MATTHEW THOMAS FERREIRA

Metabólitos da 15-lipoxigenase Influenciam Crescimento, Migração e Potencial Invasivo de Células de Glioblastoma.

Tese apresentada ao Programa de Pós-Graduação em Biologia de Sistemas do Instituto de Ciências Biomédicas da Universidade de São Paulo, para obtenção do Título de Doutor em Ciências.

São Paulo
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Orientadora: Profa. Dra. Alison Colquhoun

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Certificamos que o Protocolo CEP-ICB nº **980/2018** referente ao projeto intitulado: ***"Os efeitos dos produtos de 15-LOX-1/2 em GBM in vitro"*** sob a responsabilidade de ***Matthew Tomas Ferreira*** e orientação do(a) Prof.(a) Dr.(a) ***Alison Colquhoun,*** do Departamento de Biologia Celular e do Desenvolvimento, foi analisado pela **CEUA** - Comissão de Ética no Uso de Animais e pelo **CEPSH** – Comitê de Ética em Pesquisa com Seres Humanos, tendo sido deliberado que o referido projeto não utilizará animais que estejam sob a égide da Lei nº 11.794, de 8 de outubro de 2008, nem envolverá procedimentos regulados pela Resolução CONEP nº 466/2012.

São Paulo, 17 de agosto de 2018.

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“Keep your heart with all vigilance, for from it are the issues of life. Put away from you crooked speech and put perverse talk far from you. Let your eyes look directly ahead, and let your gaze be straight in front of you. Make straight the path of your feet, and all your ways will be established.” -Proverbs 4:23-26

ABSTRACT

Ferreira MT. **15-Lipoxygenase Metabolites Influence Growth, Migration and Invasive Potential of Glioblastoma cells** [Thesis (Ph.D. thesis in Systems Biology: Cell and Tissue Biology)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2019.

Introduction and Objectives: Understanding the relationship between extremely aggressive cancers, like glioblastoma (GBM), and fatty acid metabolism could contribute to improve anti-tumoral therapies. 15-Lipoxygenase-1 (15-LOX-1), a linoleic acid (LA) and arachidonic acid (AA) metabolizing enzyme, induces both pro- and anti-tumorigenic effects in different cancer types. Its role in glioblastoma has not yet been clearly described. The aim of this study was to describe the influence of 15-LOX and its metabolites on GBM cell growth, migration and invasive potential. ***Experimental Design***: Two GBM cell lines were cultivated and treated *in vitro* with 15-LOX metabolites (13(S)-Hydroxyoctadecadienoic Acid (13-HODE), 9-HODE and 15(S)-Hydroxyeicosatetraenoic Acid (15-HETE)) and/or 15-LOX inhibitors (LUT and NDGA). MTT viability curves (HODEs [0.1-1µM]) and dose response curves (HODEs [1-10µM]; LUT [7.5-15µM]; NDGA [20-40µM]) were performed over 24h, 48h, 72h. Next, conventional and/or quantitative RT-PCR's (15-LOX and related downstream mRNAs: PPARs, GPR132, MMPs) of the cells were performed. Wound healing assays were performed after 12h of treatment (HODEs [5µM]; LUT [15µM]; NDGA [40µM]) to examine migration. Then, gelatin zymography (10mg/mL) and western blots (40µg of protein/sample) were performed to identify the influence of these metabolites and inhibitors on GBM invasive capacity (defined here as the presence/activity of MMPs). ***Results and Discussion***: The 15-LOX pathway is present and active in the GBM cell lines. Both 13-HODE [5µM] and 9-HODE [5-10µM] increased cell count in U87MG (n=3). 13-HODE and 15-LOX-1 inhibitors, LUT [15µM] and NDGA [40µM], decreased migration in cell line T98G, and NDGA reduced matrix metalloprotease (MMP2) activity and increased the latent form of MMP2. 13-HODE/9-HODE treatments increase MMP2 mRNA in T98-G and U87-MG, respectively. All data were plotted and analyzed using GraphPad Prism 5.0. Analysis between two groups was performed with Student's T test, and a two-way ANOVA with Bonferroni post-test was used to compare multiple groups. The differences were considered statistically significant at $p<0.05$. ***Conclusions and Perspectives****:* 13-HODE and 9-HODE influence GBM cell growth, 13-HODE and 15-LOX inhibition decreased migration, and 13-HODE/9-HODE increased invasive potential, while 15-LOX inhibition decreased invasive potential. 15-LOX and its LA-derived metabolites exercise a certain pro-tumorigenic influence on GBM cells *in vitro*. Further studies will clarify if these relationships positively correlate with malignancy.

Keywords: Glioblastoma. Lipoxygenases. Lipid Mediators. Cancer. MMPs.

RESUMO

Ferreira MT. **Metabólitos da 15-lipoxigenase Influenciam Crescimento, Migração e Potencial Invasivo de Células de Glioblastoma.** [Tese (Doutorado em Biologia de Sistemas: Biologia Celular e Tecidual)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2019.

Introdução e Objetivos: Compreender o relacionamento entre cânceres extremamente agressivos, como o glioblastoma (GBM), e o metabolismo dos ácidos graxos poderia contribuir para a melhora de terapias anti-tumorais. A 15-lipoxigenase-1 (15-LOX-1), uma enzima que metaboliza o ácido linoleico (LA) e o ácido araquidônico (AA), induz efeitos pró ou anti-tumorigênicos em diferentes tipos de câncer. Seu papel no GBM ainda não foi claramente descrito. O objetivo deste estudo foi descrever a influência da 15-LOX e seus metabólitos no crescimento, migração e potencial invasivo de células de GBM. ***Delineamento Experimental***: Duas linhagens celulares de GBM foram cultivadas e tratadas *in vitro* com metabólitos da 15-LOX (Ácido 13(S)-Hidroxioctadecadienoico (13-HODE), 9-HODE e Ácido 15(S)-Hidroxieicosatetraenoico (15-HETE)) e/ou inibidores de 15-LOX (LUT e NDGA). Curvas de viabilidade com MTT (HODEs [0,1-1µM]) e curvas de dose-resposta (HODEs [1-10µM]; LUT [7,5-15µM]; NDGA [20-40µM]) foram realizadas por 24h, 48h, 72h. Em seguida, foram realizados RT-PCR convencional e/ou quantitativo das células (RNAm's de 15-LOX e seus correspondentes *downstream*: PPARs, GPR132, MMPs). Os ensaios de fechamento de ferida foram realizados após 12 horas de tratamento (HODEs [5µM]; LUT [15µM]; NDGA [40µM]) para avaliar a migração. Em seguida, foram realizadas zimografias em gelatina (10mg/mL) e western blot (40µg de proteína/amostra) para identificar a influência desses metabólitos e inibidores no potencial invasivo do GBM (definida aqui como a presença/atividade das MMPs). ***Resultados e Discussão***: A via de 15-LOX está presente e ativa nas linhas celulares de GBM. 13-HODE [5µM] e 9-HODE [5-10µM] aumentaram a contagem de células em U87MG (n = 3). Inibidores de 13-HODE e 15-LOX-1, LUT [15µM] e NDGA [40µM], diminuíram a migração na linhagem celular T98G, e NDGA reduziu a atividade de MMP2 e aumentou a forma latente de MMP2. Os tratamentos com 13-HODE / 9-HODE aumentam o RNAm de MMP2 em T98G e U87-MG, respectivamente. Todos os dados foram plotados e analisados usando o GraphPad Prism 5.0. A análise entre dois grupos foi realizada com o teste T de Student, e a ANOVA de duas vias com pós-teste de Bonferroni foi usado para comparar mais de dois grupos de dados. As diferenças foram consideradas estatisticamente significativas quando $p < 0,05$. ***Conclusões e Perspectivas:*** 13-HODE e 9-HODE influenciam o crescimento de células de GBM, 13-HODE e 15-LOX inibiram a migração, e 13-HODE/9-HODE aumentaram o potencial invasivo, enquanto a inibição de 15-LOX diminuiu o potencial invasivo. 15-LOX e seus metabólitos derivados de LA exercem uma influência pró-tumorigênica sobre as células de GBM *in vitro*. Novos estudos esclarecerão se essas relações se correlacionam positivamente com a malignidade.

Palavras-chave: Glioblastoma. Lipoxigenases. Mediadores lipídicos. Câncer. MMPs.

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LIST OF ACRONYMS

AA – Arachidonic Acid

cAMP – cyclic Adenosine Monophosphate

COX – Cyclooxygenase

CYP – Cytochrome P450

DMSO – Dimethyl sulfoxide

ECM – Extracellular Matrix

GBM – Glioblastoma

GPR 132 – G Protein Coupled Receptor 132

HETE – Hydroxyeicosatetraenoic Acid

HODE – Hydroxyoctadecadeinoic Acid

HPA – Human Protein Atlas

IDH1 – Isocitrate dehydrogenase

LA – Linoleic Acid

LOX – Lipoxygenase

LUT – Luteolin

MMP – Matrix Metalloprotease

MTT - Tetrazolium dye (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

NDGA - Nordihydroguaiaretic acid

PC – Phosphatidylcholine

PGE_2 – Prostaglandin E2

PL – Phospholipase

PPAR – Peroxisome Proliferator-Activated Receptor

ProMMP – Latent Form Matrix Metalloprotease

PUFA – Polyunsaturated Fatty Acid

RT-PCR – Reverse Transcription Polymerase Chain Reaction

TCGA – The Cancer Genome Atlas

CONTENTS

1 INTRODUCTION

Cancer is a collection of related diseases in which cellular dysregulation occurs to a point where cell death is not occurring adequately, cell proliferation is upregulated and cells begin to invade nearby tissues (1)(2). More than 18 million people were diagnosed with cancer in 2018, and the World Health Organization/ International Agency for Research on Cancer (WHO/IARC) predicts that this number will reach an estimated 24.5 million by 2030. Of these 18 million people, nearly 296 thousand were diagnosed with cancer in the brain or nervous tissue in 2018 and this number will likely increase to 350 thousand cases by 2030 (3). Gliomas, which are the most common form of brain tumors, are denominated by their similarity to glial cells in the nervous tissue, however, whether the cell of origin that produces the heterogenous tumor mass are glial cells (i.e. astrocytes, oligodendrocytes, microglia and ependymal cells) or neural stem cells or oligodendrocyte progenitor cells continues to be a topic for discussion in the literature (4).

1.1 CLASSIFICATION

The glioma grading system established by the World Health Organization (WHO) uses four grades (I, II, III, IV). Traditionally, the classification of primary brain tumors depended exclusively on a consensus reached after examining a tumor's histological characteristics (5). This histological exam first determines whether the tumor is diffuse (Grades II-IV) or not. Other aspects examined, i.e. in the case of malignant gliomas, include regions of necrosis and endothelial proliferation. Unfortunately, this type of cytological classification is prone to variability due to the subjective nature of histological interpretation.

However, in 2014, a meeting held in the Netherlands hosted by the International Society for Neuropathology gathered many of the world's experts to discuss the incorporation of molecular aspects into the diagnostic process of brain tumor classification (6). This meeting officially opened the way for a more complete, effective and modernized process of tumor classification. Hybridizing the diagnostic process, examining both genotypic and phenotypic aspects, was incorporated into the *World Health Organization Classification of Tumours of the Central Nervous System: Revised 4th Edition* (6).

The clinically less frequent Grade I gliomas, generally classified as pilocytic tumors, were not altered. However, the new classification of other astrocyte-derived tumors subdivided each

grade, diffuse gliomas (Grade II), anaplastic gliomas (Grade III), and Glioblastomas (Grade IV), into three additional subclassifications: IDH-mutant, IDH-wild type and NOS. Isocitrate dehydrogenase (IDH) exists as different isoforms within the mitochondria, cytosol and peroxisomes. IDH1, one of the main producers of cytosolic NADPH, is found in the cytosol and peroxisomes. IDH1 mutations convert α-ketoglutarate to D-2-hydroxygluterate (D-2HG). D-2HG can then increase DNA and histone methylation, thus, producing a potentially tumorigenic situation (7). IDH1 is a known biomarker of early astrocytoma development (8).

After histological examination, the status of IDH is determined through immunohistochemistry, and if the results are inconclusive the *not otherwise specified* (NOS) class is designated (6)(9). The other tumor types that were reclassified, but are not covered in the present study, can be reviewed in the WHO's 2016 publication (6). Using histology together with genomics opens the way for incorporating molecular cancer biology into clinical environments. Moreover, including proteomics and lipidomics could improve the classification process and even the therapeutic approach, however, accessible, cost-effective technologies for measuring these parameters are currently not available. Furthermore, identifying specific biomarkers and understanding their roles in and response to pharmacotherapies are still being explored.

About 28% of all central nervous system (CNS) tumors and 80% of all malignant CNS tumors are gliomas, and 45.2% of all malignant gliomas are classified as glioblastomas (GBM) (10). According to Matias *et al*. (2018) the typical characteristics that define GBM histological diagnostics are "nuclear atypia, cellular pleomorphism, mitotic activity, diffuse growth pattern, microvascular proliferation, and/or necrosis" (11). As for the IDH1 status, primary glioblastomas (90% of cases), IDH1 is not mutated, and in secondary glioblastoma (10% of cases) IDH1 mutations are identified (6).

As an attempt to treat GBM, conventional therapies applied are surgical resections, radiation therapy and chemotherapy. Unfortunately, the efficacy of these combined treatments is short-lived and the overall survival for most of these patients is between 12-15 months (11). There are a number of theories that try to explain the temporary effectiveness of current therapies, ranging from cancer stem cells (12) to tumor invasiveness (13), or from multiple drug resistance (14) to taking advantage of immune cell responses (15). Due to the heterogenous nature of glioblastoma cell composition, the involvement of all these factors, and others, have been demonstrated (16)(17).

The 2011 publication of Hanahan and Weinberg's "Hallmarks of Cancer: The Next Generation" included the tumor-promoting inflammation as an enabling characteristic of cancer (18). Later, Fouad and Aanei (2017) revisited and even reorganized these classic hallmarks proposing a new definition of a cancer hallmark as "acquired evolutionary advantageous characteristics that complementarily promote transformation of phenotypically normal cells into malignant ones, and promote progression of malignant cells while sacrificing/ exploiting host tissue" (19). One of their proposed hallmarks is called "metabolic rewiring". In a review by Pavlova and Thompson, metabolic rewiring can be further separated into 6 hallmarks, one of which is the use of opportunistic modes of nutrient acquisition (20). This opportunistic nutrient acquisition includes scavenging free fatty acids from the microenvironment (21)(22). Both tumor-promoting inflammation and metabolic rewiring involve fatty acid-derived lipid mediators.

1.2 FATTY ACIDS AND LIPID MEDIATORS

Fatty acids are one of four basic building blocks in the cell; the others are sugars, amino acids and nucleotides. Fatty acids constitute fats, lipids and membranes, and they also serve as a source of energy. For periods of long-term fasting, fatty acids are stored as triacylglycerol lipid droplets in adipocytes. When energy is needed, and glucose metabolism is not adequate, fatty acid oxidation often occurs, although not exclusively, in the mitochondria; as is the case with heart, liver (23). Panov *et al*. (2014) emphasize in their review that "20% of the brain's energy metabolism is supplied by the β-oxidation of fatty acids in astrocytic mitochondria" (24). Fatty acids used in nervous tissue metabolism for ATP synthesis can also be used, with help of certain oxidases, to produce bioactive oxidized fatty acids called lipid mediators. The lipid mediators produced in both mitochondria and cytosol of many cell types, including astrocytes, are responsible for an array of signaling cascades involved in homeostasis, stress responses and disease pathology (25). As the literature clearly demonstrates, bioactive lipid mediators are directly involved in regulating the immune response to pathogens and lesions, as well as regulating inflammation (26)(22).

Fatty acid sources, most frequently localized in lipid membranes, vary in their number of carbons, the number of double bonds they possess, the location of their unsaturated carbons (double bonds) and hydroxylation sites (sites containing an -OH group). The composition of the cell membrane is very diverse, including glycerophospholipids, sphingolipids and cholesterols (27).

According to Harayama and Riezman's review (2018), there are two types of lipid diversity: 1) chemical and 2) compositional. Chemical diversity includes the individual structure of a lipid, e.g. stereoisomers; whereas compositional diversity considers the ratio of different lipids present in the membrane. They, together with Hayashi *et al.* (2014), describe how the position of a lipid group on the glycerol backbone within the cytoplasmic membrane, can determine where a fatty acid becomes saturated, desaturated and elongated, and the subsequent biochemical signaling pathways that follow it. For example, considering Phosphatidylcholine (Figure I1), along the glycerol backbone are 3 binding sites (Sn1, Sn2, Sn3); the third of which possess a phosphate group forming a glycerol-3-phosphate moiety. The Sn1 and Sn2 sites link with fatty acids. In the Sn1 position, the linked fatty acids tend be saturated and monounsaturated fatty acids; while in the Sn2 position, unsaturated fatty acids may be found (27)(28). The enzymes involved with fatty acid remodeling in the membrane, e.g. transacylases, have been reported as being involved with polyunsaturated fatty acid accumulation (28). (Figure I1 briefly reviews biochemical principles and fatty acid synthesis cited in this thesis).

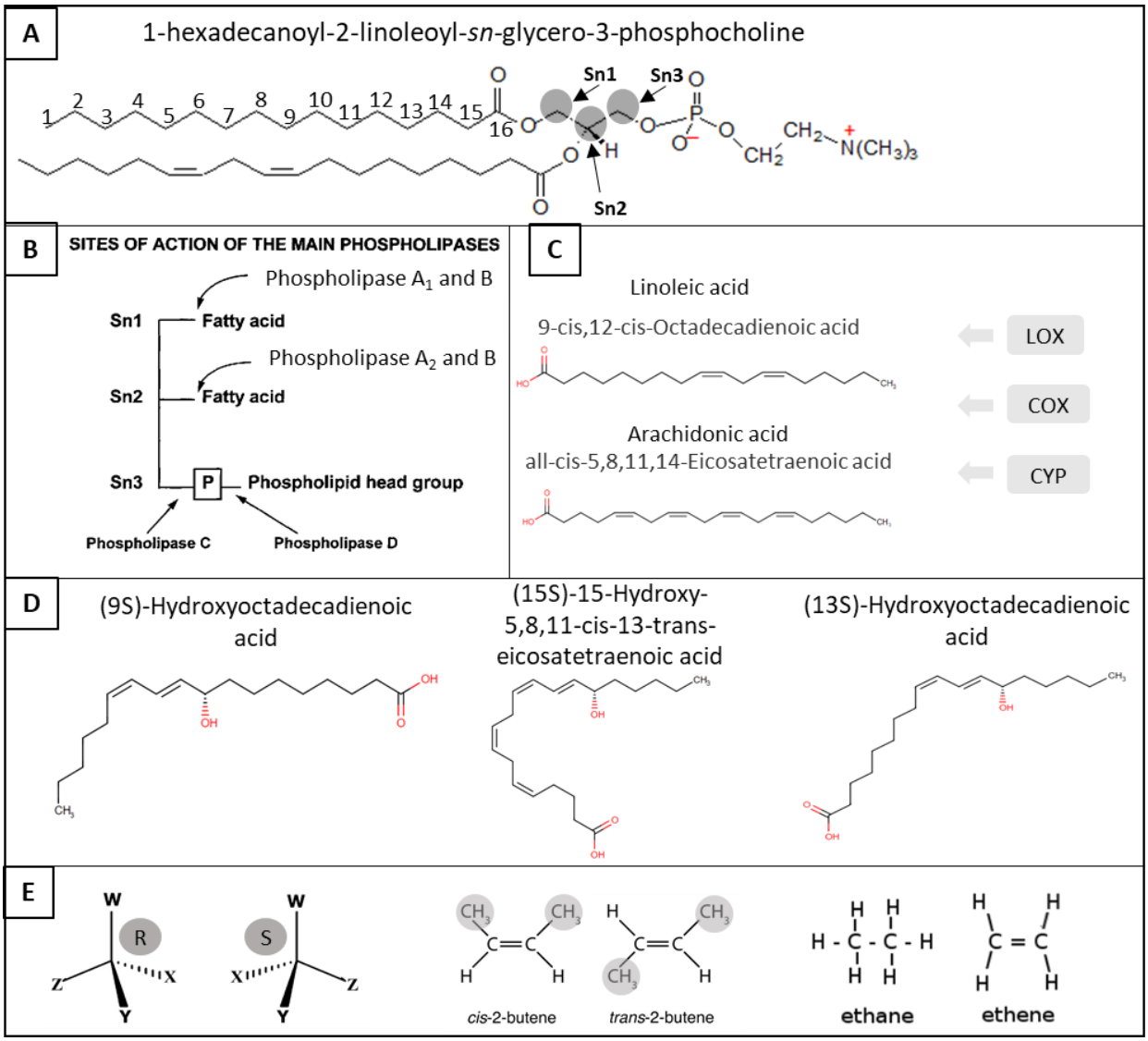

Figure I1. Review of Biochemistry and Fatty Acid Synthesis. **[A]** An example of a typical phospholipid: 1-hexadecanoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PC). At the Sn1 site is a monounsaturated fatty acid, palmitic acid (16:0) (PA), while at the Sn2 site is a PUFA, linoleic acid (18:2n6) (LA). The classification of PUFAs are based upon the first unsaturated carbon of the membrane-bound fatty acyl group [ex. omega-6 fatty acid; 6th Carbon from the methyl group]. Moreover, the numbering applied (ex. Linoleic Acid = 18:2) refers to the number of carbons (18) and the number of double bonds (2) present. **[B]** Phospholipase A_2 hydrolyzes the LA to release it from the glycerol's Sn2 position. **[C]** LA and arachidonic acid (20:4n6 AA; another common example of a Sn2 bound PUFA) become available for oxygenation by lipoxygenases (LOX), cyclooxygenase (COXs) and/or cytochrome P450 epoxygenase (CYPs). **[D]** The products yielded can be referred to as lipid mediators when examining biological activities. In this case, the denomination of these lipids depends on the carbon at which the hydroxyl group is added (ex. 9-HODE; 9th carbon from the carboxyl end). **[E]** [*Left-to-Right*] These are examples of chiral structures (S) and (R); *cis*- and *trans*- conformations, which are responsible for the bends in PUFA's structure; and unsaturated (ex. Ethene) and saturated (ex. Ethane) structures. Fatty acid chemical structures were acquired from The Human Metabolome Database (http://www.hmdb.ca/).

1.2.1 Polyunsaturated Fatty Acid Synthesis (Linoleic Acid/Arachidonic Acid)

In general, polyunsaturated fatty acids (PUFAs) cannot be synthesized, *de novo*, by mammals and, therefore, must be consumed in one's diet. The intake of PUFAs, followed by their assimilation into cellular membranes subsequently alter the bioavailability of certain fatty acid substrates and their lipid mediator products. PUFAs are fatty acids that have more than one double bond in their carbon chain. The fatty acid nomenclature depends on the carbon of the first double bond closest to the methyl terminus or the omega end, e.g. omega-3 (Carbon 3 = docosahexaenoic acid), omega-6 (Carbon 6 = arachidonic acid). PUFAs in the brain are represented by omega-3 (n3) and omega-6 (n6) fatty acids (29). (Figure I1)

Once incorporated in the cell membrane, PUFAs are made available for metabolism with the aid of a superfamily of enzymes called phospholipases (PL). There are three main classes of phospholipases according to their hydrolytic cleavage sites: PLA, PLC and PLD. PLA cleaves fatty acyl ester bonds at either the sn1 (PLA1) or sn2 (PLA2) position (30). There are four main types of PLA2's: secretory (sPLA2), calcium-independent (iPLA2), lipoprotein (Lp-PLA2) and cytosolic (cPLA2). Cytosolic PLA2 is of interest due to its role in fatty acid metabolism and lipid mediator production, as well as its association with chemoresistance in GBM (31). Membrane-bound fatty acids, like linoleic acid and arachidonic acid, can be hydrolyzed and released by cPLA2 and, with the help of other enzymes, subsequently processed into bioactive lipid mediators.

Linoleic acid (LA) is an 18 carbon, long chain fatty acid (LCFA = that which contains 14-18 carbons) with two *cis*(Z) double bonds beginning from the 6^{th} carbon (18:2n6) (Figure I1). This PUFA is the precursor of all the omega-6 (n6) fatty acids and can be synthesized *de novo* by Δ12-desaturase; however, mammals lack this enzyme. Therefore, LA must originate from the diet and is thus referred to as an essential fatty acid. LA can be found in corn and soy oils and is one of the most abundant fatty acids in the Western diet. Regarding lipid mediator production, the metabolic destiny of LA may either be hydroxylation (which will be described ahead) or further desaturation and elongation to form other n6 PUFAs. With the aid of Δ6-desaturase, LA can be converted into gamma-linolenic acid (GLA 18:3n6). Then the enzyme elongase 5 adds 2 more carbons forming dihomo-gamma-linolenic acid (DGLA 20:3n6). Next, DGLA is converted into arachidonic acid (AA 20:4n6) by means of Δ5-desaturase. AA can be synthesized *de novo* or introduced by dietary means (ex. eggs and meat). Each of these PUFAs can be metabolized to form an array of bioactive

lipid mediators. However, LA and AA are the most common PUFAs and are the focus of this study (32). Figure I2 summarizes PUFA metabolism.

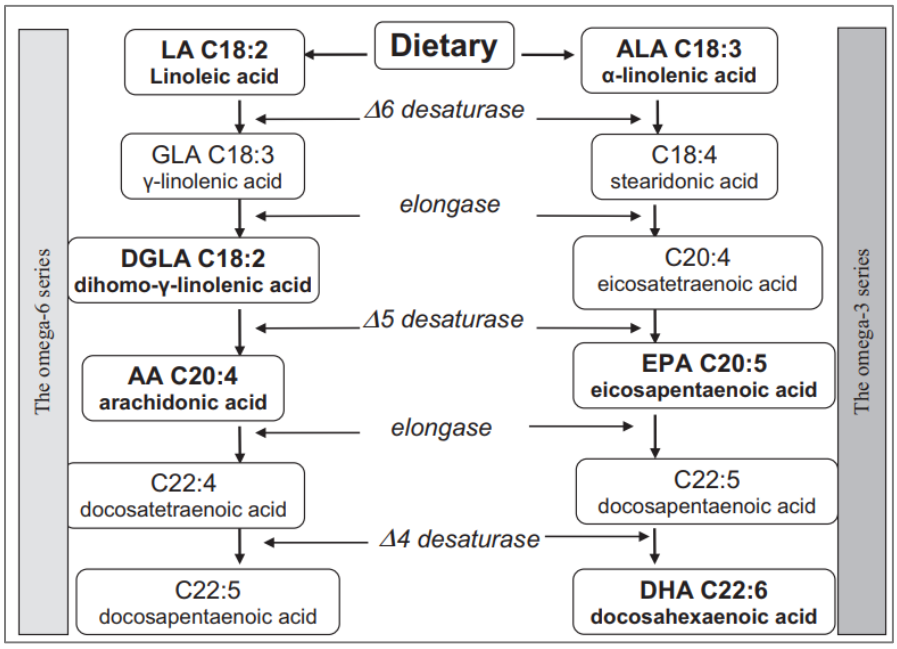

Figure I2. Summary of PUFA metabolism (32)

1.3 ENZYME FAMILIES

1.3.1 Cyclooxygenases

As previously mentioned, the transformation of LA and AA into lipid mediators occurs both enzymatically and nonenzymatically (33)(34). Due to AA's four *cis*-double bonds, it possesses a number of sites at which peroxidation may occur. There are three families of enzymes responsible for AA peroxidation. The first group is the cyclooxygenase (COX) family. There are two main isoforms of COX in humans designated as COX-1 and COX-2. Although a splice variant of COX-1, COX-3, has been discovered in human brain tissue, it is poorly understood (35)(36). These two main isoforms (COX-1 and COX-2) are responsible for converting AA into bioactive eicosanoids. These 20-carbon bioactive lipid mediators operate in an autocrine and paracrine manner with their respective receptors on the plasma membrane (G-protein couple receptors [GPRs]) as well as the nuclear envelope (peroxisome proliferator-activated receptors [PPARs]). However, these eicosanoid receptors are able to bind with more than one type of eicosanoid, thus receiving a reputation of "promiscuity" (37). The main products of COX activity are prostaglandins and thromboxane A_2 and are often referred to as "prostanoids" due to the fact that prostanoic acid is their base structure (34)(38).

First, COX introduces two O_2 molecules to arachidonic acid by hydrogen abstraction, which, after oxygenation, becomes a free radical to bind with an oxygen molecule. Next, it forms an unstable cyclic endoperoxide-hydroperoxide compound referred to as prostaglandin G2 (PGG_2). PGG_2 quickly rearranges as a less unstable prostaglandin, PGH_2, which then undergoes peroxidation by varying enzymes to produce an array of prostaglandins (PGs) (39).

COX-1 is generally considered a house-keeping enzyme that maintains the basal synthesis of prostanoids for homeostatic reasons. However, COX-2 is an enzyme that is highly expressed at sites of inflammation. Sustained COX-2 expression in tumorigenesis has been associated with tumor progression and poor prognosis. That is why COX enzymes have been studied extensively as targets for treatments related to inflammation and cancer. Non-steroid anti-inflammatory drugs (NSAIDs), like ibuprofen, inhibit COX activity effectively. However, its long-term use has proven to be harmful to the liver and GI tract and has been abandoned as a long-term chemotherapeutic approach (40). COX-targeting therapies continue to be of extreme interest since the pro-

tumorigenic lipid mediator, PGE_2, identified in many cancer types, including gliomas, has proven to be upregulated and very influential in cell growth, migration and invasion (41)(42).

1.3.2 Cytochrome P450 Epoxygenase

The second family of enzymes that metabolizes AA is the monooxygenated epoxygenase, cytochrome P450 (CYP). According to the CYP Nomenclature Committee, "CYP refers to heme-containing proteins characterized by a maximum absorption wavelength of 450 nm in the reduced state in the presence of carbon monoxide" (43). This carbon monoxide binding pigment was initially described in 1958 by Klingenberg (44). Over the following decades, the cytochrome P450 super family has been gradually elucidated, establishing over 13,000 genes represented by over 400 gene families among all the biological kingdoms (43).

Mammalian CYP450 is bound to either mitochondrial or endoplasmic reticulum membranes (43). It is suggested that the reaction mechanism of this oxygenation occurs in two steps. In the first step, P450, bound to the substrate, receives an electron reducing the heme iron. The second electron then activates the heme-bound oxygen molecule on CYP450 by splitting the O-O bond. From there, one oxygen atom becomes part of a water molecule and the other oxygen is incorporated into the substrate (45). Its general reaction with AA produces epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs), which often regulate blood pressure control (46)(47). Some of the other main eicosanoids produced by the CYP family are thromboxane A2 (by TXA_2 synthase) and prostacyclin I_2 (by PGI_2 synthase), the latter of which is an important anti-inflammatory mediator (48)(49).

1.3.3 Lipoxygenases

The third family of enzymes involved in LA and AA metabolism into lipid mediators is the lipoxygenase (LOX) family. Lipoxygenases are a family of nonheme iron-containing dioxygenases that insert, in a stereospecific way, molecular oxygen into polyunsaturated fatty acids that contain at least two *cis*-double bonds (15)(50). There are 3 main functions of lipoxygenases: 1) signaling pathways; 2) peroxidation reactions and 3) mobilization of lipids. The first function involves forming single free fatty acid products from esterified substrates, such as leukotrienes and HETEs. The second function involves the quick formation of mixed products of acids or esters. The enzymes involved in this activity have a quick turnover and the subsequent products may cause structural and pathological changes. The third function involves oxygenating unsaturated fatty

acids esterified in triglycerides. This releases the fatty acids for β-oxidation and energy production (50).

Lipoxygenase biochemistry typically depends on free-radical oxidation. Figure I3 is an example of the typical mechanism involved in lipoxygenase activity on a *cis,cis*-1,4-pentadiene (51). Lipoxygenases are denominated according to the carbon on the AA/LA chain at which oxygenation and hydroperoxyl radical formation occurs. There are 3 main human lipoxygenases: 5-LOX, 12-LOX (also called platelet-type 12-lipoxygenase) and 15-lipoxygenase (15-LOX, subtypes -1 and -2) that are accompanied by a few, less-studied isozymes. Figure I4 represents lipoxygenation of LA.

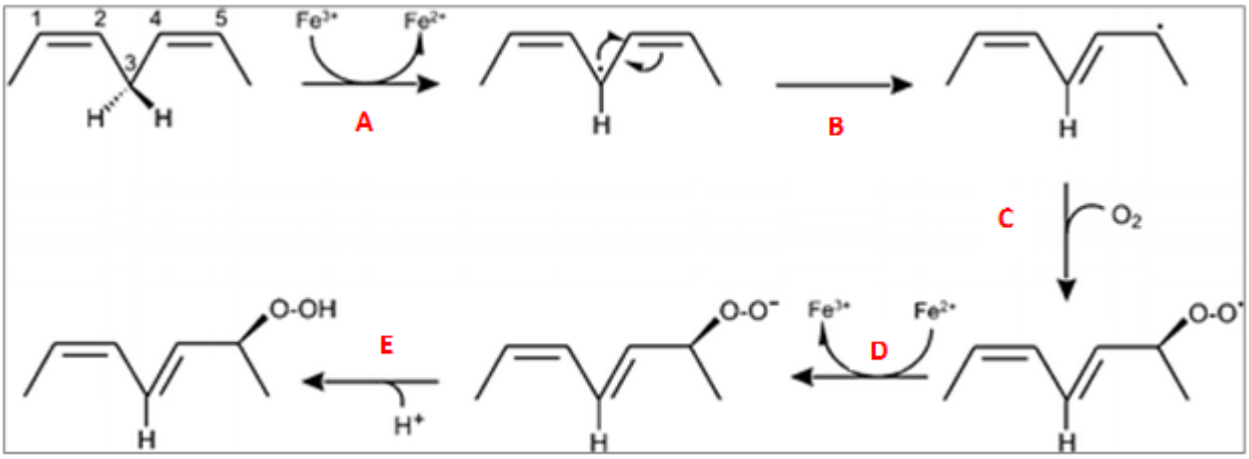

Figure I3. Mechanism for lipoxygenation of a *cis,cis*-1,4-pentadiene. **A**) First hydrogen abstraction occurs due to the activity of iron Fe^{3+} (ferric form), thus, forming a radical and Fe^{2+} (ferrous form; inactive). **B**) This radical then draws the electron of the neighboring double bond (carbons 4 – 5) forming a radical on the sixth carbon. **C**) Binding quickly with an oxygen molecule, a peroxy radical is formed. **D**) Next, the active site iron (Fe^{2+}) donates an electron forming a peroxyanion, which **E**) is then protonated to form a hydroperoxide. (Figure adapted from Haeggstrom and Funk, 2011) (51).

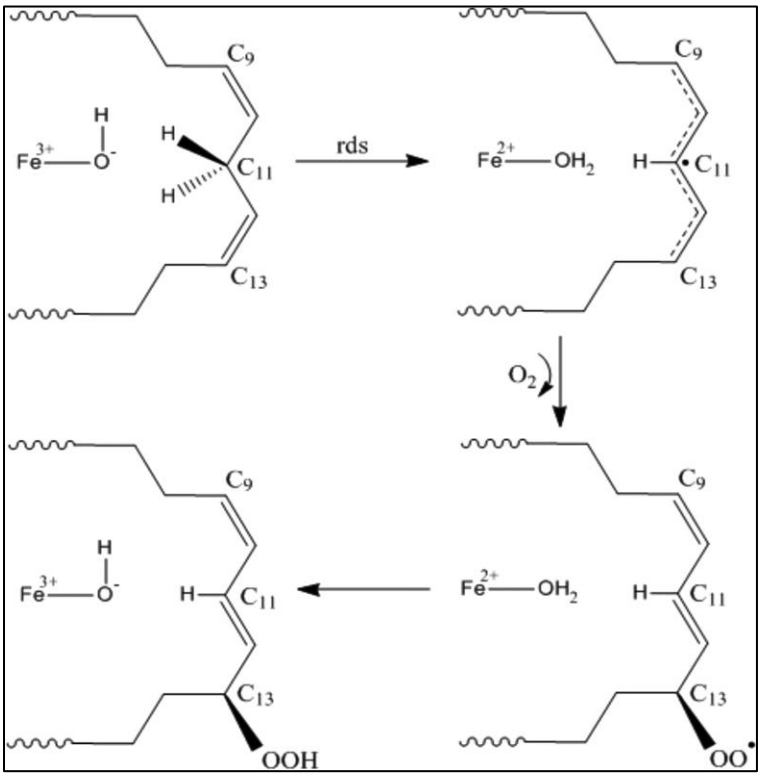

Figure I4. Schematic representation of Linoleic Acid metabolism by non-heme iron-containing lipoxygenases as designed by Soler *et al*. (160).

1.3.4 5-Lipoxygenase

The 5-LOX enzyme is the most well characterized of the LOX family. It is responsible for converting AA into 5-hydroperoxyeicosatetraenoic acid (5-HpETE), which is an unstable and inactive precursor of 5-hydroxyeicosatetraenoic acid (5-HETE) (52). The 5-LOX product, 5-HpETE, can be further processed to form Leukotrienes A4 (LTA_4) and then B4 (LTB_4), as well as the cysteinyl leukotrienes C_4 (LTC_4), D_4 (LTD_4) and E_4 (LTE_4), which are structurally similar but functionally different (53). 5-LOX's activity depends on a co-factor, 5-lipoxygenase activating protein (FLAP). FLAP is located on the nuclear membrane and is responsible for facilitating 5-LOX access to AA (54). When FLAP is inhibited, a noticeable decrease of 5-LOX products can be observed (55).

1.3.5 12-Lipoxygenase

There are three isoforms of 12-lipoxygenase in mammals and they are distinguished by their tissues of origin: platelet, leukocyte and epidermal. 12-LOX is responsible for converting AA

into 12-hydroperoxyeicosatetraenoic acid (12-HpETE) first, which is unstable and inactive, and subsequently into 12-hydroxyeicosatetraenoic acid (12-HETE) (50). 12-HETE's biological activities vary depending on the lipoxygenase and tissue, and are associated with vasoconstriction/ vasodilation, platelet aggregation, inflammatory mediator production and adipogenesis during inflammation and tissue repair (56).

1.3.6 15-Lipoxygenase

Fatty acid metabolism through 15-LOX occurs by two functional isomeric enzymes designated as either 15-LOX-1 and 15-LOX-2. The first, 15-LOX-1, is expressed principally in reticulocytes, eosinophils, macrophages, epithelial tracheobronchial cells, and in the skin (52). 15-LOX-1 metabolizes linoleic acid (LA) to hydroxyoctadecadienoic acids (13-HODE and 9-HODE), and it metabolizes AA to 15-hydroxyeicosatetraenoic acid (15-HETE). 15-LOX-1 is capable of binding to both AA and LA, however it has a much greater affinity toward LA (57). AA and LA will be described in more detail further on. 15-LOX-1 also possesses an initiating role in production of the anti-inflammatory lipoxins derived from n-6 PUFAs (58). The down-regulation of 15-LOX-1 expression has already been linked to colorectal cancer progression (59). However, the overexpression of 15-LOX-1 has been associated with prostate cancer tumorigenesis (60).

The second type of 15-lipoxygenase, 15-LOX-2, is expressed in the prostate, lung, skin, and cornea tissues (61). 15-LOX-2 also metabolizes AA to 15-HETE, but does not effectively metabolize LA (62)(50). While both 15-LOX-1 and 15-LOX-2 were downregulated in breast cancer patients with metastases (63), it has been demonstrated that the reduction of 15-LOX-2 expression in primary prostate epithelial cells is inversely correlated with the tumor`s cell cycle. This indicates that through 15-LOX-2 up-regulation the suppression of prostate cancer development could result from restricting cell cycle progression (64). Despite the conflicting reports of pro-/anti-tumorigenic activities resulting from 15-LOX metabolism, the evidence seen in GBM is limited. The relationship between 15-LOX-1 and 15-LOX-2 expression and activity in GBM, if thoroughly investigated, could serve as a therapeutic target.

1.3.7 15-Lipoxygenase Products

There are many products and intermediate products of lipoxygenase metabolism but only a few, more stable products are highlighted in this study. Both 15-LOX-1 and 15-LOX-2 are capable of converting AA into 15-hydroperoxyeicosatetraenoic acid (15-HpETE), which is less stable and

biologically inactive, and then into 15-HETE. 15-HETE is an endogenous signaling molecule that can bind to PPARγ and PPARβ/δ that repress the expression of pro-inflammatory genes and possesses potent anti-inflammatory activities (65)(66). It is involved in upregulating platelet aggregation and thrombin generation (67) and inhibits cell cycle progression in prostate cancer (64). In non-small cell lung cancer, 15-HETE levels were reduced prior to the onset of tumors. This suggests that 15-HETE may have a role in lung cancer similar to its role in prostate cancer (68).

13-hydroxyoctadecadeinoic acid (13-HODE) is an 18-carbon lipoxygenase-derived monohydroxy fatty acid and the major metabolite in LA metabolism by 15-LOX-1. 13-HODE is a ligand for the peroxisome-proliferator-activated receptor (PPARγ) (68). In colon cancer, 13-HODE has been shown to bind to PPARγ and consequently inhibiting the formation of metastatic cancer (69). Also its presence decreases PPARδ, which results in the recovery of apoptosis in human colorectal cancer (70). However, in prostate cancer the overexpression of 15-LOX-1/13-HODE is associated with tumor progression (60).

The other major metabolite of 15-LOX-1 metabolism of LA is 9-hydroxyoctadecadeinoic acid (9-HODE). 9-HODE can be synthesized at low levels from LA by 15-LOX-1. 9-HODE, along with 13-HODE, can also be generated non-enzymatically by means of heat or UV exposure (71). 9-HODE acts through PPARγ also, but the consequences are different than 13-HODE. When PPARγ was examined in mesangial cells, Negishi *et al*. determined that 9-HODE stimulates cell proliferation and extracellular matrix synthesis (72). The other 9-HODE receptor is G2A (or GPR132). Through G2A, in keratinocytes, 9-HODE produced an increased mobilization of intracellular calcium, secretion of cytokines and inhibition of accumulation (73). In addition, 9-HODE can inhibit cell proliferation of keratinocytes (71).

All three of the lipid mediators covered can be produced in their R and S conformations. Chiral isomers have identical chemical compositions but have an asymmetrical difference, also known as isomers. (refer to Figure I1). This slight conformational difference of fatty acids can result in different functions (74). However, in the present study, the lipid mediators examined were only those in their (S) conformation, thus, chiral conformation will not be included in the nomenclature used (ex. 13-HODE = 13(*S*)-HODE).

The role of 15-LOX and its products are unknown in glioblastoma metabolism and activity. Table I1 summarizes the influence of 15-LOX in different cancer types seen in the literature.

Table I1. Summary of 15-LOX`s expression and influence on tumor progression in different models of cancer.

Cancer type	15-LOX expression	Influence on Tumor	Reference
Breast Cancer	↓	**Neg**	(63)
Colon Cancer	↓	**Neg**	(59)
Prostate Cancer	↑	**Pos**	(62)
Glioblastoma	**?**	**?**	**-**

15-LOX expression: upregulated (↑), downregulated (↓).
Influence: Negative (Neg) = anti-tumorigenic; Positive (Pos) = pro-tumorigenic

1.4 TUMOR INVASION

1.4.1 Matrix Metalloproteases

One hallmark of cancer partially responsible for the lack of successful therapies, is invasiveness (18). GBM cells are capable of migrating and invading neighboring tissues quickly, and there are a number of pathways responsible for transformed cells to relocate (75). Matrix-metalloproteases (MMPs) are zinc endopeptidases mainly responsible for the alteration of the extracellular matrix (ECM) and its biophysical properties (76). The MMP family is a subgroup of proteolytic enzymes responsible for ECM remodeling. As seen in Figure I.5, MMPs can be classified by their domain organization (basic MMPs, matrilysins, gelatinases, membrane-type or furin-activatable MMPs) (76).

MMP-2 and MMP-9, are responsible for degrading type IV collagen in the ECM. Type IV Collagen is a crucial component of the basement membrane and is often up-regulated in glioma tissue (77). These two MMPs are zinc-dependent endopeptidases (78). When MMP-2 and MMP-9 are secreted, they are latent enzymes; 72kDa (proMMP2) and 92kDa (proMMP9). Both MMP-2 and MMP-9 are composed of a propeptide domain, a catalytic domain (containing three fibronectin motifs) and C-terminal hemopexin-like domain; refer to Figure I.5. Latency is believed to be maintained by the presence of Zinc on the catalytic domain bound to a sulfhydryl (SH) group present on a cysteine residue in the propeptide domain (78)(79). The disruption of this Zinc-cysteine pairing, resulting from proteolytic intervention or conformational changes, issues in the activation of these MMPs (80).

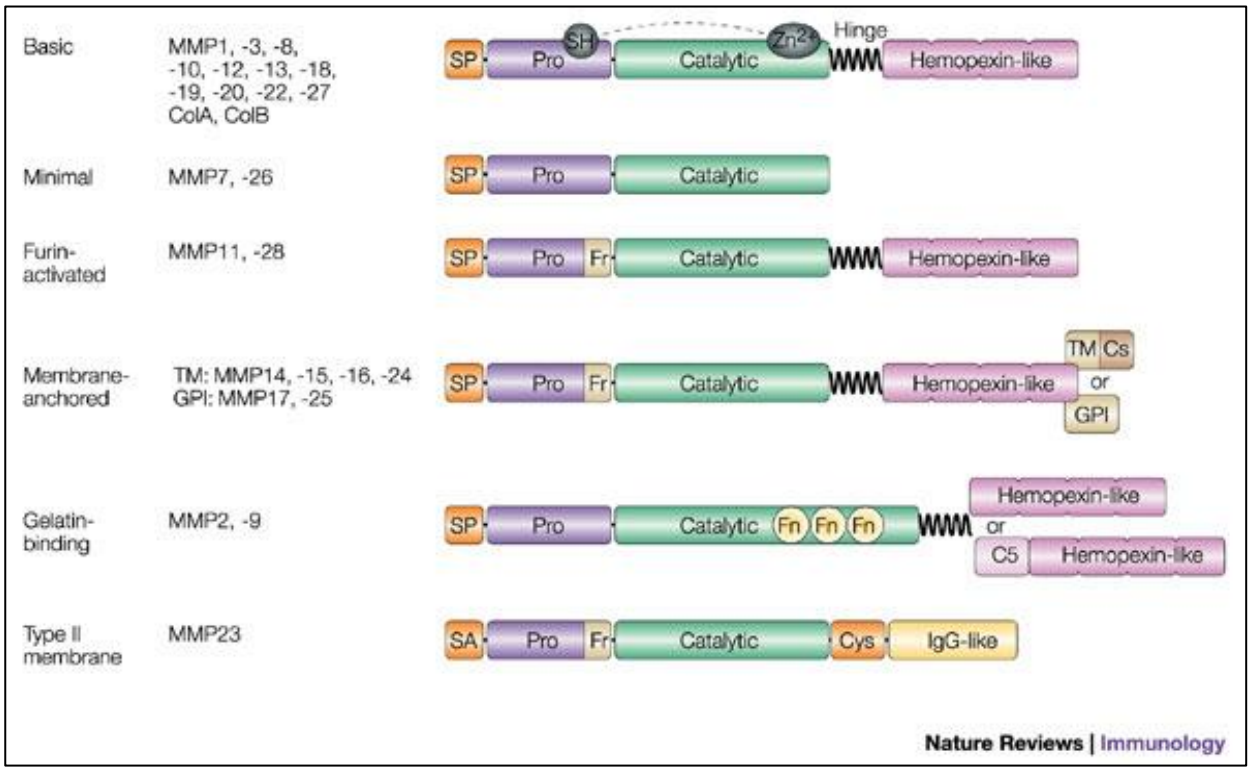

Figure I5. Summary of the classifications and structures of mammalian MMPs; created by Parks *et al.* (81).

The activation of MMP-2 and MMP-9 are slightly different. MMP-2's activation is mostly dependent upon MMP-14. MMP-14 (also known as MT1-MMP) is a membrane bound protein known for its involvement in upregulating the production of angiogenic factors (ex. vascular endothelial growth factor (VEGF)-A). It also interacts with cell adhesion molecules; for example, MMP-14 is responsible for shedding CD44, thus promoting cell migration. However, MMP-14 is most well-known for cleaving proMMP-2 into its active form, MMP-2. MMP-14 forms a homodimer and then pairs with tissue inhibitor of metalloproteases 2(TIMP2). After one MMP-14 component pairs with TIMP2, proMMP-2 binds to the TIMP-2-free MMP-14 and is cleaved in the middle of its pro-domain creating its activated form, MMP-2 (62kDa) (82).

MMP-9 activation depends on conformational adjustments to release the Zn dependent active site. The intracellular form of MMP-9 (85kDa) is partially glycosylated, when it matures it is glycosylated and secreted (92kDa). Once in the extracellular environment a number of factors, the most potent of which is MMP-3, can cleave proMMP-9 into its active form, MMP-9 (82kDa) (83)(78). MMP-9 can suffer post-conformational changes producing a 62kDa inactive form as well as forming heterodimers (with TIMPs) and homodimers (~200kDa) (83).

As is seen in various types of aggressive cancer, these three MMP's are often upregulated and associated with tumor progression and invasion (84). Although there are studies demonstrating the influence of prostaglandins on MMPs (85)(86), there are no studies investigating 13-HODE and 9-HODE's influence on MMP-2 or MMP-9 expression/activity in GBM. Currently, there are only studies demonstrating a stimulatory effect of 15-HETE on MMP-2 upregulation in rheumatoid arthritis (87) and potentially in pulmonary arterial hypertension (88), and 13-HODE/15-HETE downregulating MMP-1/MMP-13 in osteoarthritis (89).

2 JUSTIFICATION

The influence of 15-LOX and its metabolites on GBM metabolism and cellular activities remains unclear. Due to the presence of HODEs and HETEs in GBM cells, it is hypothesized that they play a role in cellular activities. Therefore, this study aimed to elucidate the influence that 15-LOX and its products 13-HODE, 9-HODE and 15-HETE, may have in GBM cell growth, migration or invasion.

3 MATERIALS AND METHODS

3.1 Cell Culture

Cell lines T98-G, U251-MG, A172, U138-MG and U87-MG were analyzed. Cell lines were kindly donated by Dr. Menck (U87-MG, U138-MG) and Dr. Costanzi-Strauss (U251-MG), Biomedical Sciences Institute and Dr. Maria-Engler (A172, T98-G), Pharmacy School, University of São Paulo. For comparative analyses at certain points of this study, a breast cancer cell line (MCF-7) was used, and a fibrosarcoma cell line (HT-1080) was kindly donated by Dr. Vanessa Freitas, Biomedical Sciences Institute, University of Sao Paulo. Cultivation was performed following a previously published protocol in our lab (90). Ideal growth concentrations were standardized for all five cell lines over 72 h and were determined to be $3x10^4$ cells. *Data not shown.* All cell lines were cultured in DMEM (Dulbecco's Modified Eagle Medium - Gibco Inc.) supplemented with 10% (v/v) FBS (fetal bovine serum - Gibco Inc.), 50 units/mL penicillin, 50 µg/mL streptomycin (Gibco Inc.). All the cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 in both $25cm^2$ and $75cm^2$ flasks, until the desired confluency.

3.2 Dose-Response Curve / Trypan Blue Staining

To determine their impact on GBM growth, exogenous 13-HODE, 9-HODE and 15-HETE, were added to the cell lines in variable concentrations. Twelve hours after plating cells in 24-well plates, the medium was changed to medium containing different concentrations of 9-HODE (1µM, 5µM, 10µM), 13-HODE (1µM, 5µM, 10µM), or 15-HETE (0.1µM, 1µM, 2.5µM). As for the lipoxygenase inhibitors treatments, Luteolin (7.5µM or 15µM) and Nordihydroguaiaretic acid (20µM or 40µM) were used. The negative controls in this experiment were cells treated with either Ethanol (HODEs) or DMSO (inhibitors). The treatments were applied in 24-hour intervals. The samples were collected at 24 h, 48 h and 72 h, and stained with 0.4% Trypan Blue. Then both the viable and unviable cells were counted in a Neubauer chamber. All treatments were tested at least three times in triplicates.

3.3 MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay

Cells were seeded at $5x10^2$ for U87-MG, in 96-well plates. After 24 hours cells were treated with 0.1 µM, 1 µM and 10 µM of the following lipid mediators: 13-HODE; 9-HODE and 100%

Ethanol (control). At 24, 48 and 72 hours of treatment, each well was incubated for 4 hours with 0.25mg/mL of tetrazolium at 37°C in a humidified atmosphere with 5% CO_2. At the end of incubation, cells were washed with warm PBS and lysed with 100 µl of 0.04 M HCl in isopropanol to solubilize the formazan. The absorbance was read at 590 nm in an Epoch Multi-Volume Spectrophotometer System (*BIOTEK, Winooski, VT, USA*).

3.4 Total RNA Extraction

For total RNA extraction, the five cell lines were processed following our previously established protocol (90) were each lysed with 1 mL of Trizol (Invitrogen) and left at room temperature for five minutes. After this period 0.2 mL of chloroform were added, and the samples were mixed for 15 seconds and left at room temperature for three minutes. Following this, the samples were centrifuged at 10,697 RCF (Relative Centrifugal Force) for 15 minutes at 4 °C. Next, the superior aqueous layer of the sample was removed and added to 0.5 mL of isopropanol to be centrifuged at 10,697 RCF for 10 minutes at 4 °C. The remaining precipitate was washed three times in 1 mL of 95% ethanol and centrifuged at 5344 RCF for five minutes at 4 °C. Finally, the precipitate was resuspended in 20 µL – 100 µL of diethylpyrocarbonate (DEPC) water that was previously deactivated by autoclaving. The concentration of RNA was determined using an Epoch microplate reader measuring the absorbency at a ratio wavelength of A260 nm/A280 nm as the standard value; all the RNAs used in this study had presented between 1.8 and 2.0 purity. The purified RNA was stored and maintained in the -80 °C freezer.

3.5 cDNA Synthesis

Complementary DNA (20 µL) was obtained by using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (*Invitrogen*) in a reverse transcription polymerase chain reaction (RT-PCR) with 2 µg of the RNA of interest. This reaction included 2 µL of a free nucleic acid mix (dNTP mix), 2 µL Random Primer, 1 µL of RNA inhibitor (RNAse OUT), 2 µL Dithiothreitol (DTT), 4 µL RT buffer, and 1 µL MMLV (all reagents were purchased from *Thermo Fisher Scientific*). An *Eppendorf MasterCycler®* thermocycler was used to amplify the cDNA. Amplification was then confirmed by electrophoresis with a 1% agarose gel containing ethidium bromide revealed in a *Syngene G-Box* (UV light) and captured by the *GeneSys* program (*Syngene*).

3.6 Conventional RT-PCR

Primers were designed using the open-sourced *Perlprimer program* (91) and the NCBI Primer-BLAST tools. The primers were then purchased from *Thermo Fisher Scientific*. The protocol followed our previously published article (90). Annealing temperatures and band sizes were determined for each primer pair (**Table R2**). Next, the analysis of mRNA expression by conventional RT-PCR was performed. The following genes were amplified: the enzymes 15-LOX-1, 15-LOX-2, MMP-2, MMP-14, MMP-9 and the receptors PPARy, PPARδ, GPR132. Amplification was confirmed by gel electrophoresis with 1% agarose containing ethidium bromide, and the product was viewed through a U.V. light capture system (*Syngene*). The internal control gene chosen was ribosomal subunit 18 (18s) due to its consistent endogenous expression in all glioma cell lines.

3.7 Quantitative Real-Time PCR

The traditional polymerase chain reaction is important for confirming the cell lines' ability to express the genes of interest and the optimal annealing temperatures at which the primer binds to the sample. To analyze the gene expression of the enzymes and receptors of interest, we used Real Time quantitative PCR (RT-qPCR). We obtained a standard curve showing the optimal quantities of cDNA and the concentrations of primers to be used in the experiments (Data not shown). Reactions were prepared containing Syber Green Mix (*Life Technologies*). The amplifications were performed through the 7300 REAL TIME PCR system (*Applied Biosystems*). Dissociation curves verifying amplification specificity were also performed. To evaluate the differential expression of the treated groups, the relative quantification method with 18s was used as a normalizer (endogenous control). The primers for MMP-2, MMP-9, and MMP-14 were first standardized, and then tested in five glioma cell lines (U87-MG, U251-MG, U138-MG, A172, T98-G) and a breast cancer cell line (MCF-7) and a fibrosarcoma cell line (HT1080), first using conventional RT-PCR and then confirmed by RT-qPCR.

3.8 Protein Extraction and Quantification

3.8.1 Sample Preparation

U87-MG and T98-G cells were cultivated in T-25 plates until they reached 80-90% confluency. At this moment, cells were treated with Luteolin (7.5µM or 15µM) or NDGA (20µM or 40µM) with their respective controls in duplicates for 24h and 72h following the methodology previously mentioned. Cells were then trypsinized and counted in a Neubauer chamber. Next a pellet was formed by centrifugation at 4°C for 3min at 410 RCF and washed with cold PBS 3x before snap-freezing the samples in liquid nitrogen. The samples were stored in a -80°C until the time of extraction.

3.8.2 Protein Extraction

The frozen pellet was slowly thawed on ice then centrifuge for 10min at room temperature and excess PBS was removed. Then the lysing buffer containing protease inhibitor was added (volume depending on the size of the pellet). The pellet was re-suspended in the buffer for 5 minutes and then sat in ice for 30 minutes. Next the sample was centrifuged at 9520 RCF in 4°C for 10min. After 10min the supernatant was remove and placed in a new tube.

3.8.3 Protein Quantification

First a concentration curve was created using 1mg/mL of Albumin in varying concentrations (see Table M1). Then 1 mL of Solution C (50:1 ratio of Solution A [Anhydrous Na_2CO_3 (w/v: 20g/L), NaOH (4g/L), NaK tartrate (0.2g/L)] and Solution B [$CuSO_4$ x 5 H_2O (5g/L)], respectively) to each sample standard, and the sample itself, for 15 minutes. After 15min, 100 µL of Folin were added and the samples rested in ice for 30 minutes. Then 100 µL of each sample was placed in a 96-well plate, in duplicate, for absorbance reading in the spectrophotometer BioTek. After recording the absorbance at 750nm, a concentration curve was calculated and the estimated protein content of the sample of interest was determined. Then the samples were aliquoted and 2x Laemmli sample buffer was added accordingly (1:1) and place in boiling water (or close to boiling) for 5 minutes. Then the samples were frozen at -80°C for long-term use.

Table M1. Protein Quantification Standard Curve

Protein Quantification Table			
Tube	**Albumin 1mg/mL**	**H_2O**	**Resulting Protein**
1	150 µL	50 µL	750
2	100 µL	100 µL	500
3	50 µL	150 µL	250
4	20 µL	180 µL	100
5	15 µL	185 µL	75
6	10 µL	190 µL	50
7	0 µL	200 µL	Blank

3.9 Western Blot

3.9.1 Gel Preparation

The gels used for electrophoresis were specifically prepared for each Western Blot. The running gel mix was prepared and added to the glass support where it was allowed to dry for 30-45 minutes (see Table M2). The loading gel was then mixed (see Table M3) with the well-forming comb and allowed to dry for 20-30 minutes. The gels were then placed inside the electrophoresis cube with fresh running buffer.

Table M2 (Gel#1) and Table M3 (Gel#2). Composition of Western Blot electrophoresis gels.

GEL #1 (Running Gel)	
	10% Bis-Acrylamide (*2 Gels*)
Distilled H_2O	7.9 mL
1.5M Tris-Buffer pH8.8	6.7 mL
40% Bis-Acrylamide	5 mL
10% SDS	200 µL
10% APS	200 µL
TEMED	8 µL

GEL #2 (Loading Gel)	
	4% Bis-Acrylamide (*2 Gels*)
Distilled H_2O	3.4 mL
1M Tris-Buffer pH6.8	830 µL
40% Bis-Acrylamide	630 µL
10% SDS	50 µL
10% APS	50 µL
TEMED	5 µL

3.9.2 Running Samples:

Samples were added to each well according to the desired protein content. The loading gel ran at 75 V and 0.25 amps for 20-30min in Running Buffer (Glycine + UltraPure™ Tris (*Invitrogen*) + H_2O; pH 8.3). When all samples reached the running gel, the current was adjusted to 100 V for 2 hours in ice. After electrophoresis, the gel was immediately placed in the transfer apparatus and rested in fresh Blotting Buffer (TBS 1X + Methanol). The transfer ran at 100 V for up to 2 hours.

3.9.3 Blocking and Antibodies:

The transfer was confirmed by staining the membrane with Ponceau Red solution. After washing the membranes with TBS 1x to remove the stain, they were blocked with 5% Fat Free Milk + TBS Tween 20 for 1 hour. After removing excess milk with a wash in TBS 1x, the primary antibodies (15-LOX-1[1:700]; MMP-2 [1:2000]; MMP-14 [1:2000]; actin [1:3000]) were added to the membranes in clean plastic sacks and gently rocked on an agitator overnight in 4°C. The next morning, the membrane was removed and washed twice with TBS Tween 20 for 10 minutes each and once with TBS 1x for 5 minutes. Then, the secondary antibody was added (anti-sheep, anti-mouse, anti-rabbit [1:2000]) and the membrane was place on an agitator for 2 hours (maximum) at room temperature. Finally, the membranes were washed twice with TBS Tween 20 for 10 minutes each and once with TBS 1x for 5 minutes and stored for revealing with enhanced chemiluminescence (ECL- *Bio-Rad Laboratories Inc., Sao Paulo, SP, Brazil*) in a Syngene G-BOX with its GeneSys program. In the case of 15-LOX-1, the secondary antibody possessed a biotinylated conjugate requiring an additional 3.5-hour incubation with streptavidin horseradish peroxidase conjugate (ExtrAvidin Peroxidase; *Sigma-Aldrich, St. Louis, MD, USA*) in order to have a reaction with the ECL solution.

3.10 Zymography Assay

Gelatin zymography assays measure degradation caused by gelatinase enzymes, such as Matrix Metalloproteases (MMPs). To identify if MMPs were produced by the cells, a zymogram was performed of the serum-free medium in which the cells were incubated. The 10% Gelatin Zymogram Gel was prepared (refer to Tables M4 and M5) and stored in Running Buffer (0.25M TrisBase pH8.3 + 10% SDS + Glycine + ddH_2O) as recommended by Toth and Fridman (83).

Fresh serum free samples were removed from cell culture after 21h of incubation and stored on ice. Older samples (no more than 1 week old) were removed from a -80°C freezer and slowly thawed on ice for 2 hours. The gels were placed in a running chamber with running buffer while the samples thawed. Once thawed, the samples were centrifuged at 10,000 RCF for 5 min at 4°C.

The supernatant was separated and used for the zymogram. Using various quantities, the samples were the diluted 1:1 with 2x Sample (Laemmli) Buffer, not containing Dithiothreitol (DTT)/ mercaptoethanol, and were not boiled; all of which may denature the MMPs beyond recovery. After resting for 5 minutes, 25 µL of the sample were placed in the well of the loading gel. The gel ran for 2-3 hours at 100 V. The gels were washed with 2.5% Triton X-100 Buffer three times for 15 min on an agitator. Triton X-100 washes serve to remove the SDS present in the samples so that the proteins can be restored to their tertiary and potentially functional conformations. Then Development Buffer (1.5M Tris-HCl, pH8.8 + 1M $CaCl_2$ + 2% NaN_3 + ddH_2O) covered the gels and they were incubated for 17 hours. The negative control was a second gel washed with 10 mM Ethylenediaminetetraacetic acid (EDTA)-containing 2.5% Triton X-100 Buffer and incubated with 10 mM EDTA-containing development buffer. EDTA serves as a protease inhibitor by chelating the metal ions present which could activate the protease active sites (92). The next day, the gels were washed with Fixation Buffer and stained using 0.1% Coomassie Blue Staining Buffer (4.5:1:4.5 ratio of Methanol, Acetic Acid, ddH_2O + Coomassie Blue) for 30 min. Then the gels were washed in a Destaining Buffer (4.5:1:4.5 ratio of Methanol, Acetic Acid, ddH_2O) until bands could be seen. Images were captured in a *Syngene* G:BOX - GeneSys program (*Maryland, USA*).

Table M4 (10% Gel) and Table M5 (4% Gel). Composition of Zymogram Gels.

Separating Gel	
	10% Zymogram Gel
ddH_2O	4.74 mL
1.5 M Tris-Buffer, pH8.8	2.50 mL
40% Bis-Acrylamide	2.51 µL
1% Gelatin (10 mg/1mL)	**100 µL**
20% SDS	50 µL
10% APS	50 µL
TEMED	5 µL

Loading Gel	
	4% Gel
ddH_2O	3.64 mL
40% Bis-Acrylamide	640 µL
0.5 M Tris-Buffer, pH6.8	625 µL
20% SDS	25 µL
10% APS	40 µL
TEMED	20 µL

3.11 Scratch Assay

Aiming to measure short-term migration in T98G cells treated with the 15-LOX products and LOX inhibitors, a wound healing assay was performed. First, the initial number of cells needed to reach 90-100% confluency in 12 h for an assay in a 24-well plate was determined ($9x10^4$ cells). Using a 1mL pipette tip, a streak was made in the middle of the well. After the medium was carefully changed to include previously determined concentrations of eicosanoids and drugs with their respective controls, then images of the plates were taken immediately (0 h), and later at 12 h. The images of the scratched sites were processed in ImageJ Software by calculating the number of cell-free pixels in the images at 0 h and comparing them with the number of pixels at 12 h. A reduction in the values between 0 h and 12 h imply that cell migration into the cell-free space occurred. The relative 0h/12h value for each treatment was then compared with the 0h/12h value of the DMSO treatment (positive control) and a value (%) was produced.

3.12 Statistical Analysis

Dose response tests are presented as the mean plus the SEM. Using the GraphPad Prism 5 software the significance was determined by a two-way ANOVA test with a Bonferroni post-test to compare between multiple data sets. Not assuming equal variances, one-tailed or two-tailed unpaired t-test was used with Welch's correction to compare specific pairs of data. Real time PCR

followed the Livak method of $2^{-\Delta\Delta CT}$(93). Differences were considered significant with $p<0.05$. The significance of the p-value is represented in the figures by "*" (*<0.05, **<0.01, ***<0.001).

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APPENDEX - SUPPLEMENTARY DATA

This project is part of a larger project examining the expression and activity of various lipid mediators present in glioblastoma cells. Previous LC/EIS-MS/MS identified the presence of 15-LOX metabolites as the most abundant lipid mediators in five GBM cell lines and patient samples compared to the other lipoxygenase isoforms (Figure S1).

Examining the Human Protein Atlas (HPA) and The Cancer Genome Atlas (TCGA) Databases, the following figures compliment and support the findings of this study. The Human Protein Atlas did not identify 15-LOX-1 or 15-LOX-2 in GBM cell lines or glioma samples with the primers and antibodies used (Figures S2 and S3). The TCGA database demonstrated a significant decrease in 15-LOX-1 mRNA between non-tumor and GBM samples, while no significant difference was observed in 15-LOX-2 mRNA (Figure S4). Survival curves did not demonstrate any changes between high and low expression of 15-LOX-1 or 15-LOX-2 among GBM patients (Figure S4). The expression of PPARβ/δ mRNA and protein was identified in GBM cell lines and glioma samples according to HPA. Decreased mRNA was also observed in GBM samples compared to non-tumor samples and was correlated with decreased survival (Figure S5).

Regarding MMP expression in GBM, the Human Protein Atlas demonstrated the presence of MMP-2 and MMP-14 mRNA and protein expression in normal brain tissue, GBM cell lines and glioma patient samples (Figures S6 and S7). MMP-9 was not identified in any of the tissues (Figure S8). The TCGA database identified a significantly increased expression of MMP-2, MMP-14 and MMP-9 mRNA between non-tumor and GBM samples. Decreased survival was observed among GBM patients with high expression of MMP-2 (Figure S9). A summary of the findings may be found in Table S1.

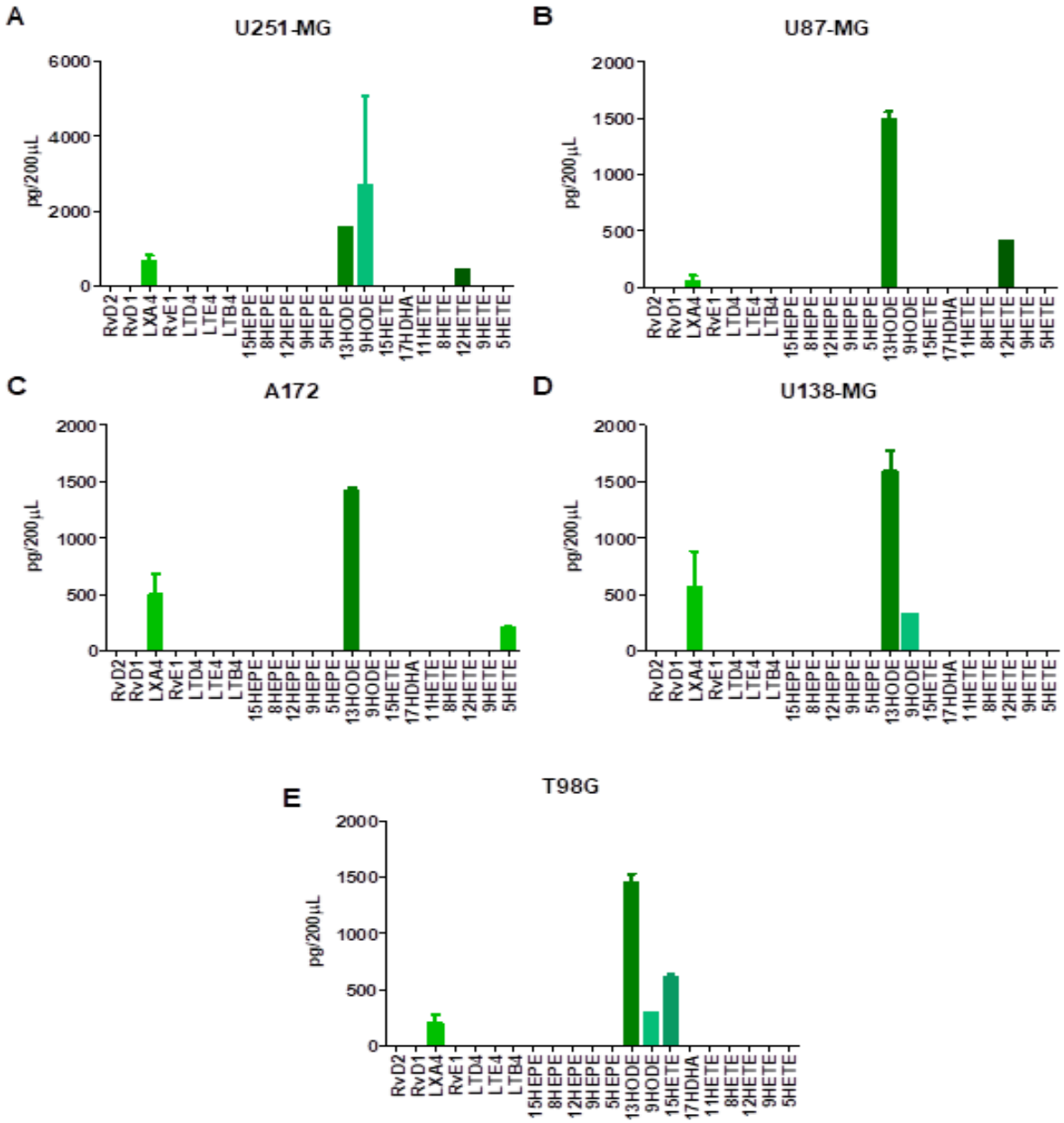

Figure S1. LC\ESI-MS/MS of the products: RvD2, RvD1, LXA4, RvE1, LTD4, LTE4, LTB4, 15-HEPE, 8-HEPE, 12-HEPE, 9-HEPE, 5-HEPE, **13-HODE, 9-HODE, 15-HETE**, 17-HDHA, 11-HETE, 8-HETE, 12-HETE, 9-HETE, 5-HETE in the cell lines U-251MG (A), U-87MG (B), A172 (C), U-138MG (D) and T98G (E). N=3.

Source: Souza, FC. Eicosanoids as new therapeutic targets in the treatment of human glioblastoma [Thesis (Doctor from Department of Cell and Developmental Biology)] – Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo; 2017.

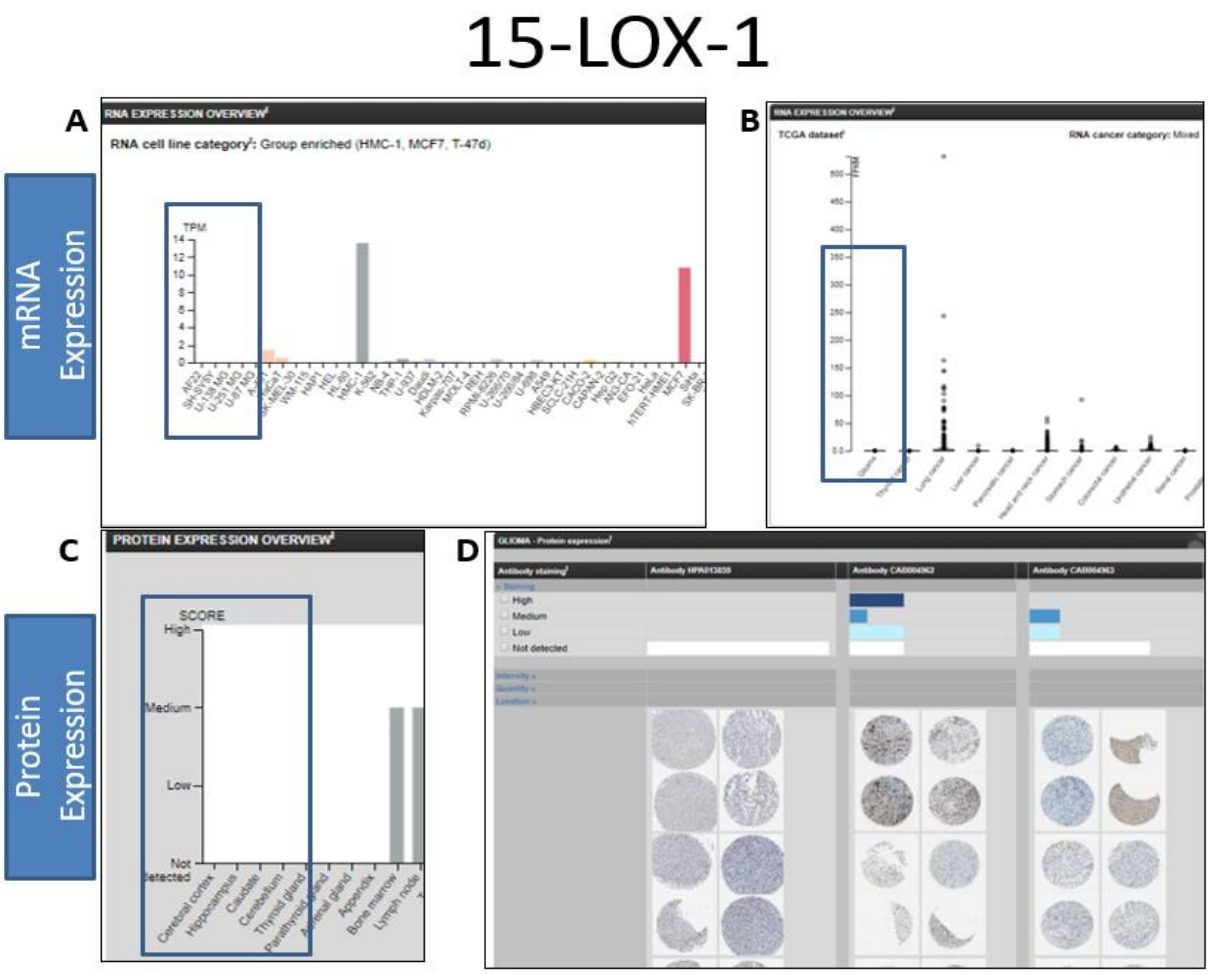

Figure S2. 15-LOX-1 mRNA and protein expression observed by the Human Protein Atlas Project. A) mRNA was not observed in GBM cell lines U138-MG, U251-MG and U87-MG. B) mRNA was not observed in glioma samples. C) Protein Expression was not observed in normal brain tissue. D) Partial protein expression was observed in GBM patient samples.

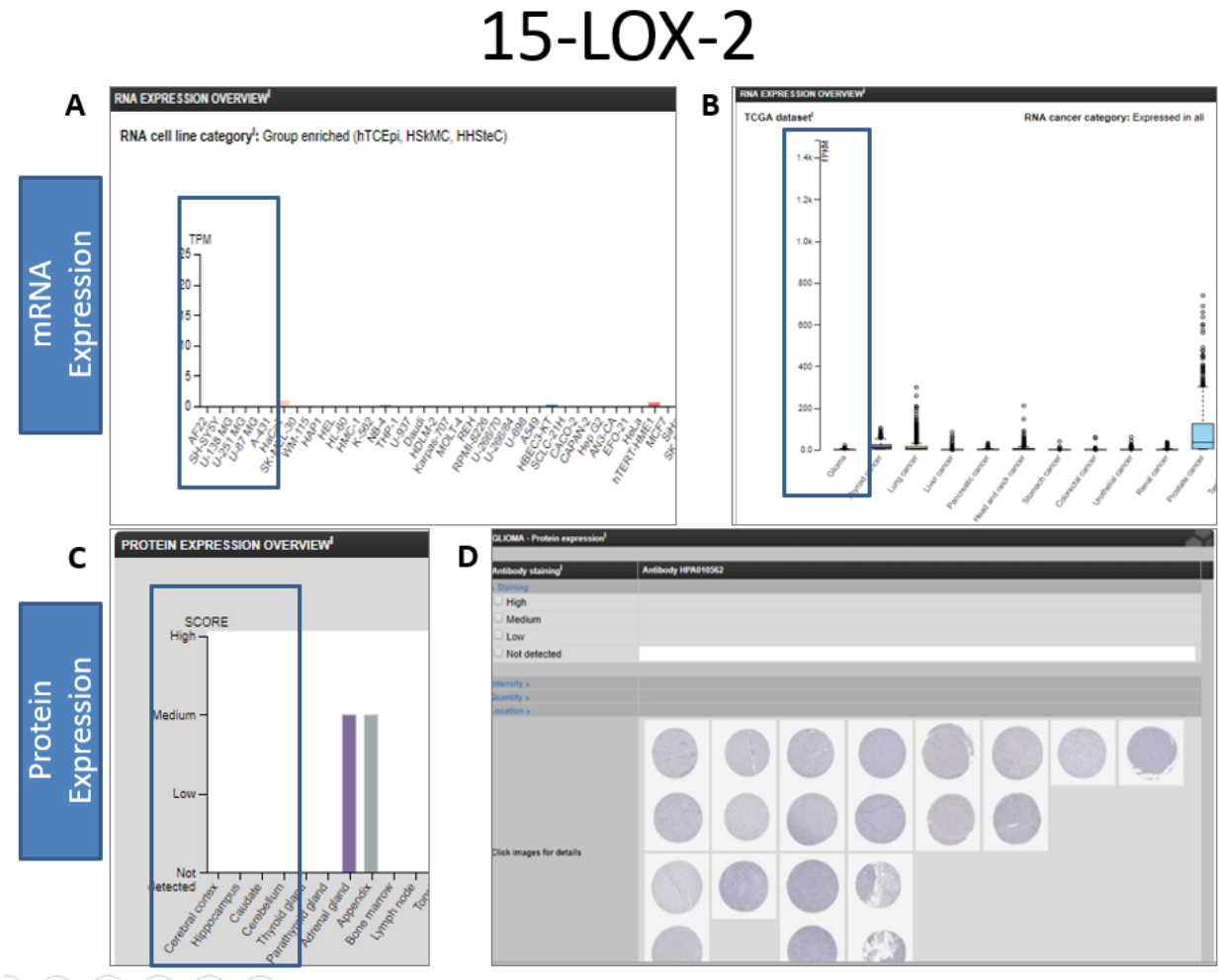

Figure S3. **15-LOX-2 mRNA and protein expression observed by the Human Protein Atlas Project**. A) mRNA was not observed in GBM cell lines U138-MG, U251-MG and U87-MG. B) mRNA was not observed in glioma samples. C) Protein expression was not observed in normal brain tissue. D) No protein expression was observed in GBM patient samples.

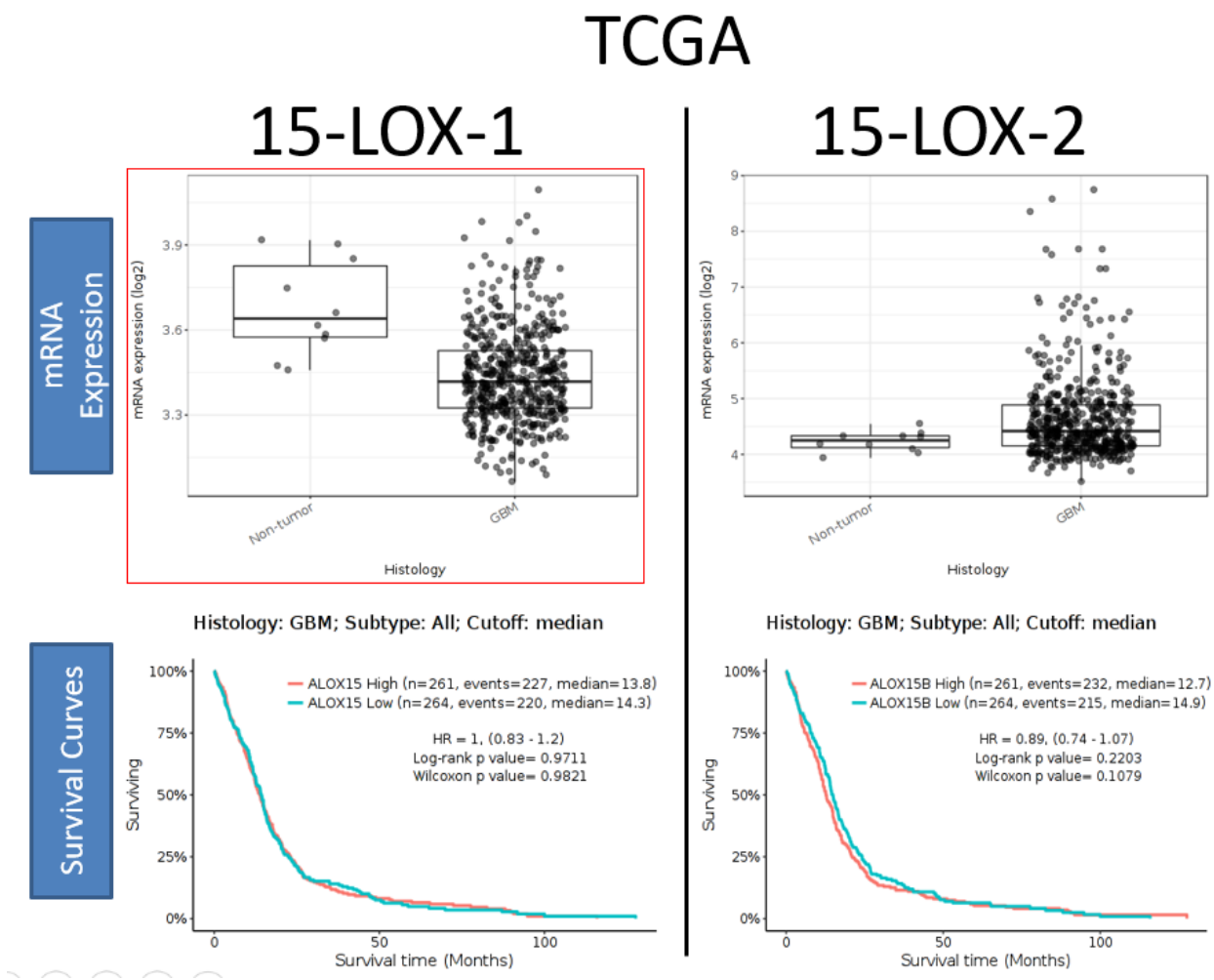

Figure S4. **TCGA database information concerning 15-LOX-1 and 15-LOX-2 mRNA expression and survival curves**. 15-LOX-1 mRNA is significantly decreased in GBM cells compared to non-tumor tissue. Survival curves were not altered by the high or low expression of 15-LOX-1 or 15-LOX-2.

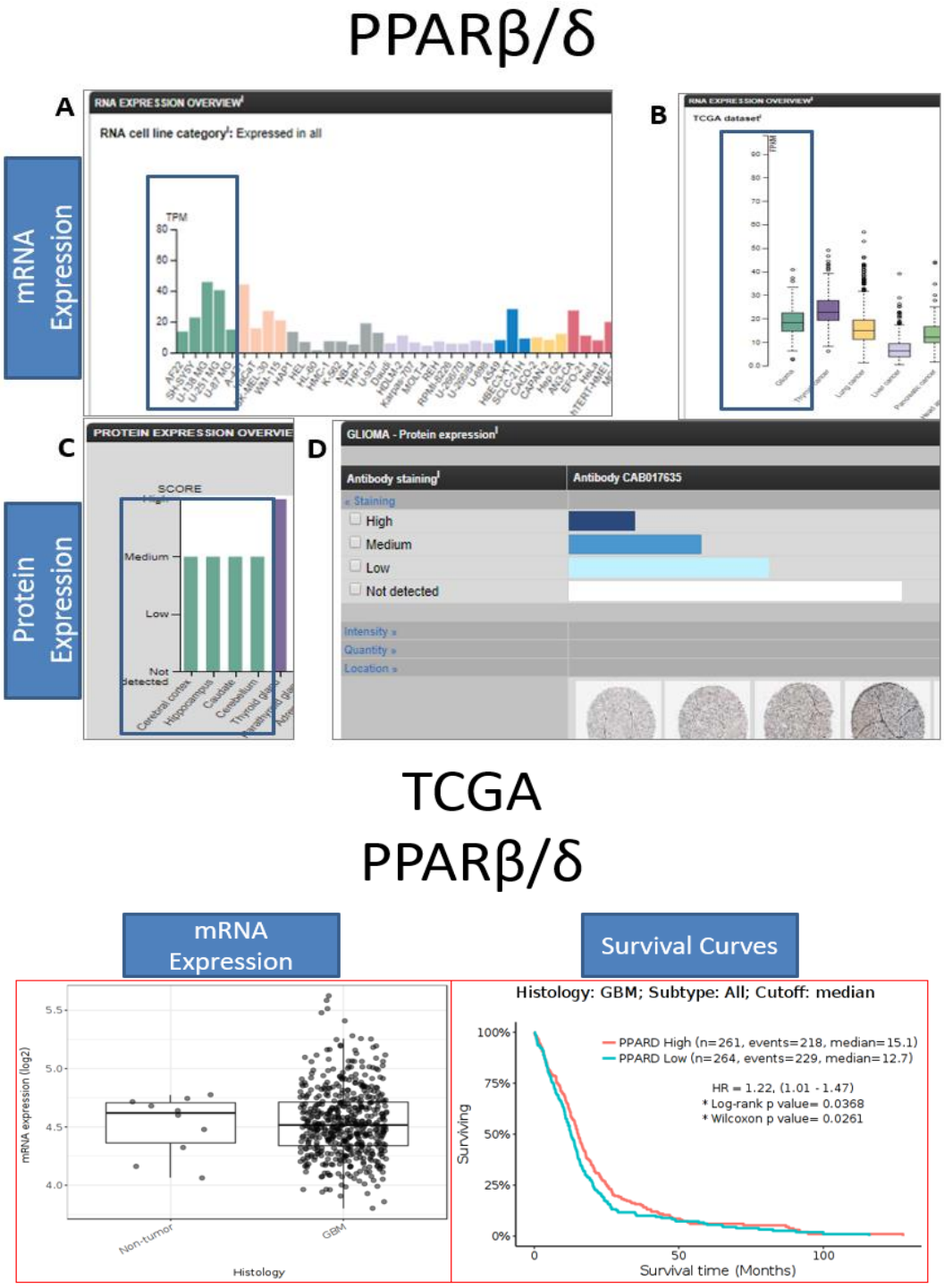

Figure S5. **PPAR beta/delta mRNA and protein expression observed by the HPA [top] and TCGA [bottom].** A) mRNA was observed in GBM cell lines U138-MG, U251-MG and U87-MG. B) mRNA was observed in glioma samples. C) Protein expression was observed in normal brain tissue. D) Protein expression was observed in GBM patient samples. TCGA: mRNA was significantly decreased in GBM samples compared to non-tumor samples and decreased expression was associated with decreased survival.

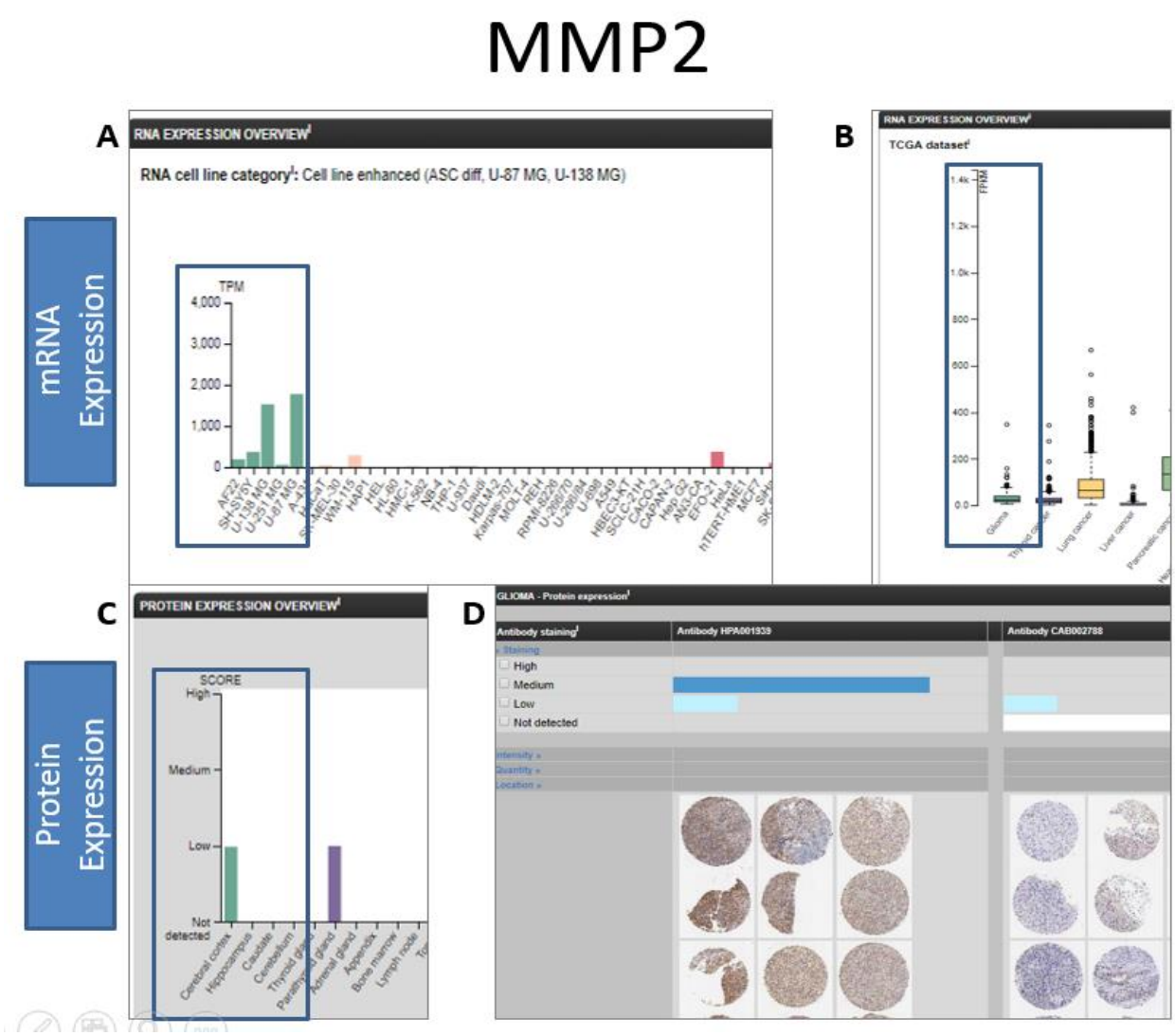

Figure S6. **MMP-2 mRNA and protein expression observed by the Human Protein Atlas Project**. A) mRNA was observed in GBM cell lines U138-MG and U87-MG, and very little was found in U251-MG. B) mRNA was identified in glioma samples. C) Protein expression was only observed in the cerebral cortex and not in other regions of normal brain tissue. D) Low to medium protein expression was observed in GBM patient samples.

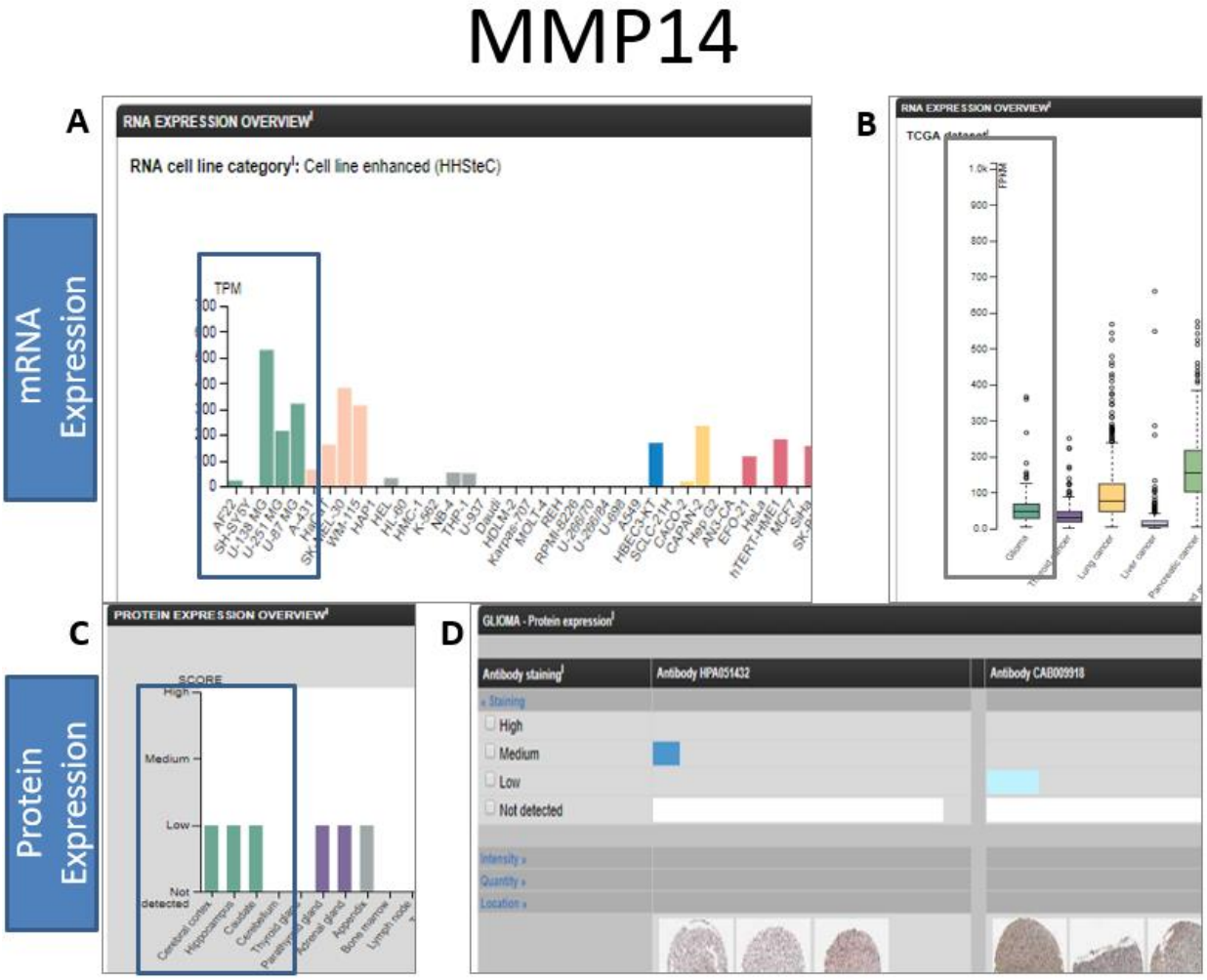

Figure S7. **MMP-14 mRNA and protein expression observed by the Human Protein Atlas Project**. A) mRNA was observed in GBM cell lines U138-MG, U251-MG and U87-MG. B) mRNA was identified in glioma samples. C) Protein expression was observed in the cerebral cortex, hippocampus and caudate, but not in the cerebellum. D) Very low to medium protein expression was observed in GBM patient samples.

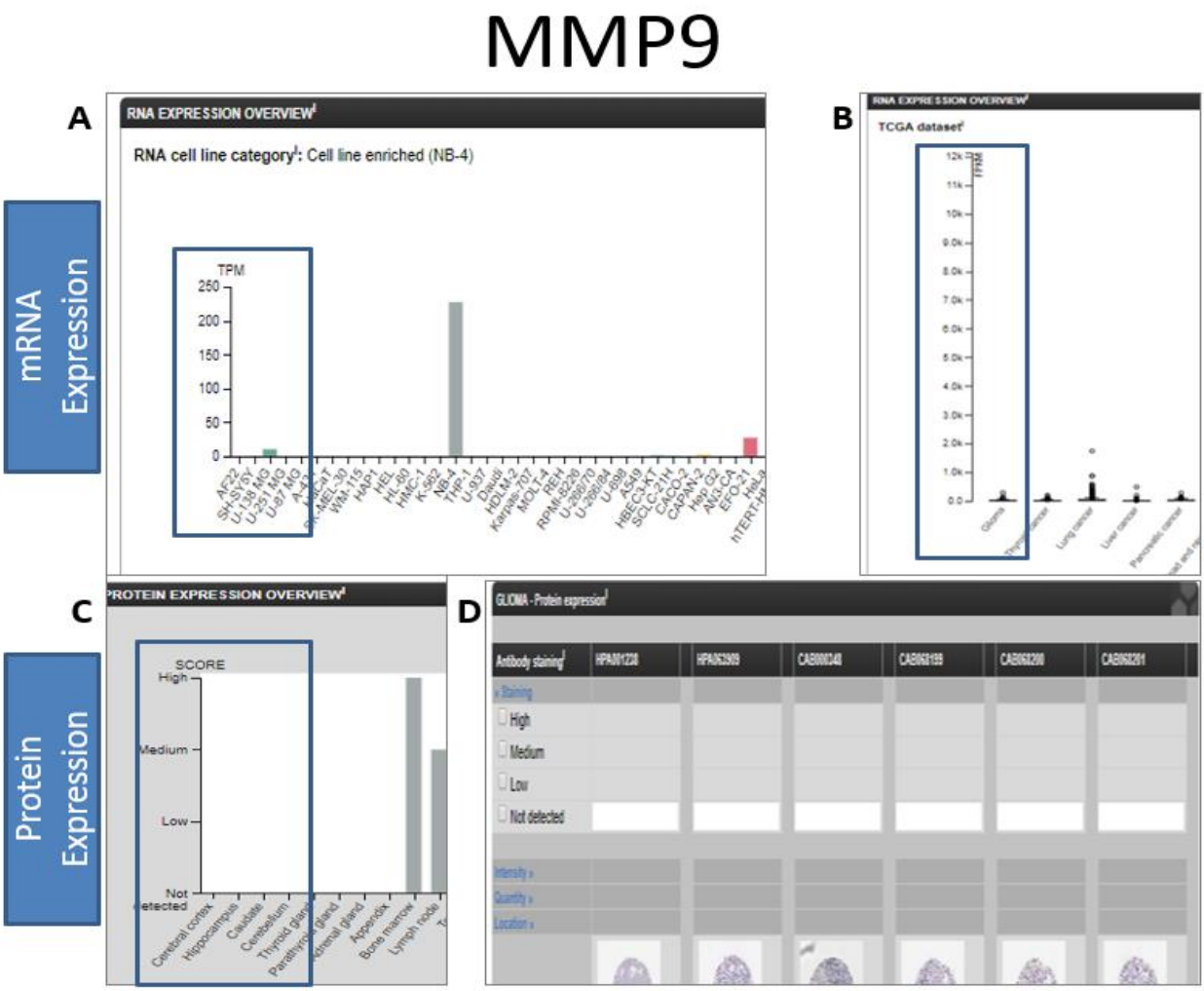

Figure S8. **MMP-9 mRNA and protein expression observed by the Human Protein Atlas Project**. A) mRNA was only observed in the GBM cell line U138-MG and not in U251-MG and U87-MG. B) mRNA was not observed in glioma samples. C) Protein expression was not observed in normal brain tissue. D) No protein expression was observed in GBM patient samples using six different antibodies.

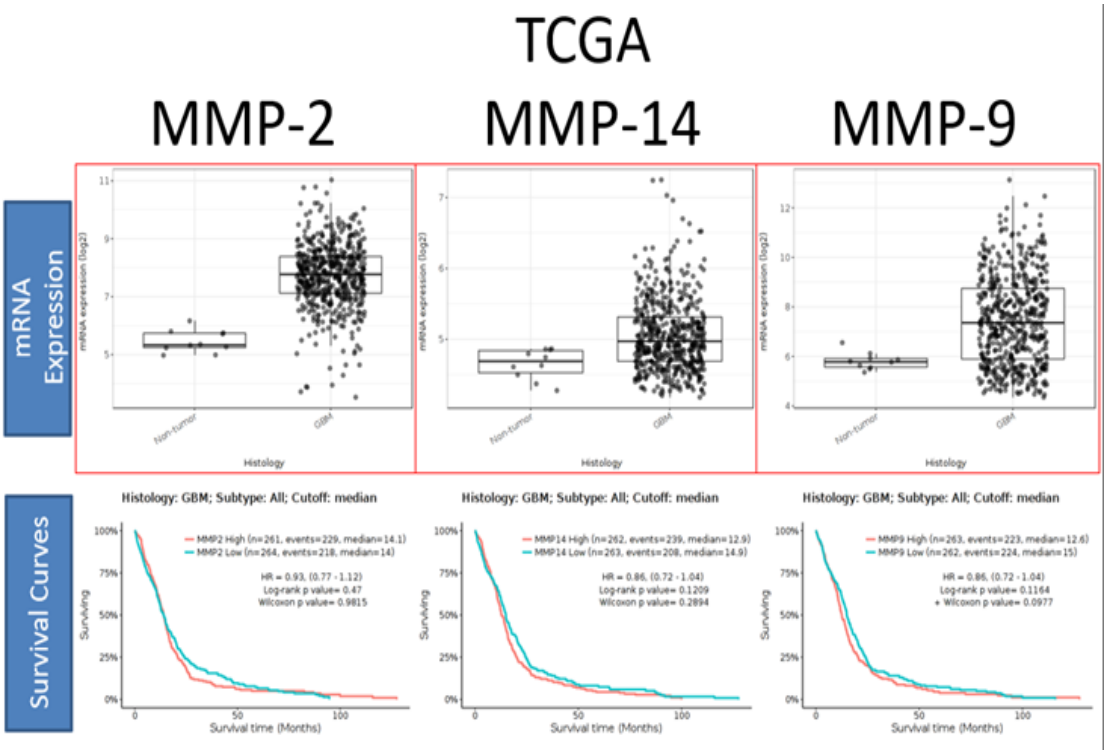

Figure S9. **TCGA database information concerning MMP-2, MMP-14, MMP-9 mRNA expression and survival curves**. The mRNAs of all three MMPs were significantly increased in GBM cells compared to non-tumor tissue [top row]. No significant correlation between MMP-2, MMP-14 and MMP-9 expression and survival were observed [bottom row].

Table S1. Summary of mRNA/ protein expression identified by the Human Protein Atlas and TCGA Databases, accessed beginning from September, 2018.

Protein	Protein Expression (Normal Brain Tissue)	mRNA present (no. / 3 GBM Cell Lines tested)	mRNA found in Glioma Samples	Protein Expression (in Glioma samples)	*TCGA: mRNA	†TCGA: Survival
15-LOX-1	-	-	-	-	↓	-
15-LOX-2	-	-	-	-	-	-
PPARγ	√	√ (1)	-	√	-	-
PPARβ/δ	√	√ (3)	√	√	↓	↑
GPR132	√	√ (1)	√	√	-	↑
MMP-2	√	√ (3)	√	√	↑	↓
MMP-14	√	√ (3)	√	√	↑	-
MMP-9	-	√ (1)	-	-	↑	-

*TCGA: significantly increased (↑) or decreased (↓) mRNA expression in GBM samples compared with non-tumor samples.
†TCGA: survival curves that displayed a significant increase (↑) or decrease (↓) in survival correlated with mRNA expression.
√ = confirmed
- = information not available