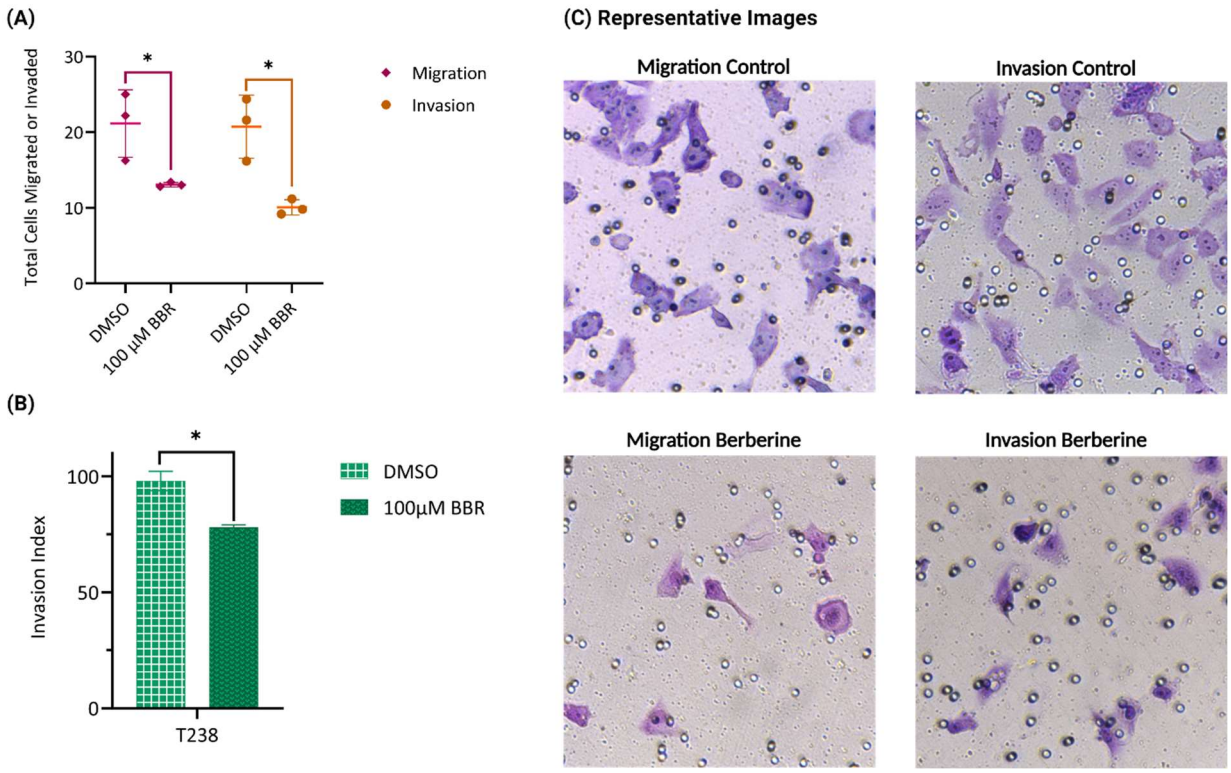


Figure 17. BBR decreases migration and invasion in ATC cells. Treatment with 100 µM BBR significantly decreases **(A)** migration compared to vehicle control (DMSO) in T238 anaplastic cells after 24 hours. 100 µM BBR significantly decreases **(A)** cells invaded compared to vehicle control in T238. **(C)** Representative images of migratory cells through a chamber and invaded cells through a Matrigel layer towards a chemoattractant. Images taken at 40X magnification. A Mann-Whitney U Test was used for n=3 independent experiments per time point with n=5 measurements per condition. *p<0.05, **p<0.01, ***p<0.001

We next asked the question— what are the cellular mechanisms and signal transduction targets behind BBR's observed activity on proliferation, apoptosis, invasion, and migration? To investigate this growth inhibitory effect of BBR at the protein level, phosphorylation of key proteins in aberrant signal transduction networks was observed. Hyperphosphorylated mitogen-activated protein kinase (MAPK) signaling, particularly in ligand-independent tumors expressing the BRAFV600E mutation common in ATC, impacts most essential cellular processes contributing to tumorigenesis – differentiation, proliferation, apoptosis, survival, and development (Guo et al., 2020). With such extensive repercussions following

hyperactivated Ras/Raf/MEK/ERK signaling, an agent with multitarget capability like BBR could be essential in fine-tuning this transduction. Phosphorylation of MEK **(Figure 18A)** and ERK1/2 **(18B)**, two crucial serine-threonine kinases mediating the aforementioned cellular processes as well as tumor extracellular matrix degradation and angiogenesis along the MEK/ERK axis (Guo et al., 2020), was markedly decreased following BBR treatment in anaplastic thyroid cancer cells (T238, SW1736) while leaving phosphorylation in immortalized normal thyroid cells (Nthy-ori-3-1) relatively unchanged **(18A,B)**. The essential impact of ERK1/2 on regulating transcription factors and gene expression in cancer makes this decreased phosphorylation by BBR necessary in regulating many of the most aggressive elements of ATC, possibly including proliferation, differentiation, invasion, and metastasis.

Western blot analyses under the same treatment conditions also revealed significantly decreased phosphorylation of 40S ribosomal protein S6 (rpS6) **(18C)**. Since phosphorylation of rpS6 is commonly used as a readout of PI3K/AKT/mTORC1 activation coupled with synergistic crosstalk that occurs between mTORC1 and the MEK and ERK signaling to control rpS6 phosphorylation (Yi et al., 2021), these findings suggest that BBR targets another critical cell regulatory pathway. BBR turns down this overactive signaling by decreasing phosphorylation of rpS6 **(18C)**, which again regulates cell proliferation and survival. With this PI3K/AKT/mTORC1 axis having shown mechanisms in controlling immune cell differentiation, essential for the anti-inflammatory tumor microenvironment, and tumor cell metabolism (Mafi et al., 2022). BBR's regulatory role in this cascade may also influence TME composition. Increased phosphorylation of AMPKα, another essential serine-threonine kinase, has an anti-tumor effect and is inextricably involved in metabolic regulation and restoring energy

balance. In a prostate cancer model, it has been demonstrated that activation of AMPK following BBR treatment led to decreased proliferation, degradation of androgen receptors, and apoptosis (Guamán Ortiz et al., 2014; Jeong et al., 2014). Additionally, Motoshima *et al.* have demonstrated that AMPK activation can lead to cell cycle arrest via p53-p21 upregulation (Motoshima et al., 2006). As increased androgen receptor levels are often associated with increased aggressiveness in thyroid carcinomas and BBR has been shown to induce cell cycle arrest in ATC, this link between BBR and AMPKα expression levels was of interest in ATC. Upon BBR treatment, AMPKα phosphorylation was increased compared to vehicle controls **(18D)**; however, we note that the change was seen more predominantly in T238 cells, whereas the marginal increase in SW1736 mirrored that of immortalized normal cells (Nthy-ori-3-1). As all of these signaling pathways need to exist in normal cells at physiological levels, it is essential that BBR treatment did not change the activation of these pathways in our immortalized, normal epithelial cell line. Taken together, BBR modulates essential pro-proliferative, pro-survival, and metabolic signaling in ATC to reduce tumor aggressiveness *in vitro*.

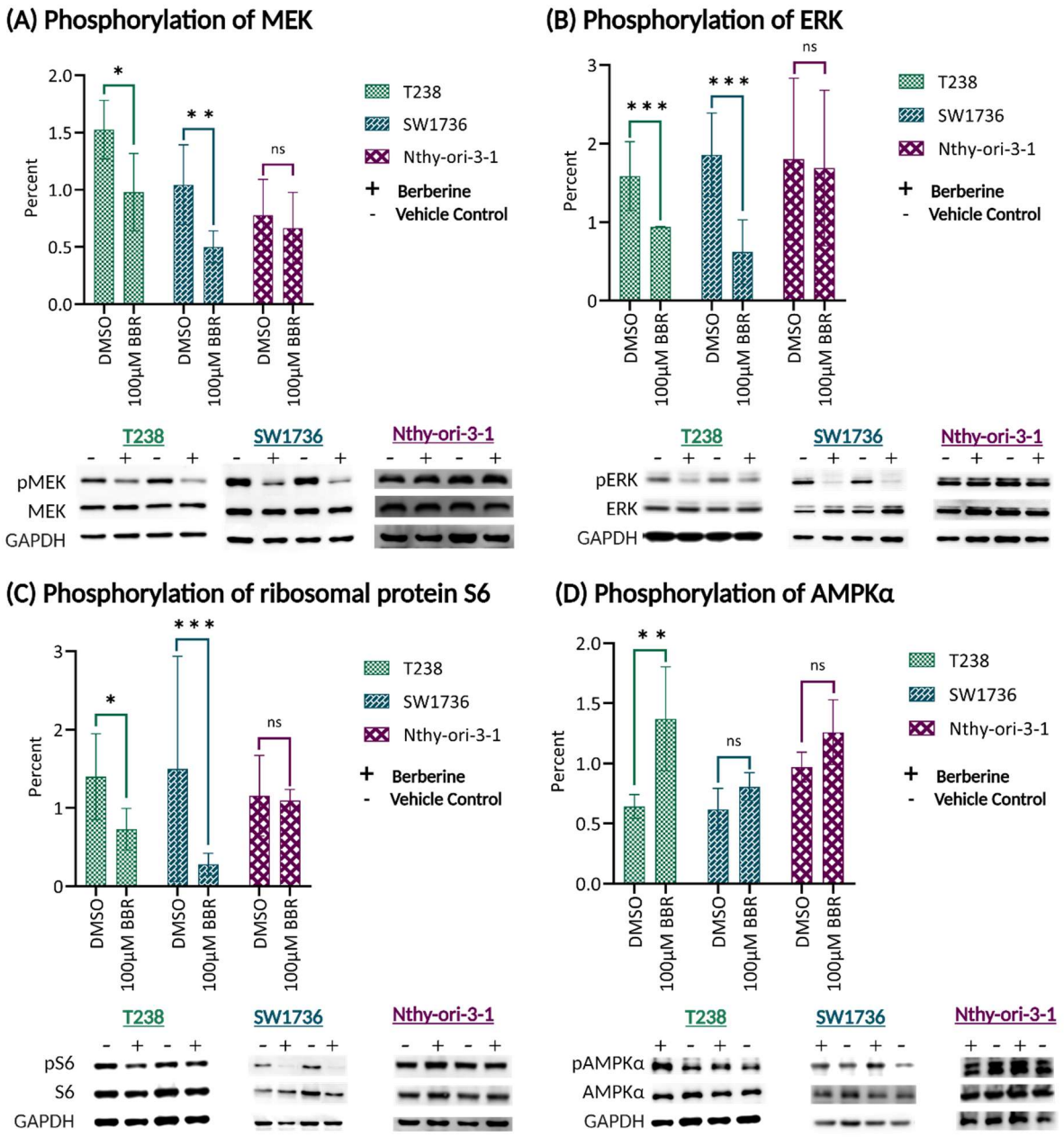


Figure 18. BBR fine-tunes phosphorylation in important downstream regulators of the pro-proliferative, pro-survival, and metabolic signaling pathways in anaplastic thyroid cancer *in vitro*. Following 24-hour 100µM BBR treatment, phosphorylation of **(A)** MEK, **(B)** ERK, and **(C)** ribosomal protein S6 in proliferating ATC (T238, SW1736) cells was significantly downregulated. **(D)** Increased AMPKα phosphorylation following BBR treatment was observed under the same conditions. BBR did not significantly impact the normal signaling of these proteins in immortalized normal thyroid epithelial cell line, Nthy-ori-3-1 **(A,B,C,D)**. A Mann-Whitney U Test was used for n=6 independent experiments per time point. *p<0.05, **p<0.01, ***p<0.001.

Summary of Results–

1. Berberine alters critical components of ATC's fatally aggressive phenotype by slowing proliferation, inducing apoptosis, decreasing motility, decreasing migratory capacity, and decreasing invasiveness.
2. Berberine inhibits overactive cell signal transduction pathways that have regulatory control over the above functional phenotypes.
3. Berberine induces necessary signaling for an anti-tumor effect.

Conclusions—

Anaplastic thyroid cancer is a particularly aggressive cancer with an extremely poor prognosis and no effective treatment strategies (Ferrari et al., 2019). Most notably, ATC's high proliferative rate and growth kinetics allow for it to expand beyond the thyroid locally and obstruct the patients' airways (Alhejaily et al., 2023). Additionally, ATC has a high propensity for local and distant invasion and metastasis (Li et al., 2023). Much of this is controlled by signaling pathways that are dysregulated in ATC, including inability of p53 to control cell cycle and apoptosis, constitutive activation of MAPK signaling contributing to rapid growth and proliferation, overactivation of PI3K/AKT signaling leading to replicative immortality and invasion, and under activation of anti-tumorigenic AMPK signaling (McFadden et al., 2014; Smallridge et al., 2009). This current study demonstrates that berberine treatment in ATC controls the phenotypic outputs that contribute to its aggressiveness. By regulating aberrant cell signal transduction pathways, BBR has a systemic downstream impact on critical drivers of ATC. We found BBR to specifically exert its control over-growth and proliferation in our ATC cells, while leaving the normal thyroid cells unscathed. Similarly, Park *et al.* found BBR to

inhibit growth in a different ATC cell line, 8505C (Park et al., 2012). From our aim 1 results, we have found that berberine reprograms the ATC phenotype by specifically targeting these hallmarks of cancer, including sustained proliferative signaling, activation of invasion and migration, and resistance of cell death mechanisms.

Specific Aim 2 – Berberine reduces the burden of soluble and cellular mediators of inflammation commonly found in the anaplastic thyroid cancer tumor microenvironment.

Experimental Design –

ATC has chronic inflammation that drives its aggressiveness that is mediated by infiltrating immune cells, cytokines, chemokines, and release of reactive oxygen species (Ferrari et al., 2019; Guarino et al., 2010; Liotti et al., 2012). Macrophages are essential players in the tumor microenvironment and have a particularly impactful role in the immune response to ATC as tumor associated macrophages (TAMs) are the most commonly observed immune cell population in the ATC TME (Ferrari et al., 2019; Guarino et al., 2010; Liotti et al., 2012; Palacios et al., 2022). TAMs account for over 50% of the ATC TME, and their infiltration is associated with worse prognosis (Jung et al., 2015; Palacios et al., 2022), so it is relevant to model how this immune cell population responds to BBR treatment.

U937 cells (human histocytic lymphoma origin) are one of the most widely used models to evaluate macrophage function and mimic inflammatory responses *in vitro* (Baek et al., 2009). Various stimuli can be used to induce terminal differentiation in U937 to commit them to a macrophage lineage, including TPA (Baek et al., 2009). Following differentiation, U937 cells can be stimulated with IFN-γ and LPS to polarize towards an inflammatory M1 macrophage phenotype (Baek et al., 2009). As previously noted, inflammation is a major issue in the ATC TME in both initiation and progression. After macrophage differentiation and polarization, we evaluate the expression of inflammatory mediators released by these cells into their conditioned media (CM) to observe their potential impact in setting up a niche in the ATC

TME. Additionally, we differentiated U937 cells and polarized them with ATC CM to evaluate the impact of the ATC cells themselves on macrophage polarization.

Rationale **–** Elevated presence of inflammatory cytokines and chemokines within the ATC microenvironment and infiltration of pro-inflammatory immune cell types serve a pro-tumorigenic role in ATC (Ferrari et al., 2019; Guarino et al., 2010; Liotti et al., 2012). ATC tissues have an extensive infiltration of a mixed population of tumor-associated macrophages (TAMs), including both pro-inflammatory M1 and pro-tumorigenic M2 macrophages, interspersed with the cancer cells. TAMs represent more than 50% of immune cells in ATC, illuminating their importance in ATC (Palacios et al., 2022). This is disparate from the focal TAMs present in PTC tissues (Kim et al., 2013). In general, a higher density of TAMs in the thyroid cancer TME correlates with a worse prognosis (Guarino et al., 2010; Kim et al., 2013; Palacios et al., 2022). Although M2 polarized macrophages are those with a classically pro-tumorigenic phenotype, aiding in immunosuppression, angiogenesis, and evading immune surveillance as tumor development advances, depending on their location and timing within the TME, M1 macrophages also have an essential pro-tumorigenic role in helping ATC to establish its inflammatory niche at the onset of tumor progression. In contrast to what is known in other cancers, in thyroid cancer, an inflammatory tumor immune microenvironment has a pro-tumorigenic role, whereas an autoimmune-like tumor immune microenvironment, typical in PTC, tends to be associated with a better prognosis. Inflammation in the context of cancer can play a further role in establishing primary and metastatic niches and enhancing tumor growth, invasive potential, and angiogenesis.

Activated pro-inflammatory M1 macrophages secrete numerous pro-inflammatory cytokines and chemokines.

Results –

We used NCBI's Gene Expression Omnibus (GEO) Database with Dataset GSE33630 to look for any differences in inflammatory mediators present in human ATC tissue vs. patient-matched normal thyroid tissue samples. Following this evaluation, elevated expression of Interleukin-1 Receptor Accessory Protein (IL-1RAP) (2.04log2 fold-change increase) **(Figure 19A)** and TNF-α induced protein 6 (TNFAIP6) (4.29log2 fold-change increase) **(Figure 19B)** was observed in patient data. IL-1RAP is a component of the IL-1 receptor complex. In other cancer models, increased IL-1α expression has been associated with dedifferentiation, malignant transformation, angiogenesis, proliferation, and activation of NF-kB and STAT3. As the receptor for this cytokine is upregulated in ATC patient samples compared to matched-normal tissue, this signaling is presumed to play an important role in ATC **(Figure 19A)**. We also found BBR can decrease IL-1α expression from activated macrophage conditioned media in our *in vitro* model **(Figure 21A)**. Further, TNFAIP6 expression was found to be upregulated in ATC patient tissue compared to matched-normal control tissue **(Figure 19B).** TNFAIP6 expression is induced by both TNF-α and IL-1 and is associated with increased inflammation—further validating that ATC tissue is rife with inflammation that contributes to its aggressiveness. As we found BBR can decrease TNF-α and IL-1α expression from activated macrophage conditioned media in our *in vitro* model **(Figure 21A)**, this could have an impact in controlling activation of TNFAIP6.

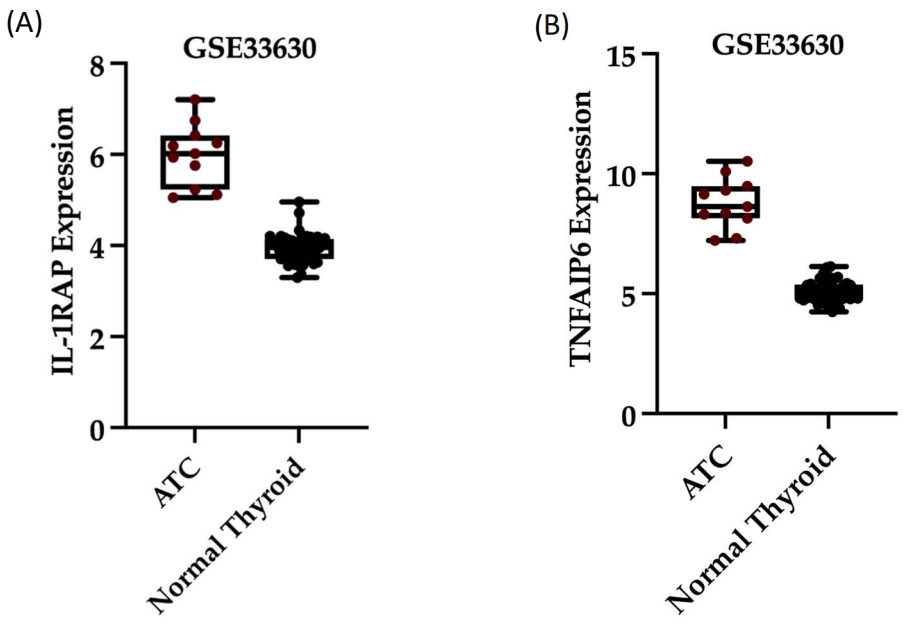


Figure 19. Increased expression of IL-1 RAP and TNF-α IP6 in ATC vs. patient-matched normal thyroid tissue. NCBI's GEO dataset GSE33630 was used to evaluate expression of (A) IL-1RAP and (B) TNFAIP6 in human ATC tissue vs. matched normal adjacent thyroid tissue.

We then assessed the secretory profile of pro-inflammatory M1 polarized macrophages in conditioned media following BBR treatment (**Figures 20, 21, 22)**. Forty pro- and anti-inflammatory cytokines and chemotactic factors were evaluated **(20A)**, and nineteen were found to be significantly differentially expressed **(Table 4)**. Low expression (categorized here as normalized relative expression less than 1000) **(21A, 22A)** and medium-to-high expression (classified here as normalized relative expression greater than 1000) **(21B, 22B)** of all forty cytokines and chemokines following normal activation and polarization steps established a baseline level of cytokine and chemokine expression *in vitro* **(20B/C, 21A/B, 22A/B).** Low and high expression levels correspond to physiologically lower and high baseline levels of the cytokines mentioned. BBR treatment significantly decreased expression of pro-inflammatory cytokines in both the low expressor (IL-1α {76% decrease}, IL-1β {97%

decrease}, IL-6 {98% decrease}, IL-11 {62% decrease}, IL-12 p40 {82% decrease}, IL-12 p70 {92% decrease}, TNF-α {93% decrease}) **(21A)** and the high expressor (IFN-γ {55% decrease}, IL-6sR {92% decrease}, sTNF RI {80% decrease}, sTNF RII {83% decrease}) **(21B)** groups. Similarly, BBR treatment decreased expression of pro-inflammatory chemokines, adhesion molecules, and other chemotactic factors in both the low expressor (eotaxin-1 {72% decrease}, eotaxin-2 {93% decrease}, ICAM-1 {77% decrease}, M-CSF {55% decrease}, PDGF-BB {83% decrease}) **(22A)** and high expressor (I-309 {95% decrease}, IP-10 {65% decrease}, MIP-1-β {79% decrease}, TIMP-2 {65% decrease}) **(22B)** groups. Ablation of this chronic inflammation in ATC is essential for advancing therapeutic and diagnostic potential in ATC. BBR's ablation of inflammation steers ATC in a less aggressive and, subsequently, more treatable direction.

(A)

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
L1	POS	POS	NEG	NEG	EOTAXIN-1	EOTAXIN-2	G-CSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
L2	POS	POS	NEG	NEG	EOTAXIN-1	EOTAXIN-2	G-CSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
L3	Il-1β	IL-2	IL-3	IL-4	IL-6	IL-6 sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
L4	Il-1β	IL-2	IL-3	IL-4	IL-6	IL-6 sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
L5	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1-α	MIP-1-β	MIP-1-δ
L6	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1-α	MIP-1-β	MIP-1-δ
L7	RANTES	TGF-β1	TNF-α	TNF-β	sTNF-R1	sTNF-R2	PDGF-BB	TIMP-2	BLANK	BLANK	NEG	POS
L8	RANTES	TGF-β1	TNF-α	TNF-β	sTNF-R1	sTNF-R2	PDGF-BB	TIMP-2	BLANK	BLANK	NEG	POS

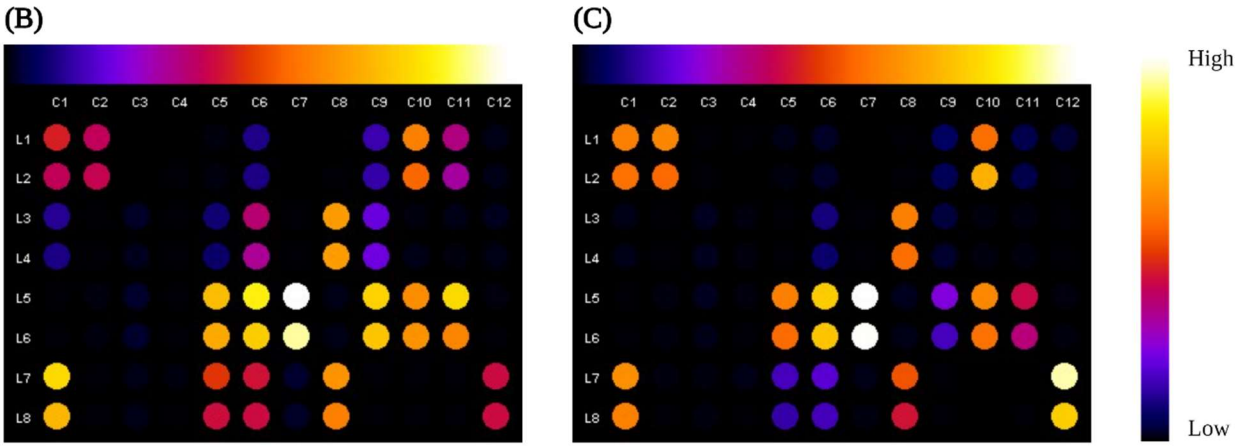


Figure 20. Berberine lessens inflammatory cytokine and chemokine expression in conditioned media of M1-activated and polarized U937 cells. (A) Map of cytokines and chemokines evaluated in order, consistent with **(B)** and **(C).** Representative densitometry expression of cytokines and chemokines in the conditioned media of U937 cells activated with TPA and polarized with IFN-γ + LPS with **(B)** DMSO vehicle control or **(C)** 100µM BBR added at the activation and polarization stages. n=3 biological replicates; n=2 technical replicated per biological replicate.

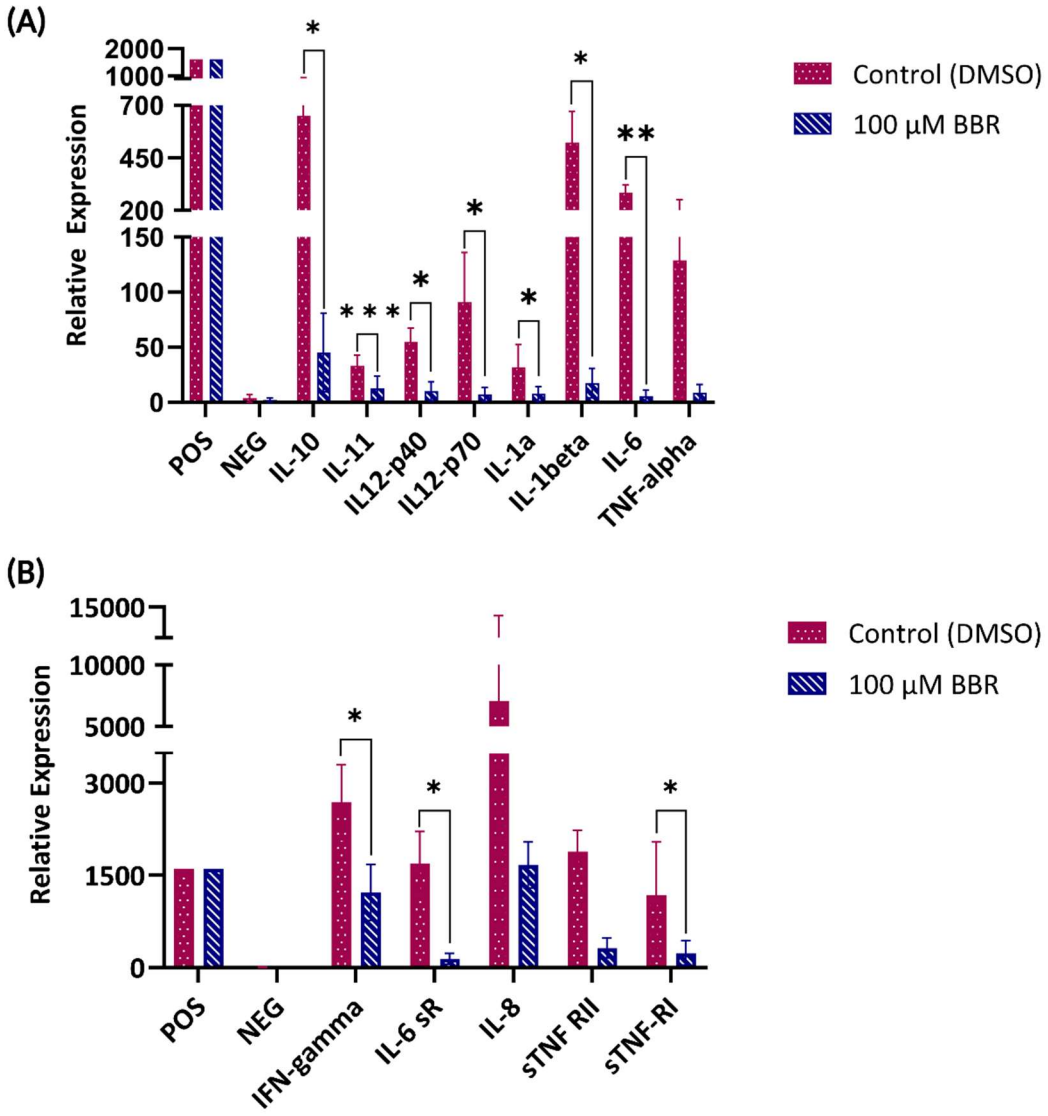


Figure 21. Berberine lessens inflammatory cytokine expression in conditioned media of M1-activated and polarized U937 cells. Expression levels of soluble pro-inflammatory cytokines with relative expression **(A)** below 1000 and **(B)** above 1000. A Mann-Whitney U Test was used for n=3 independent experiments. n=3 biological replicates; n=2 technical replicated per biological replicate. *p<0.05, **p<0.01, ***p<0.001

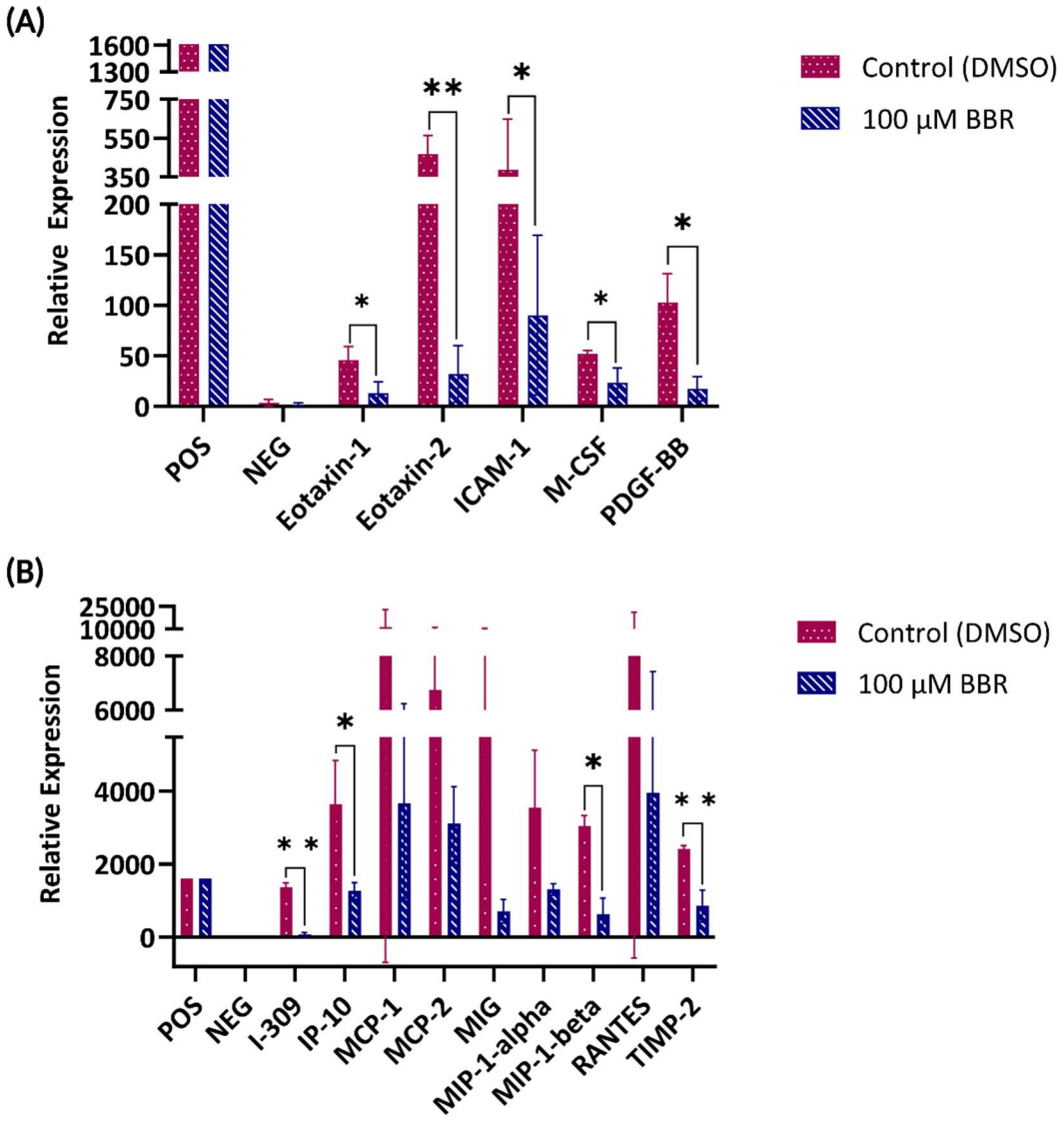


Figure 22. Berberine lessens inflammatory chemotactic factor expression in conditioned media of M1-activated and polarized U937 cells. Expression levels of soluble pro-inflammatory chemokines and adhesion molecules with relative expression **(A)** below 1000 and **(B)** above 1000. A Mann-Whitney U Test was used for n=3 independent experiments. n=3 biological replicates; n=2 technical replicated per biological replicate. *p<0.05, **p<0.01, ***p<0.001

Cytokine/ Chemokine Name	Selected Known Functions
Eotaxin-1	marker of allergic responses, but also serve as biomarkers in some cancers (Cheadle et al., 2007; Zajkowska & Mroczko, 2020, 2021)
Eotaxin-2	strongly associated with primary and metastatic tumors in colorectal cancer (Cheadle et al., 2007; Zajkowska & Mroczko, 2020, 2021)
I-309	promotes chemotaxis and invasion and prevents apoptosis (Bernardini et al., 2000; Jin et al., 2017; Louahed et al., 2003)
ICAM-1	adhesion molecule & biomarker in various cancers; can promote angiogenesis, metastasis, and weaken immune response against tumor cells (Benedicto et al., 2017; Bui et al., 2020; Lim et al., 2022; Lin et al., 2006)
IFN-γ	pro- and anti-tumorigenic functions in cancer; induces PD-L1 and IDO expression on tumors, stimulating immune-suppressive mechanisms; EMT induction through JAK/STAT1 signaling (Chen et al., 2011; Jorgovanovic et al., 2020; Lin et al., 2006; Lv et al., 2015)
IL-1α	dedifferentiation, malignant transformation, angiogenesis, proliferation, and activation of NF-kB and STAT3 (Gelfo et al., 2020; Xi et al., 2020)
IL-1β	metastasis, angiogenesis, EMT, growth, invasion, adhesion, and immune cell programming (Gelfo et al., 2020; Rébé & Ghiringhelli, 2020; Zhang & Veeramachaneni, 2022)
IL-6	associated with chronic inflammation and carcinogenesis – prolonged activation associated with cancer development and metabolic disorders – activates JAK/STAT3, PI3K/AKT and MAPK signaling (Bollrath et al., 2009; Hirano, 2021; Rašková et al., 2022; Tanaka et al., 2014)
IL-6sR	has an agonist effect with IL-6 by forming a heterodimer to promote proliferation and invasiveness (Rašková et al., 2022)
IL-8	promotes EMT, stemness of TC cells, invasiveness, motility, and angiogenesis; stimulates AKT phosphorylation and Slug expression in TC (Bauerle et al., 2014; Gelfo et al., 2020; Visciano et al., 2015)
IL-11	promotes EMT in ATC through PI3K/Akt/GSK3β signaling pathway activation (Zhong et al., 2016)
IL-12 p40/p70	serum levels in TC are positively associated with IL-1β, IL-5, and IL-6 levels (Provatopoulou et al., 2014)
IP-10	promotes tumor growth, migration, and invasion and recruits regulatory T cells (Lunardi et al., 2015)
M-CSF	associated with high tumor grade, more metastases, and poor prognosis in multiple tumor types
MIP-1-β	associated with high TAM infiltration, high tumor grade, more metastases, and poor prognosis

PDGF-BB	stimulates proliferation, migration, and apoptotic resistance; strong mitogenic agent (Paek et al., 2020)
TIMP-2	elevated expression associated with unfavorable survival outcomes in multiple cancers; associated with cancer initiation and progression (Wang et al., 2022)
TNF-α	correlated with augmented proliferation, metastasis, malignancy grade, and poor prognosis in breast cancer; co-expression with Il-6 and/or IFN-γ has been associated with increased aggressiveness in tumors (Lv et al., 2012; Tripsianis et al., 2014)

Table 4. Selected known functions from literature of cytokines and chemokines significantly downregulated by BBR treatment in our U937 model.

The levels of two important secreted inflammatory mediators, IFN-γ and TNF-α, secreted from the conditioned media of U937 cells classically activated and polarized in the presence of absence of berberine were confirmed via ELISA. BBR significantly decreased IFN-γ expression **(Figure 23)** and TNF-α expression **(Figure 24).**

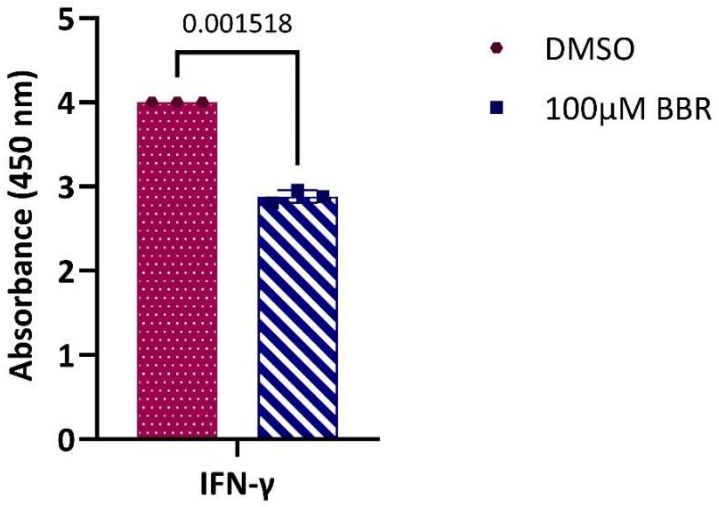


Figure 23. Berberine decreases IFN-γ expression in conditioned media of M1-activated and polarized U937 cells. (A) IFN-γ expression in conditioned media. Absorbance was measured at 450 nm. n=3, p=0.001518. A Mann-Whitney U Test was used for n=6 independent experiments. *p<0.05, **p<0.01, ***p<0.001

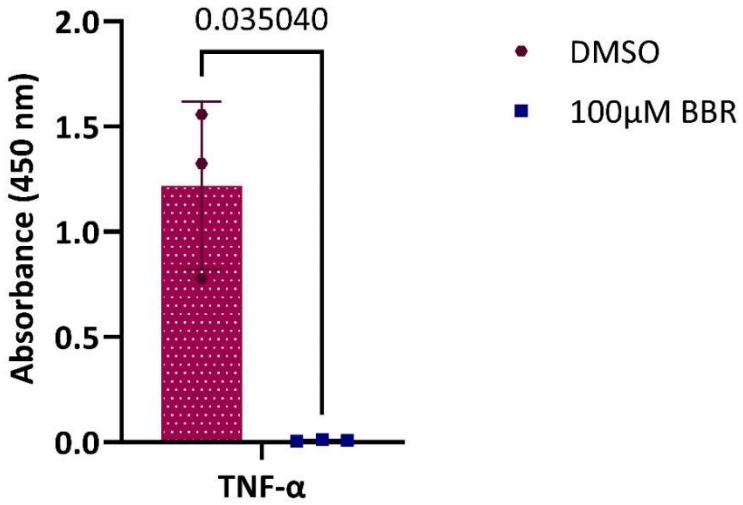


Figure 24. Berberine decreases TNF-α expression in conditioned media of M1-activated and polarized U937 cells. (A) TNF-α expression in conditioned media. Absorbance was measured at 450 nm. n=3, p=0.03504. A Mann-Whitney U Test was used for n=6 independent experiments. *p<0.05, **p<0.01, ***p<0.001

We further evaluated the ability of ATC cells treated with BBR to alter the polarization of U937 cells. Following U937 activation with TPA for 24 hours, cells were treated for 48 hours with conditioned media from control ATC cells, ATC cells treated with 100 µM BBR for 24 hours, or complete media alone. Fresh media without FBS replaced the CM and this new CM was collected after an additional 48 hours. Levels of IFN-γ and TNF-α were evaluated via ELISA. IFN-γ levels were marginally decreased in CM from U937 cells polarized with BBR-treated ATC CM compared to those polarized with control ATC CM **(Figure 25)**. TNF-α levels were significantly decreased in CM from U937 cells polarized with BBR-treated ATC CM compared to those polarized with control ATC CM **(Figure 25)**.

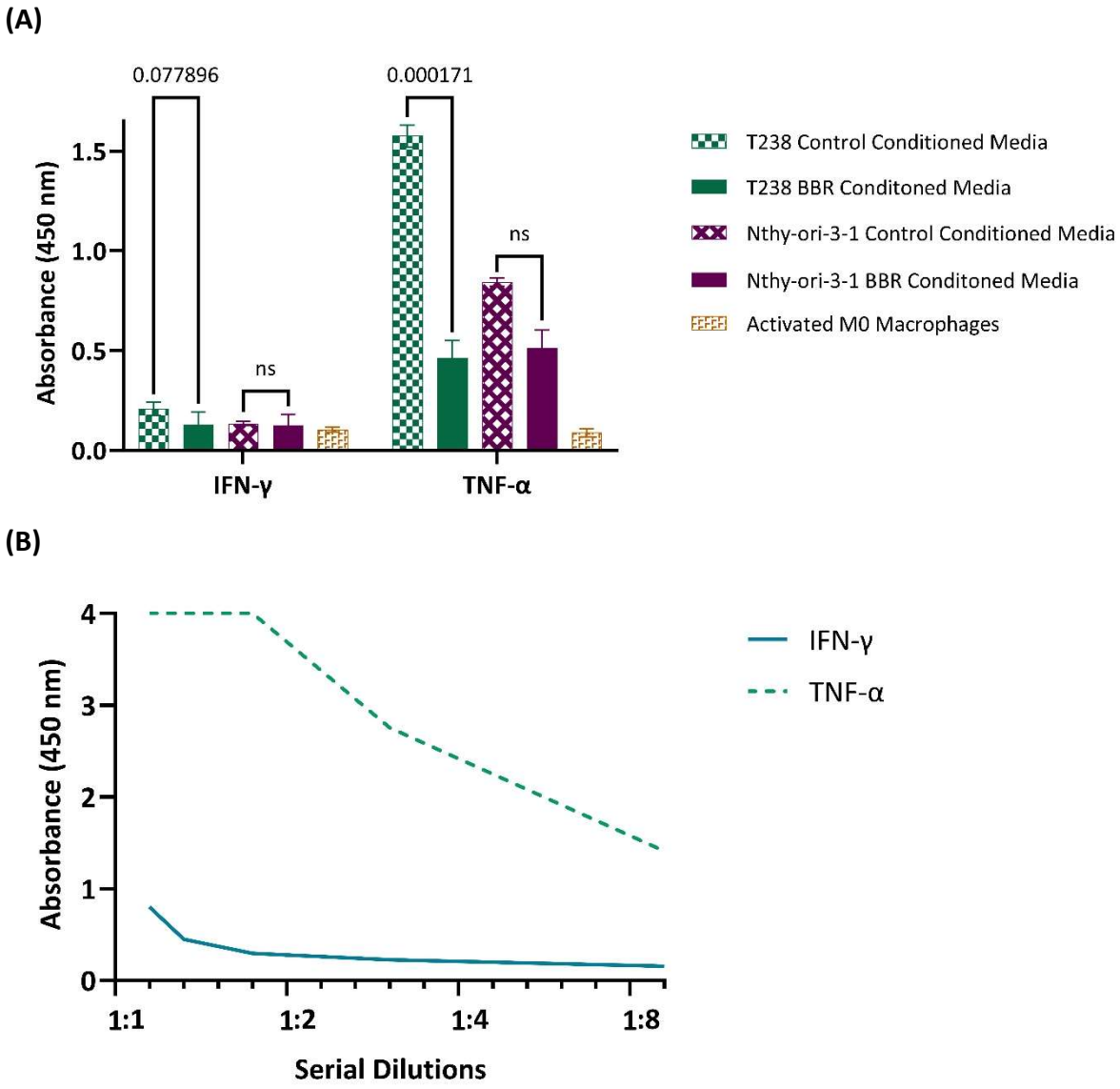


Figure 25. Conditioned media from ATC cells treated with BBR impacts the polarization and release of soluble inflammatory mediators from activated macrophages *in vitro.* (A) Berberine decreases IFN-γ and TNF-α secreted from U937 cells activated with TPA and polarized with ATC conditioned media. **(B)** IFN-γ and TNF-α standards following four serial dilutions. Absorbance was measured at 450 nm. n=6, p=0.03504. A Mann-Whitney U Test was used for n=6 independent experiments. *p<0.05, **p<0.01, ***p<0.001

Summary of Results –

1. The ATC TME has a high, diffuse expression of a mixed population of TAMs, which is associated with a poor prognosis.
2. BBR may alter TAM activation and polarization to a particular phenotype that is not as inflammatory or as immunosuppressive.
3. BBR alters the inflammatory immune infiltrates secreted by classically activated macrophages.
4. Release of soluble mediators from ATC cells treated with BBR alters macrophage polarization.

Conclusions—

The inflammatory cytokines, chemokines, and adhesion molecules observed to be downregulated in conditioned media of macrophages activated and polarized with BBR have been previously implicated in cancer initiation, progression, and poor prognosis in other cancer types (Gelfo et al., 2020). Immense inflammation promotes tumor progression, and also induces immunosuppression in the tumor microenvironment via recruitment of neutrophils, tumor-associated macrophages, myeloid-derived suppressor cells, regulatory dendritic cells, and regulatory T cells (Gelfo et al., 2020). Focusing on the molecules with the most significant decrease following BBR treatment in activated macrophage conditioned media in our study, there are evident roles in ATC aggressiveness that are regulated by these molecules.

Unlike many other cytokine expression levels, the role of IL-11 has been explored in ATC. Zhong *et al.* found IL-11 expression to be positively correlated with distant metastases in ATC (Zhong et al., 2016). This increase in IL-11 expression induces EMT, invasion, and migration in ATC cells through PI3K/AKT/GSK3β activation, thus elucidating its pro-metastatic role in ATC (Zhong et al., 2016). As this is a known functional role for IL-11 in ATC, BBR's ability to alleviate IL-11 expression levels could be an essential piece in controlling invasion and metastasis.

IL-1α and IL-1β can both stimulate angiogenesis through macrophage recruitment and other VEGF-expressing inflammatory cells (Gelfo et al., 2020; Xi et al., 2020). IL-1α specifically stimulates production of IL-8 (Gelfo et al., 2020). In other tumor models, IL-1β facilitates metastasis by inducing cytokine production, angiogenesis, proliferation, epithelial-to-mesenchymal transition, growth, invasion, and adhesion (Rébé & Ghiringhelli, 2020; Zhang & Veeramachaneni, 2022). Its role in tumor progression is partially due to its effect on other immune cells in the TME (Rébé & Ghiringhelli, 2020; Zhang & Veeramachaneni, 2022). As these are core aberrant features in ATC, BBR treatment reducing secreted IL-1α and IL-1β from macrophages could have an important role in controlling these features in a more dynamic model of the TME. Additionally, serum levels of IL-12 in TC and inflammatory thyroid diseases are positively associated with IL-1β, IL-5, and IL-6 levels (Provatopoulou et al., 2014). IL-6 has detrimental effects during carcinogenesis and in chronic inflammation as well, which is often seen prior to or in the establishment of ATC (Hirano, 2021; Rašková et al., 2022; Tanaka et al., 2014). Additionally, prolonged release of IL-6 is associated with cancer development, as well as obesity and metabolic disorders through its activation of JAK/STAT3,

PI3K/AKT and MAPK signaling (Hirano, 2021). IL-6 soluble receptor has an agonistic effect with IL-6 through formation of a heterodimer that can bind to cells that do not have IL-6 receptor through a trans mechanism that further induces the pro-proliferative and pro-survival pathways described above (Rašková et al., 2022). The initiation of IL-6-STAT3 signaling can induce EMT, tumor migration, and cancer stemness (Hirano, 2021; Rašková et al., 2022). In colitis-induced tumorigenesis, IL-6 activation of STAT3 is required for intestinal epithelial cell survival and tumor progression (Bollrath et al., 2009; Hirano, 2021). Similar to colitis-induced tumorigenesis, development of ATC is also preceded by significant inflammation. As such, BBR's role in decreasing expression of Il-6 could serve an important function on the control over ATC cells. Investigation into whether Il-6 targeting is being investigated in cancer uncovered that tocilizumab, an Il-6 inhibitor drug, is in multiple phase 1-4 clinical trials for a variety of inflammatory diseases, including recurrent ovarian cancer (Tanaka et al., 2014). Further, crosstalk of Il-6 with TNF-α and IL-1β through the p38 MAPK stress-induced pathway and NFκB leads to cancer propagation and survival (Hirano, 2021; Rašková et al., 2022).

In breast cancer, co-expression of high IL-6 and TNF-α expression correlates with increased tumor cell proliferation, higher malignancy grade, increased lymph node involvement, increased lymphovascular invasion, increased incidence of metastasis, significantly shorter survival, and overall poor prognosis (Tripsianis et al., 2014). As these are common features seen in ATC, the reduction of TNF-α levels observed in both the inflammation array and confirmed via ELISA following BBR treatment could have an influential impact on the ATC TME. Similarly, in an *in vitro* PTC model, high co-expression of TNF-α and IFN-γ increased

downregulated E-cadherin expression, upregulated N-cadherin and vimentin expression levels, and subsequently induced both EMT and invasion and migration of PTC cells (Lv et al., 2015).

IFN-γ has a complicated role in the tumor microenvironment, orchestrating both pro-tumorigenic and antitumor immunity. IFN-γ is well-established to act as a cytotoxic cytokine together with granzyme B and perforin to initiate apoptosis in tumor cells (Jorgovanovic et al., 2020). However, IFN-γ also has pro-tumorigenic functions within the TME. IFN-γ enables the synthesis of immune checkpoint inhibitory molecules and indoleamine-2,3-dioxygenase (IDO), thus stimulating other immune-suppressive mechanisms (Jorgovanovic et al., 2020). It also has essential metastatic functions in promoting EMT through JAK/STAT1 activation and interaction with IFN-induced proteins and microRNAs—these effects have been observed in prostate cancer, renal cell carcinoma, and pancreatic cancer (Chen et al., 2011; Jorgovanovic et al., 2020). IFN-γ also induces expression of IP-10, whose presence in pancreatic cancer is associated with increased regulatory T cell recruitment and poor prognosis (Lunardi et al., 2015). Although further investigation into the role of IFN-γ in ATC specifically is warranted, as BBR is still inducing apoptosis in these tumor cells by other mechanisms, IFN-γ may be playing a larger role in stimulating immune suppression in this context, particularly with its co-expression with TNF-α and induction of ICAM-1 expression and activity both leading to increased invasion and worse prognosis in many tumor types (Lin et al., 2006; Lv et al., 2015). Cancer cells demonstrate increased adhesion strength than normal cells, allowing for the formation of stable focal adhesions, the ability to survive despite mechanical stress, and increased proliferation on the extracellular matrix (Yayan et al., 2024). IFN-γ induces ICAM-1

expression in tumor models, enhancing cancer cell migration (Bui et al., 2020; Lin et al., 2006). ICAM-1 primarily acts as an adhesive molecule; however, it can also drive tumorigenesis through promotion of metastasis and angiogenesis and weakening of the immune response in cancer cells (Bui et al., 2020; Lim et al., 2022). Therefore, it is known as a biomarker in various cancer types, including colorectal cancer, melanoma, and lymphoma (Benedicto et al., 2017). This molecules expression is increased in the more invasive and aggressive variants of some cancers, including triple-negative breast cancer and non-squamous cell lung cancer (Bui et al., 2020). Further, high ICAM-1 expression in the liver may influence liver metastases in many cancers (Benedicto et al., 2017). As ICAM-1, I-309, IL-1α, IL-1β, Il-6, and Il-8 have all be observed to have pro-angiogenic functions in different tumor models, the role of BBR in suppressing the secretion of these molecules could have a similar growth-suppressive impact in ATC that was observed by Jin *et al.* using an angiogenesis inhibitor.

Eotaxin-2 is a chemokine strongly associated with primary and metastatic tumors of colorectal cancer, however, its role in ATC remains unexplored to this point (Cheadle et al., 2007; Zajkowska & Mroczko, 2020, 2021). Similarly, eotaxin-1 is a major marker of allergic responses but has also recent been observed to be an upregulated biomarker in some cancers, particularly in oral squamous cell carcinoma cells (Cheadle et al., 2007; Zajkowska & Mroczko, 2020, 2021). As these were two of the most significantly downregulated chemokines in this data following BBR treatment, it stands to question if these are also markers of aberrant inflammatory processes in ATC leading to increased aggressiveness.

I-309 further induces MAPK signaling and has anti-apoptotic functions, thus protecting the tumor cells when it is available in the TME (Louahed et al., 2003). Further, Bernardini *et al.* found I-309 to induce chemotaxis, invasion, and differentiation of human umbilical vein endothelial cells and also demonstrated this pro-angiogenic functionality in rabbit cornea and chick chorioallantoic membrane assays (Bernardini et al., 2000). A study by Jin *et al.* investigated the function of an anti-angiogenic drug in ATC and found dose-dependent effects on inhibiting ATC cell growth through suppression of AKT/GSK3β signaling (Jin et al., 2017). TIMP-2 is associated with cancer initiation and progression, and its elevated expression is further associated with unfavorable survival outcomes in multiple cancers (Wang et al., 2022). Similarly, M-CSF expression is increased in advanced stage tumors, specifically, and thus associated with worse prognosis in human cancers, such as breast cancer, ovarian epithelial tumors, endometrioid cancers, and papillary renal cell carcinoma (Laoui et al., 2014). In Ewing Sarcoma, high density of TAMs and subsequently high MIP-1-β levels are associated with poor prognosis (Fujiwara et al., 2011). As ATC also has a high proportion of TAMs in its TME, BBR's ability to mediate MIP-1-β levels could be essential. PDGF-BB can stimulate proliferation, and is also deemed to be a strong mitogenic agent (Paek et al., 2020). Overall, presence of all of these inflammatory mediators released into the ATC TME can have a deleterious effect on immune activation, induction of apoptosis, growth and proliferation, invasion and metastasis, angiogenesis, and EMT, amongst other mechanisms. The downregulation of these mediators by BBR treatment and the ability of BBR to alter the polarization of these macrophages, could serve an essential role in the dynamic TME of ATC.

Specific Aim 3 – BBR induces metabolic changes in ATC that alter the tumor's energetics.

Experimental Design –

T238 cells were treated with 100 µM BBR or equal volume of vehicle control and sent to Genewiz by Azenta Life Sciences for RNA Sequencing. RNA Sequencing data was returned with most significantly DEGs. Data was mined and Qiagen's Ingenuity Pathway Analysis (IPA) was used for further *in silico* analysis and determination of most significantly modulated pathways post-BBR treatment. Following comprehensive *in silico* analysis and pathway analysis, protein expression of oxidative phosphorylation complexes was evaluated to see if a similar effect was seen at the protein level as was demonstrated at the transcript level. Finally, production of superoxide, which results as leakage from the electron transport chain in OXPHOS complex I, was evaluated in ATC cells and normal thyroid epithelial cells with and without BBR treatment.

Rationale –

A major driver of anaplastic thyroid cancer is its ability to use a two-compartment system of metabolism to meet the tumor's extensive energetic requirements (Johnson et al., 2015; Gill et al., 2016). If a drug is able to inhibit this increased energy production and block the tumor's ability rich energy supply, it stands to reason that this could kill the tumor cells, or at least lessen the extremely rapid growth rate of these tumor cells specifically. As a person's normal cells also need to be able to utilize OXPHOS to generate energy, it is essential that using a mitochondrial inhibitor as a therapy impacts metabolism in the tumor cells, specifically. Recently, attention in berberine research has been focused on mitochondrial inhibition in

other disease mechanisms and pathologies, including diabetes and insulin resistance, cardiovascular disease, leishmania infection, chronic lymphocytic leukemia, and hepatocellular carcinoma (Fang et al., 2022; Lv et al., 2012; Ravera et al., 2020; Turner et al., 2008; Yan et al., 2017; Yin et al., 2012). In many of these pathologies, this process interconnects with AMPKα signaling, which we have previously found to be increased following BBR treatment. In diabetes specifically, BBR has a known role as an AMPK activator with mitochondrial inhibition leading to activation of the AMPK pathway via an increase in the adenosine monophosphate to adenosine triphosphate (AMP:ATP) ratio—this occurs as mitochondrial inhibition limits production of ATP (Yin et al., 2012). The AMPK energy sensing system monitors cellular energy levels via the AMP:ATP ratio and, in the context of diabetes, AMPK is activated to increase insulin sensitivity and regulate mitochondrial function (Yin et al., 2012). Following BBR treatment, AMPK phosphorylation began to increase in liver cells by 30 minutes post-exposure and sustained in cells for greater than 16 hours (Yin et al., 2012). Our findings have found this increase to remain in tumor cells for greater than 96 hours. As BBR has this mitochondrial inhibitory effect and subsequent AMPK activating effect in other models, we are curious to explore if mitochondrial inhibition in ATC can regulate its two-component metabolism and not allow the tumor cells to receive their vast energetic needs.

Results –

RNA Seq analysis **(Figure 26A)** revealed that BBR-treated T238 cells clustered away from T238 control cells in a Principal Component Analysis (**Figure 26B)**. 439 significant differentially

expressed genes were identified in this data set displayed as a volcano plot clustering significantly differentially expressed genes upregulated by berberine treatment (in red) and downregulated by berberine treatment (in blue) **(Figure 26C)**.

The thirty DEGs with the highest level of significance are shown through a heat map with difference in expression across vehicle control and BBR treated ATC cells **(Figure 27A)**. Among these 30 genes, eleven are mitochondrial-encoded genes **(Figure 27A)**. IPA was then used to map connections between the significant DEGs and the pathways they are involved in or regulate. This analysis found that mitochondrial dysfunction pathways were significantly downregulated following BBR treatment and likewise oxidative phosphorylation was significantly downregulated **(Figure 27B).** This reveals a role for BBR as a mitochondrial inhibitor in this ATC tumor model, which is a finding that has not been previously observed in literature. Additionally, p53 signaling was significantly upregulated following BBR treatment **(Figure 27B)**. As p53 mutation is an essential propagating driver of ATC initiation and progression, upregulating its signaling pathways (likely in a p53-independent fashion) is vital in controlling replicative immortality and rapid, unchecked division of ATC cells. This also essentially induced apoptosis in tumor cells. Finally, sirtuin signaling is both significantly upregulated and downregulated **(Figure 27B)**. Although initially counterintuitive, of the seven mammalian sirtuins, about half are tumor suppressors and the other half have oncogenic roles. Although the sirtuins themselves were not differentially expressed in our data set, the pathways they are involved in include AMPK, MAPK, PI3K/AKT, and p53 signaling, as well as the presence of sirtuins 3, 4, and 5 in the mitochondria interacting with OXPHOS, apoptosis, and autophagy mechanisms.

As mitochondrial-encoded genes represented many of the most significant differentially expressed genes and mitochondrial dysfunction and oxidative phosphorylation were the most significantly differentially expressed pathways, we evaluated expression of all mitochondrial-encoded genes. The human mitochondria DNA (mtDNA) sequence map **(Figure 28A)** shows all of the mitochondrial-encoded coding genes and their position within each of the five OXPHOS complex subunits. The mitochondrial-encoded tRNAs are represented in black and the rRNAs in green **(Figure 28A)**. Mitochondria encode 37 total genes: 13 protein-coding, 22 tRNAs, and 2 rRNAs. It also encodes for pseudogenes. All 13 of the mitochondrial-encoded protein-coding genes were downregulated in ATC following BBR treatment **(Figure 28B)**. Four pseudogenes and nine tRNAs are significantly downregulated in this data set as well **(Figure 28B)**.

Seven of these protein-coding genes and two pseudogenes belong to the OXPHOS Complex I **(Figure 28B)**. Complex I is the nicotinamide adenine dinucleotide + hydrogen (NADH) dehydrogenase complex. Following differential centrifugation to isolate mitochondria from T238 and Nthy-ori-3-1 cells, protein expression of the 39 kDa subunit of OXPHOS Complex I was evaluated **(Figure 29B)** following mitochondrial isolation via differential centrifugation **(Figure 29A)**. BBR treatment decreased the expression of this subunit in BBR-treated ATC cells, but not in normal thyroid cells **(Figure 29B)**. This demonstrates that expression is not just altered by berberine at the RNA level, but at the protein level as well. Importantly, this effect is seen selectively in ATC cells, allowing normal cells to still meet their necessary energy requirements via OXPHOS.

IPA was subsequently used to interrogate connections between BBR downregulation of OXPHOS components and other essential cellular pathways. In addition to the connection between decreased OXPHOS and increased AMPK signaling, which has an anti-tumorigenic role when activated in cancer, connections to ROS production and apoptotic induction were illuminated **(Figure 30)**. Induction of apoptosis, as seen following BBR treatment of ATC cells in aim 1 in this study, is essential for control of ATC spread.

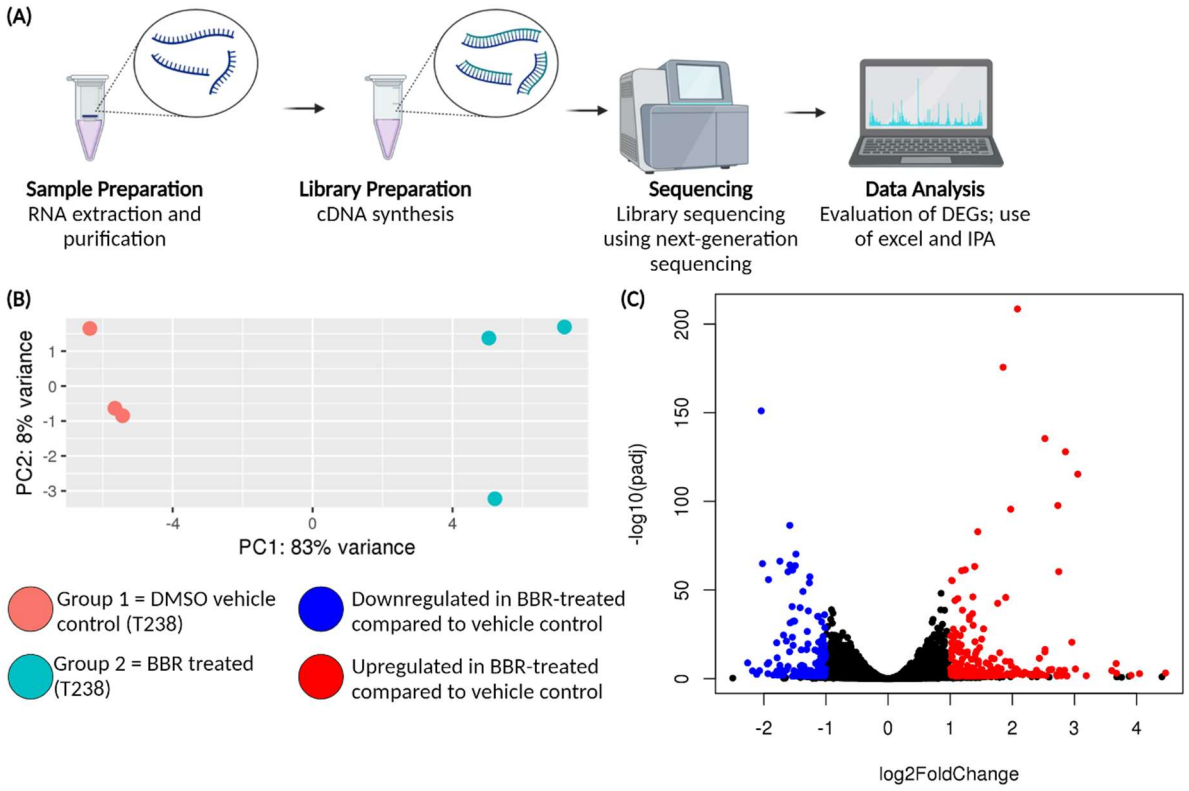


Figure 26. RNA Sequencing analysis of T238 cells treated with BBR vs. vehicle control. (A) Schematic of RNA Sequencing workflow. **(B)** Principal Component Analysis demonstrating 83% variance between vehicle control (salmon) and BBR-treated (aqua) T238 groups. **(C)** Volcano Plot of significantly downregulated (blue) and upregulated (red) transcripts in BBR-treated T238 group compared to vehicle control. n=3

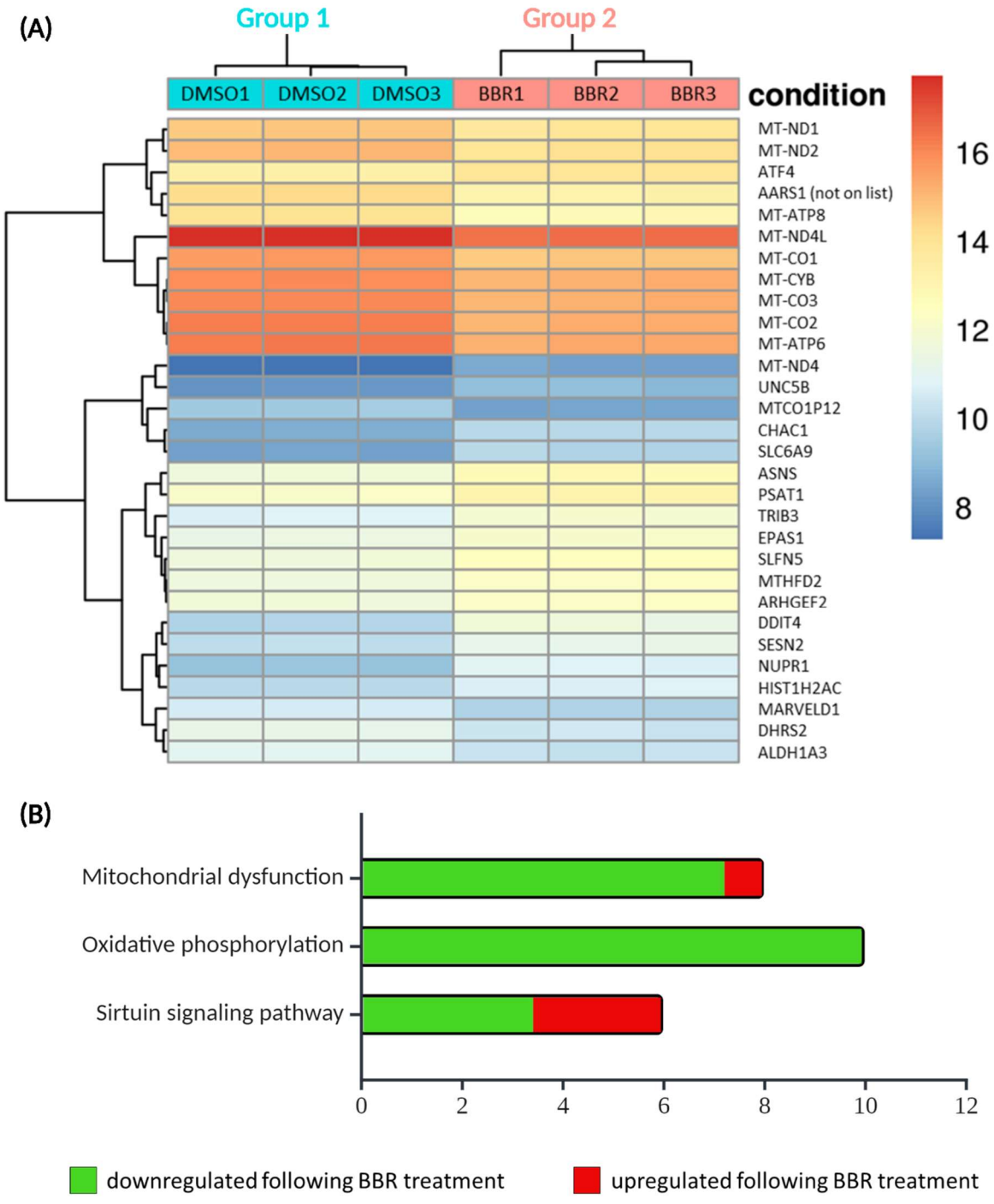
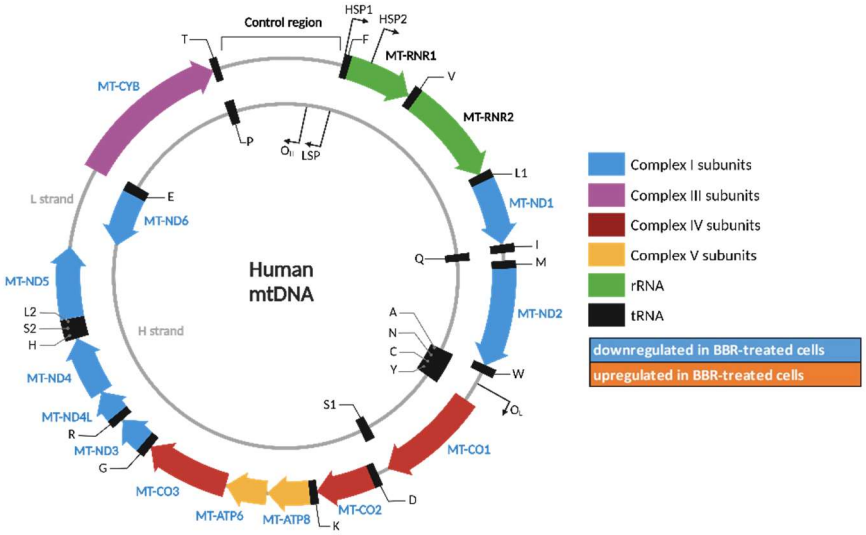


Figure 27. Differential expression of important genes and pathways of T238 cells treated with BBR vs. vehicle control. (A) Heat map of the most significantly differentially expressed genes in the RNA Seq data set of BBR-treated vs. vehicle control T238 cells. **(B)** Gene ontology analysis of most the significantly changed pathways following BBR treatment in this data set revealed via *in silico* analysis using Qiagen's Ingenuity Pathway Analysis Software.

(A)



(B)

Gene name	Complex	log2FoldChange	pvalue	padj
MT-ND4L	I	-2.02	1.1E-68	1.4E-65
MT-ND5	I	-1.61	6.2E-09	1.4E-07
MT-ND4	I	-1.61	6.9E-64	5.1E-61
MT-ND2	I	-1.53	6.6E-65	5.6E-62
MT-ND1	I	-1.49	1.7E-67	1.9E-64
MT-ND3	I	-1.13	2.2E-38	8.0E-36
MT-ND6	I	-1.12	2.0E-23	2.9E-21
MTND4P12	I (pseudogene)	-2.12	6.9E-04	4.0E-03
MTND2P28	I (pseudogene)	-1.36	3.5E-09	8.2E-08
MT-CYB	III	-1.58	2.0E-90	3.6E-87
MT-CO1	IV	-2.04	1.7E-155	9.2E-152
MT-CO2	IV	-1.37	1.2E-52	6.8E-50
MT-CO3	IV	-1.26	4.7E-61	3.4E-58
MTCO1P12	IV (pseudogene)	-1.93	2.1E-59	1.4E-56
MT-ATP8	V	-1.74	4.2E-70	5.7E-67
MT-ATP6	V	-1.58	6.7E-68	7.8E-65
MT-TS2	tRNA	-2.18	3.8E-06	4.5E-05
MT-TH	tRNA	-1.79	5.6E-06	6.3E-05
MT-TL2	tRNA	-1.79	3.4E-14	1.8E-12
MT-TN	tRNA	-1.42	2.0E-43	9.0E-41
MT-TY	tRNA	-1.40	6.5E-19	6.2E-17
MT-TG	tRNA	-1.34	2.9E-04	1.9E-03
MT-TP	tRNA	-1.30	1.1E-29	2.6E-27
MT-TT	tRNA	-1.25	9.0E-03	3.5E-02
MT-TC	tRNA	-1.10	3.7E-09	8.6E-08
MT-TM	tRNA	2.17	1.1E-03	5.8E-03
MTRNR2L12	rRNA	2.27	7.8E-04	4.4E-03

Figure 28. Differential expression of mitochondrial encoded genes in T238 cells treated with BBR vs. vehicle control. (A) Human mitochondria DNA (mtDNA) sequence map. **(B)** Differential expression of all mitochondrially-encoded transcripts, tRNAs, rRNAs and pseudogenes with this dataset sorted by complex subunit. Shades of blue represent transcripts downregulated following berberine treatment. Shades of orange represent RNAs upregulated following berberine treatment.

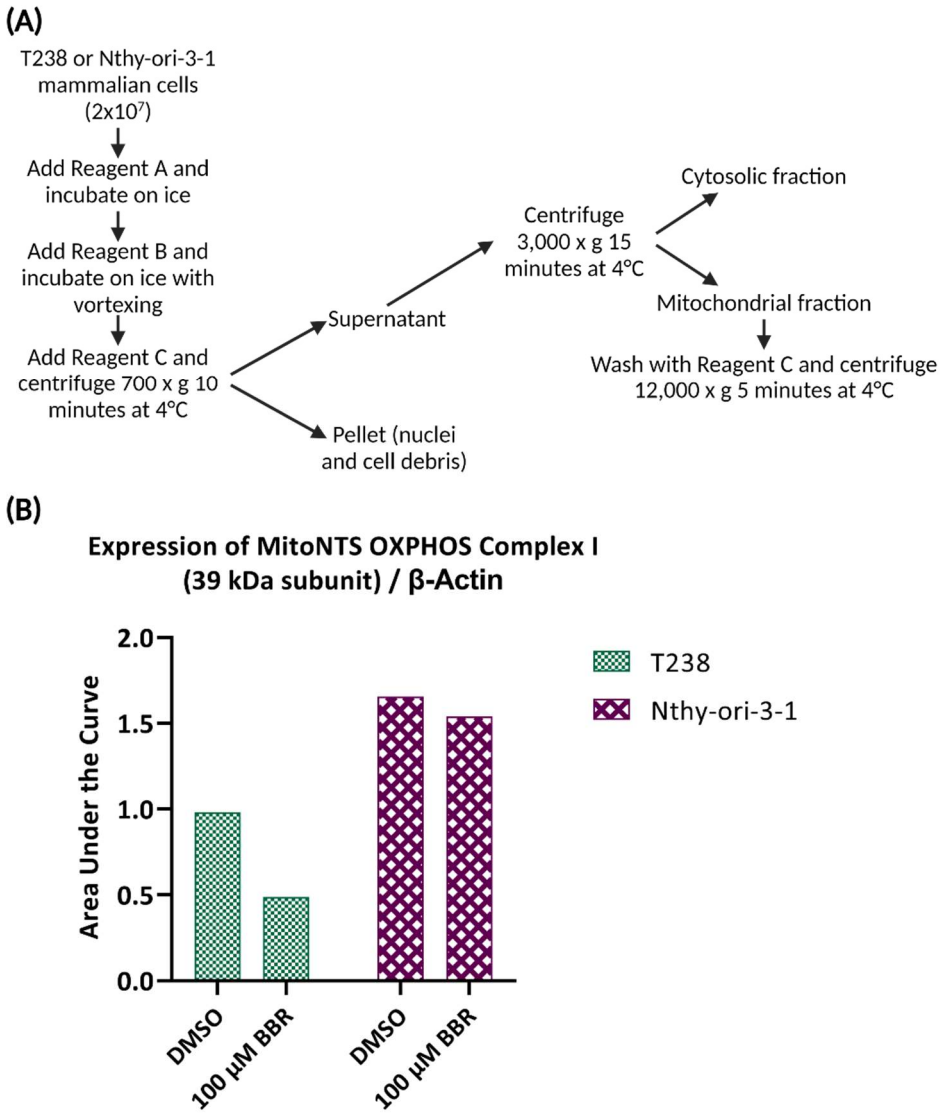
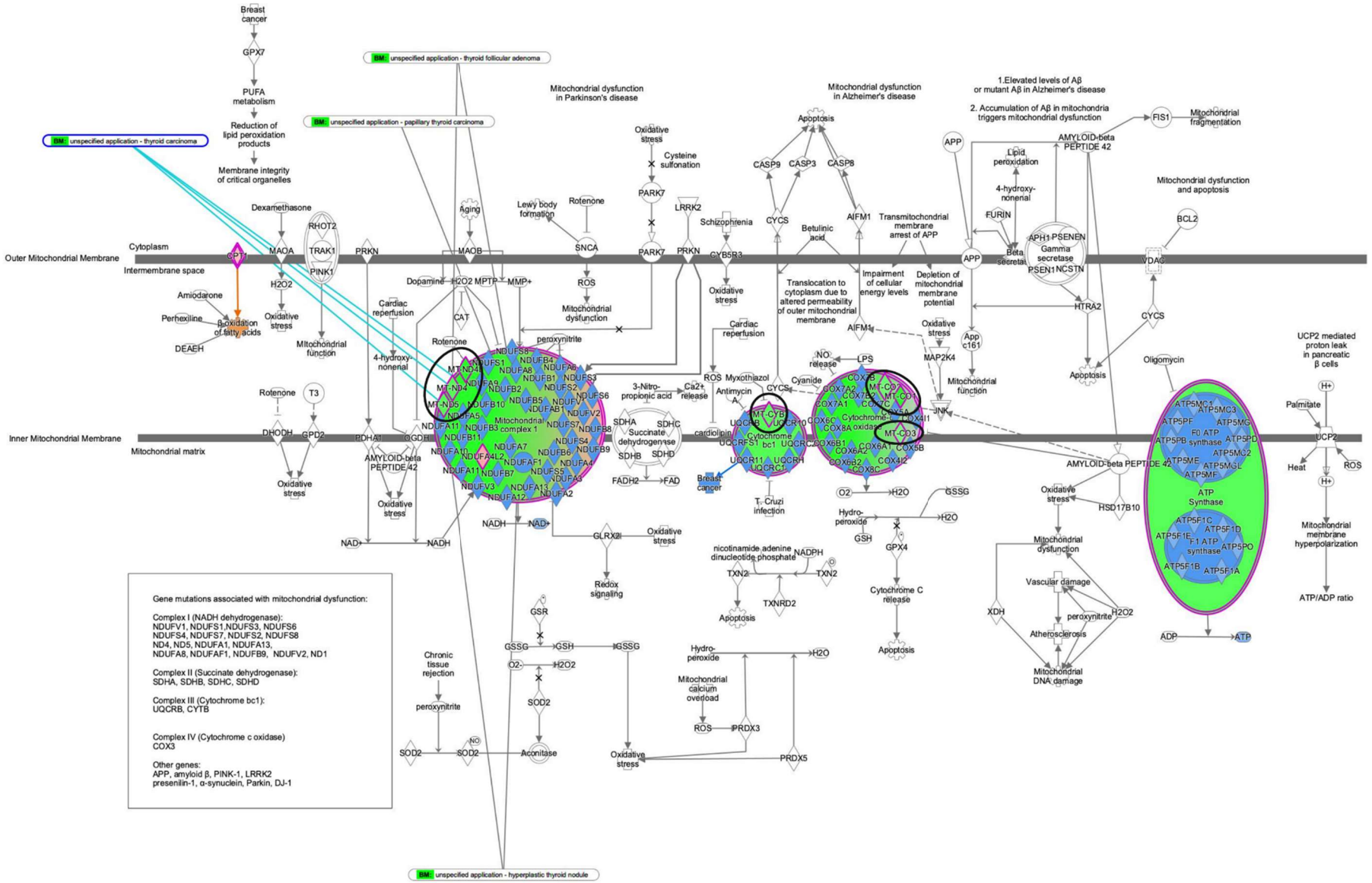


Figure 29. Downregulation of an oxidative phosphorylation protein in T238 cells treated with BBR vs. vehicle control. (A) Differential centrifugation protocol for isolating mitochondria from ATC and immortalized normal thyroid epithelial cell lines, T238 and Nthy-ori-3-1 respectively. **(B)** Berberine treatment decreases protein expression of OXPHOS Complex I (39 kDa subunit) in T238 cells, but not in Nthy-ori-3-1.


Breast cancer
GPX7
PUFA metabolism
Reduction of lipid peroxidation products
Membrane integrity of critical organelles
BM: unspecified application - thyroid carcinoma
BM: unspecified application - papillary thyroid carcinoma
BM: unspecified application - thyroid follicular adenoma
Mitochondrial dysfunction in Parkinson's disease
Mitochondrial dysfunction in Alzheimer's disease
1.Elevated levels of Aβ or mutant Aβ in Alzheimer's disease
2. Accumulation of Aβ in mitochondria triggers mitochondrial dysfunction
Mitochondrial fragmentation
Mitochondrial dysfunction and apoptosis
Dexamethasone
Aging
Lewy body formation
Rotenone
Oxidative stress
Cysteine sulfonation
PARK7
LRRK2
Schizophrenia
Apoptosis
CASP9
CASP3
CASP8
CYCS
Betulinic acid
AIFM1
Transmitochondrial membrane arrest of APP
APP
Lipid peroxidation
4-hydroxy-nonenal
FURIN
AMYLOID-beta PEPTIDE 42
APH1 PSENEN Gamma secretase PSEN1 NCSTN
BCL2
VDAC
HTRA2
Cytoplasm
Outer Mitochondrial Membrane
Intermembrane space
CPT1
MAOA
TRAK1
PINK1
PRKN
MAOB
SNCA
CYB5R3
Amiodarone
Perhexiline
β-oxidation of fatty acids
DEAEH
H2O2
Oxidative stress
Mitochondrial function
Dopamine
MPTP
MMP+
ROS
Mitochondrial dysfunction
Cardiac reprefusion
CAT
Translocation to cytoplasm due to altered permeability of outer mitochondrial membrane
Impairment of cellular energy levels
Depletion of mitochondrial membrane potential
App c161
MAP2K4
JNK
Apoptosis
Oligomycin
UCP2 mediated proton leak in pancreatic β cells
Rotenone
peroxynitrite
4-hydroxy-nonenal
NDUFS8
NDUFS1
MT-ND1
NDUFA8
NDUFA6
NDUFB1
NDUFS3
NDUFA9
NDUFB2
NDUFS2
MT-ND4
NDUFB5
NDUFS6
MT-ND5
NDUFB10
NDUFV1
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NDUFS4
NDUFA7
NDUFB6
NDUFB9
NDUFA10
NDUFA4L2
NDUFAF1
NDUFA4
NDUFA11
NDUFB7
NDUFS5
NDUFA3
NDUFV3
NDUFA13
NDUFA12
NDUFA2
NADH
NAD+
ROS
Myxothiazol
Antimycin A
Cyanide
NO release
LPS
3-Nitro-propionic acid
Ca2+ release
MT-CYB
UQCRB
UQCRC2
cardiolipin
Cytochrome bc1
UQCRFS1
UQCRC1
UQCRH
UQCRC2
UQCRB
UQCRQ
UQCR11
UQCR10
COX7B
COX7A2
MT-CO2
COX7A1
COX7C
MT-CO1
COX5A
COX4I1
Cytochrome-c oxidase
COX6C
COX8A
MT-CO3
COX6B1
COX6A2
COX6A1
COX5B
COX6B2
COX4I2
COX8C
Inner Mitochondrial Membrane
Mitochondrial matrix
DHODH
GPD2
PDHA1
OGDH
T3
Rotenone
AMYLOID-beta PEPTIDE 42
Oxidative stress
SDHA Succinate dehydrogenase SDHB
SDHC
SDHD
FADH2
FAD
Breast cancer
T. Cruzi infection
O2
H2O
GSSG
Hydro-peroxide
GSH
GPX4
Cytochrome C release
Apoptosis
HSD17B10
Mitochondrial dysfunction
Vascular damage
XDH
H2O2
peroxynitrite
Atherosclerosis
Mitochondrial DNA damage
ATP5MC1
ATP5MC3
ATP5PF
ATP5MG
F0 ATP synthase
ATP5PB
ATP5PD
ATP5ME
ATP5MC2
ATP5MGL
ATP5MF
ATP Synthase
ATP5F1C
ATP5F1D
ATP5F1E
F-1 ATP synthase
ATP5PO
ATP5F1B
ATP5F1A
ADP
ATP
Palmitate
H+
UCP2
Heat
ROS
Mitochondrial membrane hyperpolarization
ATP/ADP ratio
GLRX2
Oxidative stress
Redox signaling
nicotinamide adenine dinucleotide phosphate
NADPH
TXN2
TXNRD2
Apoptosis
GSR
GSSG
GSH
O2-
H2O2
SOD2
Chronic tissue rejection
peroxynitrite
SOD2
NO
SOD2
Aconitase
Mitochondrial calcium overload
ROS
PRDX3
PRDX5
Oxidative stress
Gene mutations associated with mitochondrial dysfunction:
Complex I (NADH dehydrogenase):
NDUFV1, NDUFS1,NDUFS3, NDUFS6
NDUFS4, NDUFS7, NDUFS2, NDUFS8
ND4, ND5, NDUFA1, NDUFA13,
NDUFA8, NDUFAF1, NDUFB9, NDUFV2, ND1
Complex II (Succinate dehydrogenase):
SDHA, SDHB, SDHC, SDHD
Complex III (Cytochrome bc1):
UQCRB, CYTB
Complex IV (Cytochrome c oxidase)
COX3
Other genes:
APP, amyloid β, PINK-1, LRRK2
presenilin-1, α-synuclein, Parkin, DJ-1
BM: unspecified application - hyperplastic thyroid nodule

Figure 30. Ingenuity Pathway Analysis of differentially expressed mitochondrial-encoded genes and the pathways that they are involved in. Components of the oxidative phosphorylation complexes that were differentially expressed are circled in black.

Mitochondria are the major site for ATP production via oxidative phosphorylation, with complexes I and III of the electron transport chain the primary site of superoxide production.(Yin et al., 2012) Although ROS are necessary for many normal physiological processes and are inextricably linked to thyroid hormone synthesis, excessive amounts are toxic to normal cells and increase tumor aggressiveness and inflammation. (Wang et al., 2015) Increased superoxide expression, a type of ROS, is associated with malignant transformation and increased proliferation in cancer cells. (Kinnula & Crapo, 2004)

As found above **(Figures 25-28)**, BBR reduces complex I components at the transcript and protein level. Here we observed production of superoxide following 100 μM BBR treatment of ATC cells (T238, SW1736) and immortalized, normal thyroid epithelial cells (Nthy-ori-3-1). We qualitatively observed decreased production of superoxide in the ATC cells **(Figure 31)** treated with BBR compared to vehicle control. This decrease of superoxide production following BBR treatment is likely a direct result of decreased OXPHOS in the tumor cells and could be participating in crosstalk with inflammatory mediators and cell signaling activation in the ATC cells.

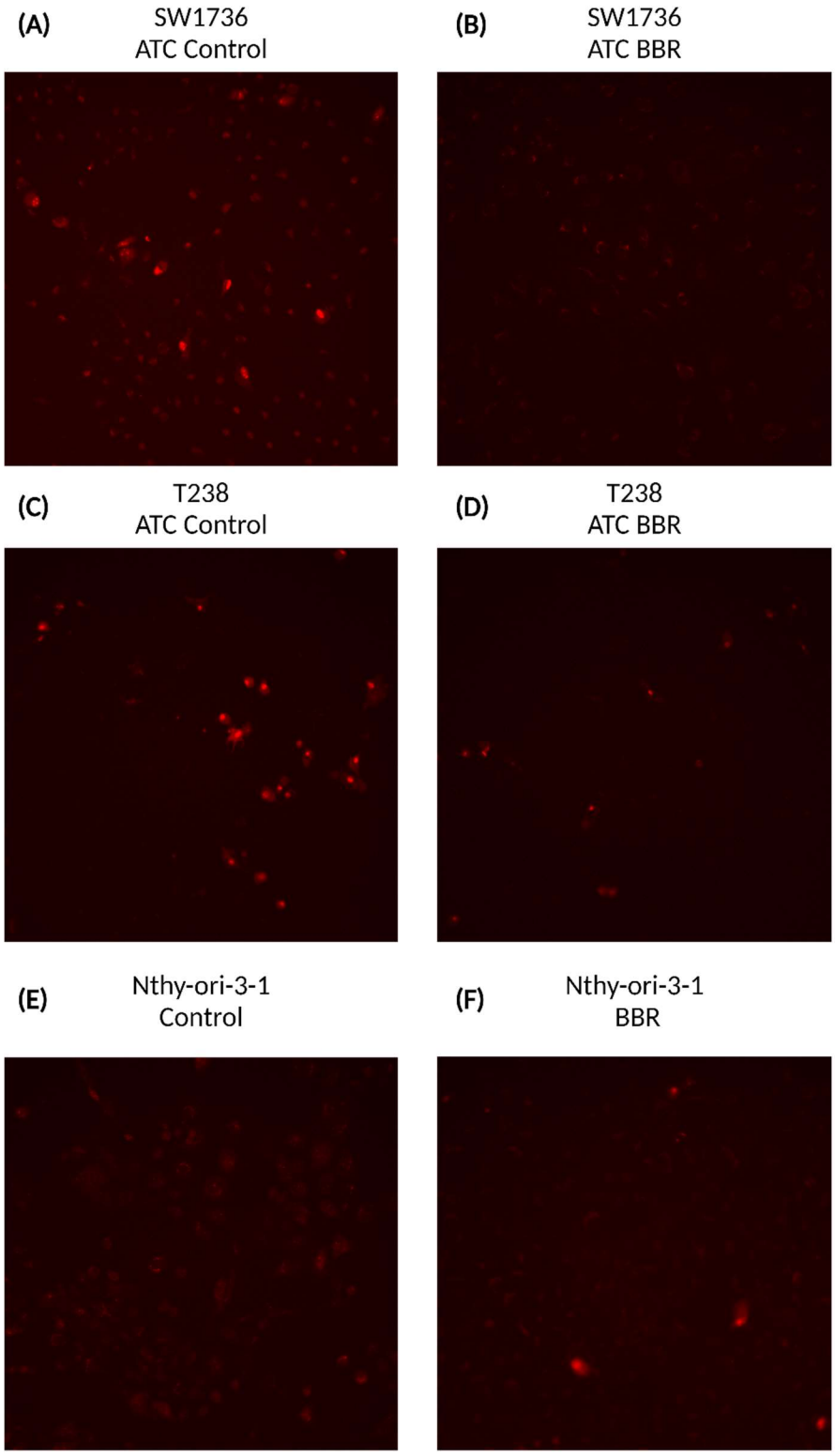

Figure 31. Production of superoxide by ATC cells with or without 100 µM BBR treatment measured by MitoSox™ Red Reagent. Superoxide production in (A,B) SW1736, (C,D) T238, and (E,F) Nthy-ori-3-1 following BBR treatment (B,D,F) or vehicle control (A,C,E). Representative images shown. n=3

Summary of Results –

1. BBR significantly decreases mitochondrial-encoded gene expression.
2. BBR plays a key role in regulating cellular energetics and behaves as an OXPHOS inhibitor in ATC.
3. BBR-induced mitochondrial dysregulation may play a role in inducing the changes in ATC aggressiveness and signaling demonstrated earlier.
4. BBR reduces production of superoxide, a particular ROS, that plays an essential role in tumor-associated inflammation and cancer cell proliferation, invasion, and metastasis.
5. We found a novel role for berberine as an oxidative phosphorylation inhibitor in anaplastic thyroid cancer.

Conclusions—

This data revealed a novel role for berberine in ATC as a mitochondrial inhibitor. ATC cells do not significantly experience the Warburg effect, but rather use a two-component metabolic system where CAFs show an increase in glycolysis, shuttling lactate into the tumor cells, and increasing OXPHOS within the ATC cells themselves (Gill et al., 2016). With inhibition of OXPHOS in these tumor cells following BBR treatment, energetic availability to the tumor significantly diminishes. In diminishing this cellular energy, processes that are upregulated in the tumor that require significant energy to carry out can no longer be completed. Additionally, BBR reduction of superoxide production helps to lessen tumor-associated inflammation and cancer cell proliferation, invasion, and metastasis (Reuter et al., 2010).

These data together reveal a potential mechanism where BBR inhibition of mitochondrial function activates AMPK signaling. Downstream effects further include an increase in apoptosis, decreased survival signaling connecting into PI3K/AKT signaling, decrease in inflammatory IL-6 and its role in JAK/Stat3 signaling and proliferation, and an increase in Sirtuin 1 and its impact on proliferation and migration (Cao et al., 2019).

Overall Summary of Results–

In the first specific aim, we found that berberine reprograms the ATC phenotype by specifically targeting major hallmarks of ATC, including sustained proliferative signaling, activation of invasion and metastasis, and resistance of cell death mechanisms **(Figure 32)**. From specific aim 2, this work demonstrated that the anti-inflammatory activity of berberine reduces the secretion of inflammatory cytokines and chemokines from activated macrophages – essential components of the tumor-initiating and tumor-promoting inflammation in ATC. Finally, from specific aim 3, we discovered a novel role for berberine as an oxidative phosphorylation inhibitor in anaplastic thyroid cancer. Taken together, berberine's ability to remodel ATC's aggressive phenotype, control overactive signaling, decrease tumor-promoting inflammation, and regulate mitochondrial dysfunction demonstrates its use as a systemic, selective mediator of tumor initiation and progression that can be used as an OXPHOS inhibitor to ameliorate the aggressiveness of fatal anaplastic thyroid cancer.

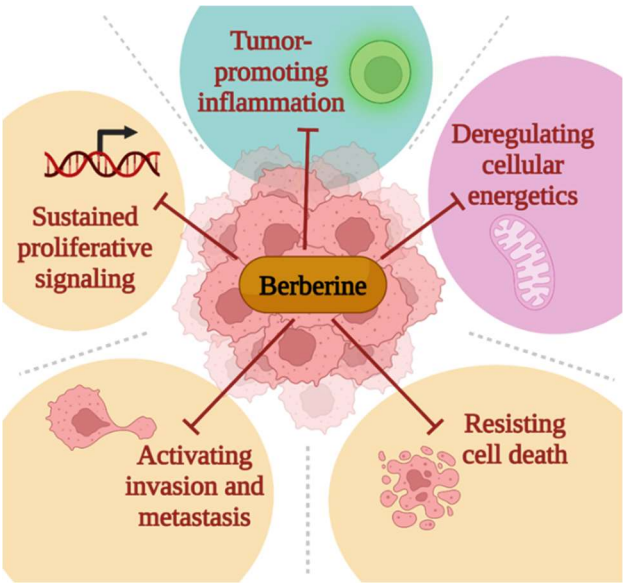

Figure 32. BBR disrupts hallmarks of ATC.

V. Discussion

Novel Targets in ATC – Mitochondrial Inhibition

ATC has a low burden of genetic mutations compared to other lethal and fast-growing cancers (Jannin et al., 2022). Additionally, its mutational burden is not any more significant than papillary thyroid cancer (Capdevila et al., 2018), however, there are obviously key differences at play between ATC and PTC that contribute to ATC's universal fatality rate, while PTC has a 95% five-year-survival rate (Jannin et al., 2022; Tuttle et al., 2010). With this comparatively low mutational burden for an aggressive cancer, there have been limited targets available in developing new therapies against ATC. For the known genetic mutations that are prevalent in many ATC cases, such as BRAFV600E and mutated TP53, their targeting has done little to improve long-term outcomes, such as overall survival, although these targets have had significant success in other cancers (Johnson et al., 2015). With this comes a lack of effective therapy and a reliance on palliative care to make these patients comfortable during their end-stage disease (O'Neill & Shaha, 2013; Smallridge & Copland, 2010). As such, one essential feature of designing an effective therapy comes into focus. ATC may require a more holistic remedy, as drugs targeted to a singular genetic lesion have not had therapeutic success thus far or patients have developed therapeutic resistance. Targeting must exert control over multiple hallmark features of ATC **(Figure 32)** to have sufficient impact. Secondly, this drug or combination therapy should work selectively in ATC tissue, while leaving normal tissue relatively unscathed. To understand which dysregulated features of ATC would best serve as

targets for a novel therapeutic, understanding its large-scale distinguishing characteristics is essential.

As described previously, the emergence of the ATC phenotype actually comes about as a result of metabolic and cellular changes with disease-preceding inflammation as a key feature (Gill et al., 2016). Observing the fundamental differences in the metabolic characteristics and inflammatory infiltrates in ATC tissue versus noncancerous tissues as well as precursor lesions and pathologies (goiter, PTC, etc.) would help to inform how to preferentially target tumor cells (Johnson et al., 2015). Under normal physiological conditions, the thyroid gland is essential for controlling growth, development, and normal adult metabolism through its production and secretion of thyroid hormone (Mullur et al., 2014). The status of thyroid hormone directly correlates to regulation of energy expenditure and storage in the body, as well as control over weight (Mullur et al., 2014). Thyroid hormone receptor isoforms TRα and TRβ undergo sumoylation as a post-translational modification necessary for TH to be able to positively and negatively regulate important genes for metabolic regulation (Mullur et al., 2014). TRβ regulates cellular growth and differentiation, and its dysregulation and diminished expression is commonly found in cancers – particularly aggressive solid tumors including thyroid and breast cancer (Bolf et al., 2020). As such, physiological TRβ could actually be acting as a tumor suppressor (Bolf et al., 2020). TRβ has been known to be silenced in ATC, although its functional role has not been fully established. Bolf *et al.* found that in ATC cell line SW1736, a cell line also used in our investigations, that restoring TRβ expression reduced aggressive phenotype, decreased cancer stem cell population, and induced T3-dependent cell death (Bolf et al., 2020). These findings highlight

that dysregulation of metabolism initiating within the thyroid itself contributes to the development and aggressiveness of ATC.

In addition to regulation of metabolism through the interplay of TH and thyroid hormone receptors, changes in metabolism within the thyrocytes themselves drive ATC progression as well (Dabravolski et al., 2022; Gill et al., 2016). Mitochondrial abnormalities actually have a crucial role in development and progression of all types of thyroid cancer (Dabravolski et al., 2022). The dynamic interplay between CAFs, the mitochondria, and the tumor-infiltrating immune cells serve an essential role in driving ATC and create a distinct situation from what is present in PTC or the normal thyroid. For example, patient samples of PTC thyrocytes have high TOMM20 expression diffusely throughout the tumor, regardless of if the disease is more advanced in stage (Johnson et al., 2015; Gill et al., 2016). This feature was a commonality between PTC and ATC, which also shows high TOMM20 staining compared to non-cancerous tissue (Gill et al., 2016). A distinguishing feature was robust MCT1 expression in ATC specimens, while MCT1 expression was low across all PTC specimens and non-cancerous tissue (Gill et al., 2016). As described previously, MCT1 allows for influx of lactate into the tumor cells after it has been produced as a byproduct of glycolysis taking place in the CAFs (Gill et al., 2016). Once lactate is within the tumor cell, TOMM20 allows for translocation into the mitochondria where it can be used to activate OXPHOS and produce ATP (Gill et al., 2016). Mitochondrial metabolism has a major link to nearly all oncogenic processes (Bueno et al., 2021). As such, it stands to reason that MCT1 inhibition would prevent lactate uptake and force aerobic cells to use glucose rather than lactate and decrease energetic access for hypoxic cells (Gill et al., 2016). To date, MCT1 inhibitors, such as α-cyanohydroxycinnamate,

are being tested for treatment of other human malignancies, such as breast cancer, and have demonstrated promise in slowing tumor growth and having a synergistic effect when given with radiotherapy (Gill et al., 2016).

Further, mitochondria specific to thyroid cancer are known to produce extremely high levels of ROS, particularly under hypoxic TME conditions, leading to further oxidative damage, genomic instability, metabolic reprogramming of various pathways (OXPHOS, fatty acid synthesis, glycolysis, TCA cycle, and carbon metabolism), tumor growth, induction of EMT, invasion, and metastasis (Dabravolski et al., 2022). Thyroid cancer disease progression can be impacted early on by oxidative damage to macromolecules; DTC has strong antioxidant defense activity, while ATC has significantly decreased expression of antioxidant enzymes (Lee et al., 2015). Further investigation into anaplastic thyroid cancer-specific mitochondria and their signal transduction is an essential area of research to improve treatment strategies in this field. As BBR functions as an antioxidant molecule, this shows promise as an area to target.

ATC has expansive energy demands as a fast-growing, highly proliferative tumor type (Dabravolski et al., 2022). This creates an extensive metabolic burden in actively growing ATC (Dabravolski et al., 2022). With that in mind, one can envision a methodology for mitochondrial inhibition as an effective regulator of ATC by shutting off or turning down its primary energy source. Resultant from lower available ATP following mitochondrial inhibition, tumor cells could experience reduced levels of ROS, inflammation, and proliferative capacity (Dabravolski et al., 2022). As such, inhibition of mitochondrial

metabolism has recently emerged as a promising new anticancer strategy (Bueno et al., 2021).

Metformin has emerged as an antitumor agent with the ability to inhibit mitochondrial metabolism (Feng et al., 2022; Johnson et al., 2015; Bueno et al., 2021; Gill et al., 2016). Currently, metformin is known as a diabetes mellitus medication that lowers blood sugar levels by improving how the body handles insulin (Feng et al., 2022). As a mitochondrial inhibitor, it is believed to inhibit complex I-dependent respiration thus blocking ATP production and activate sirtuin 1 (SIRT1) and sirtuin 3 (SIRT3) (Feng et al., 2022; Gill et al., 2016). We found sirtuin signaling pathways to be significantly modified in our data set, which could be a response to the mitochondrially inhibiting role we saw following berberine treatment. Further, metformin blocks mitochondrial-dependent ROS production (Gill et al., 2016). Preclinically, metformin shows promise as an antineoplastic agent in gastric, medullary thyroid, breast, and pancreatic cancers, effectively inhibiting cancer cell proliferation, and it is currently in a clinical trial for use in head and neck squamous cell carcinoma (Gill et al., 2016). Interestingly, epidemiological studies have observed patients receiving metformin for diabetes have decreased incidence of / or mortality from cancer (Gill et al., 2016). Unfortunately, metformin's toxicity is a problem that needs to be addressed with common side effects involving the gastrointestinal tract including diarrhea, nausea, vomiting, flatulence, and severe abdominal pain, while extremely high doses can lead to mortality (Feng et al., 2022).

Another repurposed drug, atovaquone, is an FDA-approved antimalarial drug with other antiparasitic properties (Dabravolski et al., 2022). It has been demonstrated to inhibit

mitochondrial respiration through cytochrome bc1 complex inhibition, subsequently blocking energy supply, which is important in targeting tumors (Dabravolski et al., 2022). Atovaquone has been studied in ATC and PTC *in vitro* and has been found to decrease growth, migration, and survival through inhibition of mitochondrial complex III and reduction of ATP production, while also suppressing STAT3 production, which is known to regulation growth and apoptosis, as a result of this mitochondrial inhibition (Dabravolski et al., 2022). Another anti-malarial drug, artesunate, showed similar properties against both chemo-sensitive and chemo-resistant ATC cells *in vitro* and *in vivo* through suppressing mitochondrial respiration and thus inhibiting growth and inducing apoptosis in these ATC cells (Dabravolski et al., 2022). Unfortunately, use of Artesunate is currently banned in the European Union due to its high kidney toxicity and known side effect of causing kidney failure. Atovaquone is more well-tolerated in adults with rare adverse effects including stomach pain, nausea, vomiting, and headache.

What is currently lacking in the investigation of mitochondrial inhibitors in cancer therapy for its transition to clinical use is defining the metabolic context in which the inhibitors demonstrate peak efficacy (Bueno et al., 2021). Further, the mitochondria are also essential in the orchestration of immune response, so evaluating this immunomodulatory effect is critical in optimizing clinical use (Bueno et al., 2021). Targeting metabolism in the cancer context is also not limited exclusively to the tumor cells themselves, but mitochondrial inhibitors may also work on adjacent cells in the TME as well, which could have efficacy in ATC (Gill et al., 2016).

Proposed Mechanism of Action of BBR in ATC

Our current work introduced a novel role for berberine in cancer, as an OXPHOS-inhibitor in ATC. Through our RNA Sequencing studies, and confirmed via Western blot, we observed the reduction of OXPHOS complex I, III, IV, and V components post-berberine treatment compared to ATC control *in* vitro. Further, as was observed with metformin, atovaquone, and artesunate in other cancers or ATC, we found a reduction in ATC cellular proliferation, induction of apoptosis, reduction of ROS superoxide, and decrease in *in vitro* phenotypes associated with invasion and migration. Unlike these other drugs, berberine is generally much more well-tolerated, particularly when given by an oral route of administration. Further, Turner *et al.* have also observed the mitochondrial inhibitor effects of berberine, but in a decisively different context (Turner et al., 2008). BBR has been used as a diabetes drug in humans in China for some time, and has demonstrated insulin-sensitizing properties in rodent models of diabetes (Turner et al., 2008). It has been previously reported that this sensitization to insulin happens, at least in part, via activation of AMPK (Turner et al., 2008). AMPK serves crucial roles in regulating glucose metabolism and insulin processes, as well as having an anti-tumor effect. However, it is now understood that BBR activation of AMPK in diabetes occurs through the inhibition of OXPHOS complex I in mitochondria (Turner et al., 2008). Although a diabetes model differs significantly from a lethal cancer model, the mechanisms of action by which BBR regulates metabolism and signaling appear to have striking similarities. For example, we also observed activation of AMPK in our T238 ATC cells following BBR treatment. Thus, a novel mechanism of action for BBR in ATC begins to take shape from our studies.

Berberine treatment decreases gene expression of all 13 mitochondrial-encoded protein coding genes. Functionally, this results in berberine tapering off the ability of oxidative phosphorylation to occur in ATC cells. When decreasing OXPHOS activation, production of ATP is diminished, and less energy is available to meet the tumor cells' constant energetic demands. Reduction of OXPHOS complex I signals to phosphorylate and activate AMPK. Activated AMPK has an antitumorigenic function. It participates in cellular crosstalk with the PI3K/AKT pathway by repressing mTOR signaling (Yuan et al., 2020) and subsequently ribosomal protein S6 downstream of mTOR. This has a downstream impact on cell survival, proliferation, growth, invasion, and migration– all phenotypic changes we observed following BBR treatment in aim 1 of this study. Additionally, AMPK has been shown to reversibly regulate hyperactive MAPK signaling in cancer, a pathway which is constitutively active in our ATC models (Yuan et al., 2020). MAPK signaling promotes cellular proliferation and drives carcinogenesis, and also inhibits AMPK signaling to overcome metabolic stress. In targeting OXPHOS and metabolic stress by BBR, AMPK signaling is re-activated (Yuan et al., 2020). Upon reactivation, AMPK signaling could exert its regulatory role over MAPK signaling. We observed decreased phosphorylation in MAPK pathway mediators MEK and ERK, as well as a decrease in proliferation following treatment. In other pathologies, AMPK signaling has been an important contributor to the anti-inflammatory properties observed following BBR treatment – through AMPK-dependent Nrf2 activation inflammatory macrophages, in metabolic diseases, in skeletal progenitor cells through AMPK/SIRT1/PGC-1α activation, and in adjuvant arthritis in mice through regulation of AMPK/NF-κB signaling (Wang et al., 2020). Our studies observed decreased inflammatory mediators secreted from classically activated

and polarized macrophages, as well as macrophages polarized with ATC conditioned media. Further, mitochondrial danger associated molecular patterns (mtDAMPs) also elicit inflammation through stimulating interferon responses through stimulator of interferon response cGAMP interactor 1 (STING1) via activation by mitochondrial DNA (mtDNA) and through inflammasome induction induced by mtDNA and ROS (Marchi et al., 2023). In our model, following BBR treatment, we observed a reduction in IFN-γ, as well as reduced production of superoxide, a ROS. Inflammation in the context of ATC serves some function in tumor initiation and progression, so lessening this chronic inflammatory status is critical in remodeling the aggressiveness of ATC. **(Figure 33).** Taken together from this model, the interplay between mitochondrial inhibition, upregulation of AMPK, and inflammation holds the key to understanding BBR's actions in ATC.

Our future investigations should verify this mechanism further. Additionally, this treatment strategy should be tested in a mouse model of ATC to confirm that the same results are being seen in a more complex and dynamic biological system. It should also be further explored if the combinatorial use of BBR with another therapeutic that was previously insufficient in treating ATC would have a synergistic effect against this fatal disease. Overall, if this novel role of berberine as a mitochondrial inhibitor in ATC holds true *in vivo*, it could be a hugely impactful new therapeutic modality for the treatment of ATC.

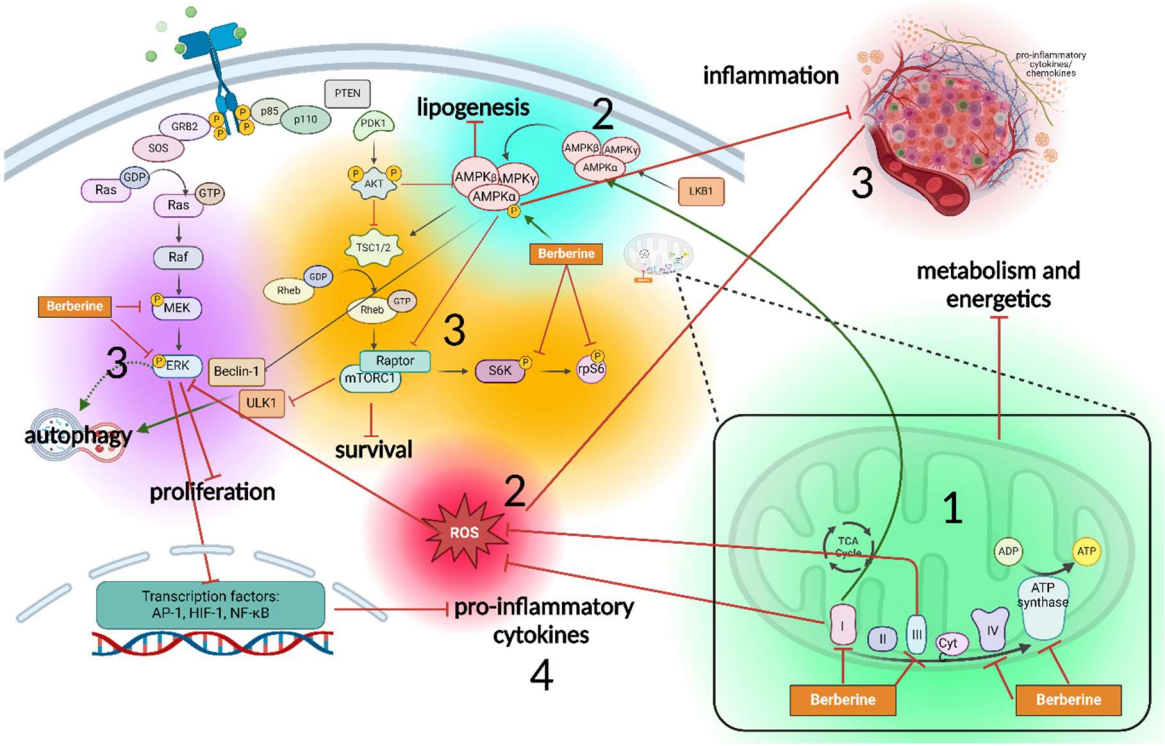

Figure 33. The proposed mechanism of action of berberine in ATC revealed from this study. Figure created on BioRender.com.

Future Directions

Currently this work lacks the introduction into a more dynamic model system. Although the *in vitro* findings were consistent, this work requires validation *in vivo.* In the instance of the TAM secretion of inflammatory cytokines in the presence or absence of BBR, it would be particularly essential to see the role of this decreased inflammation in the actual TME—both in the soluble inflammatory mediators and infiltrating immune cell populations. The technical limitations of this work were only observing the results in an *in vitro* model, which does not allow the study to mimic the dynamic context and extensive crosstalk of the ATC TME. Although the inflammatory constituents that we observed to be downregulated following BBR treatment have known roles in regulating EMT, invasion, migration, proliferation, and

angiogenesis in other cancers, and are often associated with increased tumor grade and worse overall prognosis, we would still like to see how this operates in context. Similarly, we have observed important phenotypic and cell signaling changes that control rapid growth, proliferation, invasion, and migration *in vitro*, but we would also want to see how that impacts tumor growth, tumor size, and propensity to metastasize *in vivo.* Beyond this, BBR can be poorly bioavailable in humans, so we would need to overcome this bioavailability concern. Further, treatments did not take place over extended time to test whether ATC cells develop resistance to the effects of BBR after continued use. This would need to be studied to determine if this drug can have long term efficacy in controlling ATC.

To address these holes within the study, our proposed continuation of this work exists at multiple levels: (A) further confirmation that BBR acts as a mitochondrial inhibitor in ATC, (B) that this action as a mitochondrial inhibitor contributes to the alterations in ATC's aggressive phenotypes and inflammation previously observed, (C) evaluation of other changes in energy utilization and metabolism following BBR treatment, (D) validation of this mechanism of action, and (E) validation of these findings in an *in vivo* model system utilizing a formulation of BBR with increased bioavailability, nano-particle formulated BBR (BBR-N) developed in the laboratory of Dr. Xiu-Min Li (Yang et al., 2023).

This work proposes a novel role for BBR in ATC as an OXPHOS inhibitor. To confirm that BBR truly acts as an OXPHOS inhibitor in ATC, we would treat our cells *in vitro* with other known mitochondrial inhibitors at their effective concentrations found in literature. Various OXPHOS inhibitors currently exist that target the different oxidative phosphorylation complex subunits as their site of inhibition **(Table 5)** (Blackstock, 1989)**.**

Site of inhibition	***Agent***	Empty Cell	***Comment***
Electron transport	Rotenone		Prevent reduction of ubiquinone and simultaneous oxidation of Complex I FeS centres
	Amytal		
	Antimycin A		Inhibits transfer of electron from cytochrome b_{562} to ubiquinone
	Hydrogen cyanide		
	Hydrogen sulphide		Bind to Fe^{3+} of cytochrome *a* and a_3
	Azide		
	Carbon monoxide		Binds to Fe^{2+} of cytochrome *a* and a_3
Inner membrane	2,4-Dinitrophenol		Are anionic at pH 7.0, may protonate to become lipophilic and soluble in membrane. Protons are transported through membrane and H^4 gradient is abolished
	Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone		
	Valinomycin		Renders membrane permeable to K^+ which may abolish E_m
	Nigericin		Abolishes H^4 gradient by K^+-H^+ exchange
ATP synthase	Oligomycin		Binds to OSCP in stalk and blocks H^+ pore
	DCCD		Reacts with DCCD-binding proteolipid of F_0 component and blocks H^+ pore
Adenine nucleotide carrier	Atractyloside		Binds to external conformation to preclude ADP interaction
	Bongkrekic acid		Binds to internal conformation to preclude ATP interaction
Phosphate carrier	Mercurial reagents		Bind to sulphydryl groups

Table 5. "Some inhibitors of oxidative phosphorylation." Table and Legend Taken From: Blackstock *et al.*, 1989.

We would treat our ATC cells (SW1736 and T238) as well as our immortalized, normal thyroid epithelial cells (Nthy-ori-3-1) in culture with a selection of OXPHOS inhibitors that target each OXPHOS complex, BBR, or vehicle control. Following this treatment, we would evaluate all experimental readouts that we previously observed to be altered by BBR, including trypan blue exclusion assay for evaluation of proliferation, cell death detection ELISA for evaluation of apoptotic induction, scratch wound assay and Boyden invasion (with Matrigel) and migration chambers to evaluate cell motility, invasion, and migration, Western blot to investigate activation or inhibitor of crucial cell signaling pathways, and inflammatory arrays, ELISA, and co-culture to look at differential expression of inflammatory mediators (from U937 cells).

This data would not only allow us to compare the function of BBR to other known mitochondrial inhibitors, but also substantiate that this is an innovative new methodology for treating anaplastic thyroid cancer. This would not only confirm that BBR is serving as an OXPHOS inhibitor, but also that this inhibitor of OXPHOS is what is controlling proliferation, invasion, migration, apoptosis, and inflammation downstream. Additionally, BBR treatment inhibits components of OXPHOS complexes I, III, IV, and V **(Figures 26-28).** As we would compare this role to OXPHOS inhibitors with rigorously defined targets, we could observe if BBR functionality is most closely related to the action of an inhibitor with one of these specific targets. Further, we observed that BBR was able to serve this function specifically in our ATC cells, while not impacting or harming the normal thyroid epithelial cells. This may not be the case for other OXPHOS inhibitors, and this would further validate that the specificity of BBR is what makes it such a promising therapeutic candidate.

As tumor models are dynamic, we would also want to further investigate any other changes in energy utilization and metabolism following BBR treatment. Although we have discussed that ATC uses a two-component coupling of increased glycolysis in the CAFs with increased OXPHOS in the tumor cells (Gill et al., 2016), we want to confirm energy utilization in our cells. Further, within many tumor models, increased glycolysis provides energy to support proliferation of tumor cells and produces glucose-derived metabolic intermediates crucial for essential biosynthetic mechanisms (TeSlaa & Teitell, 2014). We also want to ensure that OXPHOS inhibitor in ATC cells is inhibiting energy production completely, and not allowing for a different mechanism of ATP synthesis to take over. To investigate this, we would use the Seahorse XFe24 Analyzer (Agilent Technologies, Santa Clara, CA, USA) to investigate real-time measurements of cellular metabolism and energy utilization in our ATC cells (Caines & Barnes, 2022; TeSlaa & Teitell, 2014). This would give real-time readouts of the oxygen consumption rate which measures aerobic mitochondrial respiration through OXPHOS and the extracellular acidification rate which measures anaerobic glycolysis (Caines & Barnes, 2022; TeSlaa & Teitell, 2014). We would investigate these energetic outputs for our ATC cells without any treatment conditions every 12 hours for 96 hours, and do the same following BBR treatment and treatment with a known OXPHOS inhibitor that we observe to have efficacy in our ATC cells. This will subsequently allow for us to ensure that (A) BBR treatment *is* inhibiting OXPHOS in our ATC cells at the energy production level and (B) that increase in anaerobic energy metabolism does not occur as a result of this shift.

The above proposed experiments would be the first steps taken to validate our proposed mechanism of action **(Figure 32)**, as we would be confirming BBR's role as an OXPHOS

inhibitor and observing if other OXPHOS inhibitors also decrease proliferation, motility, invasion, migration, and inflammation in these models, while inducing apoptosis. If any of these readouts are consistent amongst mitochondrial inhibitors, then it stands to reason that the first step in BBR's mechanism is inhibiting OXPHOS in these ATC cells. We would additionally conduct further mechanistic studies to evaluate this mechanism. We could use clustered regularly interspaced short palindromic repeats interference (CRISPRi) technology to knockdown expression of different elements along this pathway and then observe if BBR treatment is still capable of having the same effect or not. For example, we would knockdown a component of OXPHOS complex I in our ATC cells, such as MT-ND4, and evaluate if BBR treatment can still increase phosphorylation of AMPKα and reduce superoxide production. If not, this would begin to confirm our mechanism of action. We could then instead knockdown AMPK in our ATC cells and evaluate if a decrease in phosphorylation of rpS6, MEK, and ERK is still observed following BBR treatment. If not, this mechanism would be further validated. Finally, we would need to put this mechanism to the test in an *in vivo* model system for further confirmation of the role of BBR in ATC.

Validation of these findings in an *in vivo* model system would use BBR-N for its increased bioavailability in tissue. We would examine the efficacy of BBR-N in an orthotopic ATC model transplanting T238 cells in Balb/c nu/nu mice and looking at endpoint parameters of tumor growth, evaluation of local and distant metastases, and harvesting of tumor tissue and blood/serum from animals for exploration of TAM infiltration, macrophage-mediated inflammation, production of ROS, and inflammatory cytokine and chemokine profiling. BBR-N would be delivered orally, as this route has the least associated side effects, while also

providing good tissue distribution due to the nanoparticle formation. Mice would also be observed for any sign of negative side effects to the treatment and liver and kidney function would be checked over the time course as well.

Overall Significance

As anaplastic thyroid cancer is an exceedingly rare cancer with limited active research towards its better control and no treatment options beyond palliative care, this work is proposing the use of a repurposed therapeutic, berberine, in ATC by a novel mechanism, as an inhibitor of oxidative phosphorylation. Patients with ATC have one of the worst prognoses of all cancer patients. Identification of a new approach to treat ATC with an agent that can act specifically on ATC cells, while not damaging normal thyroid cells holds great promise. Following further *in vitro* and *in vivo* studies, this treatment strategy could be further evaluated clinically as a novel standalone therapy or potential combination therapy for the treatment, or possibly prevention, of ATC.

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