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Telomerase Reverse Transcriptase protein expression as prognostic factor for Glioblastomas

Degree Project in Medicine

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ABSTRACT

Introduction: Glioblastoma (GBM) is the most common malignant primary tumor of the central nervous system in the adult male. The average survival is between 3 and 5 months without treatment, and between 9 and 15 months with surgical resection accompanied by chemotherapy and radiotherapy. Discovering accurate prognostic markers is important for finding the optimal treatment strategy for each patient diagnosed with GBM. **Objectives:** Evaluate the correlation between the expression of Telomerase Reverse Transcriptase protein (TERT), the magnitude of peritumoral cerebral edema and outcome in patients to identify a possible prognostic factor for GBM. **Methodology:** Patients treated by the neurosurgery department of Hospital Santa Paula, São Paulo, Brazil, between 2010 and 2018 with a diagnosis of glioblastomas were selected. The patient data was collected from the patient journals. The size of the peritumoral cerebral edema was classified from the magnetic resonance. The tissue-samples were collected after the surgery, and were submitted to a histological and immunohistochemical analysis to evaluate the level of protein and gene expression of TERT. Due to the limited samples only descriptive statistics was used. **Results:** Out of the 12 patients, 4 had a positive protein expression of TERT and the older patients generally showed a lower expression. Of the 5 deceased patients, all but one had major edema, and none showed any expression of TERT protein. In addition, all the patients with GBMs found in the parietal lobe died. **Discussion:** Lower levels of TERT may be related to advanced age. GBMs located in the parietal lobe may be more aggressive. There was a direct positive relationship between death and peritumoral edema, as well as a negative relationship between death and TERT protein expression, but the sample was too low for statistical significance to these conclusions.

Keywords: Neurosurgery, Glioblastoma, prognosis, Telomerase Reverse Transcriptase, Peritumoral edema.

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1. INTRODUCTION

Glioma is a general term for primary brain tumors, and can be derived from various brain cells. Glioblastoma (GBM) originates from astrocytes and is the most common malignant tumor in the central nervous system. It accounts for about 15% of all intracranial brain tumors and 48% of all primary malignant CNS-tumors (Ostrom et. al. 2018), and for more than 60% of all the malignant brain tumors in adults (Rock et al., 2014). It affects more men than women (1.5:1) and has a global incidence of less than 10 per 100,000. The primary type affects more the elderly. It is diffuse, invasive, and very aggressive, and has a prognosis of only 3-5% 5-year survival rate and an average survival of 12 to 15 months with the recommended multimodal treatment. The secondary type can affect people of a younger age and is associated with a higher rate of survival (Tamimi et al., 2017; Szopa et al., 2017; Yan et al., 2009).

Exposure to high levels of ionizing radiation normally used in radiotherapy is the only factor that has been confirmed to increase the risk of developing GBM. This does not include diagnostic radiation, and neither has typical environmental factors such as smoking, cell phone usage and pesticides shown an increased risk of GBMs. Heredity is not common and is only present in 5-10% (Prasad et al., 2009; Bondy et al., 2008; Inskip et al., 2001; Ohgaki, 2009; Agnihotri et al., 2013; Fisher et al., 2007). However, a meta-analysis showed that allergy can serve as a protective factor, perhaps because it is generally associated with an upregulated immune response (Linos et al., 2007). Patients with some genetic syndromes such as neurofibromatosis and tuberous sclerosis has shown a higher incidence of GBM (Johansson et. al. 2016).

1.1 Site and Symptoms

The majority, 95%, of GBMs are located in the supratentorial regions of the brain with a preference for the frontal lobe, followed by the temporal, parietal, and occipital lobes, and seldom find their way to the cerebellum or the brain stem (Nakada et al. 2011). General symptoms include intracranial hypertension, persistent headache, vomiting, papilledema, mental changes, and seizures, whereas the focal symptoms vary greatly depending on the region of the brain the tumor is located in and affects (Shapiro et al. 2011). A summary of the specific symptoms associated to each lobe can be seen below in the table.

Table 1: Symptoms related to location of brain tumor

Location	Associated Common Symptoms
Frontal lobe	Personality changes. Increased aggression or irritation. Apathy. Weakness on one side of the body. Loss of smell. Difficulty walking. Vision / Speech problems.
Temporal lobe	Forgetting words. Short-term memory loss. Seizures (often associated with strange smells/feelings).
Parietal lobe	Difficulty speaking / understanding. Problems reading / writing. Loss of feeling in part of the body.
Occipital lobe	Visual field deficits.

1.2. WHO classification and Karnofsky Performance Status

Prior to 2016, gliomas were classified by histological features such as cell atypia, the morphology of the cell nucleus, density of cells, number of mitosis, proliferation of blood vessels and tumor necrosis (Louis et. al, 2007). Since 2016, however, gliomas are classified by the World Health Organization (WHO) by a combination of histological features and genetic factors (or biomarkers) into grade I to IV depending on its malignancy. Grade I are considered curable with resection and grade II to IV are considered to be malignant and invasive. GBMs are classified grade IV Astrocytomas and can be primary tumors that arise de-novo (>90%) or secondary tumors that develop from low-grade gliomas (8%). They are further divided into GBM, IDH-wild type and GBM, IDH-mutant depending on whether a particularly important gene, isocitrate dehydrogenase (IDH) which is further discussed below, is mutated or not (Louis et. al, 2016).

Karnofsky's Performance Status (KPS) is an index that classifies the overall well-being of patients and can be used to identify high-risk patients, compare and choose different therapies and to assess the prognosis in individual patients (Crooks et. al, 1991).

Table 2: Karnofsky's Performance Status scale definitions rating (%) criteria.

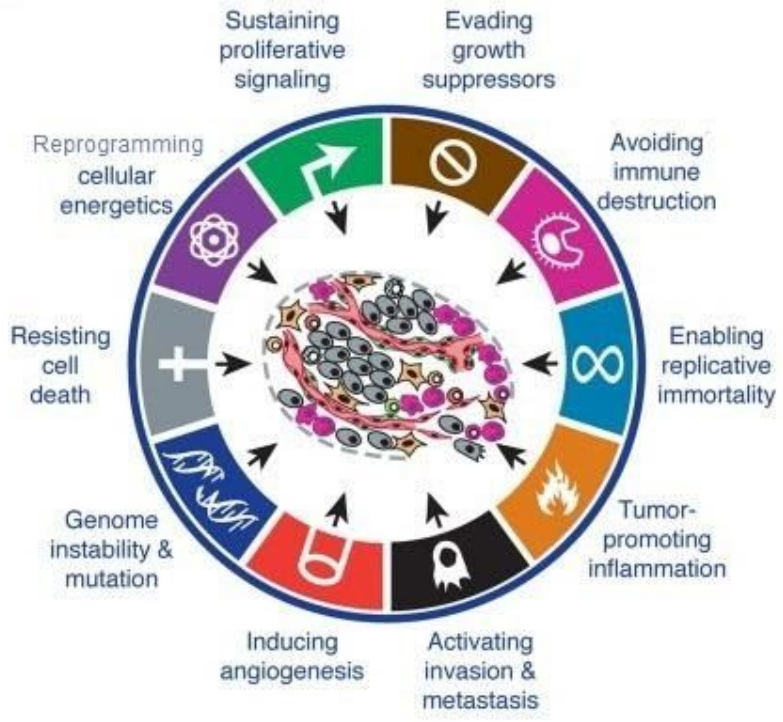
Able to carry on normal activity and to work; no special care needed.	100%	Normal no complaints; no evidence of disease.
	90%	Able to carry on normal activity; minor signs or symptoms of disease.
	80%	Normal activity with effort; some signs or symptoms of disease.
Unable to work; able to live at home and care for most personal needs; varying amount of assistance needed.	70%	Cares for self; unable to carry on normal activity or to do active work.
	60%	Requires occasional assistance but is able to care for most of his personal needs.
	50%	Requires considerable assistance and frequent medical care.
Unable to care for self; requires equivalent of institutional or hospital care; disease may be progressing rapidly.	40%	Disabled; requires special care and assistance.
	30%	Severely disabled; hospital admission is indicated although death not imminent.
	20%	Very sick; hospital admission necessary; active supportive treatment necessary.
	10%	Moribund; fatal processes progressing rapidly.
	0%	Dead

1.3. Biomarkers

Prognostic biomarkers are often genetic mutations and are of importance in determining the probable outcome, i.e. how likely the patient will suffer relapse, the progression of symptoms, the overall survival, and can serve as a tool for deciding therapeutic strategies for patients. (Pesenti et al., 2017; Szopa et al., 2017, Louis et al., 2016; Jovčevska et al., 2013). They are seldom specific and can usually be found in a variety of cancers, but this study will focus on the biomarkers that can say something about gliomas in general and

glioblastomas in particular. Many are still being developed, and they can be divided into subgroups according to what type of function they fulfil in the cancerous cells, as described in the hallmarks of cancer (Hanahan et. al, 2011). A thorough review of the particular hallmarks of glioblastomas was done in a systematic review by Nørøxe et. al, 2016, and some of the major ones that are frequently tested in routine clinical practice will be presented more in detail below.

Figure 1: Hallmarks of cancer (from Hanahan et. al, 2011).



A particularly important diagnostic biomarker for GBMs and related to the hallmark of "reprogramming cellular energetics" is the mutation of isocitrate dehydrogenase (IDH) enzymes 1 and 2. These are metabolic enzymes that have been found mutated in a wide range of cancers such as acute myeloid leukemia (AML), cholangiocarcinoma, chondrosarcoma and

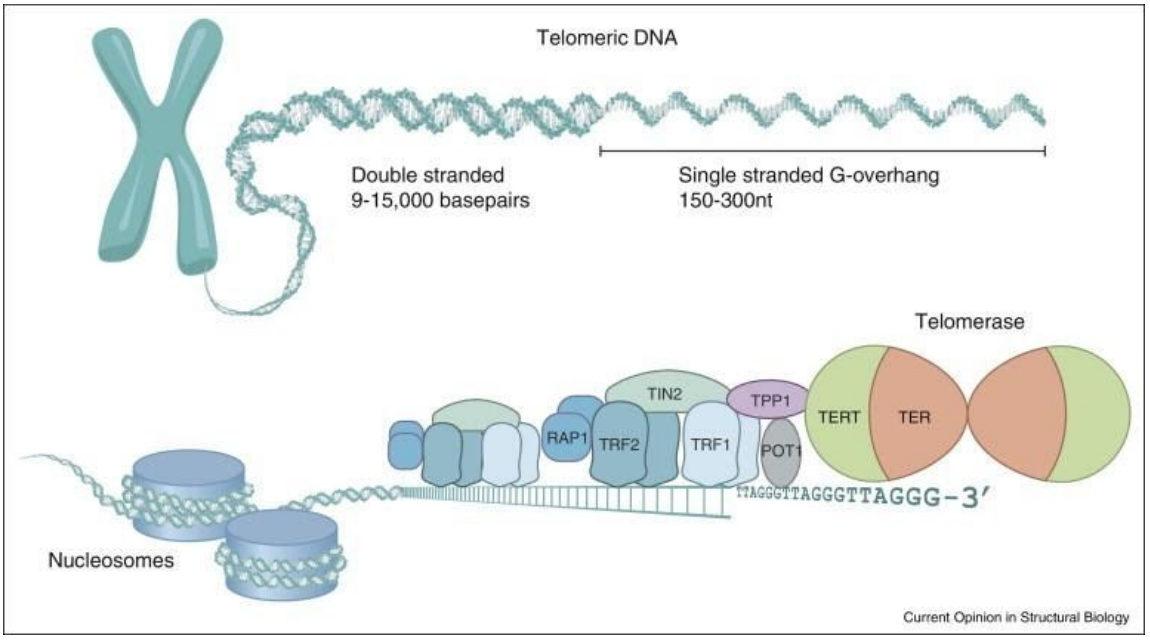
glioma. Mutations in IDH decrease its normal activity and lowers the metabolism of the cancerous cells, which means that the growth is also impaired. These mutations are thus associated with better outcome in gliomas and serve as a prognostic biomarker (Derek et al., 2017).

Another important biomarker, especially important for the selection of adjuvant chemotherapy, is the methylation of the promoter of the gene O6-methylguanine-DNA methyltransferase (MGMT). This gene codes for a DNA-repair protein that removes alkyl groups from guanine, which means that the GBM-cells that have high levels of MGMT are more resistant towards alkylating chemotherapeutic agents. Methylation of MGMT, however, silences the gene, and in such patients alkylating agents are much more efficient. (Stupp et. al, 2005, 2009).

Epidermal growth factor receptor (EGFR) normally controls various intracellular signal pathways in epithelial cells. The EGFR gene is often found upregulated and mutated in many cancers including gliomas. The most common mutation, EGFRvIII, is particularly associated with poor outcome for its ability to promote tumor growth, survival, invasion, metabolism and angiogenesis, and thus relates to many of the hallmarks of cancer. This biomarker is frequently screened for in an effort to tailor combination therapies as inefficient blood brain barrier penetration, intratumoral heterogeneity, compensatory signalling pathways and secondary mutations that all contribute to resistance (An et. al, 2018; Sigismund et. al, 2018).

Pertinent to the hallmark "enabling replicative immortality" is telomerase, an enzyme that adds protective repetitive DNA-sequences, telomeres, at the end of the chromosomes. Telomerase normally only remains active in the germ line and in activated stem cells and lymphocytes, and is suppressed during embryonic differentiation and are almost absent in normal cells. Every time a normal cell divides, the telomeres are shortened, and after about 50-70 times cell-division stops in human cells. (Nugent et al., 1998; Zhou, 2014). Telomerase is active in many types of cancers however, thus enabling these cells to replicate almost indefinitely. Telomerase reverse transcriptase (TERT) is the catalytic subunit of telomerase that actually generates the sequences of cDNA that make up the telomeres (Sandin et. al, 2014).

Figure 2: Human telomere structure and telomerase recruitment. Telomeric DNA consists of arrays of the TTAGGG telomeric repeat, forming a long region of double stranded DNA terminating in the single stranded G-rich overhang. Telomerase is recruited to the tip of telomeres by the telomerase catalytic subunit TERT and through base pairing between the template region in the telomerase RNA subunit TER and the G-overhang. (From Sandin et. al, 2014).



Some mutations of TERT, particularly mutations C228T and C250T of its promoter region, cause a two to fourfold increase in transcriptional activity and, consequently, a positive regulation of telomerase activity in cancer cells. Mutations in the promoter (TERT) are strongly present in GBMs (83%), oligodendrogliomas (78%), oligoastrocytomas (25%), astrocytomas (10%) and in many other types of cancers. (Killela et. al, 2014; Mohammad et. al, 2016; Vinagre et. al, 2013). TERT promoter mutations are believed to be associated with aggressive behaviors and unfavorable outcomes in GBM, as well as overall survival (OS) and compromised progression free survival (PFS) in patients with gliomas. However, currently TERT promoter mutations are not qualified as a predictive biomarker. (Zhou, J. et al. 2014; Ohba, J. et al, 2016). Although this study focuses on the expression of TERT, not specifically on the mutations mentioned above, Masui et. al, 2018, found that both patients with and without a mutation of the TERT promoter had a higher expression of TERT protein measured with a TERT-specific antibody (TMab-6).

1.4. Macroscopic and Histological Features

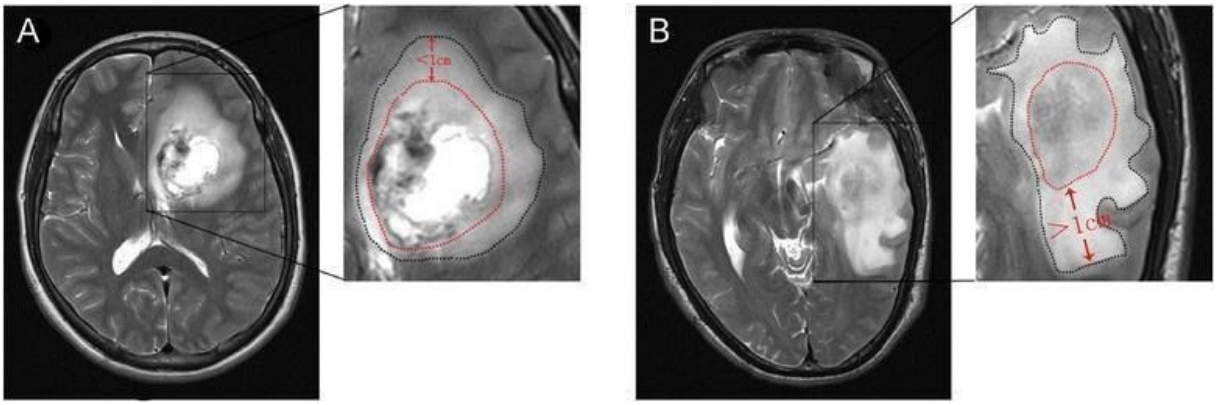
GBMs are quite heterogeneous with pleomorphic cells composed of everything between small poorly differentiated tumor cells to large multinucleate cells. They present rapid growth, high glucose consumption, intra-tumor necrosis, hypoxia, abundant microvascular proliferation, hemorrhaging and, as is the focus of our study, blood-brain barrier destruction, perivascular infiltration of glioma cells and cerebral vasogenic edema. Studies indicate that tumor vessels are abnormal, and that the BBB that protects the brain's interstitial space from plasma leakage under normal conditions appear defective with exposed endothelial cell surface and irregular basal laminae. Consequently, it leads to junction opening, increased permeability, and edema formation. (Stummer, W., 2007). Another

hypothesis proposes an increase in aquaporins (AQP) due to excessive growth factor stimulation which amplifies fluid leakage as well as tumor cell migration through the BBB. (Zador, et. al, 2016).

1.5. Imaging

Imaging can vary widely, but usually a central area of necrosis surrounded by white matter edema is present on an MR, and on a CT hypointense areas and a midline shift as a result of moderate to severe edema can be seen (Nelson et. al. 2003). The size of the edema is of prognostic value as studies have shown that the larger the edema the poorer outcome (Chen-Xing et. al, 2015). The size of the peritumoral edema is estimated as the maximum distance from the tumor margin to the outer edge of edema, as can be seen in the image below from Chen-Xing's article.

Image 1: Determining size of peritumoral edema (A) Minor edema (<1 cm) shown by T2-W MRI. (B) Major edema (>1 cm) shown by T2-W MRI. (From Chen-Xing et. al, 2015).

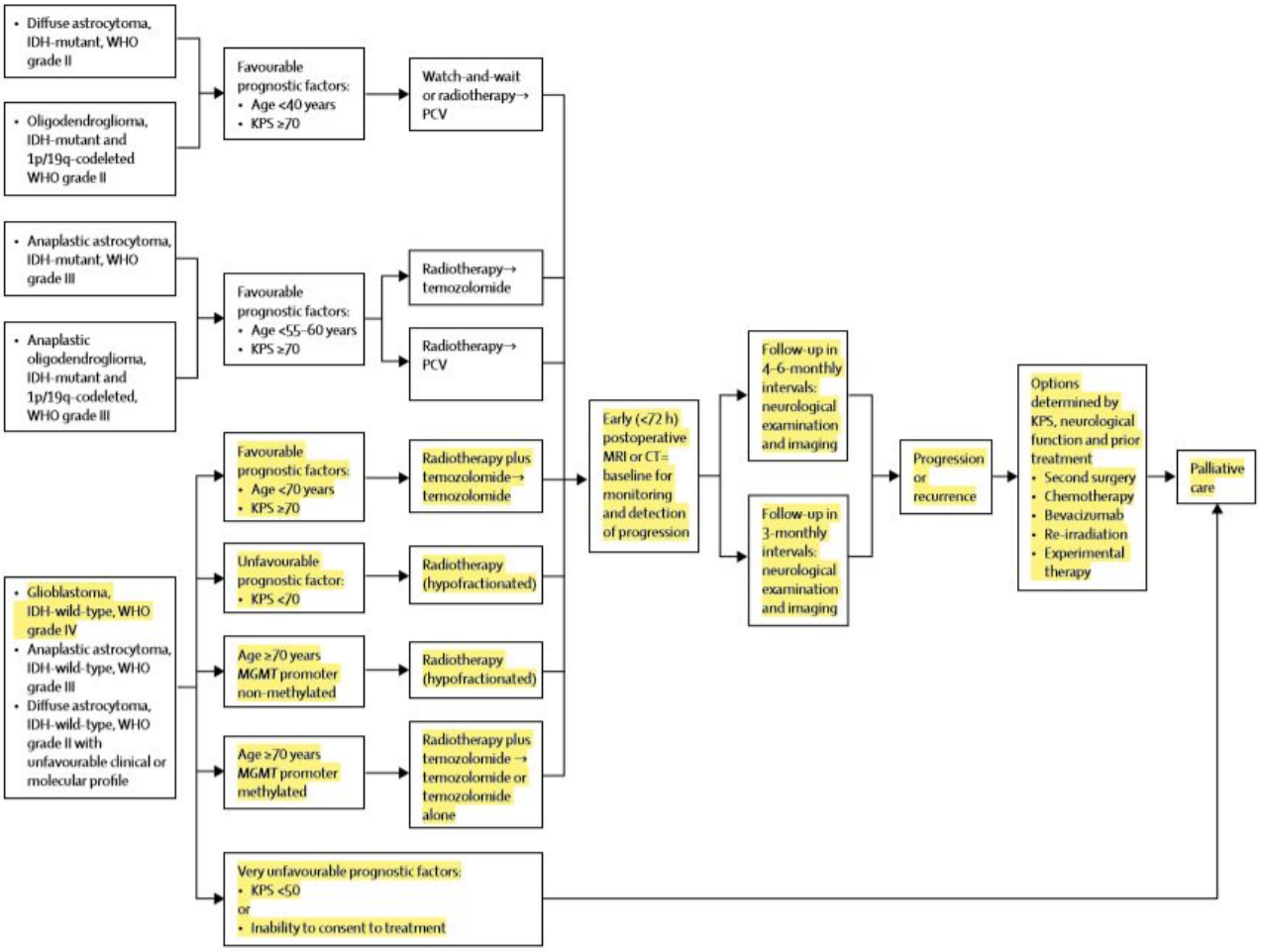


1.6. Treatment

The primary treatment of GBM is surgical resection. However, the extent of how much of the tumor can be extracted is limited by the location and the functionality of the

afflicted area, and as such about 80% of the patients suffer relapse. Adjuvant treatment in the form of radiotherapy and chemotherapy is thus used in a majority of patients, and the European Association for Neuro-Oncology (EANO) have the following guidelines that considers the patients age, KPS and MGMT-methylation, as can be seen in the flowchart below in the figure from the article from Weller et. al, 2012.

Figure 3: Clinical pathway for glioma Pathway for GBM highlighted. (From Weller et. al, 2012).



Serious side-effects such as radiation necrosis, radiation-induced permanent neuronal damage and even radio-resistance of some tumors has to be considered in the case of

radiotherapy (Iacob and Dinca, 2009). Hypofractionated stereotactic radiotherapy have shown better results than hyperfractionated radiotherapy, and some novel therapies such as Intensity-modulated radiation therapy and boron neutron capture therapy have shown to be less toxic (Norden and Wen, 2006). In addition to radiotherapy, several chemotherapeutic agents have been tested, but Temozolomide (TMZ) which targets the tumor cells DNA mismatch repair system, blocks the cell cycle and triggers apoptosis, is the standard chemotherapy for patients with GBM (Reardon and Wen, 2006; Scott et. al. 2011). An analysis of the methylation of MGMT is used to determine which chemotherapeutic agent is to be used, and as mentioned above TMZ has been shown to work better on methylated MGMT (Stupp et. al, 2005, 2009; Malmstrom et. al, 2012; Wick et. al, 2012; Hansen et. al. 2018). Common side effects include gastrointestinal symptoms, nausea, headache, fatigue and hair loss, and the patient must be vigilant of signs of febrile neutropenia.

2. JUSTIFICATION

Currently, the therapeutic arsenal of GBM is surgical resection and adjuvant radiotherapy and chemotherapy. The survival length even with these very invasive treatments, however, is still short and ranges from 9 to 15 months. Furthermore, the effectiveness of these treatments varies depending on various clinical variables as well as biomarkers. For this end, identifying diagnostic and prognostic biomarkers is of importance to find a more specific and individually tailored treatment strategy for each patient.

3. PURPOSE

The purpose of this study is to correlate the level of TERT expression and the magnitude of cerebral edema in patients with GBM to their prognosis**,** expressed as months of survival after the resection of the tumor which is the primary endpoint.

In addition, survival will be correlated to other clinical variables, such as the patient's age, gender and location of the tumor. The study will also examine which of these clinical characteristics might be associated with TERT protein expression.

In addition to these quantitative goals, several qualitative objectives for the professional development of the author were formulated and listed in Appendix A. As the results of these goals are not quantifiable but rather valuably add to the author's overall knowledge and experience, they will only be presented in Appendix A and not further scrutinized for specific results.

4. MATERIAL AND METHODS

4.1. Patients and samples

Originally 28 patients with gliomas treated by the neurosurgery department of the private Hospital Santa Paula, São Paulo, Brazil, between 2010 and 2018 were selected regardless of age, gender and comorbidity. They were all patients of the advisor of the author, neurosurgeon and Professor Paulo Henrique Pires de Aguiar. A Karnofsky's Performance Status (KPS) was estimated for each patient to evaluate their overall wellbeing and how well they would tolerate adjuvant treatment. The patients then underwent radical resection of the tumor where a sample was collected, and thereafter received adjuvant chemotherapy (temozolomide) and radiotherapy, and a histological analysis was performed. It was later decided in accordance with the Swedish advisor that only patients with a diagnosis of GBM should be selected since the primary endpoint, overall survival, differs vastly between the various grades of gliomas. This meant that only the 12 patients with GBM were selected.

4.2 Ethical considerations

The project was evaluated by the Research Ethics Committee of the Santa Paula Hospital, submitted on May 29, 2018, and approved (approval id CAAE 91035018.5.0000.5670), being appropriate and meeting the requirements of resolutions 196/96 and 466/12. Clinical data of patients enrolled in the study were collected from medical records. All patients received the same treatment as they would outside the study, and the brain samples that were analysed were part of the resections that needed to be removed. A written letter of agreement was signed by each patient.

4.3. Study period

The study began in August 2018 and ended in December 2019.

4.4. Eligibility Criteria

Patients diagnosed with GBM between 2010 and 2018 from the private Hospital Santa Paula, regardless of gender, age and comorbidity. The characterization of the type of tumor and its grade was done by a histopathological analysis according to the classification of the World Health Organization (WHO). Only Glioblastomas were accepted.

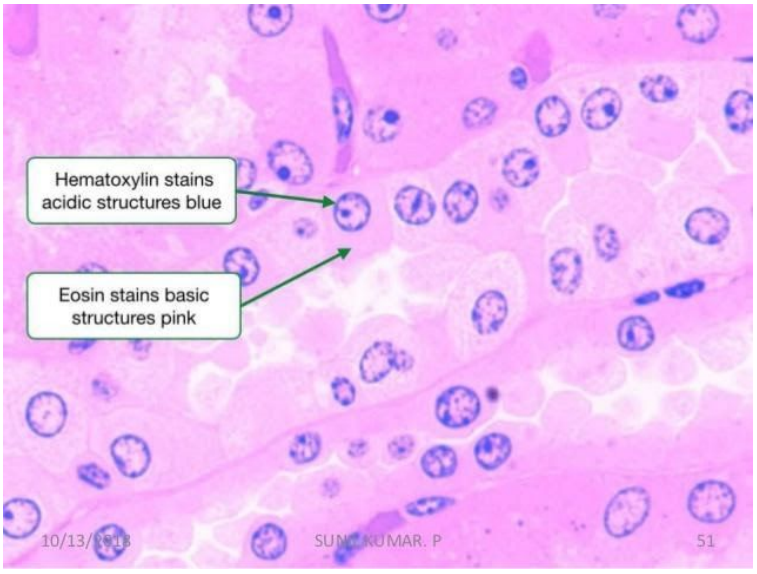
4.5. Data and sample collection

All patients underwent surgery and a tumor tissue sample was obtained. Data was collected from the medical records and from the MRIs of patients treated by the neurosurgery department of Hospital Santa Paula in August 2018.

4.6. Histopathological evaluation

This was performed by the pathology department in Hospital Santa Paula. The extracted samples were fixated in a formaldehyde 10% for 24 hours. They were then washed in running water and put in a decalcification solution. Afterwards they were embedded in paraffin wax and cut longitudinally in 4µm thick slices. Two slices were mounted on glass slides according to the Hematoxylin and Eosin (HE) staining technique for the histological analysis This stains unspecifically the nuclei of the cells blue and other tissue pink.

Image 2: Demonstration of a hematoxylin and Eosin (HE) staining from nonspecific tissue.

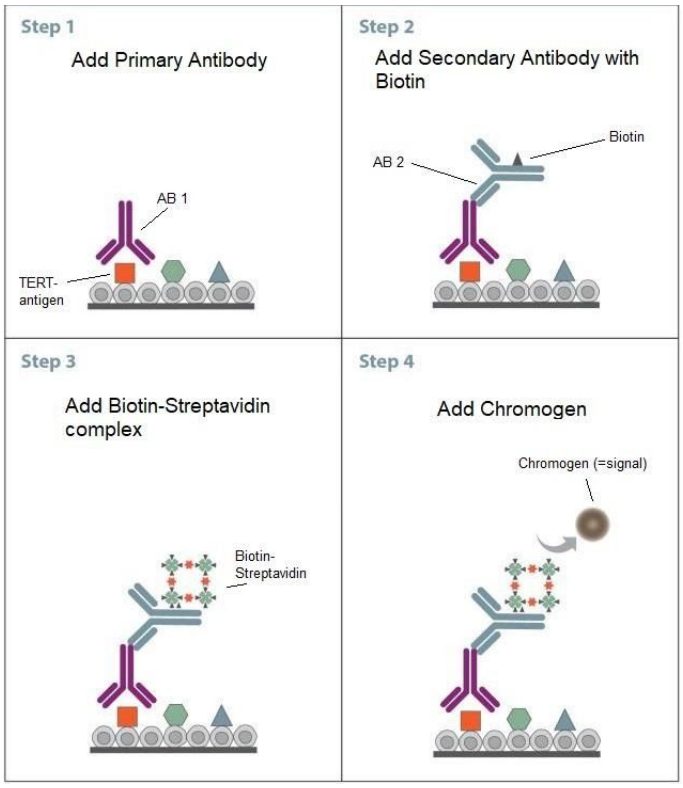


Through microscopic inspection of the slides the presence of tumor, inflammatory infiltration, necrosis and mitotic activity was estimated and the degree of malignity of the tumor could be determined according to the WHO classification of 2007 which includes a grading scheme that is a malignancy scale ranging across a wide variety of neoplasms rather than a strict histological grading system. (Louis et. al, 2007). The exact procedure and further description of the results is not available.

4.7. Immunohistochemical technique of the protein expression

Immunohistochemistry (IHC) was done at the laboratory of Clinical Analysis at FMABC. The general principle is depicted in the image below.

Figure 4: General principle of IHC.



Additional slices of the samples embedded in paraffin wax were cut with the same width, 4µm, and underwent immunohistochemistry with a specific primary antibody for TERTs, TERT (A-6) mouse monoclonal IgG2b (4 publications reference this antibody, no cited validations) from Santa Cruz Biotechnology, Inc (Figure 4, step 1). The paraffine from the slices was removed by placing them in a bath containing Trilogy IHC Pre-treatment Solution (Sigma-Aldrich Corporation®, Saint Louis, USA) for 40 minutes at 95ºC. The slices were then placed in three consecutive baths of 5 minutes each in a solution of sodium phosphate buffer (PBS), 0.05M with a pH of 7.2. The endogenous peroxidases were blocked with a 3% hydrogen peroxide 30 Vol. solution in a tank at room temperature for 40 minutes, then followed by three 5 minutes baths in PBS. Peroxide 30 Vol. means that 1 L of solution produces 30 L of O_2 via the reaction $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$. Next, incubation with the primary antibody was performed using the primary antibody diluted to the ratio recommended

by the manufacturer, 1% PBS/BSA solution (Sigma-Aldrich Corporation, Saint Louis, USA). All sections were covered with about 100µL of solution and kept in a humid chamber at room temperature for 2 hours. The slices were washed in 3 successive PBS baths for 5 minutes each. The sections were then incubated in a humid chamber at room temperature with a secondary antibody, m-IgGκ (247 publications reference this antibody, no cited validations) from Santa Cruz Biotechnology with biotin-streptavidin complex (Dako Denmark A / S®, Glostrup, DK) in the following procedure: incubation with biotin for 40 minutes; 3 successive PBS washes for 5 minutes each; and finally incubation with streptavidin for 40 minutes (Figure 4, step 2 and 3). After yet another 3 baths in PBS, the slides were developed using diaminobenzidine chromogen which should produce a brown color as a positive signal (DAB Enhancer, Dako Denmark A / S®, Glostrup, DK) (Figure 4, step 4). This solution was prepared five minutes before the end of the exposure time of the slides, pipetted over the samples and covered them for 20 minutes. The slides were then washed in 3 successive PBS baths for 5 minutes each. Counterstaining was done with Harris Hematoxylin (Sigma-Aldrich Corporation®, Saint Louis, USA) in a tank for 30 seconds. Hematoxylin is 'blued' with a weakly alkaline solution. The final dehydration process of the slides was as follows: distilled water (5 minutes), 70% ethanol (5 minutes), 80% ethanol (5 minutes), 90% ethanol (5 minutes), 100% ethanol (5 minutes)), 100% ethanol 2 (5 minutes), xylol 1 (5 minutes) and xylol 1 (5 minutes). Finally, slides were mounted with coverslip and Permount (Fisher Scientific, Pittsburgh, USA).

4.8. Determining protein expression in neoplastic tissue

This step was performed at the FMABC Clinical Analysis Laboratory. Microscopic analysis of the slides was performed with the aid of a common optical microscope, with the

aid of a 40X objective lens and a final magnification of 400X. The immunohistochemical analysis of protein expression consists of 5 photographs of different parts of each slide, one slide from each patient. With the help of three observers, including the advisor dr Paulo, the classification of the protein expression intensity in the slides. Each observer gave subjectively one or two crosses to each sample to classify them as low or high in expression. The reaction was considered positive where there was a higher intensity of purple staining the nuclei along with discreet staining of brown which signals that the DAB-chromogen (brown) has attached via the secondary and primary antibodies to the TERT-antigen, and when the expression occurred diffusely, with points of varying intensities and homogeneous distribution. Since there was no way to beforehand determine a tissue sample that undoubtedly expressed the TERT protein and could serve as a positive control, TERT (h): 293T Lysate (Santa Cruz Biotechnology, Inc) was used as a positive control to validate the primary antibody. No record of the exact procedure is available, neither is any record of a positive control of the secondary antibody. The slides were also compared to the negative control, collected from the temporal lobe during a lobectomy of an epilepsy patient (see image 3 below). The histopathological analysis also concluded the sample as void of glioma and thus was assumed to have no or very little expression of TERT which previous studies have shown is the case in normal central nervous tissue (Liu et. al, 2018). The observers were not blinded to each other.

4.9. Classification of peritumoral edema

The size of the peritumoral edemas were estimated according to Schoenegger et. al. (2009) from the MRI-scans, and classified as minor (<1 cm) and major (>1 cm) from T2-weighted images, as is illustrated in image 1. This was performed by the Brazilian advisor at Hospital Santa Paula.

4.10. Statistical analysis of the results

Initially the project analysed the samples of all of the 28 patients, even the ones with lower grade gliomas. It was later decided in accordance with the Swedish advisor, professor and neurosurgeon Bertil Rydenhag, to narrow the study to only permit glioblastomas, which meant that only 12 patients were ultimately included in the study. This however gave the study too little power for a multivariable analysis. Hence, due to the low number of samples only a descriptive analysis was performed.

5. RESULTS

The 12 samples of tissue with glioblastoma were classified according to age (in years at the moment of surgery), gender, type and grade (according to WHO), and the location of the tumor. The peritumoral edema was also classified as either minor or major. The length of survival during the study-period expressed in months after surgery was last updated in December 2019. At the end of the study-period in December 2019, the minimum time that had passed since surgery for all patients was 20 months, thus this was chosen as the censoring period. Finally, KPS was estimated for the patients, a scale that measures the patient's overall wellbeing where 100 is healthy and 0 is dead. This is summarized in the table below.

Table 3: Clinical characteristics of the patients with glioblastoma

Patient	Age	Sex	Type/ Grade	Location	Edema	Survival	KPS
1.	84	Male	ASTRO IV	Right angular gyrus and supramarginal gyrus (parietal lobe)	Major	2	-
2.	66	Male	ASTRO IV	Left middle and inferior temporal gyrus	Minor	>20	100
3.	56	Male	ASTRO IV	Left precentral gyrus (frontal lobe)	Minor	>20	80
4.	56	Male	ASTRO IV	Left angular gyrus and supramarginal gyrus (parietal lobe)	Major	4	-
8.	66	Male	ASTRO IV	Left temporal lobe	Major	>20	100
9.	66	Male	ASTRO IV	Left angular gyrus and supramarginal gyrus (parietal lobe)	Major	15	-
13.	54	Male	ASTRO IV	Right frontal lobe	Major	10	-
14.	52	Female	ASTRO IV	Basal ganglia and the left medial temporal lobe	Major	>20	60
16.	58	Male	ASTRO IV	Basal ganglia and the left medial temporal lobe	Minor	13	-
20.	58	Female	ASTRO IV	Left frontal lobe	Major	>20	70
24.	71	Male	ASTRO IV	Left temporal lobe	Major	>20	80
28.	48	Female	ASTRO IV	Left frontal lobe	Major	>20	100

The average age at the date of surgical resection was 61.2 years. Out of the 12 patients, 9 were male and only 3 were female. The location of the tumors was distributed as follows: 4

were located in the frontal lobe, 3 were located in the parietal lobe, and 5 were located in the temporal lobe. The peritumoral edemas were predominantly large: 9 were classified as major and 3 as minor. Five of the twelve patients passed away during the study and within the 20 months censoring period. The rest of the patients were followed until December 2019. KPS was only done for 7 of the 12 patients and ranged between 60 and 100. (Table 4).

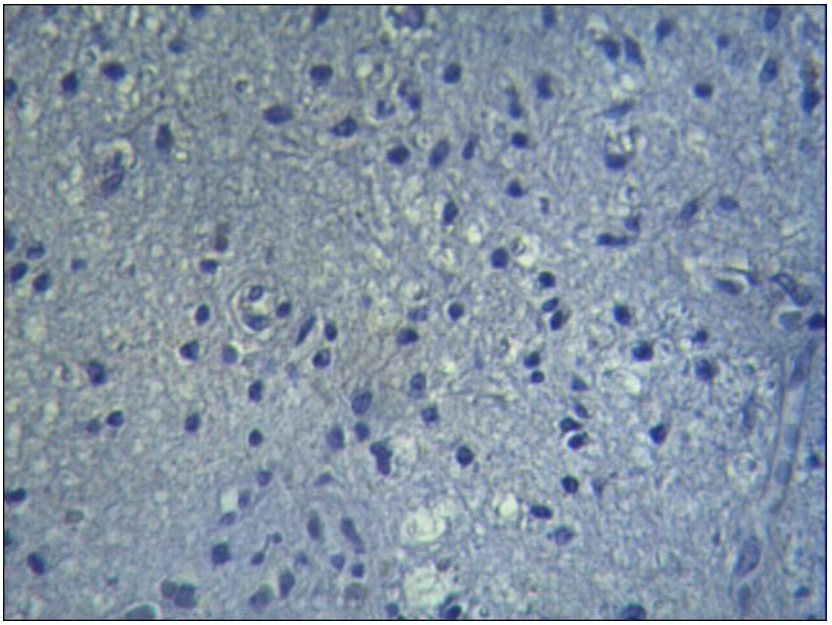
Table 4: Clinical variables from all 12 patients

Variables	Patients
Average age	61.2 years.
Sex	9 (75%) men. 3 (25%) women.
Tumor location	4 (33.3%) frontal lobe. 3 (25%) parietal lobe. 5 (41.7%) temporal lobe.
Peritumoral Edema	9 (75%) major. 3 (25%) minor.
Deceased	5 (41.7%) deceased within 20 months after resection.
KPS	Between 60 and 100.

5.1. Results from TERT protein analysis

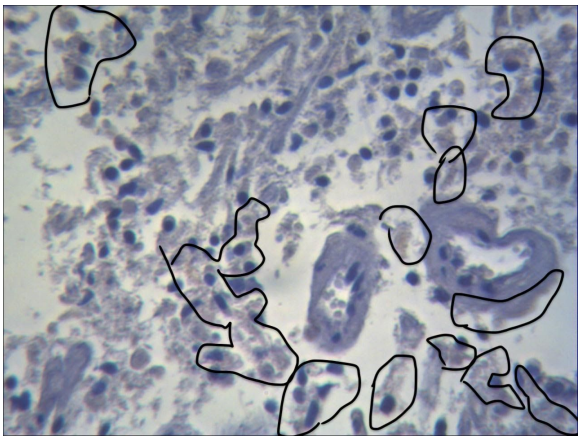
The immunohistochemical analysis of the protein expression was performed with the negative control as reference. No further reference to the positive control is found.

Image 3: Negative control of the reaction



Some of the samples (1 and 9) could not be processed and are marked as "(error)" in table 5. Among the 10 samples that could be analysed, 4 showed an elevated protein expression of TERT, as can be compared to the reference and observed in the delimited areas in the images below as a deeper purple color staining the nucleus along with staining of brown which signals that the DAB-chromogen (brown) has attached via the secondary and primary antibodies to the TERT-antigen, and is summarized in table 5.

Image 4, 5, 6, 7: Samples of tumors from patients in the study. The areas with a stronger purple from the nucleus with brown around delimited in the images below are supposed to show a higher intensity of reaction characterizing an elevated protein expression of TERT.



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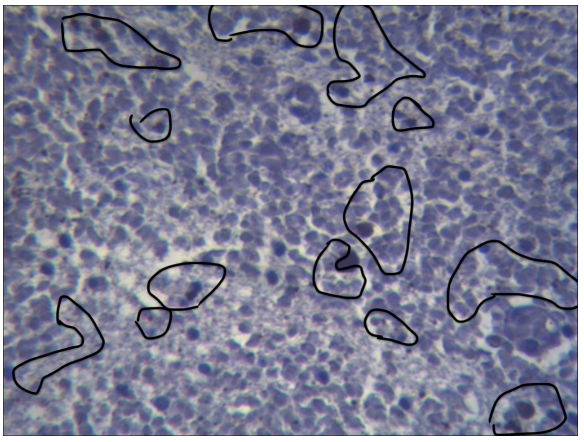

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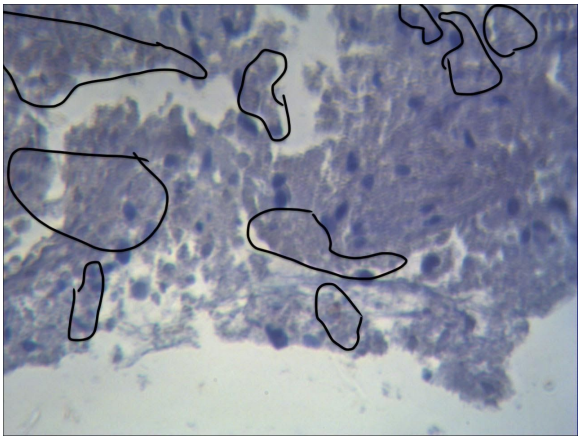

6

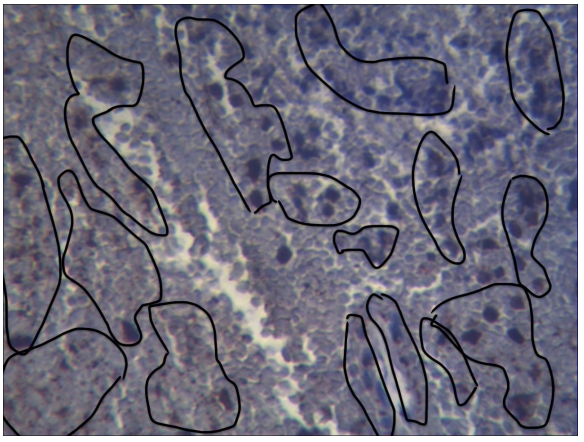

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Table 5: Table 2 updated with protein expression of TERT.

Patient	Age	Sex	Type/ Grade	Location	Edema	Survival months	Protein Expression
1.	84	Male	ASTRO IV	Right angular gyrus and supramarginal gyrus (parietal lobe)	Major	2	(error)
2.	66	Male	ASTRO IV	Left middle and inferior temporal gyrus	Minor	>20	High
3.	56	Male	ASTRO IV	Left precentral gyrus (frontal lobe)	Minor	>20	High
4.	56	Male	ASTRO IV	Left angular gyrus and supramarginal gyrus (parietal lobe)	Major	4	Low
8.	66	Male	ASTRO IV	Left temporal lobe	Major	>20	Low
9.	66	Male	ASTRO IV	Left angular gyrus and supramarginal gyrus (parietal lobe)	Major	15	(error)
13.	54	Male	ASTRO IV	Right frontal lobe	Major	10	Low
14.	52	Female	ASTRO IV	Basal ganglia and the left medial temporal lobe	Major	>20	High
16.	58	Male	ASTRO IV	Basal ganglia and the left medial temporal lobe	Minor	13	Low
20.	58	Female	ASTRO IV	Left frontal lobe	Major	>20	Low
24.	71	Male	ASTRO IV	Left temporal lobe	Major	>20	Low
28.	48	Female	ASTRO IV	Left frontal lobe	Major	>20	High

5.2. Results in patients with high TERT protein expression

The 4 patients that had a high positive expression of TERT protein was 55.5 years as compared to the average age of 61.2 years for the whole group. The gender distribution was equal with 2 women and 2 men. However, women showed a higher proportion of TERT expression than men (66.7% vs 22.2%). Half of the TERT-positive tumors were located in the frontal lobe, half in the temporal lobe, and none in the parietal lobe. Major and minor peritumoral edemas were equally common in TERT-positive tumors. However, the percentage of tumors with minor edemas that expressed TERT was higher (2 out of 3 samples, 66.7%) than for the tumors with major edemas (2 out of 9 samples, 22.2%). It should be noted that 2 of the samples from the tumors with major edemas were corrupted, so this last number could actually be higher (2-4 out of 9 samples, 22.2-44.4%). None of the four TERT-positive patients passed away during the study. However, 2 of the 5 deceased patients had their samples corrupted and could not be analysed for the presence of TERT (table below).

Table 6: Patients with high protein expression of TERT.

Variables	Patients with positive protein expression of TERT (4 patients)
Average age	55.5 (vs 61.2*) years.
Sex	2 (50% vs 75%) men. 2 (50% vs 25%) women.
Tumor location	2 (50% vs 33.3%) frontal lobe (of 4 GBMs located here). 0 (0% vs 25%) parietal lobe (of 3 GBMs located here). 2 (50% vs 41.7%) temporal lobe (of 5 GBMs located here).
Peritumoral Edema	2 (50% vs 75%) major. 2 (50% vs 25%) minor.
Deceased	0 (vs 5).

** Compared to the whole group of 12 patients.*

5.3. Results in patients with poor outcome

The average age for the 5 patients that died during the 20 months censoring period was 63.6 years as compared to the average age of 61.2 years for the whole group. These patients survived on an average for 8.8 months. The deceased were all men. The majority of the tumors in the deceased patients were located in the parietal lobe. In fact, all the patients diagnosed with GBM in the parietal lobe died, compared to 25% of the patients with tumors in the frontal lobe and 20% in the temporal lobe. The majority of the tumors (80%) had a

major peritumoral edema. This is almost the same proportion as for the entire group of patients (75%). None of the deceased patients expressed the TERT protein. Note however that the tissue samples from 2 of the deceased could not analysed as described above, and it is possible that they were positive.

6. DISCUSSION

This study contained a limited number of patients and no statistical significance can be given to any of its relationships. Hopefully however, this study and the conclusions drawn from it can be hypothesis generating and give hints to future research and how it should be improved.

In this study we concluded that in the patients with positive protein expression of TERT in GBMs, there seems to be a slight negative relationship between advanced age and intensity of protein expression. Among the patients that showed a positive protein expression of TERT, the average age was 55.5 years, compared to the average age for the whole group of 61.2 years. This could suggest that there is a higher degree of protein expression of TERT in younger patients. Also, although women are less likely to be afflicted by GBMs, they had a higher tendency to express the protein in our study. The protein expression of TERT was positive in 66.7% of the women and only 22.2 % of the men. However, the average age for these women were 50 years, whereas for the men 61 years, and as indicated above TERT seems to be expressed more in younger patients which might explain this difference. In future research a larger sample could help deduce if the actual relationship between these three factors. Also, a more careful and standardized collection of the samples is necessary as two of the samples could not be processed due to an insufficient amount of tissue.

As for any relationship between the location of the GBM and protein expression, no obvious pattern could be discerned. As for the size of the peritumoral edema, where there was

a smaller edema the expression of TERT was more frequently present than if the edema was large.

During the study, 5 of the 12 patients died within 20 the months censoring period, and on average 8.8 months after surgery. All of them were men, and they were on average 2.4 years older than the whole study group as is expected from previous studies (Fekete et. al. 2016).

None of the deceased patients expressed TERT, which is contrary to our initial assumption that a higher TERT-expression is related to a poorer prognosis as is the case with some mutated versions of TERT. This could perhaps be explained by the fact that the expression of TERT-protein seems to be higher in younger people, and old age has been shown to be correlated to poorer outcome (Stark et. al. 2012). This study also did not differentiate between mutated and unmutated TERT, neither did it analyse the existence of the mutated versions (C228T and C250T) which can co-exist and indeed tend to be associated with an upregulated level of unmutated TERT (Mausi et. al. 2018). It would have been of interest to see if any of the TERT-positive samples also sported any of these mutations despite the fact that none of these patients died. In addition to the mutated versions of TERT, the presence of mutated IDH is of particular interest as it is a recognized prognostic factor and proven to be associated with better outcome. Studies have shown that TERT promoter mutation is associated with reduced overall survival in IDH-mutant glioblastomas (Vuong et. al. 2017), and it would be interesting in future studies to see if there is a similar relationship with unmutated TERT.

All but one of the deceased had major peritumoral edema. As for the location of the tumors in the deceased patients, the majority were located in the parietal lobe. In fact, all of the patients with tumors in the parietal lobe died, as compared to 25 of the patients with GBMs in the frontal lobe, and 20% in the temporal lobe. This might suggest that GBMs located in the parietal lobe are associated with worse prognosis, perhaps due to symptoms from particularly a non-dominant parietal lobe being less obvious and more easily confused with dementia and thus leading to later diagnosis. An early study has also indicated that resections of tumors in the frontal lobe might be associated with a longer survival (Jeremic et. al. 1994), and overall tumors located supratentorially and in cerebellum are more easily accessed and removed than those located in the diencephalon or brainstem (Walid, 2008; Awad et. al, 2017).

Another weakness of the study that should be addressed in future studies is the method of visual analysis of the immunohistological samples, where using an image processing technique could assist in more accurately determine the level of protein expression. It was difficult for the untrained eye to identify the areas with more areas of deep purple staining the nuclei and brown around cells supposedly denoting accentuated TERT-protein activity. It was also difficult for the naked eye to determine what would be considered a high vs a low level of TERT-protein expression. This could be much improved by using imaging processing techniques. In addition, there was only one slide per patient, and since GBMs usually exhibit molecular heterogeneity this might have limited the ability to correctly identify areas with TERT protein expression (Parker et. al, 2015).

A lot can go wrong when analysing immunohistochemically, and the interpretation is subject to each pathologist's interpretation which studies have shown is seldom replicable (Giannini et. al, 2001). This is one of the main reasons genetic biomarkers have become more important in classifying gliomas, choose therapy strategies, and predict outcome (Szopa et. al, 2016). The actual immunohistochemical process is also prone to error if not performed perfectly, and sometimes even the datasheet with instructions that accompanies the antibodies cannot be followed blindly and preliminary tests have to be done for instance to determine the perfect concentration of the antibody. Distinguishing the positive results against the background is another problem. Monoclonal antibodies, which was chosen for this study, causes less noise as it binds more specifically to the agent, and is more easily distinguished against the background. Normally DAB-staining is colored brown which gives a quite clear contrast to the Hematoxylin-Eosin-staining, but by binding Streptavidin to the biotin attached to the secondary antibody, other colors (or chromogens), even fluorescent, can be chosen that better differentiate the positive results from the background. In this study it clearly states that DAB is used, but the positive signal is difficult to see. According to the Brazilian advisor, sometimes the the purple from the stained nuclei can almost drown out the brown from the DAB when the staining is not performed exactly as instructed and the Hematoxylin-Eosin-staining end up being too strong and the DAB-staining too weak. In this case it indeed seems there is a high background. It is also possible that the results are not positive at all, and the fact that the positive control that the study points to is no longer available makes this impossible to assess retrospectively.

Also, a problem with using biotin is that it is expressed endogenously in many cells raising the question of potential false-positive results. This is most common in the liver,

kidney and spleen, but neurons associated with the cerebellar motor system and the brainstem auditory system have also shown to express significant levels of biotin (McKay et. al, 2008). Other possible reasons for false positive results are improper handling of tissue that has left it too dry, or because the staining or incubation of the antibody lasts too long. Another of many pitfalls with IHC is that there are no universally accepted guidelines or standardized methods for determining their validity of the antibodies. The antibodies should be specific, selective, and the studies for which they are used should be reproducible. Information about validation is often lacking from the manufacturer as is also the case in this study, but the number of publications the antibody is cited in can serve as an indirect indicator of its validity (Bordeaux et. al, 2010). Another weakness of this study is the uncertainty of the primary antibody, TERT (A-6) mouse antibody IgG2b, that has only been cited in 4 publications and has no available information about validation. The secondary antibody however, m-IgGκ BP-HRP, has been cited in 247 publications giving it more credibility despite no available information about validations. Positive and negative controls of the antibodies are often not reported in publications which may lead to incorrect interpretations and irreproducible results (Hewitt et. al, 2014). There is a mention of the positive control being used for the primary antibody in this study, but the exact protocol followed is not detailed which brings into question the validity of it. Also, there is no mention of a positive control of the secondary antibody. As for the negative control, it comes from a sample that is histopathologically assumed to be devoid of cancerous cells, but as has been discussed above, histopathological evaluations are subject to the often unreproducible evaluation of the pathologist which brings into question if it is really a negative control.

Also, a more detailed inclusion criteria where relevant comorbidity is considered, as well as a more complete record of KPS, could benefit the conclusions of future studies as they are important factors for overall survival, selection of treatment and the identification of high-risk patients. It is of course important to know what actually caused the death of a patient, the glioblastoma or an unrelated condition, to avoid any false conclusions from the data. Furthermore, all patients received adjuvant radio- and chemotherapy, but detailed individual information about dosage of chemotherapy, grays administered during radiotherapy and specific method used, etc, was outside the scope of this study but could be of interest for future study. There are specific guidelines to what exact treatment should be chosen depending on the type of glioma, mutations, KPS and age of the patient, but there is no mention of such a protocol being followed in the data (Weller et. al, 2017). Neither was there information in the data about MGMT-status, which is an important factor for determining the selection of treatment and expected outcome.

Yet another great weakness was the lack of detail in the data about the selection criteria of the patients. A fairly limited number of patients (initially 28) were selected in a rather large period of time (8 years). All patients also belonged to one single private hospital which is unavailable for all but a few in a high socioeconomic group, which could mean that the results found in this study are not transferable to a normal population due to selection bias.

Perhaps the biggest weakness was that the focus of the research radically changed twice during the process. The initial project was intended to focus on the relationship between TERT promoter mutation and peritumoral edema, which was the focus of the authors bibliographical survey. When further analysing the data handed to the author, however, it

became clear that the samples had been analysed for the presence of overall TERT expression, rather than for the mutated kind. The samples also contained a large selection of patients with other types of gliomas. A paper was then written based on these findings, excluding the bibliographic survey. Finally, it was decided that since the primary objective was overall survival and patients with the various grades of gliomas have very different expected survival, the paper needed to focus solely on GBMs. These repeated changes jeopardised the integrity of the study. Since the mutations play a vital role in augmenting the expression of TERT, it would have been interesting to differentiate between mutated and unmutated expression of TERT using PCR as IHC at least in other types of cancer has been shown to be a poor method for determining the expression of TERT mutations (Paulson et. al, 2018). The study did initially try to perform a genetic analysis using PCR to determine genetic expression of TERT but the process only showed a positive result in one of the subjects, leading to the conclusion that the PCR had somehow been performed erroneously or the samples had been corrupted. The lack of results of the PCR lead the examinator to recommend it was removed from the project and it is therefore no longer present in the current version.

A strength could however be found in the fact that all the patients were treated by the same surgeon, thus minimizing the variability in quality of treatment due to differences in the skills of the surgeons. Another strength was the length that the patients were followed, a minimum of 20 months which is well beyond the estimated length of survival for the vast majority of patients with GBM.

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APPENDIX A

ACTIVITIES DURING STUDY-PERIOD CONTRIBUTING TO THE PROFESSIONAL DEVELOPMENT OF THE AUTHOR

Of the full study period the author spent 3 months in Brazil analysing the data from the study and working on this paper, as well as engaging in many activities pertinent to the qualitative objectives detailed below.

1. Qualitative objectives of study

To gain clinical experience from assisting experienced neurologists and neurosurgeons in assessing a great number of patients with wide variety of neurological conditions, and specifically pertinent to this study, identify patients with brain tumors.

To learn about the neurosurgical procedures from some of the most experienced neurosurgeons in Brazil and assist in a large number of different types of brain surgeries. Of specific interest for the project was oncological brain surgery.

To increase theoretical knowledge in the fields of neurology, neurosurgery and neuro-oncology.

To expand my knowledge in different methods of research, and if possible, to get involved in other research projects related to neurology and neurosurgery.

Networking, to get to know as many people as possible in the field of neurology and neurosurgery in Brazil as possible.

2. Activities relevant to the qualitative objectives

Mondays and fridays I would spend in an office located in the University Pontificia Universidade Catolica and the adjacent Hospital Santa Lucinda in Sorocaba, Brazil, where the advisor, neurosurgeon and professor Paulo Henrique de Pires Aguiar, also held lectures for the interns in neurology and neurosurgery. Here I would summarize and analyse the data from the patients in the study, do further research in PubMed, read from the book "Principios Tecnicos de Neurocirurgia, Atlas e texto" written by the advisor, watch a vast library of videos of surgical procedures done by the advisor, and write on the thesis. In addition, I was allowed to attend many of the lectures in neurology and neurosurgery, for instance "Neurosurgical treatment of pain" and "Neurotrauma - clinical applications". I shared an office with a research assistant, biologist and neuropsychologist, Lucia Canaberra, that coordinated many of research projects at the university, and who also set up the various experiments done on cadavers or laboratory rats in the adjacent rooms. Lucia went to great lengths to teach me about the various techniques that they used in their research, and to present and even include me in various research projects. Particularly memorable was a study about vasospasm (a condition utterly important for anybody dealing with neurosurgery) where

I was taught the use of stereotaxy to induce subarachnoid haemorrhage in a mouse brain and then measure the level of vasospasm using a transcranial doppler. Another clinical research project involved measuring various structures of the brain using precision tools on cadaver's brains, which was a very educative addition to my previous anatomy studies. All these experiments had some connection to this project, for instance by increasing the knowledge of anatomy and pathophysiology, to learn how to identify and deal with differential diagnosis, and to be able to handle various neurological complications. It also allowed me ample opportunities to network and to get to know very well the various departments at the university and the adjacent hospital with its staff. There was also a 3-day course in endoscopic surgery arranged by Johnson & Johnson and Dr Burgesi, a surgeon at Hospital Santa Lucinda, that I attended.

Tuesdays, Thursdays and Saturday mornings I spent in surgery in various hospitals in São Paulo, particularly Hospital Santa Paula and Hospital Sírio-Libanês. The patients mainly had tumors of various types, but other conditions such as for instance aneurysms, herniated discs and spinal stenosis were also treated. Most of these surgeries were performed by the advisor dr Paulo, dr Marcos Perocco or dr Marcos Vinicius. When circumstances would not allow surgery on one of these days in Sao Paulo, I would be invited to other types of surgery in the Hospital Santa Lucinda in Sorocaba. This would often involve craniofacial plastic surgery with dr Décio Portello.

A couple of Wednesdays I was privileged to spend at dr Paulos doctor's clinic in São Paulo, adjacent to both Hospital Sírio-Libanês and Hospital Santa Paula, where he and dr Marcos Perocco attended patients with all types of neurological afflictions. This allowed me

invaluable knowledge in taking anamneses, physical status and differentiating between neurological conditions, and especially identify clinical signs of patients with tumors. On a normal day each doctor would attend 15 patients or more. To illustrate the variety of patients, the diagnosis from my notes of one single day are listed as follows: hemangioma due to von Hippel-Lindau syndrome; rhizotomy for nerve pain from cervical arthrosis; neuropathic pain after medullary infarction; implantation of occipital nerve stimulation; occipital trauma; cavernoma; cholesteatoma and surgery of n. facialis; nerve grafting of n. axillaris; paraplegic after motorbike injury; subdural hematoma; aneurysm clip; stroke cerebellum; lumbago; headache after trauma; lumbar spinal fusion; spinal stenos; brain metastasis of type adenocarcinoma originating from the colon; herniated lumbar disc. On some occasions I was allowed to partake in conferences between neurosurgeons at Hospital Sírio-Libanês that was just across the street, and listen to them giving lectures about different patients of particular educational interest, for instance lectures "Neuro-oncology, meningiomas and intraventricular tumors", and "Correlation between neuroanatomy and radiology".

I also spent some of these days in FMABC in Sao Paulo observing the various research techniques described below under guidance of the advisor dr Paulo and his pupil, Anne Martinez. Because of security reasons I was not allowed there alone and could only go there on a couple occasions with the advisor and his bodyguard in his armoured car. The security-issues limited considerably my ability to move around, and I myself unfortunately became a neurology patient for a short time at the Hospital Sírio-Libanês after being assaulted and suffering head-trauma, which accounted for a period of 2 weeks of limited academic activity. At FMABC I made several contacts, in particular professor in neurology Marco Pristi and professor in statistics Luiz Carlos de Abreu.

In addition to these and other activities organized by the very enthusiastic and welcoming advisor dr Paulo was a 3-day neurosurgical conference in Curitiba, Brazil, in the beginning of November 2019. This involved ample opportunity for networking and a full schedule of lectures, most notably “Epilepsy surgery” by the advisor dr Paulo, “Endoscopic endonasal surgery of pituitary adenomas” by dr Apio Claudio Martins Antunes, and “Intracranial hypertension” by dr Carlos Tadeu Parisi de Oliveira, all of whom I had the opportunity to meet and converse further on these and other topics related to neurosurgery.

As for the selection of patients and samples, originally 28 patients with gliomas were selected and I wrote a paper that focused on all these patients. It was later decided in accordance with the Swedish advisor, professor and neurosurgeon Bertil Rydenhag, that only the 12 patients with a diagnosis of GBM should be selected so I rewrote the original paper. All patients underwent surgery and a tumor tissue sample was obtained prior to my arrival in Brazil. In later sessions of surgery of other neuro-oncological patients I observed several surgeries of gliomas and the collection of samples.

As for the data and sample collection, this was collected by the advisor upon one of the various stays at the hospital and handed to me for analysis as I was not granted direct access to the medical records due to hospital policy.

As for the histopathological evaluation, this was performed by the pathology department in Hospital Santa Paula prior to my arrival in Brazil. I was not able to assist in this procedure for future patients that I had later assisted surgery for due to the policy of the

private Hospital Santa Paula, and the procedure had to be detailed by the advisor.

As for the immunohistochemical technique of the protein expression, this was done at the laboratory of Clinical Analysis at FMABC prior to my arrival. Upon arrival, I was shown the procedure on other samples at FMABC by the advisor and staff.

As for determining protein expression in neoplastic tissue, this step was performed at the FMABC Clinical Analysis Laboratory. This had been done prior to my arrival. Upon arrival the photos of each sample were handed over to me in a session where one of the advisor's pupils, Anne Martinez, explained the results.

As for the classification of peritumoral edema, this was performed by the Brazilian advisor before I arrived in Brazil. The results were passed on to me, and the methodology was described during a session with the advisor at Hospital Santa Paula where I could observe a selection of MRI-images.

As for the statistical analysis of the results, initially the project analysed the samples of all of the 28 patients, even the ones with lower grade gliomas, and a multivariable analysis relating poor outcome to the two independent factors TERT-expression and size of the peritumoral edema was performed by me. The sample was then 28 patients which allowed sufficient power for two independent factors. I wrote a thesis based on these results, but it was later decided in accordance with the Swedish advisor to narrow the study to only permit glioblastomas, which meant that only 12 patients were ultimately included in the study. This however gave the study too little power for a multivariable analysis. Hence, due to the low

number of samples only a descriptive analysis was performed.

The study did initially try to perform a genetic analysis using PCR to determine genetic expression of TERT but the process only showed a positive result in one of the subjects, leading to the conclusion that the PCR had somehow been performed erroneously or the samples had been corrupted. The lack of results of the PCR lead the examinator to recommend it was removed from the project and it is therefore no longer present in the current version. This was done prior to my arrival in Brazil and was later summarized to me by the advisor.

The author also spent august and September 2019 doing a bibliographical survey in Gothenburg, Sweden, detailed in Appendix B.

APPENDIX B

BIBLIOGRAPHIC SURVEY

In the bibliographic survey that was done in the online database PubMed, articles that showed any relationship between TERT promoter mutations and/or peritumoral swelling as possible prognostic factors in patients with GBMs were chosen. Only articles published between 2008 and 2019 in English were considered, with a minimum number of subjects in the study being 100, and an impact factor 1.0 or above. As the study subsequently ended up not differentiating between mutated and unmutated TERT, the bibliographical survey was added as an Appendix rather than integrated into the study itself.

Table 1: Bibliographic survey

Article / Author	**Year**	**Conclusion**
TERT promoter mutations and polymorphisms as prognostic factors in primary glioblastoma Mohamed Ali Mosrati	2015	Mutation of the TERT promoter was associated with a lower length of survival (11 vs. 20 months and 12 vs. 20 months for the mutation C228T and C250T, respectively).

Combined analysis of *TERT*, *EGFR*, and *IDH* status defines distinct prognostic glioblastoma classes Marianne Labussière	2014	The mutation of the TERT promoter was associated with a bad prognosis, with an average survival of 13,8 vs 37,6 months, when IDH also was mutated. When IDH was not mutated the difference was 13,7 vs 17,5 months.
TERT promoter mutations in primary and secondary glioblastomas Naosuke Nonoguchi	2013	TERT mutations had no significant impact on patients' survival in multivariate analysis after further adjusting for other genetic alterations, or when primary and secondary glioblastomas were separately analysed. These results suggest that the prognostic value of TERT mutations for poor survival is largely due to their inverse correlation with IDH1 mutations, which are a significant prognostic marker of better survival in patients with secondary glioblastomas.
Prognostic quality of activating TERT promoter mutations in glioblastoma: interaction with the rs2853669	2015	Seventy-three percent of GBM patients harbored TERT promoter mutations. Patients with mutated tumors exhibited significantly shorter overall survival in the entire cohort (11.5 vs 23.1 months) and in the primary GBM patient subgroup lacking IDH1 mutations. This prognostic impact was confined to

polymorphism and patient age at diagnosis Sabine Spiegl-Kreinecker		younger patients (aged <65 years), while the negative prognostic power of enhanced age at diagnosis was limited to those patients lacking TERT promoter mutations. Presence of the common single nucleotide polymorphism rs2853669, disrupting an endogenous Ets2 transcription factor-binding site, was associated with improved survival exclusively in patients with a wild-type TERT promoter. On the contrary, the shortest mean overall survival was detected in those patients harbouring both an activating TERT promoter mutation and homozygous rs2853669 alleles.
TERT mutation in glioma: Frequency, prognosis and risk Yang Yuan	2016	Meta-analysis: Showed that the TERT gene is a valuable prognostic and predictive biomarker of glioma, and TERT gene polymorphisms are significantly associated with an increased risk of glioma. The study showed that a mutation in the TERT promoter is associated with a worse prognosis in patients with gliomas.
TERT promoter mutations: a novel independent	2014	The presence of *TERT* mutations was associated with poor overall survival, and the effect was confined to the patients who did not carry the variant G-allele for

prognostic factor in primary glioblastomas Matthias Simon		the rs2853669 polymorphism. An exploratory analysis suggested that *TERT* mutations might be prognostic only in patients who had incomplete resections and no temozolomide chemotherapy.
Mutations in *IDH1*, *IDH2*, and in the *TERT* promoter define clinically distinct subgroups of adult malignant gliomas Patrick J. Killela	2014	Mutations in the *TERT* promoter occurred in 74.2% of glioblastomas (GBM). Patients whose Grade III-IV gliomas exhibit *TERT* promoter mutations alone predominately have primary GBMs associated with poor median OS (11.5 months).
Expression level of *hTERT* is regulated by somatic mutation and common single nucleotide polymorphism at promoter region in glioblastoma Chul-Kee Park	2014	The somatic mutations of TERT promoter C228T and C250T were encountered in 60.4% of the GBMs and a single nucleotide polymorphism (T349C) in the hTERT promoter region was found in 66,6% of the GBMs. The somatic mutations were associated with a higher expression of TERT than the SNP T349C. There was no significant difference in the survival of the patients with the various types of mutations.

TERT promoter mutations and long telomere length predict poor survival and radiotherapy resistance in gliomas Ke Gao	215	Coexisting *TERT* promoter mutations and long telomere length were more commonly associated with poor patient survival than they were individually.
Molecular Pathways in Gliomagenesis and Their Relevance to Neuropathologic Diagnosis Christina L. Appin	2015	Primary GBMs typically lack IDH mutations and are instead characterized by EGFR, PTEN, TP53, PDGFRA, NF1, and CDKN2A/B alterations and TERT promoter mutations. Tumors without the IDH mutation were more aggressive and had a genetic profile more similar to glioblastomas.
Upregulating mutations in the *TERT* promoter commonly occur in adult malignant gliomas and are strongly associated with total 1p19q loss	2013	Found a high incidence of mutually exclusive mutations located at two hot spots, C228T and C250T, in all subtypes of gliomas (55 %). The expression level of *TERT* in tumors carrying those mutations was on average 6.1 times higher than that of wild-type tumors, indicating that the mutated promoter leads to upregulation of *TERT*. *TERT* promoter mutations were observed in almost all tumors harboring concurrent total 1p19q loss and *IDH1/2* mutations (98 %).

Hideyuki Arita		Otherwise *TERT* promoter mutations were mostly observed among *IDH* wild-type tumors. Most *EGFR* amplifications (92 %) were also associated with *TERT* promoter mutations.
The prognostic impact of TERT promoter mutations in glioblastomas is modified by the rs2853669 single nucleotide polymorphism Rui Batista	2016	The rs2853669 SNP, located in the TERT promoter region, is reported to modulate the increased TERT expression levels induced by the recurrent somatic mutations. TERT promoter mutations are a major indicator of poor survival in GBM, independently from other risk factors such as age and Karnofsky Performance Status. However, poor prognosis conferred by TERT promoter mutations was restricted to GBM patients carrying the rs2853669 A allele and not in those carrying the G allele.
TERT Promoter Mutations Lead to High Transcriptional Activity under Hypoxia and Temozolomide Treatment and	2014	*TERT* promoter mutations were specific to gliomas. *TERT* promoter mutations maintained its ability of inducing high transcriptional activity even under hypoxic and TMZ treatment conditions, and the presence of mutations was associated with poor prognosis in glioma patients.

Predict Poor Prognosis in Gliomas Chen Chen		
Brain regions associated with telomerase reverse transcriptase promoter mutations in primary glioblastomas Xing Fan	2016	*TERT* promoter mutations were detected in 46.3 % of the patients. GBMs with *TERT* promoter mutations were much more likely to locate in the right temporal lobe, while those with wild-type *TERT* promoters were more likely to occur in the anterior region of the right lateral ventricle. No significant difference was found in the lesion volumes of the tumor or in the contrast enhancement areas between the two groups. Also, the size of the peritumoral edema did not correlate to the mutation of the TERT promoter. 75.5% of the tumors that had the mutation also had a peritumoral edema larger than 1 cm, whereas 75.2% of the tumors without the mutation also showed had peritumoral edemas of similar size.
Recurrent TERT promoter mutations identified in a large-	2015	*TERT* promoter mutations were highly frequent in glioblastoma (83.9%). C228T and C250T were the most common mutations. *TERT* promoter mutations

scale study of multiple tumour types are associated with increased TERT expression and telomerase activation Dong-Sheng Huang		highly correlated with upregulated *TERT* mRNA expression and telomerase activity in adult gliomas.
TERT promoter mutation designates biologically aggressive primary glioblastoma Jason T. Huse	2014	Found a high rate of TERT mutations in their cohort (76.6%). Moreover, TERT mutations were much more common in primary versus secondary GBMs and were inversely correlated with *IDH1/2* mutations. The largest subset of lower-grade gliomas harbors virtually no *TERT* mutations and is instead highly enriched in *ATRX* mutations and the alternative lengthening of telomeres (ALT) phenotype, a telomerase-independent telomere maintenance mechanism.
TERT promoter mutated WHO grades II and III gliomas are located preferentially in the	2015	Grades II and III gliomas harboring TERT promoter mutation were located preferentially in the frontal lobe and rarely in midline. Tumors in the frontal lobe presented more frequently a TERT promoter mutation. Mutations of the TERT

frontal lobe and avoid the midline Ze-Lin Sun		promoter combined with IDH mutations were more frequently found in the frontal lobe as well. In comparison, tumors in the midline had the least frequency of both TERT promoter mutations as well as the combination of TERT promoter mutations and IDH mutations.
TERT promoter mutations occur frequently in gliomas and a subset of tumors derived from cells with low rates of self-renewal Patrick J. Killela	2013	TERT-high tumor types almost always originated in tissues with relatively low rates of self-renewal. Mutations of the telomere binding proteins alpha thalassemia/mental retardation syndrome X-linked (ATRX) has been shown to underlie a telomere maintenance mechanism not involving telomerase. TERT and ATRX mutations were mutually exclusive, suggesting that these two genetic mechanisms confer equivalent selective growth advantages.
Distribution of *TERT* promoter mutations in pediatric and adult tumors of the nervous system Christian Koelsche	2013	TERT promoter mutations were strongly associated with older age. The highest frequency of mutations was found in gliosarcomas (81%), oligodendrogliomas (78%), primary glioblastomas (54%), and fibrous tumors (50%).

		TERT promoter mutations were strongly associated with 1p/19q loss but inversely associated with loss of ATRX expression and IDH1/IDH2 mutations.
TERT promoter mutations in gliomas, genetic associations and clinico-pathological correlations M Labussie`re	2014	*TERT* promoter mutation identified in 60.8% of gliomas was globally associated with poorer outcome, as well as a higher expression of TERT mRNA, whereas a SNP rs2853669 was associated with a lower expression of TERT mRNA. The mutation of *CIC* (a repressor of ETV1-5 belonging to the Ets/TCF family) was also associated with *TERT* mRNA upregulation.
Highly prevalent *TERT* promoter mutations in bladder cancer and glioblastoma Xiaoli Liu	2013	Found highly prevalent *TERT* promoter mutations in bladder cancer, bladder cancer cell lines and glioblastoma. C228T was far more common than C250T in all cases. The two mutations were mutually exclusive in glioblastomas.
TERT promoter mutations could indicate poor	2014	In tumors without mutation in the TERT promoter, an amplification in EGFR is associated with a worse outcome. A mutation in IDH1 meant a longer survival

prognosis in glioblastoma *Hemi Malkki*		only in tumors that did not have a mutation in the TERT promoter as well.
TERT promoter mutations and rs2853669 polymorphism: prognostic impact and interactions with common alterations in glioblastomas Umberto Nencha	2015	TERT promoter mutation, but not SNP rs2853669 status, was associated with older age (61.4 vs. 52.8 years). The TERT promoter mutation was associated with EGFR amplification chromosome 10q loss, CDKN2A deletion and IDH wt. EGFR amplification was associated with a better outcome in TERT promoter mutated GBM, and a worse outcome in TERT promoter WT.
Promoting a new brain tumor mutation: *TERT* promoter mutations in CNS tumors Zachary J. Reitman	2013	Primary GBMs are known to have a high TERT activity, whereas in secondary GBMs this enzyme is rarely active. It was also noticed that a subgroup of the adults with primary GBMs who have telomerase activation do not have a mutation of the TERT promoter, raising the question of what other genetic or epigenetic mechanisms could be responsible for the activation of telomerase in these tumors.

The TERT promoter mutation status and MGMT promoter methylation status, combined with dichotomized MRI-derived and clinical features, predict adult primary glioblastoma survival Jinhuan Wang	2018	TERT promoter mutations were observed in 66.42% of primary GBMs in adults. MRI-detected necrosis is positively associated with the TERT promoter mutation status. The worst prognosis for survival was associated with TERT promoter mutation in combination with elevated peritumoral edema.
Association of Telomerase Reverse Transcriptase Promoter Mutations with the Prognosis of Glioma Patients: a Meta-Analysis Xiaogang Wang	2015	Meta-analysis of total 11 studies suggested that TERT promoter mutations were significantly associated with worse prognosis of patients with gliomas. In the article, a meta-analysis was conducted of 9 articles, where it was concluded that the mutation were independently associated with a worse prognosis. In conclusion, the mutation of the TERT promoter is a promising biomarker for predicting the prognosis in patients with gliomas.

Clinical Characteristics and Prognostic Significance of TERT Promoter Mutations in Cancer: A Cohort Study and a Meta-Analysis Ping Yuan	2016	Mutation in the TERT promoter was in this meta-analysis associated with metastasis, a higher grade of glioma, and e higher risk of death.
Elevated TERT Expression in TERT-Wildtype Adult Diffuse Gliomas: Histological Evaluation with a Novel TERT-Specific Antibody Kenta Masui	2018	Found an unexpected increase in TERT expression in *TERT*-wildtype as well as *TERT*-mutated gliomas and in tumor vasculature. This suggest that TERT protein expression may be regulated by several mechanisms in addition to its promoter mutation.

From this survey a systematic review was conducted with the objective of studying the relationship between mutations in the TERT promoter in high grade glioblastomas (WHO IV - glioblastomas) and the size of peritumoral edemas as a possible prognostic factor.

Of the selected articles, 13 (46,6%) showed a shorter survival for patients with GBMs where mutations in the TERT promoter were present, and out of these, 2 articles showed a shorter survival for patients with the mutation independent of the site of the mutation (C228T or C250T). Only 2 articles (7,1%) showed that the status of TERT had no prognostic value.

Moreover, 2 of the selected articles showed evidence of a shorter survival when TERT promoter mutations were present even when IDH was mutated as well. In the absence of TERT promoter mutations, the results diverge however. Jason T. Huse et al, show in their article "TERT promoter mutation designates biologically aggressive primary glioblastoma" that a mutation in IDH1/2 is associated with a worse prognosis in patients with GBMs, but Hemi Malkki et al., come to the opposite conclusion in their article "TERT promoter mutations could indicate poor prognosis in glioblastoma", saying that the presence of a mutation in IDH1 and the absence of mutation in TERT promoter is related to longer survival.

In 2 articles, the presence of both TERT promoter mutation and SNP of rs2853669 where the polymorphism is situated in the A allele, is associated with a worse prognosis. However, Umberto Nencha et al., in their article "TERT promoter mutations and rs2853669 polymorphism: prognostic impact and interactions with common alterations in glioblastomas" show that there is no difference in survival in patients with polymorphism in the A or G allele, independently of the status of the TERT promoter.

As for any relationship between age and the mutation of TERT promoter, Sabine Spiegl-Kreinecker in the article “Prognostic quality of activating TERT promoter mutations in glioblastoma: interaction with the rs2853669 polymorphism and patient age at diagnosis” showed that the mutation of TERT promoter was more accurate as a prognostic biomarker in younger patients, below 65 years. Christian Koelsche et al., in their paper “Distribution of TERT promoter mutations in pediatric and adult tumors of the nervous system” as well as Umberto Nencha et al., in their paper “TERT promoter mutations and rs2853669 polymorphism: prognostic impact and interactions with common alterations in glioblastomas” state that mutations in TERT promoter is strongly correlated with older age.

There were also contradictions regarding the location of tumors with the highest presence of TERT promoter mutations. Xing Fan et al., in their study “Brain regions associated with telomerase reverse transcriptase promoter mutations in primary glioblastomas” found a higher presence of mutations in tumors located in the right temporal lobe, whereas Ze-Lin Sun et al., in their article “TERT promoter mutated WHO grades II and III gliomas are located preferentially in the frontal lobe and avoid the midline” found the mutation more present in tumors in the frontal lobe.

As for the expression of TERT 2 articles found that both patients with and without a mutation of the TERT promoter had a higher expression of TERT.

As for the results regarding the size of the peritumoral edema the were some contrary conclusions. In the article by Xing Fan et al., “Brain regions associated with telomerase reverse transcriptase promoter mutations in primary glioblastomas”, no relationship was found between the size of the edema and the presence of a mutation in the TERT promoter.

However, Chang Shu et al., in their article “The TERT promoter mutation status and MGMT promoter methylation status, combined with dichotomized MRI-derived and clinical features, predict adult primary glioblastoma survival”, did find such a relationship. Particularly when comparing tumors with and without a mutation in the TERT promoter, the peritumoral edema was twice as large in tumors with the mutation. Also, the results from the study show that a lower rate of survival was found in patients with the mutation and a larger edema, and the patients with the longest survival without the mutation were young patients.

Finally, in the article by Chang Shu it is concluded that patients that showed the largest peritumoral edema on their MRI, and had a mutation in the TERT promoter, detected post-surgically in the biopsy of the tumor, were the ones with worst prognosis and had the highest mortality rate, as is shown in the figure below from Chang Shu’s article.

Figure 1: Overall survival related to size of the peritumoral edema and mutation of the TERT promoter.