EFFECT OF VITAMIN B1 SUPPLEMENTATION ON EXPRESSION AND FUNCTION OF THIAMINE DEPENDENT ENZYMES IN BREAST CANCER CELLS

by

SHIVANI C. KHATU

(Under the Direction of Jason Zastre)

ABSTRACT

Vitamin B1 (thiamine) is an essential water-soluble vitamin which is converted by Thiamine Pyrophosphokinase-1 to Thiamine pyrophosphate (TPP) which acts as a cofactor to activate enzymes in metabolic processes to generate ATP and nucleic acids. As Thiamine is not produced endogenously in the body, a regular dietary intake is essential. In cancer cells, there is an alteration of metabolic processes observed where the cells undergo anaerobic glycolysis even in the presence of oxygen, referred to as the Warburg effect, to produce ATP. The role of Thiamine dependent enzymes like Transketolase, α-Ketoglutarate dehydrogenase and Pyruvate dehydrogenase, is critical in metabolism. Thus, we wanted to investigate the effect of low dose thiamine supplementation on thiamine dependent enzymes and eventually to determine their effect on cell proliferation. The ER+ve ductal breast carcinoma cell line, MCF-7 and Triple negative MDA MB 231 breast carcinoma cell line, were used and cells were treated with 10nM Thiamine media and with 3μM Thiamine media to determine the dose effect. We also introduced these treatment groups to hypoxic environment to determine the effect of growth. We observed that there was an increase in the expression of thiamine dependent enzymes like TKT, αKGDH and PDH 293 but not in the PDHe1α enzyme in both cancer cell lines. This high expression corresponded to an elevated activity of TKT and αKGDH enzymes, however, we saw a

decline in the PDH activity which supported the protein expression. The elevated cell number observed via different cell growth assays confirmed that thiamine supplementation at low doses did have a proliferative effect on both these cancer cell lines. Thus, we concluded that thiamine supplementation at low concentrations affected the enzyme expression which could indirectly affect cell proliferation in both cell lines, irrespective of the phenotypic differences.

INDEX WORDS: Thiamine, metabolism, TKT, αKGDH, PDH, cell proliferation

EFFECT OF VITAMIN B1 SUPPLEMENTATION ON EXPRESSION AND FUNCTION OF THIAMINE DEPENDENT ENZYMES IN BREAST CANCER CELLS

by

SHIVANI C. KHATU

B. Pharmacy, Vivekanand College of Pharmacy, India, 2016

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2018

EFFECT OF VITAMIN B1 SUPPLEMENTATION ON EXPRESSION AND FUNCTION OF THIAMINE DEPENDENT ENZYMES IN BREAST CANCER CELLS

by

SHIVANI C. KHATU

Major Professor:	Jason Zastre
Committee:	Shelly Hooks
	Y. George Zheng

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
December 2018

DEDICATION

This thesis is dedicated to all the beautiful women in my life and out there in the world. When I started this project, I had little knowledge of the disease I worked with. Today, I just want every woman out there who has been taking care of every person in her life, to just take a pause, reflect and care for herself. Like others, your health and life is too precious. We together can change the world.

A special thanks to my Mother, who has strived hard and made me who I am today. She is my first teacher and I owe my life to her. Thank you for always believing in me and never leaving my hand.

ACKNOWLEDGEMENTS

This journey was not my own but also of the people who had immense faith in me and my work and who have motivated me all through my life to keep seeking knowledge.

I'd like to thank my committee members for their support and guidance throughout my project and for all the encouragement they've given me.

I'd like to thank my parents for their constant support and love in spite of being miles apart. Thank you for having faith in me that I could do this and get this far in life. Even though you had no clue what exactly I'm doing, you'd always patiently listen to me about the new experiments or cool science things I did. I love you both.

To all my aunts (second mommy's) thank you for teaching me to be patient and loving me like your own daughter. I couldn't have done it without each of your support.

To my friends who have always supported me to get through my coursework and have always motivated me to do my best. Thank you for tolerating me and my incessant talks and making me feel at home in times of despair.

My little sister, Saloni, thank you for always being the ray of sunshine in my darkest days. Our stupid talks and secrets and our fights have never made me miss home. I treasure you, my precious one, but I'm still mommy's favorite daughter!

Hunter, you've been so patient and kind to me all throughout my journey here. I have learnt a lot of things from you and I am so happy to graduate with you. Thank you for being so patient with me and for always taking care of me like a little sister. I love you and wish you all the happiness and love for your future endeavors. Love to Delilah and Bailey!

Maria, I have spent very little time with you, but these few days have been memorable. I am surely going to miss your yummy Italian baked items. Thank you for being what you are and do not change for anybody. Worry less and enjoy your PhD journey. Take care of the lab and Dr. Zastre.

Last but the greatest, a sincere and special thank you to my mentor, guide, professor, Dr. Zastre. I remember being so immature and not knowing how to do things in the lab. Thank you for being so kind and allowing me in your lab and being so patient with me. I know I have been a trouble, even though I didn't mean it. I don't know where life would take me, but I surely know one thing that I have not only gained academic knowledge from you but have also learnt important lessons for life. I can never thank you enough for everything you've done for me.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Types of Breast cancer and their clinical markers:

Breast cancer claims thousands of lives every year and is one of the fastest growing diseases affecting women . The American Cancer society predicts that in 2018, there would be approximately 266,120 new cases of invasive breast cancer development and 40,920 breast cancer deaths in women (American Cancer Society, 2018). Amongst several other types of malignancy, breast cancer is the most frequently occurring and life-threatening cancer in post-menopausal women. Women are either unaware of the underlying risks and causes of breast cancer or neglect the importance of self-inspection and clinical examinations which could lead to the diagnosis of the disease at an advanced stage (Akram M, 2017).

Age is one of the underlying risk factors as this disease has been seen to affect women above 55 years of age as well as below the age of 45 (Anastasiadi, 2017). Another possible risk factor leading to development of this disease is family history (Ripperger, Gadzicki *et al.* 2009). The proportion of women with first degree family history of breast cancer has

seen to rise in the past few years (Shiyanbola, 2017). This is an important factor leading to early breast cancer detection and optimum management of the disease.

About 5-10% of the invasive breast cancers in women are caused due to mutation in autosomal genes (Loman, Johannsson *et al.* 1998). Identification of these mutations is an important prognostic factor determining the course of treatment of breast cancer. Breast cancer is divided into several different types based on the mutations observed:

BRCA mutated breast cancers:

Inherited mutations in the BRCA1 and BRCA2 genes have been associated with certain kinds of breast cancer (Kuchenbaecker, Hopper *et al.* 2017) . Over 2000 different types of BRCA mutations leading to insertion, deletion or substitution with coding or non-coding sequence in the gene have been observed (Ha Chae *et al*, 2017, Mehdipour, 2013). Over 30% of inheritable breast cancers are caused due to BRCA1 and BRCA2 mutations (Valencia, Samuel *et al.* 2017) . The BRCA1 gene is seen to be mutated in most of the breast cancers (Futreal, Liu *et al*. 1994, Thompson, Jensen *et al.* 1995). A lower expression of mRNA of BRCA1 was seen in cells transitioning from carcinoma in situ to invasion cancer. The mutations in the BRCA genes leads to the decline in its DNA repair activity (Mehdipour, 2013). Due to its association with cell cycle regulation, DNA replication and repair, a decline in the activity of this gene has shown to induce cell growth (Deng CX, 2000, M, 2002).

Human epidermal growth factor receptor 2:

Human epidermal growth factor receptor 2 (HER2) is an important tyrosine kinase membrane receptor which shows an overexpression in breast cancer. This receptor is an oncogene and when activated passes on a stronger cell signal which further activates the growth factors and thus, induce cell proliferation and growth. An amplification in the HER2 gene has been shown to possess a tumorigenic effect (Ross and Fletcher 1999, Bacus, Altomare *et al*. 2002, Lear-Kaul, Yoon *et al*. 2003, Starczynski, Atkey *et al.* 2012). Drugs like Trastuzumab and Pertuzumab are known to target this receptor and are the preferred chemotherapeutic agents (von Minckwitz, Procter *et al.* 2017).

ER positive and ER negative breast cancer:

Estrogen receptors are G-protein coupled receptors which bind to the hormone 17β-estradiol (estrogen) (Karin Dahlman-Wright, 2006, VC, 2003). These receptors are of two kinds ERα and ERβ which are predominantly expressed in uterus, prostate, bone, ovarian and breast tissues. These receptors are responsible for the regulation of growth, development and physiology of the reproductive system (Lee, 2012). High circulating levels of sex hormones like estrogen have been suspected to be one of the major risk factors causing breast cancer (Endogenous, Breast Cancer Collaborative *et al.* 2011). During the menstrual cycles, estrogen levels supports the growth of breast and uterine tissues and thus, the body is exposed to high levels of the hormone. This could be one factor making women vulnerable to cancer (Surakasula, 2014). In some cancers, the ER receptors become hormone independent and continue their growth. These are termed as ER-ve breast cancers. The lack of receptors in ER-ve types of breast cancers, leads to inhibition in the binding of

the estradiol moiety contributing to uncontrolled growth (Crowe, Gordon *et al.* 1991, Dunnwald, Rossing *et al.* 2007). As these types of cancer cannot be targeted via hormone therapy, surgery, radiation and chemotherapy are the preferred course of treatment.

Triple negative breast cancer (TNBC):

Triple negative breast cancers are another aggressive breast cancer type claiming 15% of the total breast cancer deaths (Navrátil, 2015). This type is characterized by the absence of an estrogen receptor, progesterone receptor as well as the HER2 receptor. This is known to be one of the most aggressive cancers and are common in younger women (Navrátil, 2015). Most of the triple negative breast cancers are known to possess BRCA mutations (Peshkin, Alabek *et al.* 2010, Wong-Brown, Meldrum *et al.* 2015) . These types of cancers possess molecular heterogeneity i.e. different tumor cells display distinct types of morphology, gene expression and metastatic potential (Brian D. Lehmann 2011). Recently, it was suggested that TNBC cancers display an increased genomic instability which could contribute to its resistance to chemotherapeutic agents (Hu, Stern *et al*. 2009). Due to the aggressive nature of this type of cancer, treatment with hormonal based therapies is not recommended (Wahba and El-Hadaad 2015).

1.2 Altered cell metabolism in breast cancer:

Alteration in the metabolism of cells is one of the classical hallmarks of cancer (Hanahan and Weinberg 2011). Metabolism of glucose occurs via major pathways like glycolysis, TCA cycle, oxidative phosphorylation, etc. Cells metabolize glucose to produce ATP which is required to generate energy and thus help cells to carry out the various cellular functions (Michael and Schomburg 2013).

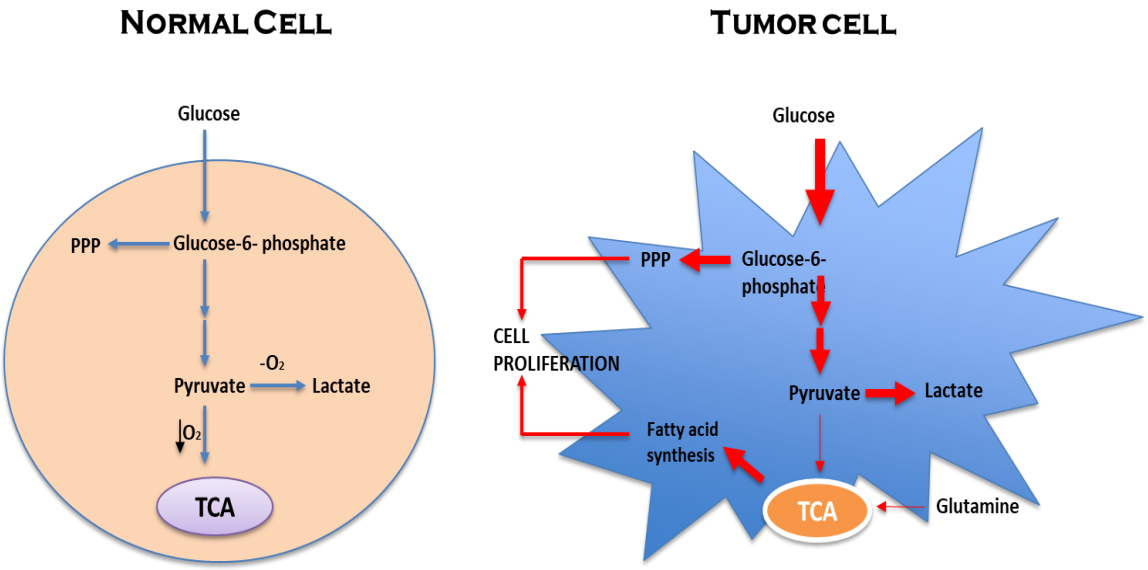

FIG 1.1 A difference in the metabolism of a normal cell and a tumor cell is shown. A normal cell catabolizes glucose to form pyruvate which enters the TCA cycle and generates ATP via the oxidative phosphorylation in mitochondria. In a tumor cell, oxidative phosphorylation is inhibited leading to an increased uptake of glucose and conversion into lactate which supports tumor progression. An increased flux of glucose forms excess amounts of ATP needed for growth. The cell also exploits glutamine as a carbon source and forms fatty acids on entering the TCA cycle. Thus, tumor cells bring a metabolic reprogramming to support continuous and rapid growth.

During glycolysis, normal cells catabolize glucose to pyruvate which is further converted to Acetyl Co-A and oxidized to carbon dioxide gas through the mitochondrial tricarboxylic acid cycle (Fig.1.1). This leads to generation of NADH and FADH2 molecules which help in the formation of 36 molecules of ATP per molecule of glucose, via oxidative phosphorylation thus acting as a major source of energy (Pawel Jóźwiak *et al*. 2014). However, tumor cells have been shown to rely majorly on the glycolytic pathway which generates 2 molecules of ATP. Recent studies reveal that the expression of glucose transporter GLUT-1 is upregulated in several breast cancers and this transporter is involved in the enhanced uptake of glucose (Kang, Chun *et al.* 2002, Oh, Kim *et al.* 2017). On silencing the GLUT-1 transporter in triple negative breast cancer cell lines, MDA MB231 and HS 578T, a reduction in cell proliferation was observed (Oh, Kim *et al.* 2017). Thus, alteration in the metabolism supports generation of abundant ATP via glycolysis, thereby supporting cell growth (Guppy, Greiner *et al*. 1993). Generation of high amounts of glycolytic byproducts were observed in more aggressive tumors types while the normal and less aggressive tumors had lower levels of these biomolecules. Thus, high proliferative cancers favor altered metabolic pathways to continue their progression (Brauer, Makowski *et al.* 2013, Ganapathy-Kanniappan and Geschwind 2013). Malignant cells also exploit glutamine, as a source of carbon molecules which enters the TCA cycle, and forms biomolecules (Pawel Jóźwiak *et al*. 2014). When breast cancer tissues were examined via metabolomic analysis, it was revealed that about 56% of the ER+ve and 88% of the ER-ve breast cancer tumor tissues had abundant glutamate reserves as compared to normal tissues (Budczies, Pfitzner *et al*. 2015). Cao *et al* observed that TNBC tumors had lower glutamine

levels but a relatively high glutamate level suggesting that glutamine was used for excessive glutaminolysis as observed in malignancy (Cao, Lamichhane *et al.* 2014). Glutamine and glutamate levels have been suggested to be a critical factor for monitoring the progression of cancer as well as can be a therapeutic target (Geck and Toker 2016). Thus, alteration in the cellular metabolic processes contributes to increased production of energy and biomass, which supports cell growth and proliferation and have prime importance in cancer progression.

1.3 Nutritional status in Breast cancer:

Due to enhanced nutrient uptake in malignancy, malnutrition is common in cancer patients (Mantzorou, 2017). Malignant cells exploit all the sources of nutrition and energy to fulfill the requirements for continuous proliferation (Adewale Fadaka 2017). Several multivitamins and minerals are considered necessary after a patient is diagnosed with breast cancer since they act as antioxidants (Bright-Gbebry M 2011). The inclusion of nutritional supplements in the diet could benefit the health of a cancer patient. It would help to overcome diseases like dementia, cardiovascular diseases or neurological diseases which would eventually assure good health (Harvie M 2015).

Institutions like the American Cancer Society, the World Cancer Research Fund, and American Institute for Cancer Research, suggest that supplementation with lost vitamins and minerals may not be beneficial to cancer patients (C. M. Lopes 2017). Supplementation with certain vitamins and minerals provide coenzymes or cofactors which play a critical role in cell metabolism (Rabhi, Hannou *et al.* 2017, Reiter, Wienerroither *et al.* 2017). Some of the enzymes involved metabolic processes are apoenzymes, i.e. they are inactive.

They possess a binding site in their structure and bind to non-protein organic molecules called cofactors. On binding, these enzymes form holoenzymes i.e. they become active and carry out their respective functions (Fig 1.2). The table below illustrates a list of vitamins and minerals which act as cofactors either themselves or provide other molecules (Table 1.1)

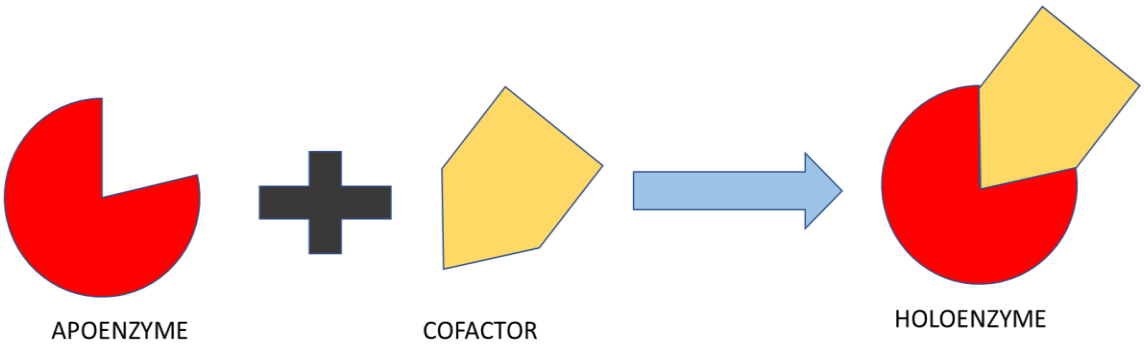

FIG 1.2 A representation of the binding site present on the apoenzyme to which a cofactor can bind. On binding, it results in the formation of holoenzyme, which is the active form of the enzyme.

Table 1.1 Vitamins and cofactors: The table below illustrates few of the cofactors or coenzymes. These cofactors help in the activation of apoenzymes and hence, are a necessary component for many biochemical reactions occurring in the body.

Cofactors	Enzymes	Function
Zn^{2+}	Carboxypeptidase	Couples to NAD+
Ni^{2+}	Urease	Hydrolysis of amides
Mn^{2+}	Arginase	Removal of electrons
Tetrahydrofolate	Thymidylate synthase	Synthesis of thymidine
Flavin adenine nucleotide	Monoamine oxidase	Inhibition of neurotransmitters
Thiamine pyrophosphate	Transketolase	PPP and TCA cycle

The B-vitamins play an important role in nucleotide synthesis and other metabolic processes and have been implicated in malignancy (Morris 1943, Scott, 1998, Kim, 2007). In a study with mice having mammary tumors, a riboflavin deficient diet slowed the growth of these tumors. But inclusion of this vitamin in the diet, restored the tumor growth process (Morris 1943). It has been demonstrated that elevated plasma folate levels correspond to increased cancer risk (Kim SJ 2016). It was observed that mammary tumors in rats displayed an increase in cell proliferation rate when supplemented with 5mg of folic acid in their daily diet (Deghan Manshadi, Ishiguro *et al.* 2014) . Even though, deficiency of these essential vitamins could affect normal health, the intake of these essential nutritional factors should be controlled to reduce risk of cancer growth. Thus, there arises a dilemma as to supplementation of certain nutrients to cancer patients would be beneficial or does it further induce tumorigenesis.

1.4 Thiamine (B_1) and homeostasis:

Source and functions of thiamine:

Vitamin-B1, thiamine, belongs to the class of B-complex vitamins and contains an aminopyrimidine and a thiazolium ring linked by a methylene bridge (Fig 1.4.A). It is an essential vitamin which is not produced by the body. Being water soluble, it is not retained in the body and gets eliminated via bodily fluids. Thus, a regular dietary intake is a must to replenish the stores of this vitamin. Thiamine is found in many sources of food i.e. fish, legumes, oils, nuts, milk, etc. and can be taken through inclusion in our diet or is available as dietary supplements. Thiamine plays an important role as a cofactor to support many

metabolic reactions in the body. It is an important factor involved in the normal functioning of the nervous system. There has been a lot of focus on intake of thiamine supplements for a number of diseases like cardiovascular diseases and is a risk factor in type 1 and 2 diabetes along with obesity, dyslipidemia, heart failure caused due to thiamine deficiency (Eshak and Arafa 2018). Thiamine deficiency is also a major cause of diseases like wet and dry Beri-Beri and Wernicke Korsakoff syndrome (Peter R. Martin 2004). Thiamine deficiency has also been shown to play a major role in the development of cardiovascular diseases (Eshak ES 2018). Due to its effect on the nervous system, thiamine deficiency is implicated in the pathogenesis of Alzheimer's disease (Koh, 2015, Gibson, 2016, Pan, 2016).

Thiamine uptake:

Being an essential vitamin for regulation of many body functions, the transport of thiamine in the cell is a very important process. Entry of thiamine into the cell is facilitated by thiamine transporters, SLC19A2(THTR1) and SLC19A3(THTR2) present on the cell surface. SLC19 is a folate/thiamine transporter family consisting of the two thiamine transporters along with Reduced folate carrier (RFC1). The RFC1 transporter functions by an anion exchange with organic phosphates and does not depend on sodium or protons for its activity (Rongbao Zhao 2013). This transporter is not involved in the transport of thiamine but helps in the uptake of folate molecules.

THTR1 and THTR2 share a 48% structural similarity with RFC1 (Rongbao Zhao 2013). In murine leukemia cells a high RFC1 density was observed which did not affect the thiamine uptake however, thiamine was effluxed out via RFC1 transporter due to its accumulation inside the cell (Zhao R 2001). In cases having a deficiency of THTR1 or THTR2 transporter, a low thiamine level leading to thiamine responsive megaloblastic anemia has been observed (Reidling, Lambrecht *et al*. 2010, Brown 2014). THTR2 deficiency was also a factor in the pathogenesis of biotin responsive basal ganglia disease (Brown, 2014). To meet the enhanced needs of malignant cells, an increase in the uptake of thiamine has been observed. A study suggested that a down regulation of the SLC19A3 thiamine transporter in breast cancer cells could be associated with the ability of malignant cells to resist apoptosis (Liu, Huang *et al.* 2003).

Once inside the cell, thiamine gets phosphorylated by an enzyme thiamine pyrophosphokinase-1(TPK1), to generate its active form, Thiamine pyrophosphate (TPP) (Fig 1.3). TPP is a cofactor responsible for the activation of three essential enzymes, transketolase (TKT), pyruvate dehydrogenase (PDH) and alpha ketoglutarate dehydrogenase (AKGDH), which play a critical role in metabolic pathways (Manzetti, Zhang *et al*. 2014).

The TPP formed inside the cell, if not used as a cofactor, can get dephosphorylated back into Thiamine Monophosphate (TMP) by an enzyme thiamine pyrophosphatase. This TMP can either be effluxed out of the cell via RFC1 along with excess TPP or gets converted back into Thiamine. Thiamine monophosphatase is the enzyme which helps in this

conversion and the thiamine gets accumulated inside the cell for further use (Rindi and LAforenza, 2000, Zhao, Gao *et al.* 2002). TPP is transported into the mitochondria by a carrier called thiamine pyrophosphate carrier (TPC) or SLC25A19, where it performs its cofactor function to help the mitochondrial enzymes PDH and AKGDH (Lindhurst, Fiermonte *et al.* 2006).

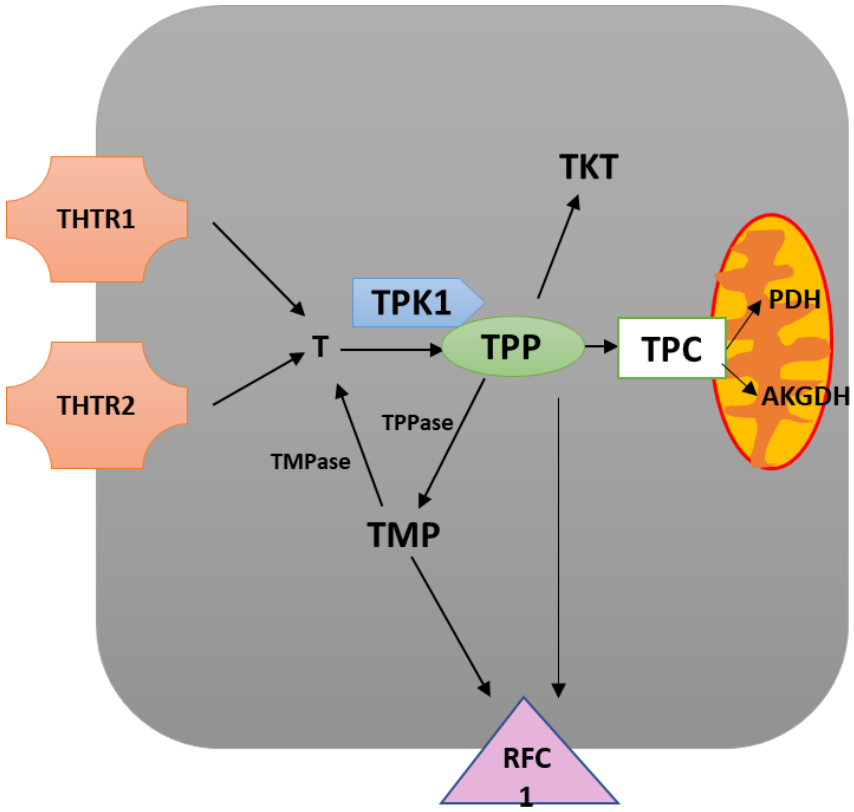

Fig 1.3 Thiamine homeostasis: Being a water-soluble vitamin, transporters THTR1 and THTR2 aid in the uptake of thiamine into the cell. Thiamine gets broken down to Thiamine pyrophosphate, its active form which then functions as a coenzyme for transketolase, pyruvate dehydrogenase and alpha ketoglutarate dehydrogenase enzymes. The unused TPP can be again converted back to thiamine or it can be taken out from the cell via RFC-1 carrier.

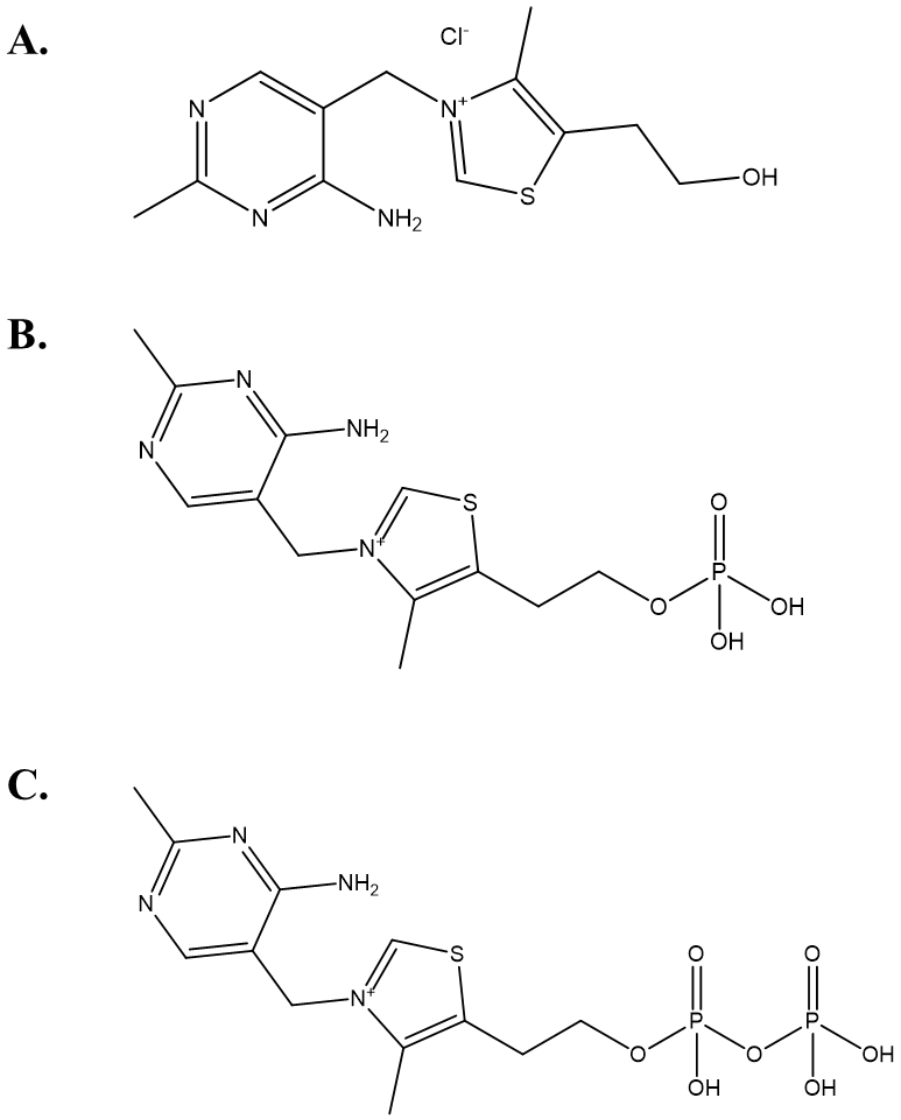

Fig 1.4 Thiamine analogues: Above is a list of all the thiamine analogues formed in the body. A) Thiamine, B) Thiamine Monophosphate, C) Thiamine pyrophosphate.

1.5 Role of Thiamine in cell metabolism:

1.5.1 Pentose phosphate pathway:

TPP is involved in the pentose phosphate pathway (PPP) where it binds to enzymes and performs its functions as a cofactor. The PPP consists of two phases: oxidative phase and the non-oxidative phase. Glucose is broken down to form ribose-5-phosphate in the oxidative phase of the PPP which is a major source of ribose sugar for the formation of nucleotides. The oxidative phase also provides NADPH which is a precursor in the synthesis of fatty acids. If not consumed, R5P further enters the non-oxidative phase of the PPP and via a series of reversible reactions gets converted to glyceraldehyde-3-phospahte (G3P) and fructose-6-phosphate (F6P). In malignancy, transketolase enzyme facilitates the conversion of G3P and F6P back to R5P to provide sugars for the synthesis of nucleotides (Peng Jiang 2014).

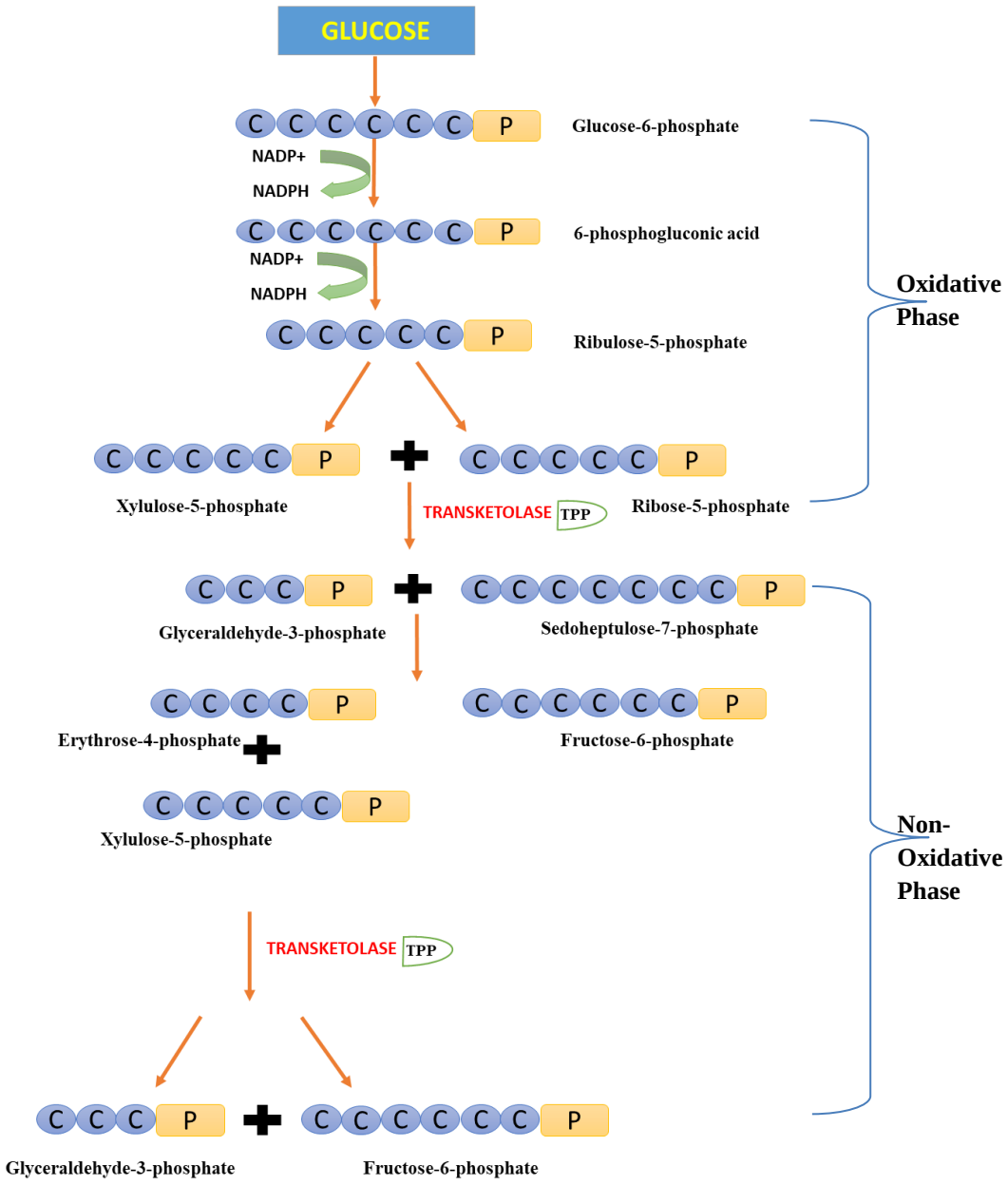

FIG 1.6 The diagram illustrates the pentose phosphate pathway. It represents the catabolism of glucose and the two phases of the PPP. Transketolase is an essential enzyme in the non-oxidative phase which helps in the conversion of intermediates and relies on thiamine pyrophosphate, a cofactor provided by thiamine.

1.5.1.1 Transketolase:

Transketolase is an enzyme encoded by the TKT gene and plays a catalytic role in the non-oxidative phase of the PPP in humans. The non-oxidative pathway carries out several reversible reactions which ultimately leads to the generation of Ribose-5-Phospahte (R5P). This substrate either gets utilized to form nucleotides via de novo pathway or enters the EMP pathway to form ATP (Berg 2002).

Cancer cells show an enhanced uptake of glucose as well as rely majorly on glycolysis for nucleotide formation. Several studies have revealed an up regulation of expression of TKT in malignant cells. A higher expression of TKT was observed in tissues isolated from hepatocellular carcinoma patients when compared with tissue samples from normal patients (Shimizu T 2014). Thus, it was suggested that this high expression of the enzyme correlated with the higher dependence on the non-oxidative pathway of the PPP to produce more Ribose-5-phosphate. For the culturing of NCIH-441 cells, a stable ^{13}C glucose isotope labelled media was used and later analyzed to detect the carbon fragments using mass spectrometry. A decreased production of CO_2 via direct oxidation was observed as well as the nucleic acid ribose synthesis levels were elevated. This confirmed of the metabolic adaptations occurring in malignant cells which further lead to metabolic disturbances in the body (Boros LG 2000). A study reveals that TKT is upregulated in metastatic peritoneal implants and increases the proliferation rate in ovarian cancer (Ricciardelli C 2015). An elevated level of nuclear TKT was also observed in peritoneal metastatic tissues as compared to the primary tumors. This high level of nuclear TKT was correlated to be associated with a reduced survival rate or an increased death rate in ovarian cancer. However, on knocking down the TKT enzyme, they found a significant reduction

in tumors and it suggested that this could be explored as a target in the treatment of the disease (Ricciardelli C 2015). TKT plays a critical role in the PPP and leads to the generation of NADPH which acts as an antioxidant against several reactive oxygen species (ROS). It was observed that upregulation of TKT enzyme in malignant cells lead to a higher production of the antioxidant which could further help to scavenge the ROS. Thus, TKT could be involved in overcoming the oxidative stress and further promoting proliferation (Xu IM 2016). An enhanced uptake of thiamine, leading to an increased generation to TPP, shows an upregulation of the TKT enzyme in cancer (Patra KC 2014). However, the effect of supplementation of thiamine on enzyme activity is not fully understood and could be a critical prognostic factor towards cancer progression.

TKT is known to have two isoforms- TKTL1 and TKTL2. They are almost structurally identical, but no evidence has yet been confirmed regarding the dependence on TPP for their activity by these isoforms. TKTL1 is a critical factor contributing to malignant growth and an increase in cell growth is observed (Zhao J 2009). Also, a high expression of TKTL1 correlated with the invasiveness and poor survival rate of patients with urothelial and colon carcinomas (S Langbein 2006). A study demonstrated that knockdown of TKTL1 using SiRNA lead to an inhibition in tumor proliferation in gastric cell lines as well as an inhibition of tumor growth in mice models. Thus, the inhibition of this gene could possibly be an important therapeutic target (Weijie Yuan 2010).

1.5.2 Tricarboxylic acid cycle (TCA cycle):

Reprogramming of metabolism during malignancy majorly affects the TCA cycle and it could be possible targets for chemotherapeutic agents (Chen JQ 2012) . During glycolysis, glucose is broken down to form pyruvate through a series of biochemical reactions. Pyruvate then gets broken down by a thiamine dependent enzyme Pyruvate dehydrogenase, which uses TPP as its cofactor and converts pyruvate to acetyl co-A. the TCA cycle forms several biomolecules along with energy in the form of $NADH_2$ (Vander Heiden and DeBerardinis 2017). PDH enzyme is a link between glycolysis and TCA cycle and acts as a gatekeeper in regulating the two pathways. Cancer cells exhibit an upregulation of an enzyme pyruvate dehydrogenase kinase (PDK). This enzyme regulates the functioning of PDH. The elevated levels of PDKs, inhibits PDH activity via phosphorylation of the enzyme leading to an accumulation of pyruvate. Pyruvate then gets converted to lactate by an enzyme lactate dehydrogenase (LDHA). Malignant cells utilize the formed lactate for further cellular proliferation (McFate, Mohyeldin *et al.* 2008).

As the TCA cycle is disrupted due to inhibition of PDH enzyme, malignant cells exploit other sources of carbon like glutamine. Glutamine converts into glutamate which enters the TCA cycle on formation of alpha ketoglutarate. Alpha ketoglutarate then forms succinyl co-A which then forms other intermediates of the TCA cycle to eventually form the necessary biomolecules and energy. Thus, the TCA cycle is completed due to the completion of anapleurotic reactions.

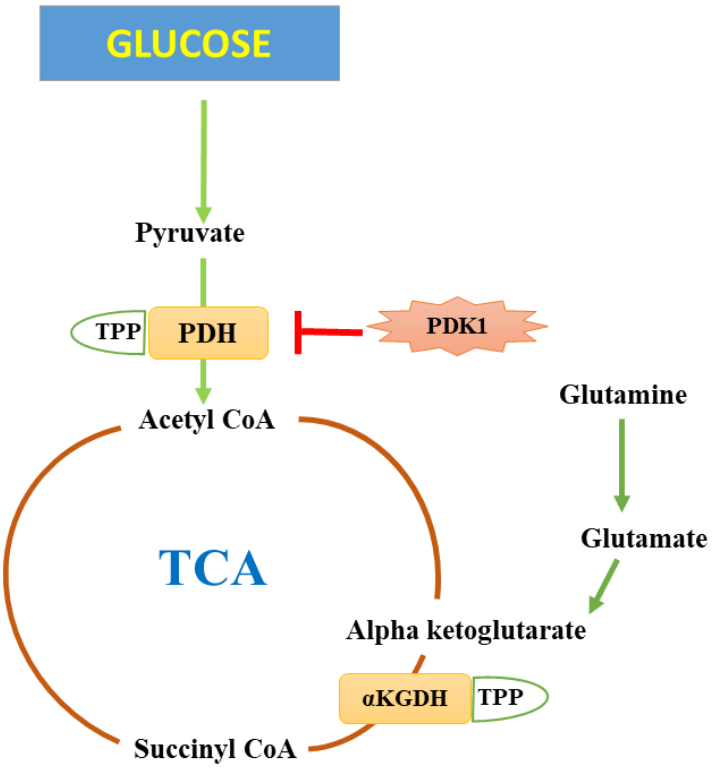

FIG 1.7 The TCA cycle: The figure above depicts the tricarboxylic acid cycle. Here pyruvate formed as a product of glycolysis, enters the cycle to produce acetyl Co-A which then facilitates the formation of amino acids and other biomolecules. In malignancy, the enzyme which helps in the conversion of PDH to acetyl Co-A is inhibited by PDK and thus other sources like glutamine get exploited to gain carbons to continue the TCA cycle and thereby form molecules which help in cell growth.

1.5.2.1 Pyruvate dehydrogenase:

PDH is an enzyme belonging to the family of Pyruvate dehydrogenase complex (PDC). It consists of three components, PDH(E1), dihydroprolyl transacetylase (E2), and dihydroprolyl dehydrogenase (E3) (Zhou, McCarthy et al. 2001). Being at the junction of glycolysis and TCA cycle, it acts as a gatekeeper in regulation of these pathways. It plays a critical role in metabolism of glucose as well as oxidation of fatty acids. PDH converts pyruvate to acetyl Co-A in an irreversible reaction and then enters the TCA cycle to form amino acids and nucleotides required for the cell cycle regulation. Two enzymes, PDKs and PDPs help in the regulation by inhibiting the PDH activity by phosphorylation, when PDH is abundant and dephosphorylating the enzyme to maintain normal levels to keep the TCA cycle moving forward. PDKs are a group of four isozymes which work to inhibit PDH at its serine residues. They target three serine residues in general: Ser300, Ser293, Ser232. On binding to these residues, they inhibit the enzyme via ATP dependent phosphorylation. It is observed that only PDK1 can specifically bind to all the serine residues and inactivate the enzyme (Yeaman SJ 1978, Korotchkina LG 2001).

Malignant cells have been observed to show an overexpression of PDK1 enzyme which leads to the phosphorylation of PDH. The expression of the PDK1 enzyme was observed to be upregulated in the head and neck squamous carcinoma cells along with an increase in expression of the phosphorylated PDH enzyme and total PDH (McFate, Mohyeldin *et al.* 2008). In the tumor associated stromal tissue, an upregulation in the expression of the

PDH enzyme has also been observed with a subsequent decrease in the PDK isoforms (Koukourakis, 2005, Koukourakis, 2006). Thiamine can function as an antioxidant thereby inhibiting the function of PDK1 enzyme (Hanberry, Berger *et al*. 2014). This could benefit in the reactivation of the PDH enzyme as well as reducing the cell proliferation (Hanberry, Berger *et al.* 2014). However, the effect of thiamine supplementation on the mRNA levels of PDH enzyme is seen to be consistent in lymphoblasts cultured in different thiamine concentration (Pekovich, 1998). Thus, an association between thiamine supplementation and its subsequent effect on expression and activity of PDH enzyme must be developed.

1.5.2.2 Alpha ketoglutarate dehydrogenase:

Another enzyme found in the mitochondrial matrix is alpha ketoglutarate dehydrogenase (AKGDH) and relies on TPP as its cofactor. It has three subunits E1, E2 and E3. These three subunits are different and the E3 subunit is identical to the pyruvate dehydrogenase complex. AKGDH facilitates the conversion of alpha ketoglutarate to succinyl co-a which is a precursor to produce various biomolecules (Koike K 1974). It also is a source of NADH which is required for regulating the ROS. It carries out the conversion of alpha ketoglutarate to succinyl co-A and forms NADH which then enters the electron transport chain. It is also a crucial target for oxidative stress since it is shown to form H_2O_2 and form reactive oxygen species (Tretter and Adam-Vizi 2004). Thiamine and its cofactor are known to possess radical scavenging effects on the ROS (Okai, Higashi-Okai *et al*. 2007). However, a correlation between the functioning of αKGDH enzyme and thiamine supplementation and its possible role in cancer must be established.

Cancer cells majorly rely on fatty acid molecules, synthesized from precursors such as citrate, which contributes in biomass expansion thereby supporting cell growth (Renaud Vatrinet 2017). Due to truncation of the TCA cycle, malignant cells have been demonstrated to exploit another carbon source like glutamine to further support the TCA cycle. This facilitates the formation of alpha ketoglutarate and regulates the anaplerotic reactions to continue the TCA cycle (Renaud Vatrinet 2017). However, there is not enough data demonstrating the change in the expression and activity of this enzyme in malignancy.

1.6 Thiamine and tumor microenvironment:

Solid tumors are deprived of oxygen due to lack of vascularization (Brown, 2007). This leads to the stabilization of a transcription factor, hypoxia inducible factor-1 (HIF-1) (GL., 2009). The activation of HIF-1 leads to the activation of certain target genes which play a critical role in supporting the reprogramming of metabolic processes (De Huang 2014). HIF dimerizes to HIF-1 α or HIF-1β subunit. HIF-1α has various isoforms consisting of Hif1α, Hif2α and Hif3α (Wang, Jiang *et al*. 1995). HIF-1α is shown to upregulate LDHA expression which is responsible for the production of lactate. Lactate is responsible for maintaining the equilibrium status of the redox- pair (NADH/NAD+) which helps in the continuation of glycolysis (San-Millan, 2017). It also produces an acidic environment which is favorable for the growth of cells. Elevated levels of lactate thereby contribute in promoting cell growth. HIF-1α has been shown to induce an increase in the expression of the thiamine transporter SLC19A3 in hypoxic environments, indicating a possible role of HIF-1α in the regulation of thiamine homeostasis (Zera, 2016). The expression of TPK1 enzyme is also shown to be upregulated in hypoxic conditions which could facilitate in the

continuous production of TPP (Jonus, 2018). HIF-1 gene is known to be involved in activation of certain glycolytic genes and thiamine dependent enzymes which support metabolic reprogramming (Kim, Tchernyshyov *et al.* 2006). An increase in the TKT flux was observed when the imatinib resistant cells were exposed to hypoxic environments (Zhao, Mancuso *et al*. 2010). A sharp decline in the PDH activity was observed in the metabolic models used to demonstrate the alteration in metabolism seen in malignancy (Eyassu and Angione 2017). This decline in activity of the enzyme in hypoxia suggested that HIF-1α activated its target gene PDK, which could have an inhibitory effect on PDH. Kim *et al* observed that hypoxia upregulates the PDK1 expression which further leads to the inhibition of pyruvate and triggers a Warburg effect (Kim, Tchernyshyov *et al.* 2006). Hypoxia is also associated with an elevated expression of TKTL1 enzyme in colorectal cancer (Bentz, Cee *et al.* 2013). It was suggested that hypoxic conditions could facilitate more energy supply to the cells through upregulating this enzyme. Recent evidences reveal that malignant cells use reductive carboxylation of alpha ketoglutarate to form isocitrate i.e. reverse of TCA cycle (Metallo, Gameiro *et al*. 2011). The group demonstrated the formation of fatty acids which thereby contributed in promotion of cell proliferation. Thus, tumor microenvironment is a critical factor to be considered in understanding the adaptive metabolic reprogramming cells undergo to facilitate enhanced cell growth.

1.7 Thiamine and Cancer:

Lifestyle is known to influence the health and quality of life. Due to increasing changes in the way of living, people adapt to unhealthy habits which leads to diseases, malnutrition etc (Farhud 2015). Deficiency of vitamins is a major issue affecting people. It is known

that vitamin b1 deficiency is associated with many diseases (Eshak and Arafa 2018). Consumption of this vitamin through diet is essential and a dose of 1-1.5mg is recommended in the daily dietary intake (The National Academies Collection, 1998) . Due to the availability of supplements over the counter, people rely in using those to maintain good health. However, it is observed that the supplements available in the market contain about 100-6000% of the dietary value of thiamine (Kim, Williamson *et al.* 1993, Dietary Supplement Label Database, 2014). This high amount of thaimine has been shown to be associated with cancer risk. Thiamine supplementation is known to be double-edged sword since treatment with varying doses of this vitamin have been shown to have a growth promoting as well as growth inhibiting effect on cancer cells (Boros, 1998, Comin-Anduix, 2001)**.** In Ehrlich's ascites tumor mice, supplementing with 25 times the required dietary intake led to an increase in the size of tumor (Comin-Anduix, 2001)**.**

Supplementation effects on cancer:

A normal thiamine diet has been shown to support the growth of mammary tumors in the MMTV-neu mice models. A normal thiamine diet helped in increasing the proliferation rate of tumor cells, thus indicating a role of thiamine supplementation in cancer (Daily, 2012). Supplementation with this vitamin has been shown to affect the expression levels of the thiamine dependent enzymes which could be associated with cancer growth. An increasing dose of thiamine is shown to upregulate the mRNA levels and activity of TKT enzyme in vein endothelial cells and bovine retinal pericytes when subject to a high glucose treatment (Elena Beltramo 2006). Human lymphoblasts when supplemented with a thiamine deficient media (0.1nmol/L) and compared using northern analysis with a control

media (10µM/L), showed an increase in the mRNA levels of the TKT enzyme in the control media (Singleton 1998). On performing the RT-PCR analysis, a higher mRNA levels in the control media for TKT was observed, but thiamine supplementation did not have any effect on the mRNA levels of other thiamine dependent enzymes like PDHe1α and AKGDH (Singleton 1998). Thus, a correlation between supplementation of thiamine and its possible effect on the enzymes as well as cell growth should be considered.

Hypothesis and aims:

We hypothesized that thiamine supplementation would have an effect on the growth of cancer cells and would also affect the expression and activity of thiamine dependent enzymes. Here we studied two breast cancer cell lines, MCF-7 and MDA MB 231 which are ER +ve and triple negative respectively. We used these cells to determine the effect of thiamine supplementation on phenotypically different cancer cells. To study the effect of varying concentrations of thiamine, we supplemented them with thiamine at doses slightly higher to that of the plasma levels. We wanted to examine the effect of thiamine supplementation on cell growth in a normoxic and hypoxic environment and determine the effect on the expression and activity of the thiamine dependent enzymes. This would prove beneficial to understand whether low doses of thiamine would directly affect cell growth and if so, its potential role on the metabolic pathways due to upregulation of the enzymes involved.

CHAPTER 2

EFFECT OF SUPPLEMENTATION OF VITAMIN B1 ON THIAMINE DEPENDENT ENZYMES AND CELL PROLIFERATION

2.1 INTRODUCTION:

Reprogramming of cellular metabolism is one of the critical hallmarks of cancer. This alteration in the metabolic pathways leads to the production of metabolites which support proliferation of malignant cells (Michael and Schomburg, 2013). Thiamine is converted to TPP, a cofactor which is a major player in different metabolic processes. However, a correlation between thiamine supplementation and its effect on enzyme activity is not fully understood. It has been observed that thiamine in low doses has proliferative effects on cells (Comin-Anduix, 2001). Thiamine supplementation in concentrations 25 times the required dietary intake has been shown to increase the tumor growth (Comin-Anduix, 2001).

The expression of thiamine dependent enzymes like TKT, PDH and AKGDH have been shown to be upregulated in malignancy (Tretter and Adam-Vizi 2005, McFate, Mohyeldin *et al.* 2008, Shimizu, Inoue *et al.* 2014). Transketolase is an essential enzyme involved in the non-oxidative pathway of the PPP and has a major role towards the process of cell growth. It is also known that malignant cells have a decreased production of CO_2 via direct

oxidation and higher ribose production confirming of metabolic adaptations in malignant cells (Boros LG 2000). An upregulation in the mRNA levels of TKT enzyme was observed in hepatocellular carcinoma cells when compared to normal cells (Kubota 2014). Also, the mRNA levels of TKT enzyme were higher in human lymphoblast cells which received a higher dose of thiamine compared to the cells treated with thiamine deficient media (Singleton, 1998). However, a correlation between thiamine supplementation and its effect on TKT activity has not established.

Head and neck squamous cell carcinomas show an upregulation in the expression of PDK1 enzyme (McFate T, 2008). An elevated expression of phosphorylated PDH and total PDH enzyme is also observed, which supports the theory that the PDK enzyme could have inactivated PDH via phosphorylation. At high concentrations, thiamine is known to possess antioxidant properties which could affect the activity of PDK enzyme (Hanberry, Berger *et al.* 2014). However, thiamine supplementation does not have an effect on the mRNA levels of PDH enzyme. Thus, the correlation between thiamine supplementation and its effect on the activity of PDH enzyme must be determined.

Alpha ketoglutarate dehydrogenase enzyme is known to affect the level of reactive oxygen species in the body, thus playing a major role in regulating the TCA cycle and metabolic flux (Laszlo Tretter 2005). Even though there is no change observed in the mRNA levels of this enzyme in the human lymphoblast cells following thiamine supplementation, the effect of thiamine on the expression of this enzyme as well as on its activity is not fully studied.

Solid tumors have been known to lack vascularization resulting in hypoxia conditions and lead to the stabilization of an oncogene, HIF-1α (Brown 2007, Semenza 2009). The stabilization of this oncogene is known to support the activation of target which have affected the activity of thiamine dependent enzymes. The PDH activity has been shown to reduce significantly in hypoxic environment (Eyassu and Angione 2017). Also, the TKT flux is observed to be much higher in tumor cells subjected to hypoxia demonstrating a critical role of hypoxia in metabolic pathways (Zhao, Mancuso *et al*. 2010). However, there is not much evidence demonstrating the combined effects of thiamine supplementation and hypoxic environment on cell growth as well as altered metabolism.

Therefore, the objective of this study was to assess the effects of thiamine supplementation on growth of phenotypically different breast cancer cells and also study the effect of thiamine supplementation on the functional activity of thiamine dependent enzymes.

2.2 MATERIALS AND METHODS:

Cell culture:

MCF-7 and MDA-MB 231 (breast cancer adenocarcinoma) cell lines were obtained from ATCC (Manassas, VA) and were maintained in RPMI 1640 media containing a thiamine concentration of 3µM (Corning Life Sciences, New York) along with 10% Fetal bovine serum (Seradigm, Radnor, PA), 1% penicillin/streptomycin (Corning Life Sciences, New York) and 0.01% mycozap antibiotics (Lonza, Walkersville, MD) to avoid mycoplasma contamination. The maintenance flasks were cultured at 37°C in an incubator with 5% CO_2 air atmosphere. The cells were seeded at a density of 50,000 cells/cm^2 in maintenance flasks and grown to 80% confluency and then split for experimental treatments using 0.25% trypsin containing 2.1mM of EDTA (Mediatech INC., Manassas, VA).

Treatments:

To determine the effects of thiamine supplementation, two concentrations of thiamine was used to culture the cells. The thiamine concentration in plasma is observed to be in the range of 10-20nM . We used 10nM thiamine concentration as a control group to simulate the physiological levels of thiamine observed in human plasma. Custom formulated thiamine deficient RPMI 1640 media (Corning life sciences, New York) was utilized, and the media was prepared by adding 10% Fetal bovine serum (Seradigm, Radnor, PA), 1% penicillin/streptomycin (Corning life sciences, New York) and 0.01% mycozap antibiotics

(Lonza, Walkersville, MD) with an addition of 1% L-glutamine (Corning Life Sciences, New York). The RPM1 1640 media contains 3µM of thiamine i.e. 300 times the plasma thiamine concentration and is used for culturing of cells. A concentration of 3µM thiamine hydrochloride was spiked in the 10nM media.

Cells seeded in both 10nM and 3µM thiamine media were cultured in normoxia i.e. at 37°C in an incubator with 5% CO_2 air atmosphere. Another set of cells seeded in 10nM and 3µM thiamine media were cultured in hypoxia at 37° C in an incubator with 5% CO_2 and 1% O_2 for a period of 24h and 7d. The ProOX oxygen controller (Biospherix, Lacona, NY) supplied nitrogen gas for controlling the O_2 levels in the hypoxia incubator. The incubator was calibrated once a week, and oxygen levels were monitored. The media was pretreated in hypoxia 24h prior to treatments for equilibration.

Cell proliferation Assays:

The effect of thiamine supplementation on cell proliferation was determined by performing three different methods. Two of these methods were used to determine the changes in cell growth after receiving the different treatments for 7d while the third method determined the number of newly formed cells.

Cell count method:

The effect of thiamine supplementation on cell growth was determined by the cell count method. Cells were seeded at 1000 cells/cm^2 in 60 mm dishes in 10nM and 3µM thiamine media and cultured in normoxia and other set in hypoxia for 7 days. On the day of harvest,

the media was aspirated, and dishes were trypsinized using 0.25% trypsin with 2.1mM EDTA and neutralized with the equal volume of media. After thorough mixing, cells were counted on a Bio-Rad TC 20 (Bio-Rad Laboratories, Hercules, CA) automated cell counter. The change in cell growth was determined by obtaining the live cell count of all the treatment dishes incubated in normoxia and hypoxia and compared to the cell count obtained from 10nM thiamine dish in normoxia.

Crystal Violet Assay:

The Crystal Violet assay was used to determine the changes in cell growth (Journe *et al*. 2008). The cells were seeded in a 6-well plate at a density of 500 cells/cm^2 and cultured as mentioned above. Media was changed the following day after the cells had attached and fresh media was supplemented 24h prior to harvest. On the day of harvest, the media was aspirated, and the wells were washed with Phosphate Buffer Saline (PBS) and then fixed with 10% formalin (Harleco, Denmark) for 1 h. Cells were stained using 0.1% of crystal violet for 30 min and then washed with water and set aside to dry overnight. To quantify the results, crystal violet was de-stained from each well using 1% Triton X-100 and agitated on a shaker at 150rpm for 30min to solubilize the stain. The absorbance was measured at 550nm using the SpectraMax M2 Spectrophotometer (Molecular Devices, Sunnyvale, CA). The change in cell growth was calculated as a measure of fold change compared to the cells in 10nM thiamine media in normoxia.

BrdU cell proliferation assay:

Colorimetric BrdU cell proliferation assay kit (Exalpha biologicals INC, Shirley, MA) was used to measure the amount of newly formed cells. The cells were seeded in a 96 well plate at a density of 1000cells/cm^2 in dishes containing 10nM and 3µM thiamine media and cultured in normoxia and hypoxia for 7d. BrdU was incorporated 12h before harvesting the samples. After 7d, media was aspirated from the treatment dishes and cells were fixed using the denaturing solution provided in the kit. The fixed cells were rinsed with wash buffer and treated with a detector antibody and incubated for 1 hour at room temperature. The wells were washed and a 1X peroxidase goat anti-mouse IgG conjugate solution was added and incubated for 30min. After a last wash, a development solution was added, and plates were incubated in dark for 30min at room temperature. An ELISA stop solution was added to each well and the intensity of color change of the dye was detected using the SpectraMax M2 Spectrophotometer at 450/550nm.

Western Blot analysis:

To determine the changes in protein expression of thiamine dependent enzymes TKT, PDH and AKGDH following thiamine supplementation, Western blot analysis was used. Cells were seeded at a density of 1000cells/cm^2 150cm dishes and cultured in normoxia and hypoxia as mentioned above. Cells were harvested using a lysis buffer consisting of 1% Nonidet P-40 (NP40), 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 0.01% sodium azide, 50 mM Tris, 250 mM NaCl, and 1 mM ethylenediaminetetraacetic acid (EDTA) at pH 8.5 with an addition of protease and phosphatase inhibitors along with phenyl methyl sulfonyl fluoride (Sigma Aldrich, St. Louis, MO) and incubated on ice for

10 min. Cells were scraped off and collected in tubes and centrifuged at 4°C at 14,000xg using a Microfuge 22R centrifuge (Beckman Coulter, Brea, CA) for 15 minutes. Supernatant was collected, and protein concentration was determined using BCA protein assay kit (Thermo Scientific, Rockford, IL).

Whole cell lysates containing 50µg of total protein were boiled for 5 minutes and gel electrophoresis was performed on a 12% SDS-PAGE gel. The gels were then transferred to a polyvinyl difluoride membrane (Hybond-P, GE Healthcare, Piscataway, NJ). The membranes were blocked in 5% skim milk solution in Tris buffer saline tween 20 (TBST) for one hour for TKT, AKGDH and PDH and 3% Bovine Serum albumin (KSE Scientific, Durham, NC) in TBST for PDH293 protein respectively. The blots were washed using TBST and blotted for the thiamine dependent enzymes- TKT (Sigma Aldrich, St. Louis, MO), AKGDH (Genetex, Irvine, CA) and PDH (Genetex, Irvine, CA), PDH293 (CalBiochem, San Diego, CA) having a concentration of 1:1000 in TBST at 4°C for 24h. β-Actin (Sigma Aldrich, St. Louis, MO) was used as the loading control. Blots were washed 3 times for 15 minutes using TBST and incubated for 1 hour with their respective rabbit (for TKT, PDHe1α, α-KDGH and PDH 293) or mouse secondary antibody (for β-actin) (Millipore, MA). Blots were washed with TBST 3 times for 10 minutes. Membranes were treated with 1:1 mixture of Supersignal West Pico luminol/enhancer solution and stable peroxide solution from Thermo Scientific and imaged using FluorChem SP (Alpha Innotech, San Leandro, CA) using the chemiluminescence.

Transketolase activity assay:

The transketolase activity assay is a colorimetric assay which involves the conversion of Ribose-5-phosphate (R5P) and xylulose-5-phosphate (X5P) to form glycerol-3-phosphate(G3P). β-Nicotinamide adenine dinucleotide (NAD+) is added along with phosphotriose isomerase (TIM) and glycerol 3 phosphate dehydrogenase (G3PD) which are used to aid in carrying out the reaction. The TKT enzyme present in the sample along with TPP, facilitates the breakdown of R5P. The activity of the enzyme can be measured by correlating it to the amount of Nicotinamide adenine dinucleotide hydrogen-reduced (NADH) generated by the reduction of NAD+. Cells were seeded at 1000 cells/cm^2 in 150cm dishes and incubated for 7 days at 37°C with 5% CO_2 air atmosphere. One set of the dishes containing cells in 10nM and 3µM thiamine media were incubated in hypoxia for 7d after supplementing them with fresh media on the 2^{nd} and 6^{th} day and another set was given a hypoxia exposure for 24h. Whole cell lysates were obtained using lysis buffer along with phosphatase and protease inhibitors and PMSF (Sigma Aldrich, St. Louis, MO).

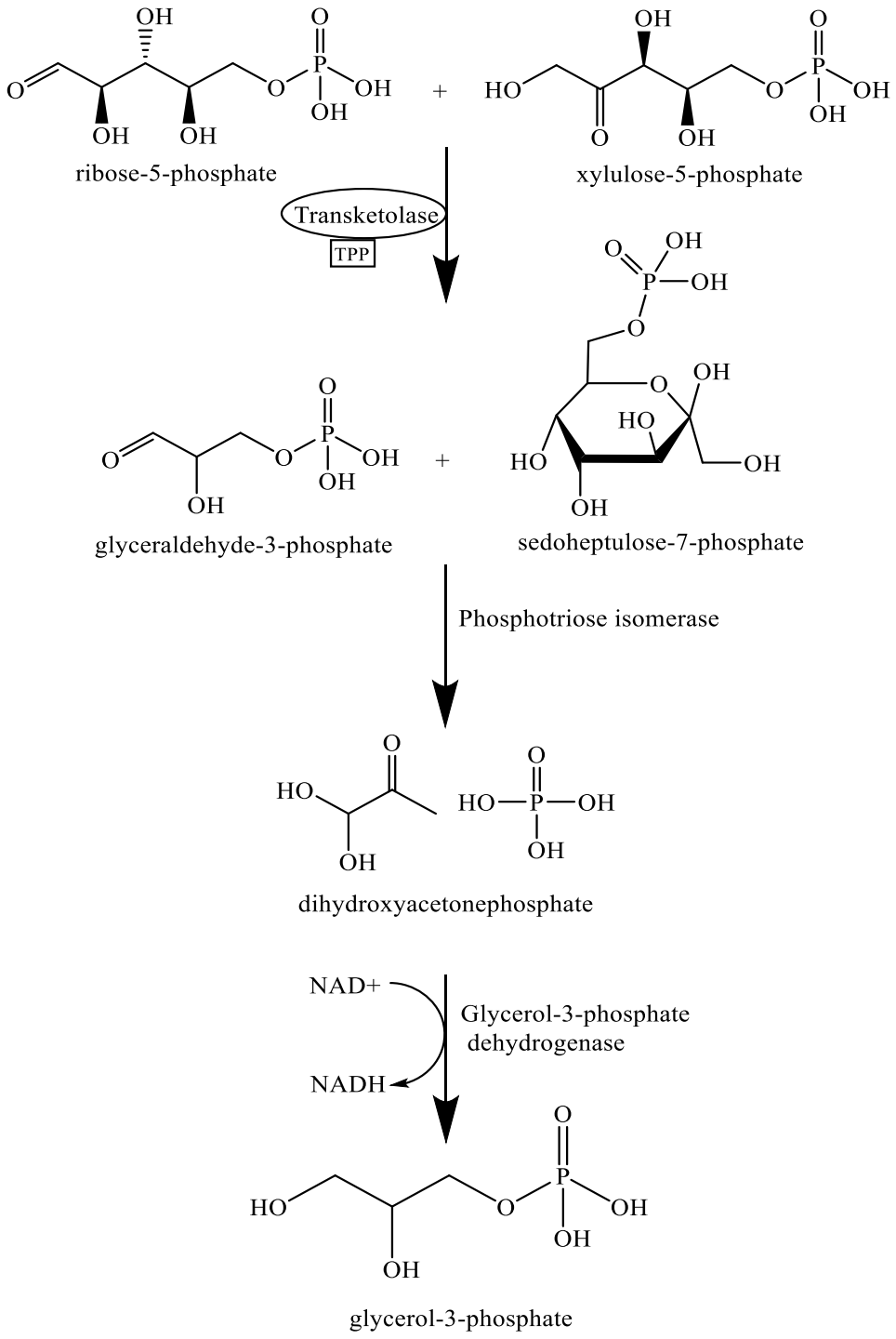

Fig. 2.1: The above figure demonstrates the series of biochemical reactions which occur during the Transketolase Activity assay. The reaction is favored by certain enzymes and cofactors and the activity of the enzyme is measured by determining the amount of NADH released.

We used 50µg of total protein for this assay. One set of the dishes received 100µM Thiamine Pyrophosphate (Sigma Aldrich, St. Louis, MO) dissolved in Tris-HCL (J.T Baker, Allentown, PA) and samples were incubated at 37°C for 30 minutes. Ribose-5-phosphate dihydrate salt (Sigma Aldrich, St. Louis, MO) at a concentration of 5mM was used as the substrate and 5 U/mL of Triosephosphate isomerase (Sigma Aldrich, St. Louis, MO) and 4.2 U/mL of α-glycerophosphate dehydrogenase (Sigma Aldrich, St. Louis, MO) were added to the reaction mix to aid this reaction. Freshly prepared 2.5 mM of β-nicotinamide adenine dinucleotide disodium salt (Sigma Aldrich, St. Louis, MO) was added to the reaction mixture and the plate was read immediately on addition of the solution mixture every 15minutes for two hours on the SpectraMax M2 spectrophotometer at 340 nm at 37°C to measure the amount of NADH released.

Alpha ketoglutarate dehydrogenase activity assay:

This enzymatic assay involves the decarboxylation of alpha ketoglutaric acid to form succinyl co-A along with the release of NADH. Alpha ketoglutarate dehydrogenase enzyme aids in carrying out this decarboxylation reaction and its activity is measured by calculating the color change in the redox dye whose concentration can be monitored by measuring the fluorescence. Cells were seeded at 1000cells/cm2 in 150cm dishes containing 10nM and 3µM thiamine media in separate dishes. One set was incubated in normoxia and other in hypoxia for 7 days. An additional set was exposed to hypoxia for 24h. The cells were lysed using lysis buffer along with 1% phosphatase and protease inhibitors and 20% apyrase (Sigma Aldrich, St. Louis, MO). Whole cell lysates were stored in ice for 15 mins and then centrifuged at 14000xg using a Microfuge 22R centrifuge

(Beckman Coulter, Brea, CA) for 15 minutes at 4°C. Protein concentration of the supernatant was determined using BCA assay. 200µg of sample was used for this assay and a developing solution (50mM Tris at pH 8.0, 5mM sodium pyruvate, 0.15mM sodium co-A, 2.5mM NAD^+, 1mM $MgCl_2$, 300mM cysteine, 15µM

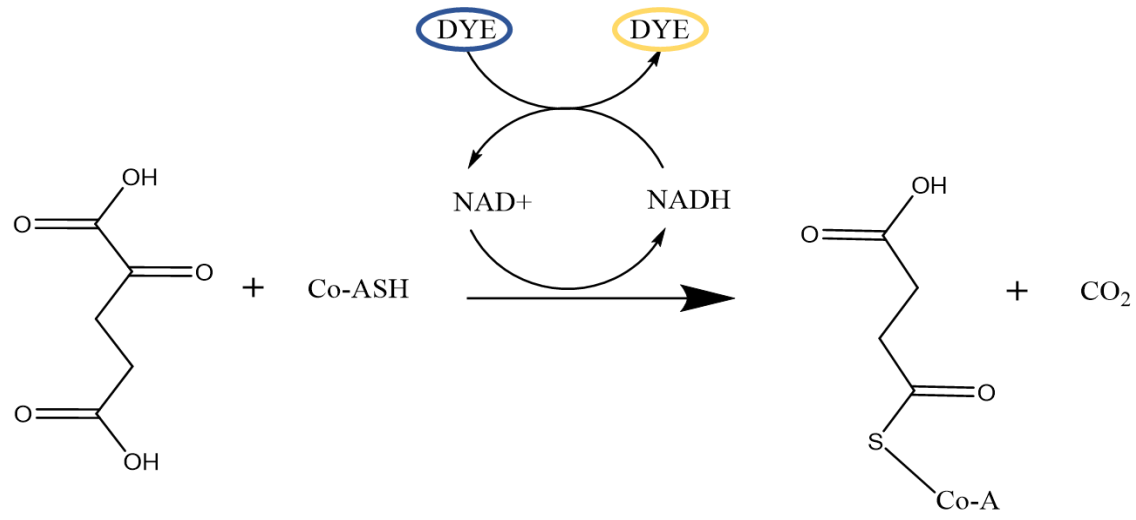

Fig 2.2: The above reaction scheme denotes the reactions occurring in the AKGDH assay. This assay measures the activity of the AKGDH enzyme by determining the fluorescence value of the redox dye present in the solution.

resazurin and 0.5U/mL diaphorase) was added to each well after warming it to 37°C. A 1mM solution of alpha ketoglutaric acid was added in the last step and plate was incubated in dark for 10 mins at 37°C. Following incubation, the fluorescence was measured after a 5 sec auto mix at ex:530nm and em:590nm every 10 min for 70 min using a SpectraMax M2 spectrophotometer at 37°C.

Pyruvate dehydrogenase assay:

The PDH assay is a colorimetric assay involving the reaction between the substrate, pyruvate and coenzyme-A due to the presence of the pyruvate dehydrogenase enzyme. This reaction is a reduction reaction denoted by the addition of a hydrogen molecule to NAD+ generating NADH which can be detected by measuring the intensity of color change observed in the redox dyes added in the reaction mixture. For this assay, 96 well plates coated with anti-mouse IgG (Abcam, Cambridge, UK) were obtained and washed with phosphate buffer saline (1XPBS). The wells were then incubated with anti-pyruvate dehydrogenase E2/E3bp antibody (Abcam, Cambridge, UK) provided in the ratio 1:2000 in PBS for 1 hour at room temperature. For the lysate samples, cells were seeded at 1000cells/cm2 in 150cm dishes and given a 10nM and 3µM thiamine dose. One set was incubated in normoxia and other in hypoxia for 7 days and an additional set was given a 24hr hypoxia treatment. They were then lysed using lysis buffer containing 10% detergent from the PDH assay kit (Abcam, Cambridge, UK) in PBS, along with 1% phosphatase and protease inhibitors and 20% apyrase (Sigma Aldrich, St. Louis, MO). Whole cell lysates were incubated at 4°C in the refrigerator for 15 mins and then centrifuged at 14000xg using Microfuge 22R centrifuge (Beckman Coulter, Brea, CA) for 15 minutes at 4°C. Protein concentration of the supernatant was determined using BCA assay.

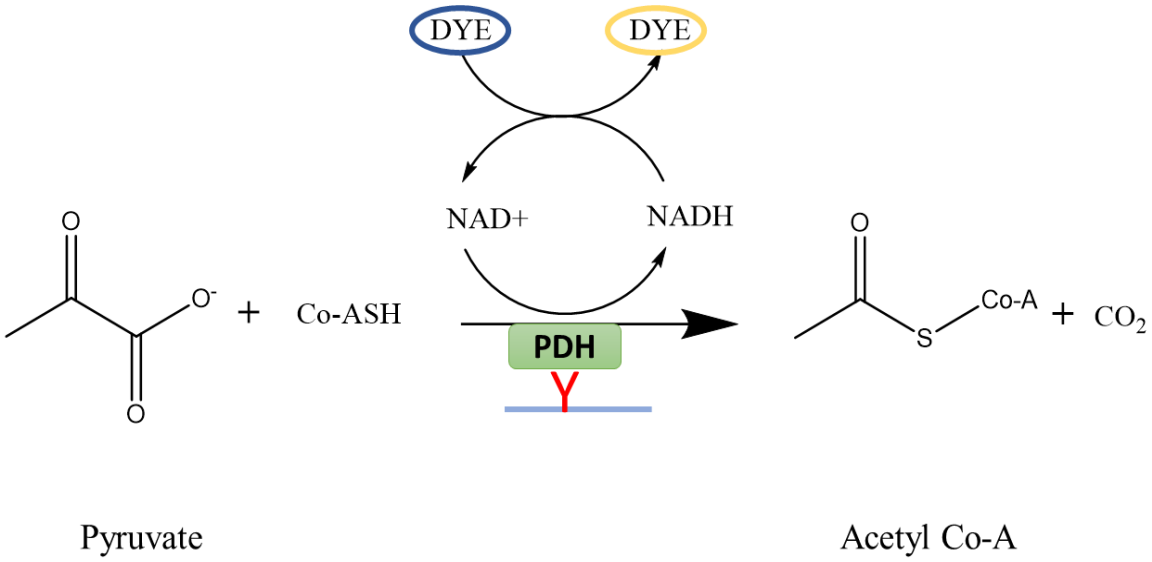

Fig 2.3: The above reaction scheme denotes the reactions occurring in the PDH activity assay. In this assay, a pre-coated anti-mouse IgG plate is used which is then bound by a secondary antibody and the substrate. This assay measures the activity of the PDH enzyme by determining the fluorescence value of the redox dye present in the solution.

We used 200µg of sample for this assay and diluted using a protein dilution buffer (1:4 ratio of detergent and PBS along with 5% of protease and phosphatase inhibitors). The precoated wells were washed with PBS and the protein sample was added and the plate was incubated in dark at room temperature with light shaking for 2.5hours. After incubation, the wells were given a PBS wash and then treated with PDP1 protein (17µg in 850µL of PBS) and incubated for 10 minutes at 37°C in the dark. Freshly made developing solution (50mM Tris at pH 8.0, 5mM sodium pyruvate, 0.15mM sodium CoA, 2.5mM NAD+, 1mM $MgCl_2$, 300mM cysteine, 15µM resazurin and 0.5U/mL diaphorase) was added to each well after warming it to 37°C. Following addition of developing solution, the plate was read using a SpectraMax M2 spectrophotometer at 37°C to measure the fluorescence at ex: 530nm and em:590 nm every 10 minutes for 70 min with a 5 sec auto mix between every read.

Statistical Analysis:

All the experiments had four independent data sets, unless otherwise noted. GraphPad Prism 6 was used for the analysis and two-way Anova and t-tests were used with a significance level of $p<0.05$ to determine the statistical significance. Tukey's test was used along with ANOVA to determine if the means were statistically different.

2.3 RESULTS:

Effect of thiamine supplementation on cell growth:

The cell count method was used to determine the difference in cell growth in media containing thiamine at different concentrations. On trypsinizing the MCF-7 cells in the 10nM and 3µM thiamine media in normoxia as well as hypoxia, we observed that the cells cultured in 3µM thiamine media in normoxia had a significant increase in the amount of live cells as compared to the cells in 10nM thiamine media in normoxia (fig 2.4.A). Also, a significant difference in growth was observed in the number of live cells formed in the 10nM and 3µM thiamine dishes in hypoxia when compared to 10nM normoxia dish. Cells seeded in the 3µM thiamine media and given a hypoxia exposure for 7d had two times higher number of cells when compared with the live cell count in 10nM thiamine dish in normoxia.

To validate the results from the cell count method, the crystal violet assay was performed. After a period of 7 days, some evident differences in the growth were observed. To confirm it, the stain was solubilized and quantified using the spectrophotometer. A two-fold increase was observed in the MCF-7 cells seeded in the 3µM thiamine media in normoxia as compared to the cells cultured in 10nM thiamine media dishes in normoxia (Fig 2.4.B/D).

The BrdU assay was another method which determined the amount of newly formed cells in different thiamine media and environmental conditions. In MCF-7 cells, we observed that there was an increase in the cells grown in the 3µM thiamine media grown in normoxia (Fig.2.4.C). A 3-fold and 5-fold increase was observed in the cells in 10nM and 3µM thiamine media, exposed to hypoxia for 7d (Fig 2.4.C).

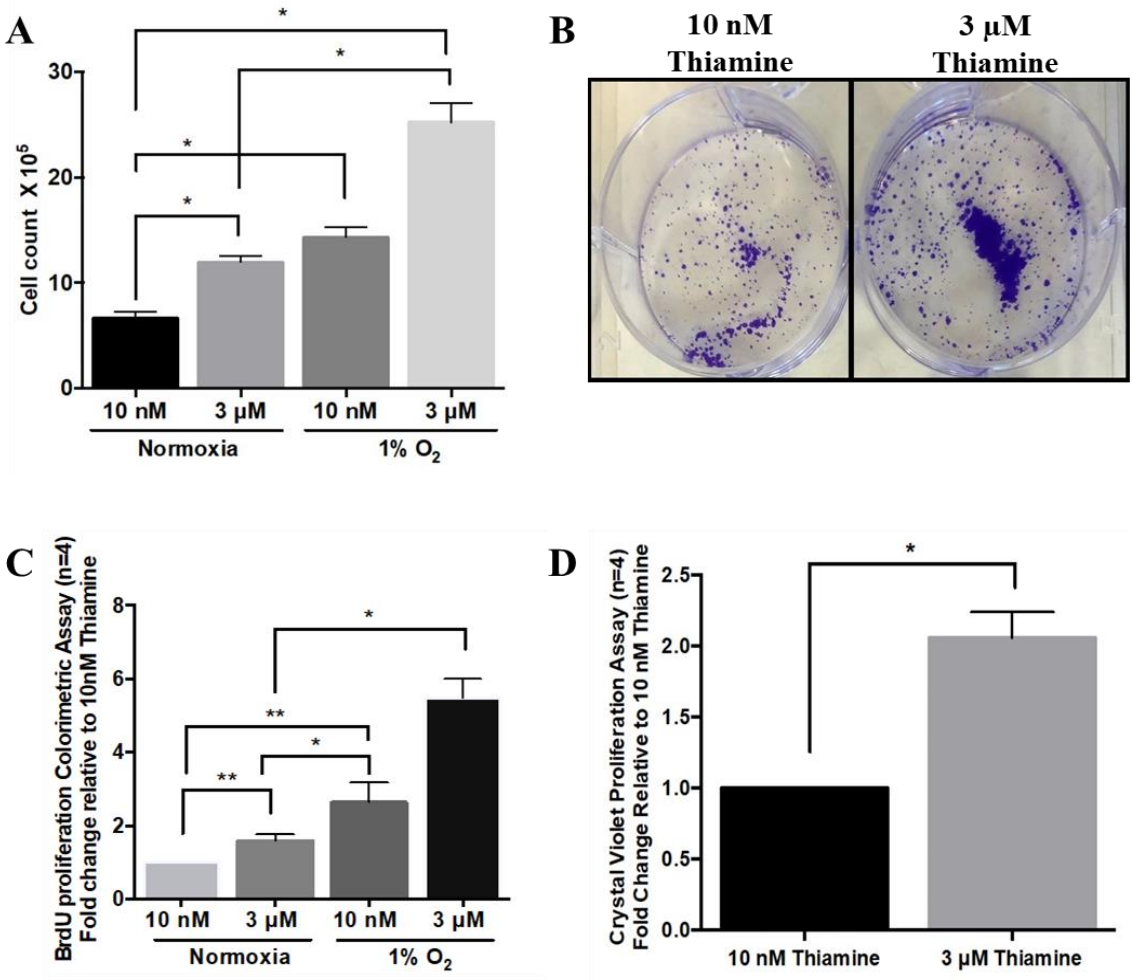

Fig.2.4 Effect of thiamine supplementation on cell growth: (A) Changes in cell growth observed in MCF-7 cells seeded in 10nM and 3µM thiamine media, using the cell count method following treatment with 1% O_2 for 7d relative to normoxic control respectively. **(B)** Crystal violet staining of MCF-7 cells cultured in 10nM and 3µM thiamine media at a density of 500cells/cm^2 to observe changes in cell proliferation. **(C)** this graph represents the BrdU cell proliferation assay performed on MCF-7 cells seeded at 500 cells/cm^2 in 10nM and 3µM thiamine media and cultured in normoxia and hypoxia for 7d. **(D)** Quantification of the crystal violet stained wells of MCF-7 cells following solubilization of stain with Triton X. **(*)** Represents statistically significant difference (p<0.0001) based on two way ANOVA **(A,C)** and Tukey's post hoc test or unpaired student's t-test **(D).**

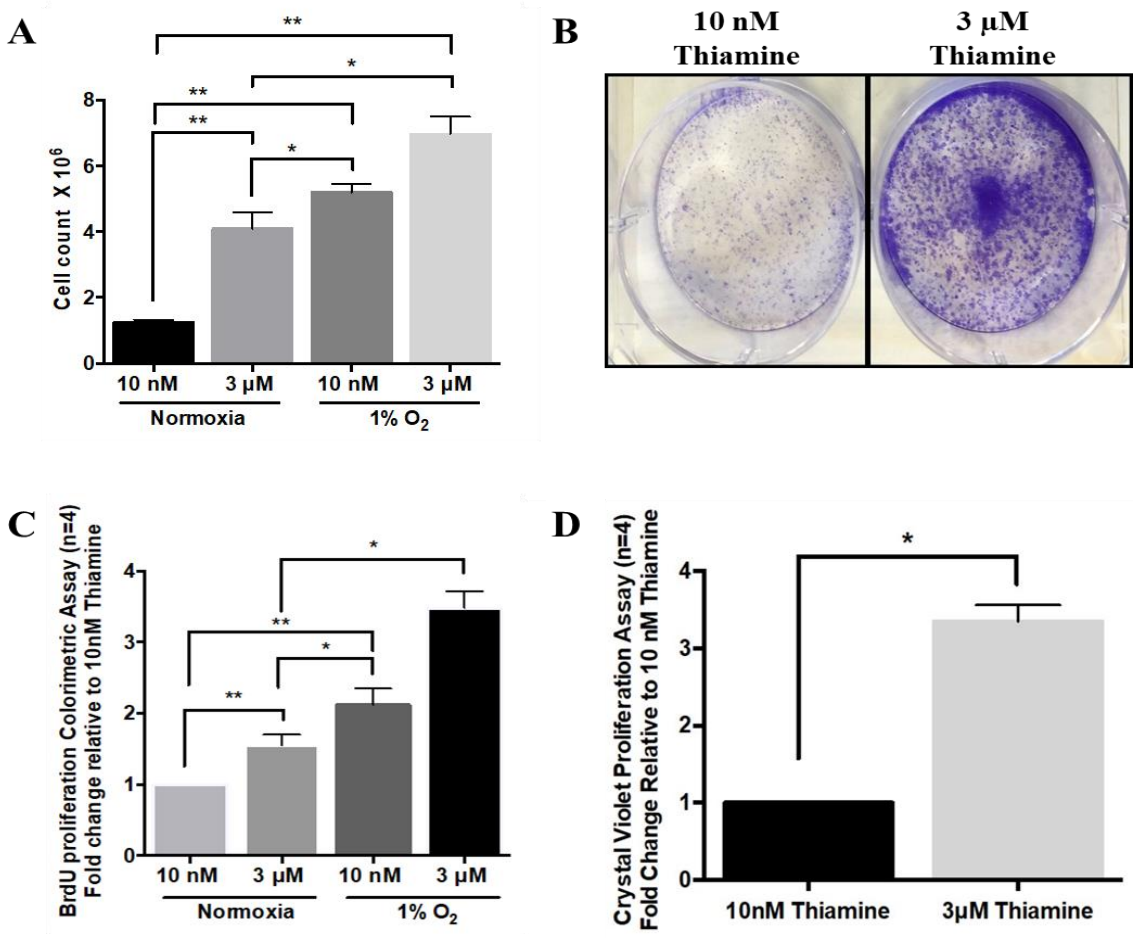

Fig.2.5 Effect of thiamine supplementation on cell growth: (A) Changes in cell growth observed in MDA MB-231 cells seeded in 10nM and 3µM thiamine media, using the cell count method following treatment with 1% O_2 for 7d relative to normoxic control respectively. **(B)** Crystal violet staining of MDA MB-231 cells cultured in 10nM and 3µM thiamine media at a density of 500cells/cm^2 to observe changes in cell proliferation. **(C)** this graph represents the BrdU cell proliferation assay performed on MDA MB-231 cells seeded at 500 cells/cm^2 in 10nM and 3µM thiamine media and cultured in normoxia and hypoxia for 7d. **(D)** Quantification of the crystal violet stained wells of MDA MB-231 cells following solubilization of stain with Triton X. **(*)** Represents statistically significant difference (p<0.0001) based on two way ANOVA **(A,C)** and Tukey's post hoc test or unpaired student's t-test **(D).**

On performing the cell count method for assessing the live cell count, the MDA MB-231 cells showed almost 2 times greater number of cells in the 3µM thiamine dish kept in normoxia when compared with the 10nM thiamine dish in normoxia (Fig 2.5.A). Similarly, a greater number of cells was observed in the 10nM thiamine dish exposed to hypoxia for 7d. The 3µM thiamine dish which was exposed to hypoxia for 7d had almost three times greater number of cells than the 10nM thiamine dish kept in normoxia.

The crystal violet assay performed on MDA MB-231 cells showed a similar result with a 1.5-fold increase observed in the cells seeded in the 3µM thiamine media when compared with the cells seeded in 10nM thiamine media and cultured in normoxia (Fig 2.5.B/D)

BrdU cell proliferation assay also revealed that MDA MB-231 cells showed a 1.5-fold increase in the 3µM thiamine normoxia dish as compared to the 10nM dish (Fig 2.5.C). However, both the 10nM and 3µM hypoxia samples showed an enhanced cell growth which was almost 2-fold and 4-fold greater when compared to the 10nM dish in normoxia (Fig 2.5.C).

Effect of Thiamine supplementation on expression of enzymes:

To assess the changes in the expression levels of proteins, the samples were quantified using Western blot analysis. In the MCF-7 cell line, similar expression levels of the α-KGDH enzyme were observed in the cell lysates of the 10nM thiamine and 3µM thiamine media cultured in normoxia for 7d (Fig 2.6.A/C). However, a significant increase in the expression levels of this enzyme was observed in the samples obtained from cells seeded

in 10nM and 3µM thiamine media cultured in hypoxia for 24h and 7d, when compared with 10nM thiamine sample in normoxia. The expression of the TKT enzyme was observed to be significantly higher in the cell lysates cultured in 3µM thiamine media in hypoxia for 7d when compared to the 10nM thiamine sample in normoxia (Fig. 2.6.B/D). The expression levels of the PDHe1α enzyme were similar in all the treatment groups cultured in normoxia and hypoxia and showed no significance (Fig.2.6.F/H). However, an increase in the expression levels of the PDH293 protein was observed in the cell lysates obtained from treatment dishes cultured in hypoxia with lower protein expression levels in the treatment groups cultured in normoxia (Fig.2.6.E/G).

In the MDA MB-231 cells, a significant increase in the enzyme expression was observed in the cells seeded in 10nM and 3µM thiamine media and cultured in hypoxia for 24h. However, a higher expression level was observed in the cell lysates obtained from the 10nM and 3µM thiamine samples cultured in hypoxia for 7d when compared to 10nM thiamine samples in normoxia (Fig 2.7.A/C). The TKT enzyme showed no change in expression in all groups except a significant increase was observed in the cell lysates obtained from 10nM and 3µM thiamine media after exposing to hypoxia for 7d (Fig 2.7.B/D). The PDHe1α enzyme showed no change in expression levels throughout all the groups (Fig 2.7.F/H). However, an increase in the expression levels of the PDH293 protein was observed in the cell lysates obtained from the 10nM and 3µM thiamine dishes cultured in hypoxia for 24h and 7d respectively (Fig. 2.7.E/G).

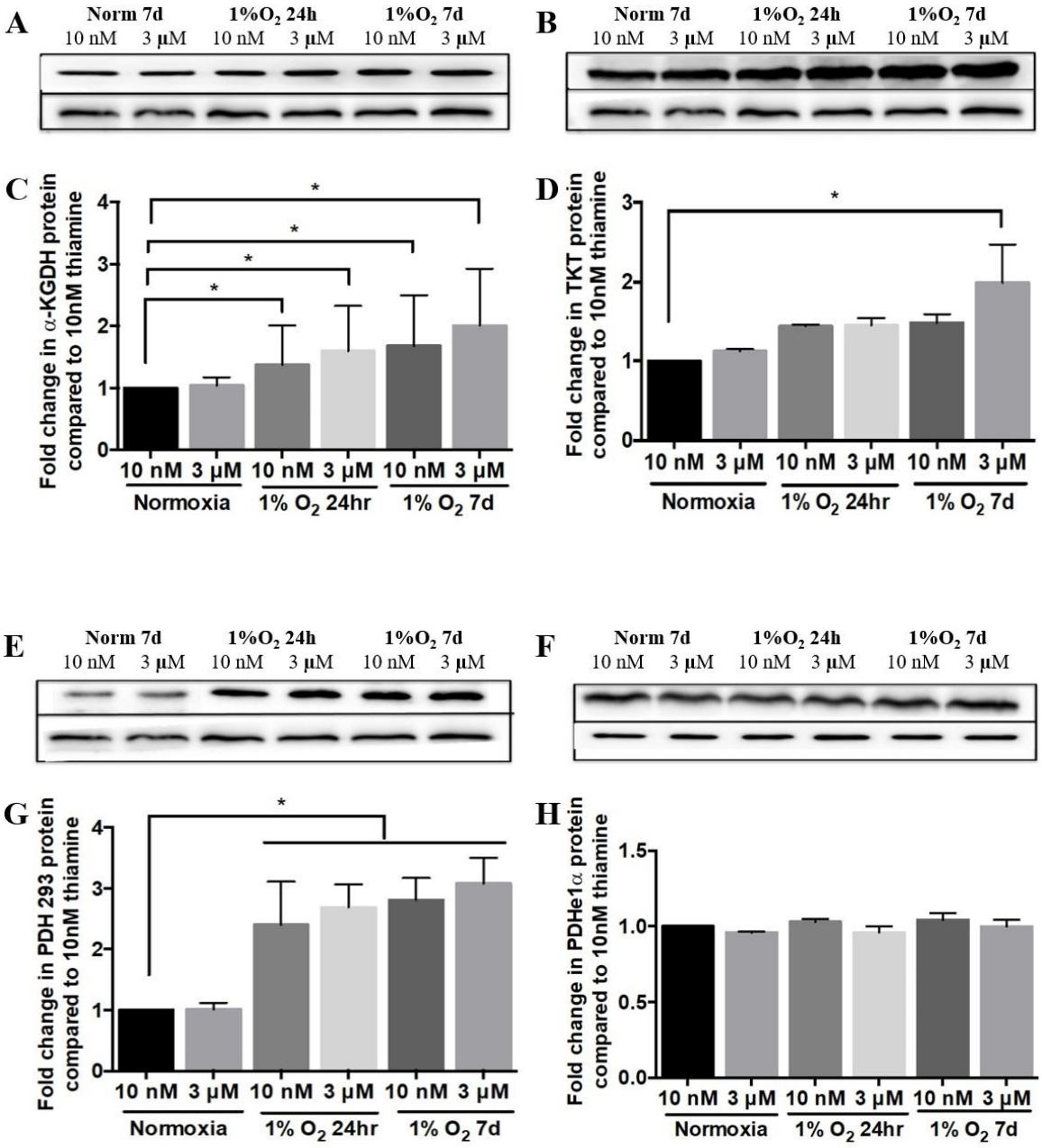

Fig 2.6 Effect of thiamine supplementation on expression of thiamine dependent enzymes: (A) Representative Western blot demonstrating expression of α-KGDH enzyme in MCF-7 cells seeded in 10nM and 3µM thiamine media following a treatment of 1% O_2 for 24h and 7d and normoxia treatment for 7d. **(B)** Representative Western blot demonstrating expression of TKT enzyme in MCF-7 cells seeded in 10nM and 3µM thiamine media following a treatment of 1% O_2 for 24h and 7d and normoxia treatment for 7d. **(C,D)** Densitometry analysis in fold change of α-KGDH and TKT protein levels in all treatment groups relative to normoxic control and including n=4 individual experiments. **(E)** Representative Western blot demonstrating expression of PDH293 enzyme in MCF-7 cells seeded in 10nM and 3µM thiamine media following a treatment of 1% O_2 for 24h and 7d and normoxia treatment for 7d. **(F)** Representative Western blot demonstrating expression of PDHe1α enzyme in MCF-7 cells seeded in 10nM and 3µM thiamine media following a treatment of 1% O_2 for 24h and 7d and normoxia treatment for 7d. **(G.H)** Densitometry analysis in fold change of PDH293 and PDHe1α protein levels in all treatment groups relative to normoxic control and including n=4 individual experiments. **(*)** Represents statistically significant (p<0.01) using two way ANOVA **(C,D,G,H)** and Tukey's post hoc test.

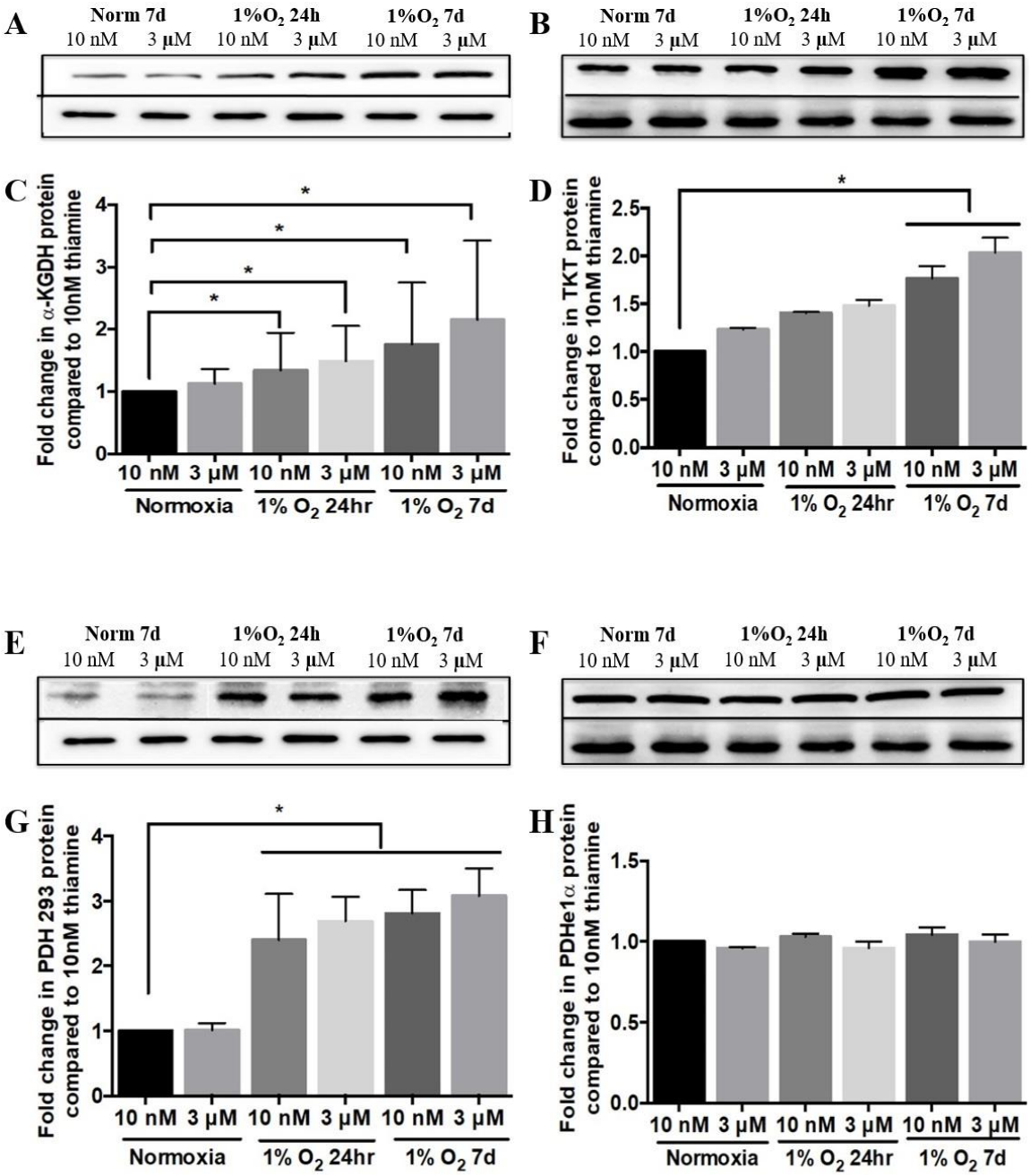

Fig 2.7 Effect of thiamine supplementation on expression of thiamine dependent enzymes: (A) Representative Western blot demonstrating expression of α-KGDH enzyme in MDA MB-231 cells seeded in 10nM and 3µM thiamine media following a treatment of 1% O_2 for 24h and 7d and normoxia treatment for 7d. **(B)** Representative Western blot demonstrating expression of TKT enzyme in MCF-7 cells seeded in 10nM and 3µM thiamine media following a treatment of 1% O_2 for 24h and 7d and normoxia treatment for 7d. **(C,D)** Densitometry analysis in fold change of α-KGDH and TKT protein levels in all treatment groups relative to normoxic control and including n=4 individual experiments. **(E)** Representative Western blot demonstrating expression of PDH293 enzyme in MCF-7 cells seeded in 10nM and 3µM thiamine media following a treatment of 1% O_2 for 24h and 7d and normoxia treatment for 7d. **(F)** Representative Western blot demonstrating expression of PDHe1α enzyme in MCF-7 cells seeded in 10nM and 3µM thiamine media following a treatment of 1% O_2 for 24h and 7d and normoxia treatment for 7d. **(G.H)** Densitometry analysis in fold change of PDH293 and PDHe1α protein levels in all treatment groups relative to normoxic control and including n=4 individual experiments. **(*)** Represents statistically significant (p<0.01) using two way ANOVA **(C,D,G,H)** and Tukey's post hoc test.

The activity of TKT enzyme was further investigated to determine the effects of thiamine supplementation. On performing the linear regression analysis, the change in the rate of activity of the TKT enzyme was calculated. A higher rate of TKT activity was observed in the samples obtained from cells seeded in 10nM thiamine as well as the 3µM thiamine media cultured in hypoxia for 7d (Fig. 2.8.B). On supplementation with 100µM of exogenous cofactor, TPP, the change in activity of TKT enzyme in these samples was observed. A TPP effect was seen in the samples from 10nM and 3µM thiamine dishes cultured in hypoxia for 24h (Fig 2.8.C). However, no other groups displayed a significant change in the rate of reaction.

The MDA MB-231 cell line displayed no significant change in the TKT activity and had similar rates of TKT activity in all the groups (Fig.2.8.D). However, a TPP effect was observed in the 10nM and 3µM thiamine samples cultured in hypoxia for 24h and 7d when compared with 10nM thiamine sample in normoxia (Fig.2.8.E).

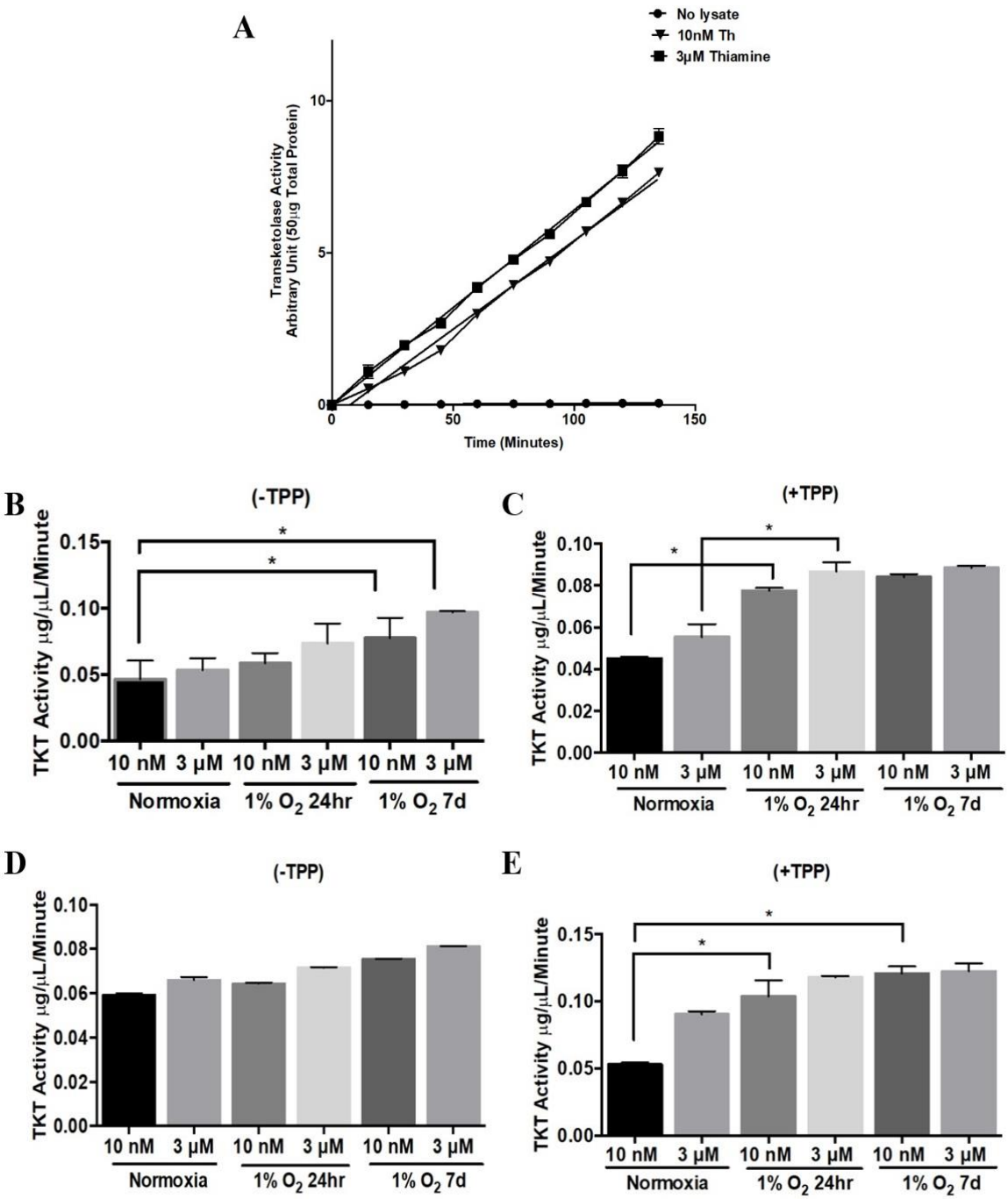

Fig 2.8 Effect of thiamine supplementation on activity of TKT enzyme: (A) This figure represents the linear regression analysis performed on TKT activity observed in two treatment groups of 10nM and 3µM in normoxia and similar analysis was performed on other treatment groups to obtain the rate of activity data**. (B,D)** Represents a change in the rate of activity of TKT enzyme in MCF-7 and MDA MB-231 cells seeded in 10nM and 3µM thiamine media following a treatment of 1% O_2 for 24h and 7d and normoxia treatment for 7d. **(C,E)** This set of data represents change in the rate of TKT activity in the corresponding treatments groups, when 100µM of TPP was spiked in to observe a TPP effect.

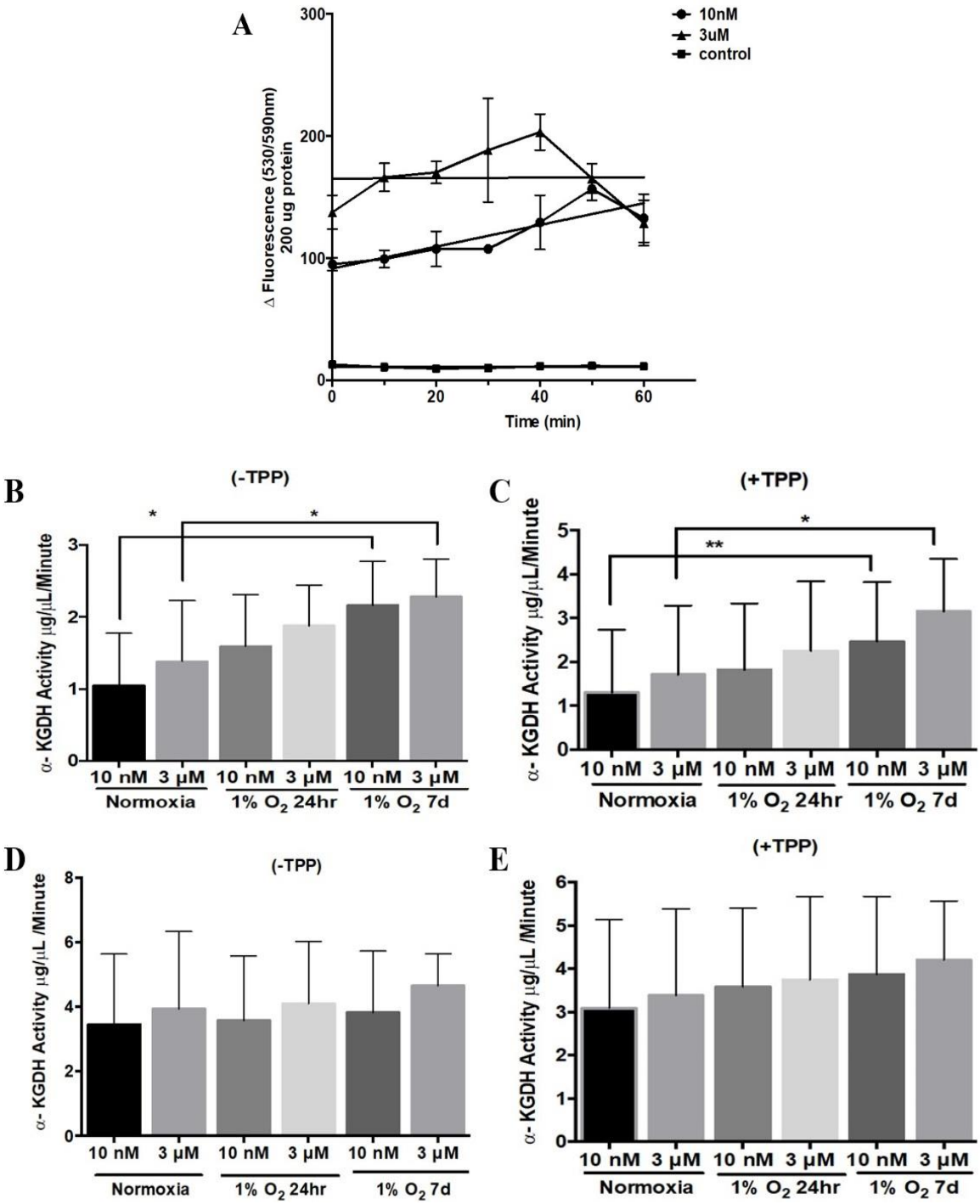

Fig 2.9 Effect of thiamine supplementation on activity of AKGDH enzyme: (A) This figure represents the linear regression analysis performed on α-KGDH activity observed in two treatment groups of 10nM and 3μM in normoxia and similar analysis was performed on other treatment groups to obtain the rate of activity data**. (B,D)** Represents a change in the rate of activity of AKGDH enzyme in MCF-7 and MDA MB-231 cells seeded in 10nM and 3μM thiamine media following a treatment of 1% O_2 for 24h and 7d and normoxia treatment for 7d. **(C,E)** This set of data represents change in the rate of AKGDH activity in the corresponding treatments groups, when 100μM of TPP was spiked in to observe a TPP effect.

AKGDH activity in thiamine supplementation:

To determine the change in the rate of activity of the AKGDH enzyme, the AKGDH assay was designed, and the amount of color change in the redox dye was measured using fluorescence. In the MCF- 7 cells, a significant increase in the rate of reaction was observed in the cell lysates obtained from the 10nM and 3µM thiamine dishes cultured in hypoxia for 7d when compared with the samples cultured in 10nM and 3µM thiamine media in normoxia respectively (Fig 2.9.B). A TPP effect was also seen in the cell lysates obtained from 10nM and 3µM thiamine dishes cultured in hypoxia for 7d when compared with same groups as above (Fig 2.9.C).

The MDA MB-231 cells, however, did not show any significant changes in the rate of the AKGDH activity among different treatment groups (Fig 2.9.D). Also, no TPP effect was observed in any of the treatment groups after addition of 100µM of TPP (Fig.2.9.E).

Effect of thiamine supplementation on PDH activity:

On performing the PDH assay in MCF-7 cells, no significant difference in the rate of activity was observed in different groups (Fig 2.10.B). When supplied with exogenous 100μM TPP, no TPP effect was observed, and the groups showed a similar rate of PDH activity (Fig 2.10.C). A similar result was observed in the MDA MB-231 cells which showed no change in the activity of the PDH enzyme in the different groups (Fig 2.10.D). On supplying with exogenous TPP, the rate of activity was similar in all the treatment groups showing no TPP effect (Fig 2.10.E).

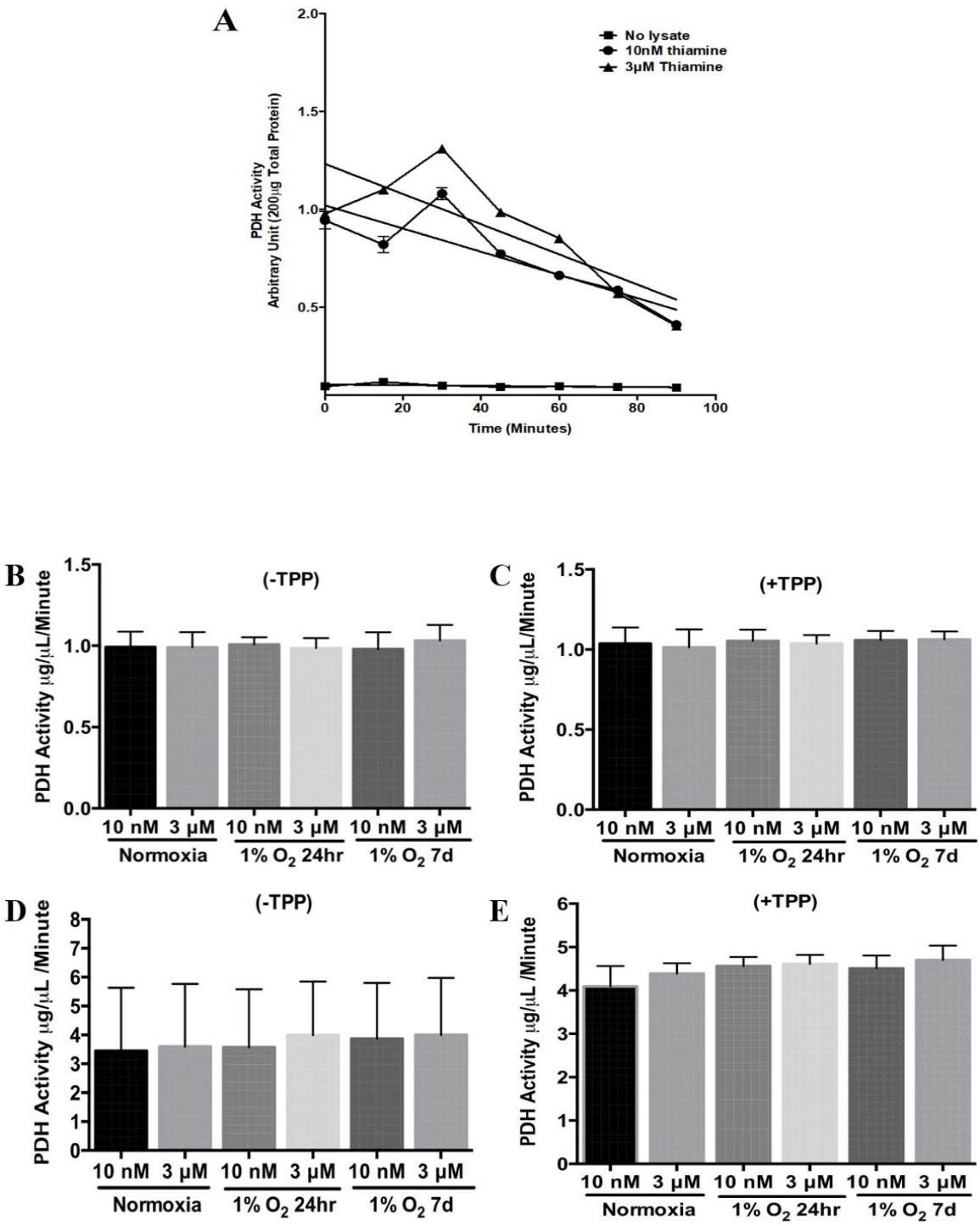

Fig 2.10 Effect of thiamine supplementation on activity of total PDH enzyme: (A) This figure represents the linear regression analysis performed on PDH activity observed in two treatment groups of 10nM and 3µM in normoxia and similar analysis was performed on other treatment groups to obtain the rate of activity data**. (B,D)** Represents a change in the rate of activity of PDH enzyme in MCF-7 and MDA MB-231 cells seeded in 10nM and 3µM thiamine media following a treatment of 1% O_2 for 24h and 7d and normoxia treatment for 7d. **(C,E)** This set of data represents change in the rate of PDH activity in the corresponding treatments groups, when 100µM of TPP was spiked in to observe a TPP effect.

2.4 DISCUSSION:

Thiamine supplementation leads to increased cell growth:

Recent evidences have revealed that thiamine supplementation has a tumor proliferating effect when supplemented in small doses (Boros, Brandes *et al.* 1998, Comin-Anduix, Boren *et al.* 2001, Daily, Liu *et al.* 2012). In the MMTV neu mice model, a normal thiamine diet could reduce the tumor latency thus promoting malignant growth (Liu, 2012). The Ehrlich's ascites model was another classic model which demonstrated the effect of supplmentation of different concentrations of thiamine on the growth of tumors (Comin-Anduix, 2001). Here we demonstrated that a similar increase in cell growth is observed in both ER+ve and triple -ve breast cancer cell lines when supplementated with 300 times that of plasma level of thiamine. On assessing the newly formed cells, we observed an increase in the cells cultured in 3μM thiamine as well as cells cultured in hypoxia. It has been shown that mRNA levels of TKT is upregulated on supplementation of thiamine (Pekovich, Martin *et al.* 1998). Thus, supplementation with thiamine could contribute in increase in activity of TKT enzyme in normoxia as well as hypoxia, thereby, promoting cell growth. It is also known that solid tumors favor a hypoxic environment due to the stabilisation of the HIF-1α oncogene (Muz, de la Puente *et al.* 2015). It plays a critical role in the metabolic reprogramming by acting on the LDHA enzyme and promoting the production of lactic acid which further supports cell growth (San-Millan, 2017). The increase in cell growth observed in the hypoxia treated cells could be due to higher expression of TKT enzyme observed in hypoxia which is a critical enzyme promoting cell growth (Haseloff, Krause *et al.* 2006). The TKT flux is also shown to be higher in hypoxic condition further supporting the above data (Zhao, Mancuso *et al.* 2010). Also, recent evidences have shown that the

TPK1 expression and activity is elevated when malignant cells are exposed to hypoxia Jonus, Hanberry *et al.* 2018). Thus, hypoxia could be another underlying factor promoting cell growth. Hence, cell growth is affected when malignant cells are supplied with thiamine and it is further boosted when thiamine treated cells are exposed to a hypoxic environment.

Thiamine supplementation affects the expression and activity of thiamine dependent enzymes:

Zastre *et al*., have demonstrated that breast cancer cells show an upregulation of homeostasis genes in different breast cancer cell lines (Zastre *et al*., 2013). Supplementation with thiamine has been shown to upregulate the mRNA levels of TKT enzyme in malignant cells (Elena Beltramo, 2006). An increase in the TKT flux is also observed when malignant cells were subjected to hypoxic environment (Zhao, Mancuso *et al.* 2010). Our data demonstrated an increase in the expression of the TKT enzyme after supplementing the cells with thiamine and culturing them in hypoxic environments which corresponded with the results above. We could not correlate the increase in expression with activity since no significant change was observed. Although, addition of TPP exogenously showed a TPP effect. Also, we found an upregulation in the expression of AKGDH enzyme which has not been observed earlier. This suggested that hypoxia could have generated oxidative stress thus inducing the expression of this enzyme which ultimately results in more ROS which induce cell proliferation (Tretter and Adam-Vizi, 2004, Day and Suzuki, 2006). A significant increase in the activity of AKGDH enzyme was also observed but addition of excess TPP had no effect. This suggested that all of the AKGDH enzyme present in the cell lysate was saturated with TPP and there was no unused enzyme in the

pool which could have been activated with the addition of TPP. However, hypoxic environment led to a decline in the PDH activity of malignant cells suggesting its role in the activation of PDK enzymes (Eyassu and Angione, 2017). We did not see any change in the expression of total PDH enzyme, however, its serine 293 residue showed phosphorylation in hypoxia. Also, PDH enzyme had no activity which suggested that there could have been an upregulation of PDK1 enzyme and hypoxia could play a critical role in further promoting the inactivation (Kim, Tchernyshyov *et al.* 2006). Thus, thiamine supplementation can lead to the increase in expression of TKT and AKGDH enzymes. However, it did not have an effect on PDH expression although, cells exposed to hypoxia showed a high expression of the phosphorylated PDH residue suggesting inactivation of the enzyme.

In conclusion, our findings suggests that a thiamine dose 300 times the normal plasma value could lead to increased cell growth. Hypoxia along with supplemental thiamine could serve as a suitable environment for promoting cell proliferation. Thiamine has also been shown to act as an antioxidant against ROS and contributing to enhance cell growth. However, there is not enough data available to justify this non-coenzyme role of thiamine. Hypoxia affects the functional activity of certain thiamine dependent enzymes which play a critical role in metabolic pathways. Thus, thiamine supplementation and hypoxia are critical controlling factors involved in regulating the altered metabolic activities of the malignant cells, thereby contributing to enhanced cell growth.

CHAPTER 3

SUMMARY AND FUTURE DIRECTION

3.1 SUMMARY:

Breast cancer is one of the rapidly growing diseases affecting women all over the world. It has not only been seen in post-menopausal women but is now diagnosed in young women due to genetic inheritance. The occurrence of this disease is widespread due to the lack of proper nutrition, unhealthy lifestyle and irregular screenings (Carol DeSantis MPH 2013, Akram M 2017, Shiyanbola, Arao *et al.* 2017). Alteration in the cellular metabolic processes is one of the classical hallmarks seen in breast cancer. Reprogramming of metabolism occurs to fulfill the requirements of energy and biomolecules needed by the cells to proliferate. This leads to an enhanced uptake of nutrient molecules from the body and generation of ATP via a non-oxidative pathway even in the presence of oxygen, called Warburg effect (Warburg 1923; Jóźwiak *et al.* 2014). The enhanced catabolism of glucose into pyruvate, allows for the formation of more ATP and lactate which aid in tumor cell proliferation. On entering the PPP, glucose forms abundant of ribose-5-phosphate which acts as a sugar backbone for the nucleic acids. All these processes ultimately boosts the cell growth process (Jóźwiak *et al.* 2014). Studies have shown that the nutritional status gets highly affected in malignancy and causes deficiency of many vitamins and minerals (Ostrovskiĭ IuM, 1979, Zaanen and Lelie, 1992). The reliance on nutritional supplements

has been on the rise due to its easy availability and to achieve a proper nutritional status. However, a higher content of vitamins present in these supplements may not be suitable for the body causing several other risks.

Recent studies have shown that thiamine supplementation has been associated in cancer risk. It was seen that Ehrlich's ascites tumor mice showed an increase in tumor growth when they were supplemented with 25 times the normal dietary intake levels of thiamine (Comin-Anduix, 2001, Brewer, Jones *et al.* 2017). Also, a normal thiamine diet reduced the mammary tumor latency and promoted cell growth in MMTV- neu mice which suggested that thiamine supplementation had a critical role in cell growth (Daily, Liu, 2012). Thus, it became very critical to determine the possible role of thiamine supplementation on the growth of breast cancer cells.

Since there was no direct link formed between thiamine supplementation and the different types of breast cancer, we used an ER +ve and a triple negative breast cancer cell lines to study the effect of thiamine supplementation on two phenotypically different cells. We supplemented a group with 10nM thiamine which is the normal plasma level of thiamine and another group with 3µM thiamine dose, a standard dose used for culturing of cells. On performing the different growth assays, we found a two-fold increase in the MCF-7 cells treated with the 3µM thiamine dose in comparison to the 10nM thiamine group. The MDA cells showed a 3.5-fold increase in the cell proliferation in the 3µM thiamine group thereby suggesting that thiamine supplementation promoted cell proliferation. This correlates with the evidence suggesting that a higher expression of TPK1 enzyme in

malignant cells could have led to enhanced conversion of thiamine to its active form which would then support metabolic reactions to promote cell growth. It is also observed that malignant cells show change in the expression of certain thiamine dependent enzymes. To determine the effect of thiamine supplementation on the functional activity of these enzymes, we quantified their expression and observed an increase in the expression levels of TKT and αKGDH enzyme in the 3μM thiamine groups in both cell lines. When we subjected to hypoxia for 24hr and 7d, MCF-7 and MDA MB-231 cells showed an increase in expression of these enzymes. This data correlated to the study which suggested that hypoxia led to upregulation of TKT enzyme and activity thus, supporting enhanced production of R5P. This substrate is utilized for production of nucleic acids which further support cell proliferation (Zhao *et al.* 2010). The AKGDH enzyme expression was also elevated suggesting a critical role of hypoxia in inducing the enzyme function leading to generation of ROS which could support malignancy (Tretter and Adam-Vizi, 2004, Day and Suzuki, 2006). However, we observed no change in the expression level of the total PDH enzyme in both the cell lines but a high expression in the phosphorylated PDH fraction in the hypoxia samples. This suggested that as observed before, HIF-1α could have led to activation of PDK1 enzyme thereby promoting inactivation of PDH via phosphorylation (Dupuy F 2015).

To confirm if the expression of these enzymes corresponded to their activities, we performed various enzyme assays. The TKT assay corresponded with the expression levels observed and showed a TPP effect when an exogenous TPP was added to ensure the maximum use of all the TKT enzyme in the pool. The AKGDH assay, showed similar results, however, the 10nM samples both in normoxia and hypoxia always had lower

activity when compared to their 3µM groups. Also, they did not show much difference in their activity on addition of exogenous TPP levels. The PDH assay however, showed a decline in the assay which supported that the enzyme was inhibited and thus showed poor activity. Addition of exogenous TPP did not reverse the inhibition indicating complete saturation of enzyme thereby showing no TPP effect.

In conclusion, all of the above data indicates a possible role of thiamine in cell proliferation. The increased proliferation rate could be due to the coenzyme function of thiamine. When exposed to hypoxic environment, malignant cells show enhanced proliferation due to stabilization of enzymes involved in metabolism to produce new cells. Also, the change in the expression of thiamine dependent enzymes suggested that hypoxia affected the functional activity of enzymes which could ultimately alter metabolic pathways. Thus, we conclude that thiamine supplementation at low doses leads to enhanced cell growth in both phenotypes of breast cancer as well as hypoxia plays a critical role in activating certain thiamine dependent enzymes which could further help in regulating the altered metabolic processes.

3.2 EXPERIMENTAL LIMITATIONS:

The following section lists the limitations in the experimental data relating to the cell culture systems utilized as also with the various techniques.

3.2.1 *Use of in vitro models*:

The above work made use of the in vitro cell models to portray the effects of thiamine supplementation. We used two phenotypically different breast cancer cell lines. All of the above data to determine the expression of the enzymes and the cell growth analysis was performed utilizing these cell lines. However, we cannot comment about the cell growth in in-vivo models since, proliferation in vitro occurs in a 2-dimensional manner and the in vivo models use a 3-dimensional approach. Our studies also assessed the enzyme expression and activities which are easily affected and degrade overtime in cell lysates than in in-vivo models.

3.2.2 *Thiamine Dosing*:

The cell lines were cultured using the 3µM thiamine media before seeding them for experiments. They were then seeded into a dish with 10nM thiamine media and then thiamine hydrochloride was spiked into the 10nM media for the 3µM treatment group. Since the in vitro models have 2-dimension, the cells are exposed to maximum amount of thiamine. However, the in vivo models due to their 3-dimensional structure, the cells closer to the vasculature receive more nutrients as compared to the farthest away. Also, the

number of transporters facilitating thiamine uptake would be another critical factor to be considered while supplementing with thiamine to ensure intracellular bioavailability. Thus, nutrient dosing can be a limiting factor which should be considered while assessing the effects in animal models.

3.2.3 *Hypoxic environment*:

Hypoxia is simulated in the lab by incubating the cells in incubators which are supplied with 1% O_2 at 37°C. The exposure to this environment is for a duration of 7 days and this creates a possibility of exposure of the treatment groups to reoxygenation when the incubator is opened to introduce other treatment groups. The reoxygenation could lead to several changes in the gene alterations giving different results. Thus, during in vivo studies, this parameter should be kept in mind and the animal tumors should be exposed to minimal amount of oxygen using different techniques.

3.3 FUTURE DIRECTIONS:

3.3.1 Determination RNA levels of enzymes:

RNAs are known to be involved in regulation of cellular processes like cell growth, ageing and cell death. It is a component which is involved in translation of genetic code into proteins (Clancy, S. 2008). All of our experiments aimed at assessing the protein levels of enzymes which required TPP as cofactor. For future considerations, we should look at mRNA levels of these enzymes as to get an understanding of how thiamine supplementation affects these enzymes at a transcription level and correlating with the effects observed in our study.

3.3.2 Determination of intracellular levels of thiamine dependent enzymes:

All our experiments aimed at assessing the effect of thiamine supplementation were carried out in in-vitro conditions and have helped in detection of expression and activity of enzymes present extracellularly. For our future work, we should consider detecting the levels of these enzymes and their activities intracellularly as to get a clear idea about how thiamine supplementation affects the enzyme function intracellularly.

3.3.3 Determination of effect of thiamine supplementation on cells from different origins:

In our studies, we have determined the effect of thiamine supplementation on two phenotypically different breast cancer cell lines. This has helped us to investigate the changes in different phenotype and whether there is a difference in the changes seen in both these cell lines. Future work should consider looking into cancer which arise from

different origins. This would be helpful to conclude whether thiamine supplementation has a different effect on those cell lines or not. It will also help to generalize whether thiamine supplementation in low doses affects a few or several different types of cancer.

3.3.4 Determination of thiamine uptake and metabolism:

Since we observed an increase in expression of thiamine dependent enzymes on supplementation with thiamine, its effect on metabolic pathways could be an area which has to be assessed. There is not much evidence which demonstrates that the thiamine that is supplemented is completely taken up and what is its possible contribution to metabolism. Quantifying the amount of thiamine which has been taken up and assessing its fate could be beneficial to prove that the elevated enzyme levels were due to the supplemented thiamine and no other underlying factor was involved. Also, more work can be done to explore the fate of the enzymes which are involved in these metabolism processes.

REFERNCES

Adewale Fadaka, Basiru Ajiboye, Oluwafemi Ojo, OlusolaAdewale, Israel Olayide, RosemaryEmuowhochere. "Biology of Glucose Metabolization in Cancer Cells." *Journal of Oncological Sciences* (2017).

Akram M, Iqbal M, Daniyal M, Khan AU. "Awareness and Current Knowledge of Breast Cancer.". *Biol Res* (2017).

American Cancer Society. (n.d.). Retrieved from https://www.cancer.org/cancer/cancer-causes/genetics/genes-and-cancer/oncogenes-tumor-suppressor-genes.html

Bacus, S. S., D. A. Altomare, L. Lyass, D. M. Chin, M. P. Farrell, K. Gurova, A. Gudkov, and J. R. Testa. "Akt2 Is Frequently Upregulated in Her-2/Neu-Positive Breast Cancers and May Contribute to Tumor Aggressiveness by Enhancing Cell Survival." *Oncogene* 21, no. 22 (May 16 2002): 3532-40. https://doi.org/10.1038/sj.onc.1205438.

Bentz, S., A. Cee, E. Endlicher, K. A. Wojtal, A. Naami, T. Pesch, S. Lang, *et al.* "Hypoxia Induces the Expression of Transketolase-Like 1 in Human Colorectal Cancer." *Digestion* 88, no. 3 (2013): 182-92. https://doi.org/10.1159/000355015.

Berg, Tymocko , L. Strayer. *Biochemistry.* New York: W.H Freeman, 2002.

Boros, L. G., J. L. Brandes, W. N. Lee, M. Cascante, J. Puigjaner, E. Revesz, T. M. Bray, W. J. Schirmer, and W. S. Melvin. "Thiamine Supplementation to Cancer Patients: A Double Edged Sword." *Anticancer Res* 18, no. 1B (Jan-Feb 1998): 595-602.

Boros LG, Torday JS, Lim S, Bassilian S, Cascante M, Lee WN. "Transforming Growth Factor B2 Promotes Glucose Carbon Incorporation into Nucleic Acid Ribose through the Nonoxidative Pentose Cycle in Lung Epithelial Carcinoma Cells." *Cancer Research* (2000).

Brauer, H. A., L. Makowski, K. A. Hoadley, P. Casbas-Hernandez, L. J. Lang, E. Roman-Perez, M. D'Arcy, *et al.* "Impact of Tumor Microenvironment and Epithelial Phenotypes on Metabolism in Breast Cancer." *Clin Cancer Res* 19, no. 3 (Feb 1 2013): 571-85. https://doi.org/10.1158/1078-0432.CCR-12-2123.

Brian D. Lehmann, Joshua A. Bauer, Xi Chen, Melinda E. Sanders, A. Bapsi Chakravarthy, Yu Shyr, and Jennifer A. Pietenpol. "Identification of Human Triple-Negative Breast Cancer Subtypes and Preclinical Models for Selection of Targeted Therapies." *J Clinical Invest* (2011).

Bright-Gbebry M, Makambi KH, Rohan JP, Llanos AA, Rosenberg L, Palmer JR, Adams-Campbell LL. "Use of Multivitamins, Folic Acid and Herbal Supplements

among Breast Cancer Survivors: The Black Women's Health Study.". *BMC complement alternate medicine* (2011).

Brown, G. "Defects of Thiamine Transport and Metabolism." *J Inherit Metab Dis* 37, no. 4 (Jul 2014): 577-85. https://doi.org/10.1007/s10545-014-9712-9.

Brown, J. M. "Tumor Hypoxia in Cancer Therapy." *Methods Enzymol* 435 (2007): 297-321. https://doi.org/10.1016/S0076-6879(07)35015-5.

Budczies, J., B. M. Pfitzner, B. Gyorffy, K. J. Winzer, C. Radke, M. Dietel, O. Fiehn, and C. Denkert. "Glutamate Enrichment as New Diagnostic Opportunity in Breast Cancer." *Int J Cancer* 136, no. 7 (Apr 1 2015): 1619-28. https://doi.org/10.1002/ijc.29152.

C. M. Lopes, A. Dourado, and R. Oliveira. "Phytotherapy and Nutritional Supplements on Breast Cancer." *Biomed Res Int* (2017).

Cao, M. D., S. Lamichhane, S. Lundgren, A. Bofin, H. Fjosne, G. F. Giskeodegard, and T. F. Bathen. "Metabolic Characterization of Triple Negative Breast Cancer." *BMC Cancer* 14 (Dec 12 2014): 941. https://doi.org/10.1186/1471-2407-14-941.

Chen JQ, Russo J. "Dysregulation of Glucose Transport, Glycolysis, Tca Cycle and Glutaminolysis by Oncogenes and Tumor Suppressors in Cancer Cells.". *Biochim Biphysi Acta* (2012).

Comin-Anduix, B., J. Boren, S. Martinez, C. Moro, J. J. Centelles, R. Trebukhina, N. Petushok, *et al.* "The Effect of Thiamine Supplementation on Tumour Proliferation. A Metabolic Control Analysis Study." *Eur J Biochem* 268, no. 15 (Aug 2001): 4177-82.

Crowe, J. P., Jr., N. H. Gordon, C. A. Hubay, R. R. Shenk, R. M. Zollinger, D. J. Brumberg, W. L. McGuire, and J. M. Shuck. "Estrogen Receptor Determination and Long Term Survival of Patients with Carcinoma of the Breast." *Surg Gynecol Obstet* 173, no. 4 (Oct 1991): 273-8.

Daily, A., S. Liu, S. Bhatnagar, R. G. Karabakhtsian, and J. A. Moscow. "Low-Thiamine Diet Increases Mammary Tumor Latency in Fvb/N-Tg(Mmtvneu) Mice." *Int J Vitam Nutr Res* 82, no. 4 (Aug 2012): 298-302. https://doi.org/10.1024/0300-9831/a000122.

De Huang, Chenchen Li, Huafeng Zhang. "Hypoxia and Cancer Cell Metabolism." *ABBS* (2014).

Deghan Manshadi, S., L. Ishiguro, K. J. Sohn, A. Medline, R. Renlund, R. Croxford, and Y. I. Kim. "Folic Acid Supplementation Promotes Mammary Tumor Progression in a Rat Model." *PLoS One* 9, no. 1 (2014): e84635. https://doi.org/10.1371/journal.pone.0084635.

Dunnwald, L. K., M. A. Rossing, and C. I. Li. "Hormone Receptor Status, Tumor Characteristics, and Prognosis: A Prospective Cohort of Breast Cancer Patients." *Breast Cancer Res* 9, no. 1 (2007): R6. https://doi.org/10.1186/bcr1639.

Elena Beltramo, Carmela Solimine, Alessandro Ubertalli Ape and Massimo Porta. "Regulation of Intracellular Glucose and Polyol Pathway by Thiamine and Benfotiamine in Vascular Cells Cultured in High Glucose." *Journal of Biological Chemistry* (2006).

Endogenous, Hormones, Group Breast Cancer Collaborative, T. J. Key, P. N. Appleby, G. K. Reeves, A. W. Roddam, K. J. Helzlsouer, *et al.* "Circulating Sex Hormones and Breast Cancer Risk Factors in Postmenopausal Women: Reanalysis of 13 Studies." *Br J Cancer* 105, no. 5 (Aug 23 2011): 709-22. https://doi.org/10.1038/bjc.2011.254.

Eshak, E. S., and A. E. Arafa. "Thiamine Deficiency and Cardiovascular Disorders." *Nutr Metab Cardiovasc Dis* 28, no. 10 (Oct 2018): 965-72. https://doi.org/10.1016/j.numecd.2018.06.013.

Eshak ES, Arafa AE. "Thiamine Deficiency and Cardiovascular Disorders.". *Nutr. metabolism cardiovascular disease* (2018).

Eyassu, F., and C. Angione. "Modelling Pyruvate Dehydrogenase under Hypoxia and Its Role in Cancer Metabolism." *R Soc Open Sci* 4, no. 10 (Oct 2017): 170360. https://doi.org/10.1098/rsos.170360.

Farhud, D. D. "Impact of Lifestyle on Health." *Iran J Public Health* 44, no. 11 (Nov 2015): 1442-4.

Futreal, P. A., Q. Liu, D. Shattuck-Eidens, C. Cochran, K. Harshman, S. Tavtigian, L. M. Bennett, *et al.* "Brca1 Mutations in Primary Breast and Ovarian Carcinomas." *Science* 266, no. 5182 (Oct 7 1994): 120-2.

Ganapathy-Kanniappan, S., and J. F. Geschwind. "Tumor Glycolysis as a Target for Cancer Therapy: Progress and Prospects." *Mol Cancer* 12 (Dec 3 2013): 152. https://doi.org/10.1186/1476-4598-12-152.

Geck, R. C., and A. Toker. "Nonessential Amino Acid Metabolism in Breast Cancer." *Adv Biol Regul* 62 (Sep 2016): 11-17. https://doi.org/10.1016/j.jbior.2016.01.001.

GL., Semenza. "Oxygen Homeostasis. ." *Systems Biol Med* (2009).

Ha, S. M., E. Y. Chae, J. H. Cha, H. H. Kim, H. J. Shin, and W. J. Choi. "Association of Brca Mutation Types, Imaging Features, and Pathologic Findings in Patients with Breast Cancer with Brca1 and Brca2 Mutations." *AJR Am J Roentgenol* 209, no. 4 (Oct 2017): 920-28. https://doi.org/10.2214/AJR.16.16957.

Hanahan, D., and R. A. Weinberg. "Hallmarks of Cancer: The Next Generation." *Cell* 144, no. 5 (Mar 4 2011): 646-74. https://doi.org/10.1016/j.cell.2011.02.013.

Hanberry, B. S., R. Berger, and J. A. Zastre. "High-Dose Vitamin B1 Reduces Proliferation in Cancer Cell Lines Analogous to Dichloroacetate." *Cancer Chemother Pharmacol* 73, no. 3 (Mar 2014): 585-94. https://doi.org/10.1007/s00280-014-2386-z.

Harvie M, Howell A, Evans DG. "Can Diet and Lifestyle Prevent Breast Cancer: What Is the Evidence?". *Am Soc Clin Oncol Edu book* (2015).

Haseloff, R. F., E. Krause, M. Bigl, K. Mikoteit, D. Stanimirovic, and I. E. Blasig. "Differential Protein Expression in Brain Capillary Endothelial Cells Induced by Hypoxia and Posthypoxic Reoxygenation." *Proteomics* 6, no. 6 (Mar 2006): 1803-9. https://doi.org/10.1002/pmic.200500182.

Hu, X., H. M. Stern, L. Ge, C. O'Brien, L. Haydu, C. D. Honchell, P. M. Haverty, *et al.* "Genetic Alterations and Oncogenic Pathways Associated with Breast Cancer Subtypes." *Mol Cancer Res* 7, no. 4 (Apr 2009): 511-22. https://doi.org/10.1158/1541-7786.MCR-08-0107.

In *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline*. The National Academies Collection: Reports Funded by National Institutes of Health. Washington (DC), 1998.

Jonus, H. C., B. S. Hanberry, S. Khatu, J. Kim, H. Luesch, L. H. Dang, M. G. Bartlett, and J. A. Zastre. "The Adaptive Regulation of Thiamine Pyrophosphokinase-1 Facilitates Malignant Growth During Supplemental Thiamine Conditions." *Oncotarget* 9, no. 83 (Oct 23 2018): 35422-38. https://doi.org/10.18632/oncotarget.26259.

Jóźwiak, Paweł, Ewa Forma, Magdalena Bryś, and Anna Krześlak. "O-Glcnacylation and Metabolic Reprograming in Cancer.". *Frontiers in Endocrinology* 5 (2014): 145-45.

Kang, S. S., Y. K. Chun, M. H. Hur, H. K. Lee, Y. J. Kim, S. R. Hong, J. H. Lee, S. G. Lee, and Y. K. Park. "Clinical Significance of Glucose Transporter 1 (Glut1) Expression in Human Breast Carcinoma." *Jpn J Cancer Res* 93, no. 10 (Oct 2002): 1123-8.

Kim, I., D. F. Williamson, T. Byers, and J. P. Koplan. "Vitamin and Mineral Supplement Use and Mortality in a Us Cohort." *Am J Public Health* 83, no. 4 (Apr 1993): 546-50.

Kim, J. W., I. Tchernyshyov, G. L. Semenza, and C. V. Dang. "Hif-1-Mediated Expression of Pyruvate Dehydrogenase Kinase: A Metabolic Switch Required for

Cellular Adaptation to Hypoxia." *Cell Metab* 3, no. 3 (Mar 2006): 177-85. https://doi.org/10.1016/j.cmet.2006.02.002.

Kim SJ, Zuchniak A, Sohn KJ, Lubinski J, Demsky R, Eisen A, Akbari MR, Kim YI, Narod SA, Kotsopoulos J. "Plasma Folate, Vitamin B-6, and Vitamin B-12 and Breast Cancer Risk in Brca1- and Brca2-Mutation Carriers: A Prospective Study.". *Am J Clin Nutr* (2016).

Koike K, Hamada M, Tanaka N, Otsuka KI, Ogasahara K, Koike M. "Properties and Subunit Composition of the Pig Heart 2-Oxoglutarate Dehydrogenase.". *J biol chem* (1974).

Korotchkina LG, Patel MS. "Site Specificity of Four Pyruvate Dehydrogenase Kinase Isoenzymes toward the Three Phosphorylation Sites of Human Pyruvate Dehydrogenase.". *J Biol Chem* (2001).

Koukourakis, M. I., A. Giatromanolaki, A. L. Harris, and E. Sivridis. "Comparison of Metabolic Pathways between Cancer Cells and Stromal Cells in Colorectal Carcinomas: A Metabolic Survival Role for Tumor-Associated Stroma." *Cancer Res* 66, no. 2 (Jan 15 2006): 632-7. https://doi.org/10.1158/0008-5472.CAN-05-3260.

Kubota, Takayuki ShimizuKen-ichi InoueHiroyuki HachiyaNorisuke ShibuyaMitsugi ShimodaKeiichi. "Frequent Alteration of the Protein Synthesis of Enzymes for Glucose Metabolism in Hepatocellular Carcinomas." *Journal of Gastroenterology* (2014).

Kuchenbaecker, K. B., J. L. Hopper, D. R. Barnes, K. A. Phillips, T. M. Mooij, M. J. Roos-Blom, S. Jervis, *et al.* "Risks of Breast, Ovarian, and Contralateral Breast Cancer for Brca1 and Brca2 Mutation Carriers." *JAMA* 317, no. 23 (Jun 20 2017): 2402-16. https://doi.org/10.1001/jama.2017.7112.

Laszlo Tretter, Vera Adam-Vizi. "Alpha-Ketoglutarate Dehydrogenase: A Target and Generator of Oxidative Stress." *Philos Trans R Soc Lond B Biol Sci* (2005).

Lear-Kaul, K. C., H. R. Yoon, B. K. Kleinschmidt-DeMasters, L. McGavran, and M. Singh. "Her-2/Neu Status in Breast Cancer Metastases to the Central Nervous System." *Arch Pathol Lab Med* 127, no. 11 (Nov 2003): 1451-7. https://doi.org/10.1043/1543-2165(2003)127<1451:NSIBCM>2.0.CO;2.

Lee, H. R., T. H. Kim, and K. C. Choi. "Functions and Physiological Roles of Two Types of Estrogen Receptors, Eralpha and Erbeta, Identified by Estrogen Receptor Knockout Mouse." *Lab Anim Res* 28, no. 2 (Jun 2012): 71-6. https://doi.org/10.5625/lar.2012.28.2.71.

Lindhurst, M. J., G. Fiermonte, S. Song, E. Struys, F. De Leonardis, P. L. Schwartzberg, A. Chen, *et al.* "Knockout of Slc25a19 Causes Mitochondrial Thiamine Pyrophosphate Depletion, Embryonic Lethality, Cns Malformations, and

Anemia." *Proc Natl Acad Sci U S A* 103, no. 43 (Oct 24 2006): 15927-32. https://doi.org/10.1073/pnas.0607661103.

Liu, S., H. Huang, X. Lu, M. Golinski, S. Comesse, D. Watt, R. B. Grossman, and J. A. Moscow. "Down-Regulation of Thiamine Transporter Thtr2 Gene Expression in Breast Cancer and Its Association with Resistance to Apoptosis." *Mol Cancer Res* 1, no. 9 (Jul 2003): 665-73.

Loman, N., O. Johannsson, P. O. Bendahl, A. Borg, M. Ferno, and H. Olsson. "Steroid Receptors in Hereditary Breast Carcinomas Associated with Brca1 or Brca2 Mutations or Unknown Susceptibility Genes." *Cancer* 83, no. 2 (Jul 15 1998): 310-9.

Mantzorou, M., A. Koutelidakis, S. Theocharis, and C. Giaginis. "Clinical Value of Nutritional Status in Cancer: What Is Its Impact and How It Affects Disease Progression and Prognosis?". *Nutr Cancer* 69, no. 8 (Nov-Dec 2017): 1151-76. https://doi.org/10.1080/01635581.2017.1367947.

Manzetti, Sergio, Jin Zhang, and David van der Spoel. "Thiamin Function, Metabolism, Uptake, and Transport.". *Biochemistry* 53, no. 5 (2014): 821-35.

Marjorie Gangolf, Jan Czerniecki, Marc Radermecker, Olivier Detry, Michelle Nisolle, Caroline Jouan, Didier Martin, Frédéric Chantraine, Bernard Lakaye, Pierre Wins, Thierry Grisar, and Lucien Bettendorff. "Thiamine Status in Humans and Content of Phosphorylated Thiamine Derivatives in Biopsies and Cultured Cells." *PLoS one* (2010).

McFate, T., A. Mohyeldin, H. Lu, J. Thakar, J. Henriques, N. D. Halim, H. Wu, *et al.* "Pyruvate Dehydrogenase Complex Activity Controls Metabolic and Malignant Phenotype in Cancer Cells." *J Biol Chem* 283, no. 33 (Aug 15 2008): 22700-8. https://doi.org/10.1074/jbc.M801765200.

Metallo, C. M., P. A. Gameiro, E. L. Bell, K. R. Mattaini, J. Yang, K. Hiller, C. M. Jewell, *et al.* "Reductive Glutamine Metabolism by Idh1 Mediates Lipogenesis under Hypoxia." *Nature* 481, no. 7381 (Nov 20 2011): 380-4. https://doi.org/10.1038/nature10602.

Morris, Robertson and. "Growth Rate and Number of Spontaneous Mammary Carcinomas and Riboflavin Concentrations of Liver, Muscle and Tumor of C3h Mice as Influenced by Dietary Riboflavin." *J Natl Cancer Inst* (1943).

Muz, B., P. de la Puente, F. Azab, and A. K. Azab. "The Role of Hypoxia in Cancer Progression, Angiogenesis, Metastasis, and Resistance to Therapy." *Hypoxia (Auckl)* 3 (2015): 83-92. https://doi.org/10.2147/HP.S93413.

Oh, S., H. Kim, K. Nam, and I. Shin. "Glut1 Promotes Cell Proliferation, Migration and Invasion by Regulating Epidermal Growth Factor Receptor and Integrin Signaling in Triple-Negative Breast Cancer Cells." *BMB Rep* 50, no. 3 (Mar 2017): 132-37.

Oh, S., H. Kim, K. Nam, and I. Shin. "Silencing of Glut1 Induces Chemoresistance Via Modulation of Akt/Gsk-3beta/Beta-Catenin/Survivin Signaling Pathway in Breast Cancer Cells." *Arch Biochem Biophys* 636 (Dec 15 2017): 110-22. https://doi.org/10.1016/j.abb.2017.08.009.

Okai, Y., K. Higashi-Okai, F. Sato E, R. Konaka, and M. Inoue. "Potent Radical-Scavenging Activities of Thiamin and Thiamin Diphosphate." *J Clin Biochem Nutr* 40, no. 1 (Jan 2007): 42-8. https://doi.org/10.3164/jcbn.40.42.

Patra KC, Hay N. "The Pentose Phosphate Pathway and Cancer.". *Trends Biochem Sci* (2014).

Pekovich, S. R., P. R. Martin, and C. K. Singleton. "Thiamine Deficiency Decreases Steady-State Transketolase and Pyruvate Dehydrogenase but Not Alpha-Ketoglutarate Dehydrogenase Mrna Levels in Three Human Cell Types." *J Nutr* 128, no. 4 (Apr 1998): 683-7. https://doi.org/10.1093/jn/128.4.683.

Peng Jiang, Wenjing Du, Mian Wu. "Regulation of the Pentose Phosphate Pathway in Cancer." *Protein Cell* (2014).

Peshkin, B. N., M. L. Alabek, and C. Isaacs. "Brca1/2 Mutations and Triple Negative Breast Cancers." *Breast Dis* 32, no. 1-2 (2010): 25-33. https://doi.org/10.3233/BD-2010-0306.

Peter R. Martin, Charles K. Singleton, and Susanne Hiller–Sturmhöfel. "The Role of Thiamine Deficiency in Alcoholic Brain Disease." *NIHAA* (2004).

Rabhi, N., S. A. Hannou, P. Froguel, and J. S. Annicotte. "Cofactors as Metabolic Sensors Driving Cell Adaptation in Physiology and Disease." *Front Endocrinol (Lausanne)* 8 (2017): 304. https://doi.org/10.3389/fendo.2017.00304.

Reidling, J. C., N. Lambrecht, M. Kassir, and H. M. Said. "Impaired Intestinal Vitamin B1 (Thiamin) Uptake in Thiamin Transporter-2-Deficient Mice." *Gastroenterology* 138, no. 5 (May 2010): 1802-9. https://doi.org/10.1053/j.gastro.2009.10.042.

Reiter, F., S. Wienerroither, and A. Stark. "Combinatorial Function of Transcription Factors and Cofactors." *Curr Opin Genet Dev* 43 (Apr 2017): 73-81. https://doi.org/10.1016/j.gde.2016.12.007.

Renaud Vatrinet, Giulia Leone, Monica De Luise, Giulia Girolimetti, Michele Vidone, Giuseppe Gasparrer and Anna Maria Porcelli. "The A-Ketoglutarate Dehydrogenase Complex in Cancer Metabolic Plasticity." *Cancer and Metabolism* (2017).

Ricciardelli C, Lokman NA, Cheruvu S, Tan I, Ween MP, Pyragius CE, Ruszkiewicz A, Hoffmann P, Oehler MK. "Transketolase Is Upregulated in Metastatic Peritoneal

Implants and Promotes Ovarian Cancer Cell Proliferation.". *Clin Exp metastasis* (2015).

Rindi, G., and U. Laforenza. "Thiamine Intestinal Transport and Related Issues: Recent Aspects." *Proc Soc Exp Biol Med* 224, no. 4 (Sep 2000): 246-55.

Ripperger, T., D. Gadzicki, A. Meindl, and B. Schlegelberger. "Breast Cancer Susceptibility: Current Knowledge and Implications for Genetic Counselling." *Eur J Hum Genet* 17, no. 6 (Jun 2009): 722-31. https://doi.org/10.1038/ejhg.2008.212.

Rongbao Zhao, David Goldman. "Folate and Thiamine Transporters Mediated by Facilitative Carriers (Slc19a1-3 and Slc46a1) and Folate Receptors." *Molecular Aspects of Medicine* (2013).

Ross, J. S., and J. A. Fletcher. "The Her-2/Neu Oncogene: Prognostic Factor, Predictive Factor and Target for Therapy." *Semin Cancer Biol* 9, no. 2 (Apr 1999): 125-38. https://doi.org/10.1006/scbi.1998.0083.

S Langbein, M Zerilli, A zur Hausen, W Staiger, K Rensch-Boschert, N Lukan, J Popa, M P Ternullo, A Steidler, C Weiss, R Grobholz, F Willeke, P Alken, G Stassi, P Schubert, and J F Coy*. "Expression of Transketolase Tktl1 Predicts Colon and Urothelial Cancer Patient Survival: Warburg Effect Reinterpreted." *Br J cancer* (2006).

San-Millan, I., and G. A. Brooks. "Reexamining Cancer Metabolism: Lactate Production for Carcinogenesis Could Be the Purpose and Explanation of the Warburg Effect." *Carcinogenesis* 38, no. 2 (Feb 1 2017): 119-33. https://doi.org/10.1093/carcin/bgw127.

Semenza, G. L. "Regulation of Oxygen Homeostasis by Hypoxia-Inducible Factor 1." *Physiology (Bethesda)* 24 (Apr 2009): 97-106. https://doi.org/10.1152/physiol.00045.2008.

Shimizu T, Inoue K, Hachiya H, Shibuya N, Shimoda M, Kubota K. "Frequent Alteration of the Protein Synthesis of Enzymes for Glucose Metabolism in Hepatocellular Carcinomas.". *Journal of gastroenterology* (2014).

Shimizu, T., K. Inoue, H. Hachiya, N. Shibuya, M. Shimoda, and K. Kubota. "Frequent Alteration of the Protein Synthesis of Enzymes for Glucose Metabolism in Hepatocellular Carcinomas." *J Gastroenterol* 49, no. 9 (Sep 2014): 1324-32. https://doi.org/10.1007/s00535-013-0895-x.

Shiyanbola, O. O., R. F. Arao, D. L. Miglioretti, B. L. Sprague, J. M. Hampton, N. K. Stout, K. Kerlikowske, *et al.* "Emerging Trends in Family History of Breast Cancer and Associated Risk." *Cancer Epidemiol Biomarkers Prev* 26, no. 12 (Dec 2017): 1753-60. https://doi.org/10.1158/1055-9965.EPI-17-0531.

Singleton, Stevan R. Pekovich Peter R. Martin Charles K. "Thiamine Deficiency Decreases Steady-State Transketolase and Pyruvate Dehydrogenase but Not A-Ketoglutarate Dehydrogenase Mrna Levels in Three Human Cell Types ". *Journal of Nutrition* (1998).

Starczynski, J., N. Atkey, Y. Connelly, T. O'Grady, F. M. Campbell, S. di Palma, P. Wencyk, *et al.* "Her2 Gene Amplification in Breast Cancer: A Rogues' Gallery of Challenging Diagnostic Cases: Ukneqas Interpretation Guidelines and Research Recommendations." *Am J Clin Pathol* 137, no. 4 (Apr 2012): 595-605. https://doi.org/10.1309/AJCPATBZ2JFN1QQC.

Thompson, M. E., R. A. Jensen, P. S. Obermiller, D. L. Page, and J. T. Holt. "Decreased Expression of Brca1 Accelerates Growth and Is Often Present During Sporadic Breast Cancer Progression." *Nat Genet* 9, no. 4 (Apr 1995): 444-50. https://doi.org/10.1038/ng0495-444.

Tretter, L., and V. Adam-Vizi. "Alpha-Ketoglutarate Dehydrogenase: A Target and Generator of Oxidative Stress." *Philos Trans R Soc Lond B Biol Sci* 360, no. 1464 (Dec 29 2005): 2335-45. https://doi.org/10.1098/rstb.2005.1764.

Tretter, Laszlo, and Vera Adam-Vizi. "Generation of Reactive Oxygen Species in the Reaction Catalyzed by A-Ketoglutarate Dehydrogenase." *The Journal of Neuroscience* 24, no. 36 (2004): 7771-78.

Valencia, O. M., S. E. Samuel, R. K. Viscusi, T. S. Riall, L. A. Neumayer, and H. Aziz. "The Role of Genetic Testing in Patients with Breast Cancer: A Review." *JAMA Surg* 152, no. 6 (Jun 1 2017): 589-94. https://doi.org/10.1001/jamasurg.2017.0552.

Vander Heiden, M. G., and R. J. DeBerardinis. "Understanding the Intersections between Metabolism and Cancer Biology." *Cell* 168, no. 4 (Feb 9 2017): 657-69. https://doi.org/10.1016/j.cell.2016.12.039.

von Minckwitz, G., M. Procter, E. de Azambuja, D. Zardavas, M. Benyunes, G. Viale, T. Suter, *et al.* "Adjuvant Pertuzumab and Trastuzumab in Early Her2-Positive Breast Cancer." *N Engl J Med* 377, no. 2 (Jul 13 2017): 122-31. https://doi.org/10.1056/NEJMoa1703643.

Wahba, H. A., and H. A. El-Hadaad. "Current Approaches in Treatment of Triple-Negative Breast Cancer." *Cancer Biol Med* 12, no. 2 (Jun 2015): 106-16. https://doi.org/10.7497/j.issn.2095-3941.2015.0030.

Wang, G. L., B. H. Jiang, E. A. Rue, and G. L. Semenza. "Hypoxia-Inducible Factor 1 Is a Basic-Helix-Loop-Helix-Pas Heterodimer Regulated by Cellular O2 Tension." *Proc Natl Acad Sci U S A* 92, no. 12 (Jun 6 1995): 5510-4.

Warburg, Otto. "The Metabolism of Tumors." *Biochem* (1923).

Weijie Yuan, Shaobin Wu, Jianwu Guo, Zhikang Chen, Jie Ge, Pu Yang,. "Silencing of Tktl1 by Sirna Inhibits Proliferation of Human Gastric Cancer Cells in Vitro and in Vivo." *Cancer biology and therapy* (2010).

Wong-Brown, M. W., C. J. Meldrum, J. E. Carpenter, C. L. Clarke, S. A. Narod, A. Jakubowska, H. Rudnicka, J. Lubinski, and R. J. Scott. "Prevalence of Brca1 and Brca2 Germline Mutations in Patients with Triple-Negative Breast Cancer." *Breast Cancer Res Treat* 150, no. 1 (Feb 2015): 71-80. https://doi.org/10.1007/s10549-015-3293-7.

Xu IM, Lai RK, Lin SH, Tse AP, Chiu DK1, Koh HY, Law CT, Wong CM, Cai Z, Wong CC, Ng IO. "Transketolase Counteracts Oxidative Stress to Drive Cancer Development.". *Proc Natl Acad Sci USA* (2016).

Yeaman SJ, Hutcheson ET, Roche TE, Pettit FH, Brown JR, Reed LJ, Watson DC, Dixon GH. "Sites of Phosphorylation on Pyruvate Dehydrogenase from Bovine Kidney and Heart.". *Biochemistry* (1978).

Zhao, F., A. Mancuso, T. V. Bui, X. Tong, J. J. Gruber, C. R. Swider, P. V. Sanchez, *et al.* "Imatinib Resistance Associated with Bcr-Abl Upregulation Is Dependent on Hif-1alpha-Induced Metabolic Reprograming." *Oncogene* 29, no. 20 (May 20 2010): 2962-72. https://doi.org/10.1038/onc.2010.67.

Zhao J, Zhong CJ. "A Review on Research Progress of Transketolase.". *Neurosci Bull* (2009).

Zhao R, Gao F, Wang Y, Diaz GA, Gelb BD, Goldman ID. "Impact of the Reduced Folate Carrier on the Accumulation of Active Thiamin Metabolites in Murine Leukemia Cells.". *J biol Chem* (2001).

Zhao, R., F. Gao, and I. D. Goldman. "Reduced Folate Carrier Transports Thiamine Monophosphate: An Alternative Route for Thiamine Delivery into Mammalian Cells." *Am J Physiol Cell Physiol* 282, no. 6 (Jun 2002): C1512-7. https://doi.org/10.1152/ajpcell.00547.2001.

Zhou, Z. H., D. B. McCarthy, C. M. O'Connor, L. J. Reed, and J. K. Stoops. "The Remarkable Structural and Functional Organization of the Eukaryotic Pyruvate Dehydrogenase Complexes." *Proc Natl Acad Sci U S A* 98, no. 26 (Dec 18 2001): 14802-7. https://doi.org/10.1073/pnas.011597698.