THE USE OF KI-67MMUNOHISTOCHEMISTRY IN THE CLASSIFICATION AND GRADING OF CENTRAL NERVOUS SYSTEM (CNS) TUMORS IN TANZANIA

Francis Zerd, MD

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By

Francis Zerd,

Report Submitted in Partial Fulfillment of the Requirement for the Degree of Master of Medicine (Anatomical Pathology) of the Muhimbili University of Health and Allied Science

October, 2020

CERTIFICATION

The undersigned certify that, she has read and hereby recommend for acceptance by Muhimbili University of Health and Allied Sciences a dissertation entitled: **"*The use of Ki-67 Immunohistochemistry in the classification and grading of central nervous system (CNS) tumors in Tanzania*"**, in (partial) fulfillment of the requirements for the degree of Master of Medicine (Anatomical Pathology) of the Muhimbili University of Health and Allied Sciences.

_____

Dr Edda Vuhahula

(DDs, PhD, FC – Path ECSA)

Supervisor

_____

Date

DECLARATION AND COPYRIGHT

I, Dr Francis Zerd, declare that this **dissertation** is my own original work and that it has not been presented and will not be presented to any other University for a similar or any other degree award.

Signature: _____ **Date:** _____

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DEDICATION

To my son, Ethan

To my father, Zerd Kilenga

To my mother, Genoveva Zerd

To my sister, Selestina Zerd

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LIST OF ABBREVIATIONS

CNS	Central Nervous System
DAB	3, 3'-Diaminobenzidine
DPX	Distyrene Plasticizer Xylene
H&E	Haematoxylin and Eosin
HRP	Horseradish Peroxidase
IHC	Immunohistochemistry
IRB	Institutional Review Board
kDa	kilo Daltons
LI	labelling index
MNH	Muhimbili National Hospital
MUHAS	Muhimbili University of Health and Allied Sciences
OS	Overall survival
REDCap	Research Electronic Data Capture
SSA	Sub-Saharan Africa
TBS	Tris-Buffered Saline
TMA	Tissue Micro Array
USA	United States of America
WHO	World Health Organization

ABSTRACT

Background:

The 2016 World Health Organization classification for central nervous system tumors includes assessment of molecular markers along with histology. As a consequence, some new diagnostic entities have been added, but on the other hand, variants and patterns that have no diagnostic and/or biological relevance are no longer recognized. However, this classification cannot absolutely be implemented without modification in majority of low-and middle-income countries such as Tanzania because of unavailability of molecular testing such as isocitrate dehydrogenase wild and mutant types which are among of essential parameters for the classification. Therefore, in resource constrained countries like Tanzania, an additional simple and affordable method of grading the central nervous system tumors is inevitable to compliment the current classification for prognostication purposes and for planning of appropriate patient's management. Conversely, the immunoreactivity of Ki-67 protein has been associated with the proliferation of cell populations in various tumors permitting it to be used as a marker of tumor aggressiveness. This study aims at demonstrating the applicability of Ki-67 labelling index as an added tool to support the 2016 World Health Organisation classification of Central Nervous System tumors in centers where facilities for molecular testing are lacking.

Objective:

To evaluate the use of Ki-67 immunohistochemistry in the classification and histological grading of central nervous system tumors.

Materials and methods:

This was a retrospective cross-sectional study where all central nervous system tumors diagnosed at Muhimbili National Hospital between June 2009 and June 2019 were studied. All cases with retrievable patient information as well as anatomical location were included. Each tumor was reviewed histologically and classified according to 2016 WHO grading system. Ki-67 immunohistochemistry was done by utilizing tissue micro-array method where the most hyper-cellular area with minimal or no necrosis was sampled for tissue microarray processing.

Ki-67 labelling index was analyzed by choosing an area with high proliferation index as assessed by Ki-67 positive tumor cells with nuclear stain counted in 100 tumor cells in a continuous manner. The number of positive cells was considered to be the Ki-67 labelling index of the case, where a <5% was considered low proliferation corresponding to grade I tumors, 5% and above was considered high proliferation, however it was categorized at different levels correlating with different grades; 5-9% grade II, 10-20 grade III and >20% grade IV respectively.

Results:

A total of 141central nervous system tumor cases were enrolled in this study. There were 77 (54.6%) males and 64 (45.6%) females with male to female ratio of 1.2:1. The age ranged from 1 to 77 years with overall mean age of 37.6 $\pm$ 18.8 years, and median of 40 years. Children below 10 years old contributed to 11.3% of all cases. The frontal lobe was mostly affected consisting of 26/141 (18.4%) cases, followed by temporal lobe 19/141 (13.5%) and dura-based lesions 16/141 (11.3%). About 55.3% (78/141) of all tumors were meningiomas of which grade I comprised of 85.9% (67/78), followed by astrocytic tumors 22.7% (32/141) and ependymal tumors 4.9% (7/141). About 64.5% tumors were grade I histologically, 22.7% grade II, 0.7% grade III and 12.1% grade IV, however Ki-67 LI index showed grade I tumors to be 52.4% with a KI-67 LI range of 0 – 8%, grade II, 31.4% with Ki-67 LI ranging from 3% – 14%, grade III composed of 6.4% with Ki-67 LI range from 7% - 28% and grade IV 9.3% with Ki-67 LI of 14% - 90%. There was about 22.2% of grade I tumors with higher proliferation index, furthermore about 71.4% of all primary tumors which initially was diagnosed to be grade I histologically had a Ki-67 LI of 5-9% whereas 85.7% of their counterpart recurrent tumors had a Ki-67 LI of 5-9% and they were all grade I histologically.

Conclusion:

Ki-67 labelling index has the potential of detecting grade I tumors with aggressive behaviors and therefore subjecting these patients to a more appropriate treatment hence significantly reducing the risks of recurrence and improving the overall patient survival. Furthermore, the study showed that in Tanzania the most common central nervous system tumors are meningiomas followed by astrocytomas. The most common affected region was

supratentorial with frontal lobe leading as the most affected anatomical site. This is to our knowledge the first study in Tanzania to analyze all central nervous system tumor types involving patients of all age groups consequently forming a baseline ‘data and a reference for further epidemiological and clinico-pathological studies in the country and the region at large.

CHAPTER ONE

1.1 Introduction

1.1.1 Background

For years, the histogenesis and classification of CNS tumors have been almost exclusively an area of surgical pathologists whose main focus was the establishment of clinic pathological correlations which would help in guiding decision-making concerning adjuvant radiotherapy or chemotherapy and allow prognostication of these tumors. In recent years, CNS tumors have become a focus of medical research. In order to assure reproducible correlations between phenotypic and genetic alterations occurring during tumour initiation and progression, a reliable, morphology-based classification is of paramount importance (1).

As in other tissues, the classification of CNS tumors is based on histogenetic principles, i.e., the medical terminology relates to their cell of origin while recognizing that this approach may, in some occasions, it may represent an oversimplification. Central nervous neoplasms display a wide spectrum of different histopathological features which in turn are associated with greater differences in incidence, location, preferential age of affected patients, clinical course, and clinical outcomes. Substantial progress has been made towards a globally accepted classification of tumors of CNS since this is a prerequisite for the comparative evaluation of international treatment trials. The histopathological classification proposed by the WHO is generally recommended and widely used as a reference, and this will be our study classification reference (2).

Tumors arising from the same cell of origin may exhibit significant variation in biologic behavior, ranging from a slowly growing semi benign lesion to a highly malignant tumor rapidly invading surrounding brain tissue. This tendency for malignant progression is particularly evident in diffusely infiltrating astrocytomas. Important indicators of tumor anaplasia in CNS tumors include cytological atypia, mitotic activity, high cellularity, vascular proliferation, and necrosis (3).

The magnitude of these individual features varies considerably in different tumor types. For example, nuclear atypia and extensive vascular proliferations indicate tumor anaplasia and poor

clinical prognosis in diffuse fibrillary astrocytomas of the cerebral hemispheres but bear no such inference in pilocytic astrocytomas. For practical purposes, several of these histopathological characteristics are condensed into a grading scheme, and therefore today, the malignancy scale of the WHO classification is more widely accepted despite some challenges in grading of these tumors, in this study we will put forward the use of Ki-67 as an assistive marker that simplifies the identification of rapidly proliferating cells that may be challenging to pick on H&E alone and also in some biopsies with various artefacts.

Ki-67 is an antigen that encipher for two protein isoforms with molecular weights of 345 and 395 kDa, it was originally discovered by Scholzer and Gerdes in the early 1980s (4). The primary structure of pKi-67 has been acknowledged as a vast protein encoded for by almost 30,000 base pairs inside the human genome. The protein goes through phosphorylation and dephosphorylation during cell division process (mitosis) and it is predisposed to proteases, its structure suggests that its expression is regulated by proteolytic pathways (5).

Rapid and uncontrolled proliferation is a characteristic of cancer (6), the delineation of the Ki-67 antibody revealed unique staining pattern which was reactivity with a nuclear structure present solely in proliferating cells (7). With a half-life of about 1-1.5h, Ki-67 is expressed during all active phases of the cell cycle (G1, S, G2 and M), but is absent in resting cells (G0) (8,9). During the later phases of mitosis (anaphase and telophase) there is a sharp decrease in Ki-67 levels (10). The immunoreactivity of the Ki-67 protein (pKi-67) is associated with the escalation of intrinsic cell populations in malignant tumor permitting it to be used as a marker of tumor aggressiveness (11,12).

The primordial Ki-67 antibody could only be used on fresh or frozen tissue, since fixation greatly reduced or eradicated immunostaining. This prototypic antibody was superseded by other antibodies which could recognize pKi-67 in traditional fixed tissue sections, under gone heat induced epitope retrieval (13). The commonly used of these is MIB-1. It is without a doubt that this antibody has been the single largest spur to the analysis of pKi-67's prognostic potential.

The extent of expression of Ki-67 is proportional to the histologic grade (14–17) and tumor progression (9). Therefore, the prognostic value of pKi-67 has been investigated in a number of

studies with its potential as a reliable marker having been shown in cancers of the breast, soft tissue, lung, prostate, cervix and central nervous system (18,19)

As an increasing number of studies have suggested that Ki-67 may be an important factor in cancer grading and prognostic evaluation. It has been shown that Ki-67 Immunohistochemical (IHC) staining is an effective method of assessing the prognosis in a number of tumor types (20) and also a potential biomarker for prognosis and overall survival (21,22). However while some studies showed pKi-67 to have a strong prognostic value there were others that proved it to have little or none after all (23).

For the past century, the process of CNS tumors classification has been based broadly on concepts of histogenesis that tumors can be classified according to their microscopic resemblance with different apparent cells of origin and their supposed levels of differentiation. The characterization of such histological similarities has been primarily based on light microscopic features in H&E stained sections, Immunohistochemical expression of ancestry analogous proteins and ultrastructural characterization. (24)

The 2016 WHO Classification of tumors of the CNS is both ideal and practical advance over its 2007 antecedent through the use of molecular parameters in addition to histology to define many tumor entities, thus formulating a concept for how CNS tumor diagnoses should be structured in the molecular era (25). However, when considering grading of CNS tumors, the WHO recommends the count of mitoses per 10 high-power fields but it has been suggested that additional studies be done to fine-tune this grading system due to challenges in WHO grading alone (26,27).

For example, a distinction between astrocytomas with a good prognosis and astrocytomas with a poor prognosis can often be made on histological features; for some tumours histological differentiation is not clear, especially when only small fragments of tumor biopsies are available. It is for this reason that more objective criteria for predicting the prognosis of CNS tumours are required, and it's for this reason the role of Ki-67 immunostaining has been employed (28).

The WHO 2016 recommended that additional studies to be done so that to help with fine-tuning the CNS this grading system and hence molecular studies have been incorporated in this

classification system (25). Nonetheless, in our set up it's difficult to classify these tumors at molecular level but we have identified a molecular marker that can help in classifying CNS tumors according to their biological behaviour similar to other molecular studies incorporated into 2016 WHO classification. The aim of this study was to apply Ki-67 as an assistive immunohistochemistry method in grading the CNS neoplasms in our set up.

1.1.2 Problem Statement

CNS tumors have always been a concern to the pathologist due to the diversity in their histomorphology. Grading of CNS neoplasms is crucial for optimal prognostication and determination on the choice of therapy. Ki-67 is also an accustomed prognostic and predictive indicator for the assessment of biopsies from patients with cancer (29). The 2016 WHO classification system has added molecular tests that helps in further and easily grading of tumors, unfortunately these molecular tests cannot be done in low income countries due to economic constrains.

Histological grading of CNS tumors can be strenuous despite criteria given by WHO more often due to finite tumor material submitted. The number of mitosis is of foremost importance but can be hard to identify in the H&E stained sections. Ki -67 is a novel non-histone nuclear protein that is expressed in all active phases of the cell cycle and thus labelling with the monoclonal antibody against this antigen readily identifies cells that are actively proliferating and hence identification is simplified (14,30). This grading system has been utilized by the 2007 WHO system that we are currently using in our setup.

Studies has shown that Ki-67 expression is significantly higher in malignant tissues with poorly differentiated tumor cells, as compared with tissue with well differentiated tumors. Therefore, due to its predictive role, Ki-67 expression identifies subpopulations of patients who are more likely to respond to a given therapy consequently Ki-67 becomes a potential molecular therapeutic target for cancer therapy and may be a promising factor for targeted molecular therapies (31).

Also due to limitations on available data regarding CNS tumors owing to the fact that very few studies have focused on CNS tumor prevalence, leading to inability to assess the full impact of

primary CNS tumors on the healthcare system in Tanzania. Hence, the present study will provide an estimate of the prevalence of CNS tumors, updating any earlier prevalence study done.

1.1.3 Rationale

The diagnosis and grading of tumors of the CNS, for the most part, are morphologically based and predicated on the interpretation of descriptions of what the H&E findings are for a particular tumor type. It is astonishing that the application of various grading systems in CNS neoplasia has been limited somewhat by interobserver unevenness (32).

Since the designation of grade and tumor type is the basis upon which therapeutic intervention is grounded, investigators have been searching for ancillary means by which histomorphological based systems can be improved. The avail of cell proliferation markers in the evaluation of tumors can be potentially useful in this venture. The study will aid the analysis on the importance of the index of cell proliferation measured by Ki-67 as an adjunct in grading and predicting prognosis of CNS tumors in addition to 2007 WHO classification system that is still practiced in our set up.

Tanzania is amongst the countries that cannot yet use the WHO 2016 classification system due to lack of molecular tests that are required, the benefit of the use of Ki-67 which is available in our country and it's relatively cheaper as compared to sophisticated molecular studies incorporated in 2016 WHO classification and grading system cannot be over empathized.

The findings of this research work are also the prerequisite for the accomplishment of my studies in Master of Medicine degree program in Anatomical Pathology.

1.2 Work flow

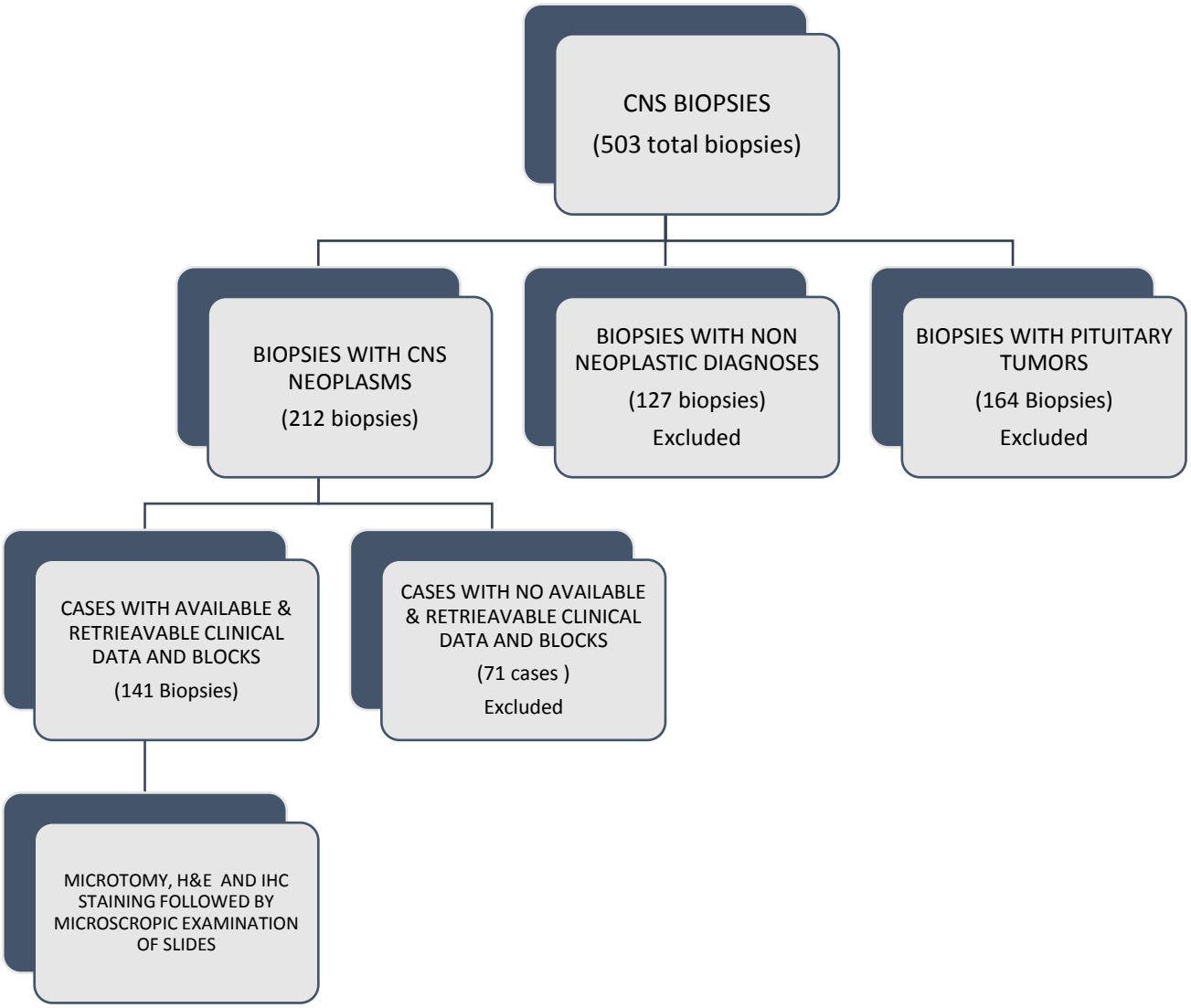


Figure 1. Diagram illustrating the conceptual framework of this study.

1.3 Hypothesis:

Assessment of Ki-67 expression by IHC is a useful test in grading of CNS tumors when age of the patient and location of tumor is known, thus improving the 2016 WHO CNS classification and grading in our setup.

1.4 Research Question

1. What is the distribution of CNS tumors according to 2016 WHO classification in our setup?
2. Will Ki-67 Immunohistochemistry be helpful in improving grading of CNS tumors in our setup?

1.5 Objectives

1.5.1 Broad Objective

To evaluate the use of Ki-67 Immunohistochemistry in histological classification and grading of central nervous system (CNS) tumors in Tanzania

1.5.2 Specific Objectives

1. To determine the histomorphological classification and grading of CNS tumors according to 2016 WHO grading system
2. To evaluate the Ki-67 Immunohistochemistry in different CNS tumors in relation to sex, age and anatomic location
3. To determine the correlation of Ki-67 Immunohistochemistry with 2016 WHO grading system.

1.6 Literature Review

Despite major advances in neuroimaging techniques and molecular biology, histopathological assessment of central nervous system tumors remains the primary means of establishing a definitive diagnosis, predicting patient outcome, and, more recently, predicting responsiveness to adjuvant therapy (33). Grading of CNS neoplasms is fundamental for optimal prognostication and deciding on the choice of therapy. Histological grading of CNS tumors can be challenging despite criteria given by WHO, accurate pathological grading is essential because it defines treatment and prognosis (34). The following are different studies that reported the use of Ki-67 in aiding the grading of CNS tumors;

A study done in USA on the practical value of Ki-67 in diagnosis of astrocytomas showed that there was a strong correlation with poor outcome when Ki-67 and p53 labeling index was higher than 15% for diffuse astrocytomas, and this was statistically significant. This level of expression also indicated a worse outcome than suggested by the histologic grading. Therefore, this study proposed that a realistic prognostic up grading of diffuse astrocytomas should be made only when labeling indexes for both Ki-67 and p53 are greater than 15% (35).

A study done on the use of Ki-67 as a predictor of relapse of mantle cell lymphoma in USA, a multivariate analysis revealed that Ki-67 of greater than 30% was the only significant risk factor for CNS relapse. A two-year cumulative incidence of CNS relapse in patients with Ki-67 greater than 30% was 25.4%, while that in the patients with Ki-67 less than 30 was 1.6%. There was no statistical significant difference in the survival by the site of CNS involvement (36).

Another study done in USA on Value of Ki-67 LI in Predicting Recurrence of WHO Grade I Meningiomas, the average age of patient was 61.8 years; whereby 72% were females. The Ki-67 LI was low (1–4%) in 78.7%, intermediate (5–9%) in 16.2%, and high (>10%) in 5.2%. when compared with tumors with low Ki-67LI, those with intermediate Ki-67 LI had 2.47 times and those with high Ki-67 LI had 3.38 times higher risk of recurrence or progression. Tumors with Ki-67 LI > 4% had a shorter time to recurrence or progression. Therefore, for WHO grade I cranial base meningiomas, Ki-67 LI > 4% may predict an increased risk of recurrence or progression of residual tumor (37).

In a study done in Finland, it was shown that Ki-67 expression in formalin fixed, paraffin embedded tissue of 15.3% resulted in significant differences in survival (38). However another study done in Bulgaria showed that Ki-67 percentage of positive nuclei in GBM tumor samples cannot be used to estimate the survival of patients despite Ki-67 being a valuable IHC pathological tool (39).

In a literature review study done in Brazil on expression of Ki-67, five studies showed an association between cell proliferation and survival time. Four articles mentioned a Ki-67 of 10% as cut-off point for survival and a fifth article a Ki-67 of 14.3%. A study showed an association between treatment failure and Ki-67. Four studies established the association between Ki-67 and WHO classification. High levels of Ki-67 are associated to high grade astrocytomas and to a decreased survival time. A value greater than 10% is a cut-off point reasonable to indicate a greater potential for malignancy and worse prognosis (40).

A study on prognostication of glioblastomas with Ki-67 in Australia, showed that there was a statistically significance between Ki-67 LI and overall survival showed that patients with Ki-67 of $\geq$ 22% had a 5-year survival was approximately 30% as compared to 5% in those with Ki-67 $\leq$ 22%. Hence this study demonstrated a positive correlation between Ki-67 index and overall survival in patients with glioblastomas (41).

In a study done in Turkey on prognostic importance of Ki-67 in patients with grade II glial tumors, patients aged $\geqslant$40 years had a poorer overall survival than those aged <40 years and these were statistically significant. Patients with a Ki-67 LI greater than 4% had a poorer overall survival than those with Ki-67 LI less than 4% with strong statistical significance. Ki-67 $\geqslant$4% was an independent prognostic factors for overall survival, however it cannot be used alone but in combination with other prognostic factors (42).

In a study done in Thailand on subtyping glioblastomas according to WHO 2007, there was significant increased Ki-67 indices in the large cell anaplastic variant compared to either classic medulloblastoma or desmoplastic/nodular variant. The statistical significance of Ki-67 indices suggests its applicable adjuvant diagnostic tool to distinguish the large cell/anaplastic variant from other subtypes (43).

In Korea, a total 45 cases of primary central nervous system lymphoma were selected, and immunohistochemistry for CD10, Bcl-6, MUM1, and Ki-67 were performed. A high Ki-67 (90%) was an independent prognostic factor for a poor OS in the whole cohort and in the patients with non-GCB subtype tumors and it's an independent prognostic marker that could be used to stratify patients for more suitable management (44).

In a systematic review and meta-analysis study done in China, a total of 51 studies involving 4,307 patients, were included. The results showed that overexpression of Ki-67 can predict poor OS, moreover, subgroup analyses also indicated that high level of Ki-67 expression was related to poor OS regardless of region, pathology type, cut-off value and statistical method. Therefore, this study emphasized the importance of Ki-67 overexpression as a OS predictor (45).

In Pakistan, a study on comparison between Ki-67 expression and histological grading of astrocytomas showed that pilocytic astrocytoma (WHO grade-1) had a mean Ki-67 LI of 0.7±1.4 (range 0.6–0.8%), diffuse astrocytoma (WHO grade II), had a mean Ki-67 LI of 3.07±3.7 (range 1–15%), anaplastic astrocytoma (WHO grade III) had a mean Ki-67 LI of 11.55±7.8 (range 2–35%) and glioblastoma multiforme had a mean Ki-67 labelling Index of 14.1±8.9 (range 5–36%), and there was a statistical significant difference between four compared groups. Therefore, Ki-67 labelling index can be used as an adjuvant to histopathological grading for proper diagnosis and grading of astrocytoma especially in borderline cases and in small biopsies (15).

In a study done in India on Ki-67 immunoprofile in CNS tumors showed that grade I, and II astrocytomas showed a very low expression of the antigen (<1 % and 2% respectively). Whereas the grade III and grade IV astrocytomas showed more than 50 % positivity. Likewise, in oligodendrogliomas and meningiomas as the grade increased a significant increase in the Ki-67 expression was observed with highest expression of 20% in rhabdoid meningioma. In medulloblastomas, grade IV tumor showed an expression of 5%. Other low-grade tumors like ependymoma (LI 1%), hemangioblastoma (LI < 1%) and schwannoma (LI <0.1%) showed very little expression of Ki-67 indicating very low mitotic activity and less aggressive nature of these tumors. Ki-67 labelling index was negative in ganglioglioma and in pituitary adenomas (29).

In India, the common age group for all grades of meningioma was 41-60 years with a female gender predominance, 19.6% cases of grade I meningiomas showed a high Ki-67 LI expression, of which 10.7% presented with recurrence. All recurrent cases were histologically WHO grade I and showed no histological features of atypia and there was no evidence of brain invasion. However, these cases showed focal hypercellular areas. The mean Ki-67 LI expression of recurrent meningiomas was 8.6%. All recurrent cases showed a high Ki-67 LI expression. Mean Ki-67 labeling index was 3.8%, 13.7%, 19.4% for grade I, II, and III cases, respectively. Multivariate analysis of mean Ki-67 LI showed statistically significant difference between subgroups., All recurrent cases were histologically WHO grade I (46).

Another study done in India on Ki-67 staining and association of histological grading of astrocytomas, a male predominance with a mean age of 48.1 years was observed. The mean Ki‐67 labeling index in grades I, II, III, and IV was 0.02%, 0.81%, 9.14%, and 17.81%, respectively. Statistical significance was observed in the Ki‐67 LI of low‐grade and high‐grade astrocytomas. There was concordance between histopathological grading and Ki‐67 LI in 90% and discordance in 10% cases. Therefore, Ki‐67 LI can be of great help in situations where there is a lack of correlation between clinical parameters and histopathological diagnosis. Determination of Ki‐67 LI should constitute a part of routine investigations in patients with astrocytomas (47).

In a prognostication study done in Beijing on about 51 primary medulloblastomas, patients were followed up ranging from 12 months up to 15 years, the mean Ki-67 value was 30.0 ± 11.3%. Patients with Ki-67 index large than 30% displayed poorer overall survival and progression free survival than those with Ki-67 less than 30% was identified as an independent significant prognostic factor by using multivariate Cox proportional hazards model. Therefore this study found out that Ki-67 index can be considered as a valuable independent prognostic biomarker for patients with medulloblastomas(48).

In Nigeria, meningioma was the most common primary CNS tumour seen. The male to female ratio was 1:3.8; with utmost in the 4^{th} decade. Majority of the cases were WHO Grade I tumours (86.1%) and transitional histologic subtype (31.8%). Ki-67 index varied widely within WHO

Grade I tumours and overlapped considerably with Grade II tumours and its expression increased with increasing WHO grade (49,50).

Since there are no studies done on Ki-67 expression in all CNS tumors in our set up that we can use for comparison, various tumors will be compared with individual studies that has been done on specific tumor type.

CHAPTER TWO

2. Materials and methods

2.1.1 Study design

This was a retrospective study design, in which all CNS biopsies between 2009 to June 2019 was enrolled.

2.1.2 Study set up

The study was conducted in the Histopathology unit, sub-section histopathology unit at Muhimbili National Hospital. MNH is a national referral hospital affiliated with the Muhimbili University of Health and Allied Sciences in Dar es Salaam, Tanzania. MNH is among few public hospitals in Tanzania that provide pathology services and receives more than 30 tissue biopsies per day where a pathology resident performs grossing under the supervision of senior pathologists. About 3 – 5 CNS biopsies are sent histopathology unit each week.

2.1.3 Study Population

All biopsies from the CNS received from January 2009 to June 2019 at MNH.

2.1.3.1 Inclusion Criteria

All tissues CNS biopsies diagnosed to have a neoplasm with retrievable patient information and anatomical location (intraoperative or radiological).

2.1.3.2 Exclusion Criteria

1. CNS biopsies with other diagnoses apart from neoplasms (i.e. inflammatory, infections, foreign body, etc.) and biopsies with missing either; tissue blocks, patient information and intraoperative or radiological findings on tumor location.
2. CNS biopsies with neoplasm but missing blocks or important clinical information's (i.e. age, sex, anatomical site of the tumor)

2.1.4 Sampling Technique

Convenient sampling method was used to recruit available tissue blocks during the study period

2.1.5 Sample size

Sample size was calculated using the following formula;

$$N = \frac{Z^2 * P * (1 - P)}{\delta^2}$$

whereas;

Z= 1.96

P = is the estimated overall proportion of CNS tumors (P = 7.19%)

(Calculated based on number of biopsies received at MNH where all biopsies received in the past 6 months were counted and CNS biopsies with diagnosis of neoplasm were identified, the ratio was used as prevalence of CNS tumors in our setup)

δ = is the estimated level of precision= 5%

N= is the number of tissue biopsies required.

N = 109 cases

2.1.6 Data variables

In this study variables were as follows;

1. Independent variables are;
    - Age
    - Sex
    - Anatomical site of the tumours
    - Histomorphological classification and grading

2. Dependent/Outcome variables are
    - Ki-67 immuno staining (Ki-67 LI)

2.1.7 Laboratory methods

2.1.7.1 Microtome

Sections of 5 micrometers for immunohistochemistry and 3 micrometers for H&E was cut from paraffin embedded blocks and put on ice blocks. Using rotary microtome (Sakura model) SRM 200 CW. Three sections were cut per block, the sections were placed in floating water bath at 45^0C and then mounted on standard frosted glass slides each with ID number slides and were allowed to drain before being put in hot plate at 60^0c for minimum of 50 minutes.

2.1.7.2 H&E staining

Sections was dew axed in three changes of Xylene for three minutes each and brought down to water through descending grades of ethyl alcohol (absolute, 95%, 80% and 70%), After that they were stained in Harris hematoxylin for five minutes, washed in tap water, differentiate in 1% aqueous eosin for 5 minutes , washed in tap water, dehydrated in ascending grades of ethanol (70%,80%,95%) 10 dips each clear in changes of xylene and mounted in DPX and labelled accordingly (protocols for H &E at MNH)

2.1.7.3 Tissue Microarray

TMA was done manually where a hollow needle was used to remove tissue cores as small as 0.6 mm in diameter from regions of interest in paraffin-embedded tissues samples. These tissue cores were then be inserted in a recipient paraffin block in a precisely spaced, array pattern. Sections from this block followed the microtomy stage. There was a total of 10 TMA slides, each composed of 15 cores, each tumor was represented by a single core. Placenta tissue was used for both control, navigation and orientation marker.

2.1.7.4 Immunohistochemistry staining

IHC by using Ki-67 was performed by the use of Dako Flex, Monoclonal Mouse, Anti Human antigen, clone MIB-1 ready to use. Retrieval solution used was CC1 solution, 32 minutes retrieval time, optiview detection kit supplied by Roche Ventana, the slides used were super frosted supplied by Thermoscientific company.

Tissue sections were deparaffinized in xylene then hydrated in 100%, 95%, and 70% thereafter put slides in antigen retrieval solution and heat in a pressure cooker.

After that the endogenous peroxidase activity was inhibited by incubating the sections with hydrogen peroxidase for 8minutes. The slides were treated with anti MIB-1 antibody at 1/250 dilution for one hour. Then the sections were incubated with anti-rabbit dextran polymer conjugated to horseradish peroxidase containing substrate and containing substrate and counterstained with hematoxylin. All incubations were carried out at room temperature and sections were washed thoroughly in between incubations. There was one positive control and one negative control for every experiment.

The Ki-67 immunostaining was evaluated by using light microscopy model Olympus CX 41 and few images from the result were taken by using Olympus soft imagine solutions by USB SC30 camera (100pixels).

2.1.8 Data collection

Study data were collected and managed using REDCap electronic data capture tools hosted at MUHAS (51,52) REDCap (Research Electronic Data Capture) is a secure, web-based software platform designed to support data capture for research studies, providing 1) an intuitive interface for validated data capture; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for data integration and interoperability with external sources.

Designed tables according to age, sex, anatomical site of biopsy, morphology of the lesion and Ki-67 Immunohistochemical results was made. Microscopy was used to determine morphological histopathology and Immunohistochemical staining by Ki-67 antibody. The microscopic interpretation was done by the principal investigator and reviewed by two experienced pathologists. The Ki-67 antigen was detected as brown granular nuclear staining in tumor cells.

2.1.9 Evaluation of Ki-67 Immunostaining

Each Ki-67 slide was evaluated on areas of high immunoreactivity with high power field (40X) objective, which included counting the number of positive and negative nuclei. Based on the

average number of nuclei evaluated in each microscopic field, we estimated that at least 100 invasive tumor nuclei were counted for each case. Every brown stained nucleus was considered positive irrespective for intensity. The estimated number of positive nuclei was divided by the total estimated number of counted nuclei, which resulted in the proliferative or Ki-67 index for each case. Necrotic or thick areas and severely overlapping tumor cells were avoided. Ki-67 scoring was recorded as the percentage of invasive tumor cells positively stained in the examined field(s).

The results were as follows;

< 5%	very low expression
5 – 9%	low expression
10 – 20%	intermediate expression
> 20%	high expression

2.1.10 Validity and Reliability of the study

The slides with diagnosis of CNS tumors were confirmed through review by principal investigator and confirmed by two experienced pathologists. The tissue blocks for H&E and immunohistochemistry was prepared by following the standard operating procedures (Protocols for each procedure attached on annexes). The results for Ki-67 immunohistochemistry was also be interpreted by principal investigator and reviewed by two experience pathologists. Also the positive and negative controls were stained together with the cases during each experiment of immunohistochemistry staining.

2.1.11 Data Analysis Plan

Data were analysed electronically by Statistical Package for Social Sciences (SPSS) version 20 computer software (SPSS Inc., Chicago Ill) after cross checking the filled data collection templates and the data will be managed electronically in the computerized software program after cross checking the filled data collection sheets.

Age was analysed using mean, median, frequency and percentages. Sex, anatomical location and histomorphological classification and grading were determined by using frequency and percentages, Ki-67 immunostaining was determined by using frequency and percentages, correlation of Ki-67 and tumour grade was determined by the use of chi square test, Pearson's correlation and a p value of <0.05 will be considered significant.

2.1.12 Limitation of Study

1. The study was not be able to evaluate patients with CNS tumours but could not be biopsied for various reasons.
2. The clinical information was limited only to the documented information; any undocumented clinical information were not able to be retrieved.
3. Information regarding site of the tumour that was not retrievable, the term other unspecified site was used.

2.1.13 Ethical consideration

Ethical clearance was obtained from MUHAS-IRB (Institutional Review Board) and authorization to execute study at MNH and Central Pathology Laboratory department was obtained from the Director of the Muhimbili National Hospital. Confidentiality of the findings was highly observed.

CHAPTER THREE
3. Results

3.1 Demographics and anatomical location of tumors

During the study period from January 2009 to June 2019, a total of 503 cases were retrieved whereby 141 cases were enrolled in this study, there were more males than females with a proportion of 54.6% males and 45.6% females with male to female ratio of 1.2:1. Males had a mean age of 37 years and females mean age was 38 years. The age ranged from 1 to 77 years, overall mean age was 37.6 $\pm$ 18.8 years, with similarities in mean ages between males and females, the age distribution followed a normal distribution curve as seen per figure 1, with the most overall affected age group being 21 – 45 years 60/141 (42.6%) (Table 1)

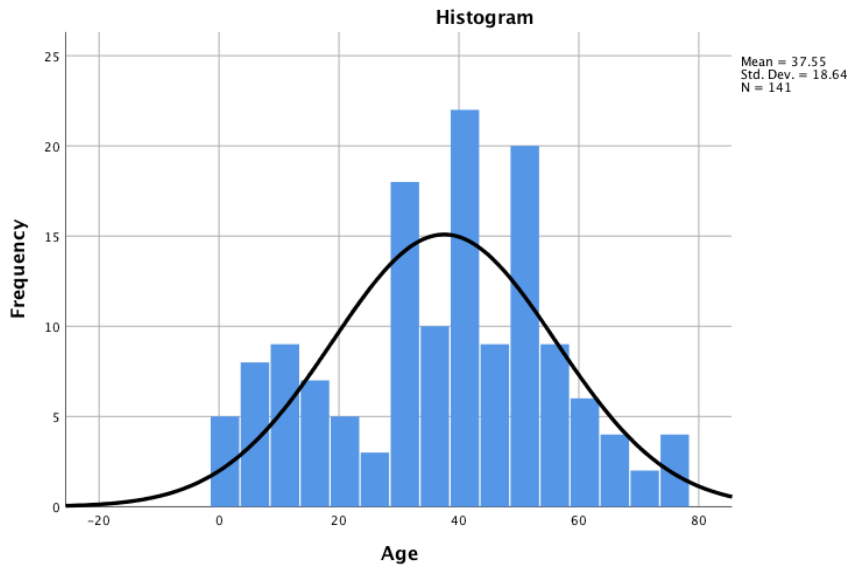
Table: 1. Demographic characteristics

Demographic data		Frequency (%) N=141	Mean age	STD deviation
Sex	Male	77 (54.6%)	37	20
	Female	64 (45.4%)	38	18
Age	1 – 10	16 (11.3%)	6	3
	11 – 20	14 (9.9%)	15	3
	21 – 45	60 (42.6%)	26	4
	> 45	51 (36.2%)	56	3

Abbreviations: STD – Standard, 1 – 10 children, 11 – 20 adolescents, 21 – 45 young adults, > 45 adults

Children (1 – 10 years) were also involved with mean age of 6 $\pm$ 3 years, they contributed to 11.3% of total cases involved in the study.

Figure: 1. Age group distribution

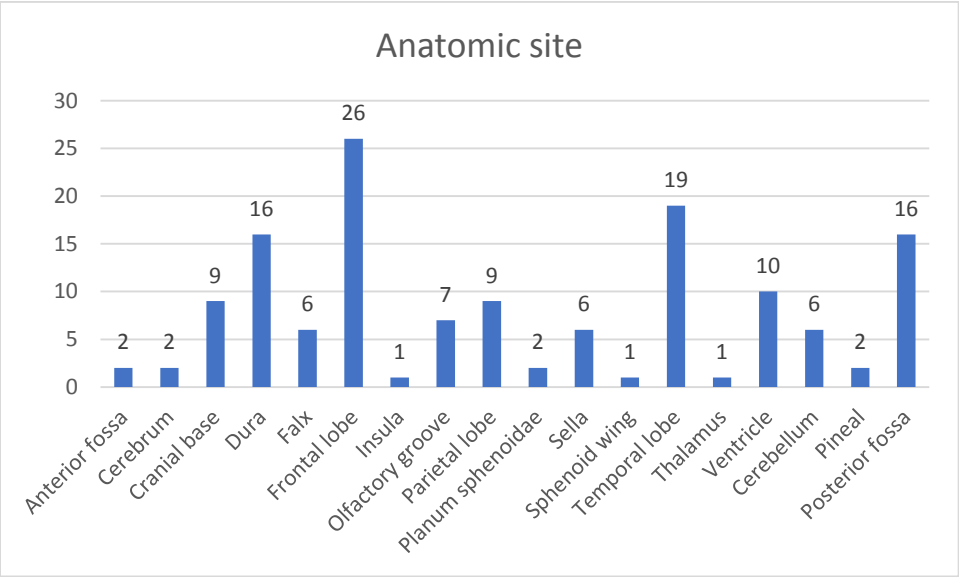


Majority of patients were seen between the age of 20 to 60 years of age, very few cases (4.3%) were seen in patients who were above 70 years of age, and no infants case enrolled in this study. Majority of the biopsies were from the supratentorium 117/141 (83.0%) and 24/141 (17.0%) from infratentorium. For the supratentorial tumors the frontal lobe consisted of most of cases 26/141 (18.4%), followed by temporal lobe 19/141 (13.5%) and dura-based lesions 16/141 (11.3%), Infratentorial tumors had posterior fossa being the most affected site 16/141 (11.3%) followed by cerebellum 6/141 (4.3%).

Table: 2. Grade and Ki-67 LI distribution

Grade	WHO (%)	Ki – 67 (%)	Ki-67 LI range %
I	91 (64.5%)	74 (52.4%)	0 – 8
II	32 (22.7%)	45 (31.9%)	3 – 14
III	1 (0.7%)	9 (6.4%)	7 – 28
IV	17 (12.1%)	13 (9.3%)	14 – 90
Total	**141 (100%)**	**141 (100%)**	

Figure: 2. Anatomic location of tumors



3.2 Histomorphological classification and grading of CNS tumors according to 2016 WHO grading system.

Majority of tumors were meningiomas which constituted of about 55.3% (78/141) of all cases followed by Astrocytic and oligodendroglial tumors 22.7% (32/141) and ependymal tumors 4.9% (7/141). Pilocytic astrocytoma and glioblastoma NOS was the leading diagnoses in astrocytic tumors with 34.4% (11/32) and 31.3% (10/32) respectively. Diffuse astrocytoma NOS and Oligodendroglioma NOS contributed to 5.0% (7/32) and 1.4% (2/32) of all astrocytic tumors (Table 3)

Ependymomas contributed to about 5% (7/141) of all cases, of all embryonal tumors, majority (5/6) were CNS embryonal tumors NOS and one was a Medulloblastoma. Other mesenchymal tumors excluding meningothelial tumors were hemangioblastomas (2/6), hemangioma (1/6), Lipoma (1/6), Gliosarcoma (1/6) and a benign spindle cell tumor NOS (1/6). There were 2.1% craniopharyngiomas and a single metastatic adenocarcinoma seen. (Table 3)

Pilocytic astrocytoma and diffuse astroctytomas were relatively more common in males than in females 1.2:1 and 2.5:1 respectively, whereas glioblastoma was relatively equally distributed within the males and females 1:1.

Ependymomas, gliomas, choroid plexus papilloma and atypical choroid plexus papilloma were seen only in males. With regards to embryonal tumors medullobalstoma was seen in a female while majority of CNS embryonal tumors were seen in males (4cases vs 1 case). Grade I meningiomas were common in females (47/78) as compared to males (20/78), this was also seen in atypical meningiomas where a slightly more cases were seen in females (5 cases in males' vs 6 cases in females). Lower grade mesenchymal tumors were seen in males and a higher grade (Gliosarcoma) was seen in female patient. One metastatic tumor was from a female patient. (Table 3)

Table: 3. WHO 2016 classification and grading

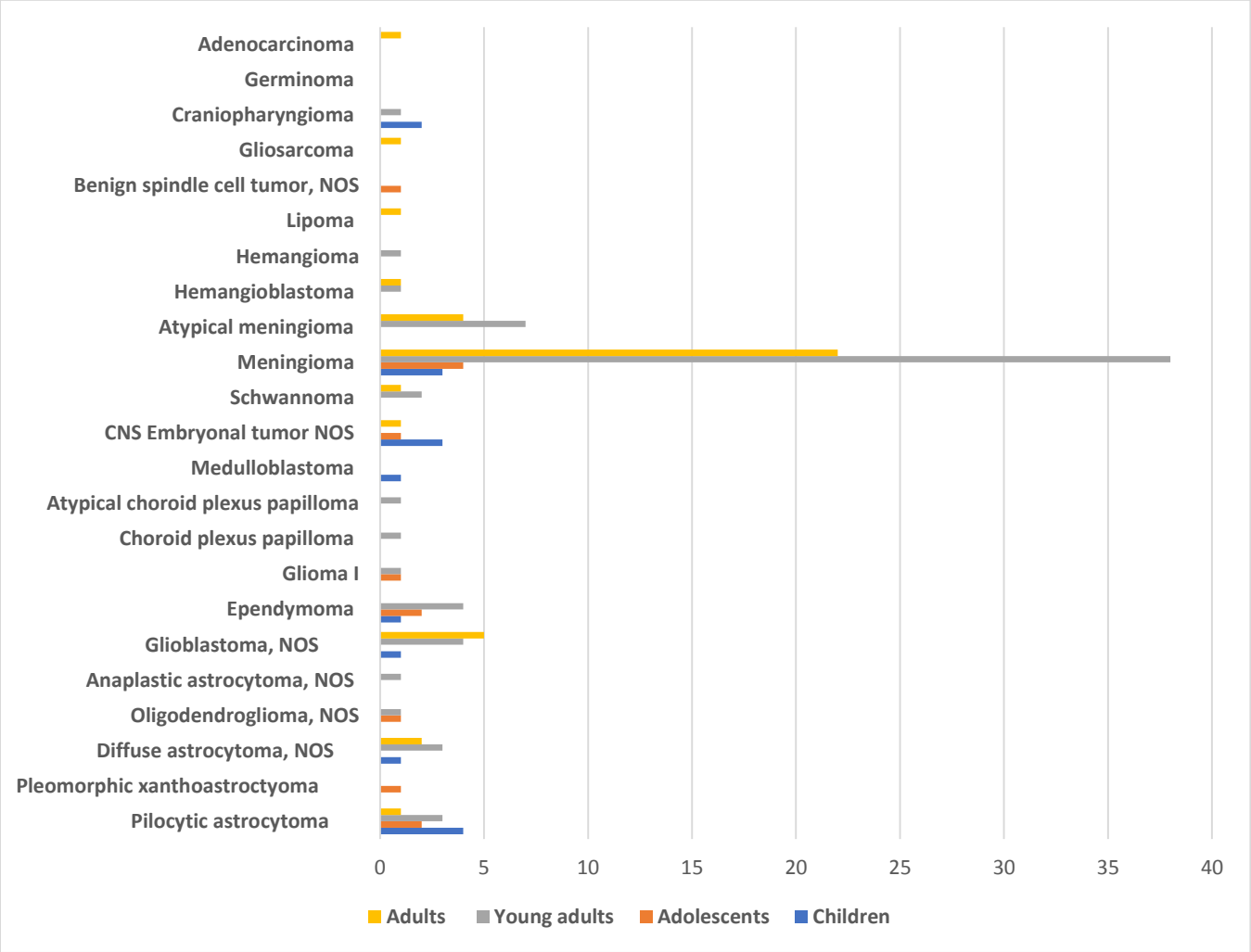
WHO 2016 classification and grading		
Diagnosis	**Grade**	**Frequency**
Astrocytic and Oligodendroglial tumors (N= 32)		
Pilocytic astrocytoma	I	11 (7.8%)
Pleomorphic xanthoastroctyoma	II	1 (0.7%)
Diffuse astrocytoma, NOS	II	7 (5.0%)
Oligodendroglioma, NOS	II	2 (1.4%)
Anaplastic astrocytoma, NOS	III	1 (0.7%)
Glioblastoma, NOS	IV	10 (7.1%)
Ependymal tumors (N=7)		
Ependymoma	II	7 (5.0%)
Other gliomas (N=2)		
Glioma I	I	2 (1.4%)
Choroid plexus tumors (N=2)		
Choroid plexus papilloma	I	1 (0.7%)
Atypical choroid plexus papilloma	II	1 (0.7%)
Embryonal tumors (N=6)		
Medulloblastoma	IV	1 (0.7%)
CNS Embryonal tumor NOS	IV	5 (3.5%)
Tumors of cranial and paraspinal nerves (N=3)		
Schwannoma	I	3 (2.1%)
Meningiomas (N=78)		
Meningioma	I	67 (47.5%)
Atypical meningioma	II	11 (7.8%)
Mesenchymal, non-meningothelial tumors (N=6)		
Hemangioblastoma	I	2 (1.4%)
Hemangioma	I	1 (0.7%)
Lipoma	I	1 (0.7%)
Gliosarcoma	IV	1 (0.7%)
Benign spindle cell tumor, NOS	I	1 (0.7%)
Tumors of sellar region (N=3)		
Craniopharyngioma	I	3 (2.1%)
Germ cell tumors (N=1)		
Germinoma	I	1 (0.7%)
Metastatic tumors (N=1)		
Adenocarcinoma	II	1 (0.7%)
Total		**141 (100.0%)**

Table: 4. WHO 2016 classification and grading vs sex distribution

WHO 2016 classification vs Sex distribution			
Diagnosis	**Grade**	**Male**	**Female**
Astrocytic and Oligodendroglial tumors (N=32)			
Pilocytic astrocytoma	I	6	5
Pleomorphic xanthoastroctyoma	II	1	-
Diffuse astrocytoma, NOS	II	5	2
Oligodendroglioma, NOS	II	1	1
Anaplastic astrocytoma, NOS	III	-	1
Glioblastoma, NOS	IV	5	5
Ependymal tumors (N=7)			
Ependymoma	II	6	1
Other gliomas (N=2)			
Glioma I	I	2	-
Choroid plexus tumors (N=2)			
Choroid plexus papilloma	I	1	-
Atypical choroid plexus papilloma	II	1	-
Embryonal tumors (N=6)			
Medulloblastoma	IV	-	1
CNS Embryonal tumor NOS	IV	4	1
Tumors of cranial and paraspinal nerves (N=3)			
Schwannoma	I	1	2
Meningiomas (N=78)			
Meningioma	I	20	47
Atypical meningioma	II	5	6
Mesenchymal, non-meningothelial tumors (N=6)			
Hemangioblastoma	I	2	-
Hemangioma	I	1	-
Lipoma	I	-	1
Gliosarcoma	IV		
Benign spindle cell tumor, NOS	I	1	-
Tumors of sellar region (N=3)			
Craniopharyngioma	I	1	2
Germ cell tumors (N=1)			
Germinoma	I	1	-
Metastatic tumors (N=1)			
Adenocarcinoma	II	-	1
Total		**77**	**64**

Abbreviations: N = Total number of cases

Figure: 3. WHO 2016 classification and grading vs Age groups distribution



NOS – not otherwise specified, 1 – 10 children, 11 – 20 adolescents, 21 – 45 young adults, > 45 adults

Most of tumors were distributed fairly across all age groups with slight differences, Pilocytic astrocytoma was seen more in children of 1 – 10 years of age, glioblastoma was seen more in adults. Meningiomas were more prevalent in young adults 38/78 (48.7%) followed by adults of >45 years 22/78 (28.2%). Most of craniopharyngiomas were seen in children below 10 years of age (2/3) and a metastatic adenocarcinoma was seen in patient who were over 45 years of age. (Figure 4)

3.3 Ki-67 Immunohistochemistry with reference to anatomic location, recurrence status, tumor grade sex and age distribution.

Both supratentorial and Infratentorial tumors had most of tumors with lower proliferation, where a 10% of supratentorial tumors showed a Ki LI of >20% as compared to 4.2% of Infratentorial tumors. About 71.4% of all primary tumors had a Ki LI of 5-9% whereas 85.7% of recurrent tumors had a Ki-67 LI of 5-9%. There was about 77.8 concordance between WHO 2016 with Ki-67 LI in grade I tumors, 78.8% in grade II, 100% in grade III and 76.5% in grade IV tumors, and this was statistically significant. (Table 5)

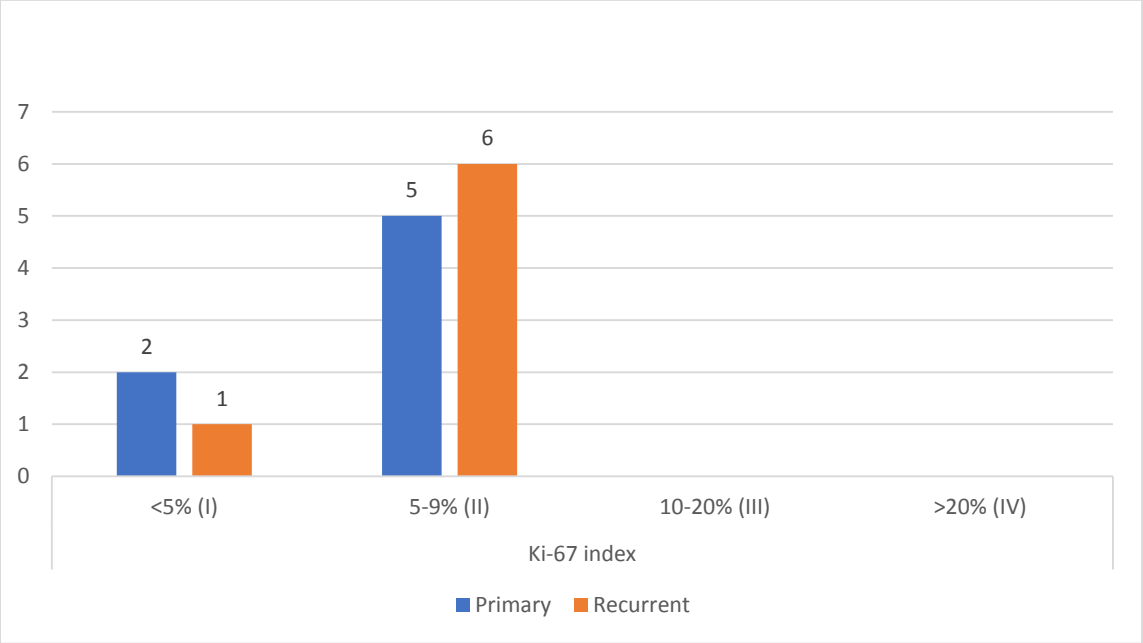
Males and Females had a lower proliferation index relatively, however about 10.4% of females had a higher Ki-67 LI of over 20% whereas males only 7.8% had a higher Ki-67 LI of over 20% and these results were not statistically significant. Children had a fairly higher Ki-67 LI with 31.2% of proliferation index of over 20%, other age groups showed a tumor with relatively lower LI, these results showed a statistical significance. (Table 5)

Table: 5. Ki-67 index

Variable	Ki-67 index				P value
	<5% (I)	5-9% (II)	10-20% (III)	>20% (IV)	
Site					
Supratentorial	59 (50.4%)	39 (33.3%)	7 (6.0%)	12 (10.3%)	
Infratentorial	14 (58.3%)	7 (29.2%)	2 (8.3%)	1 (4.2%)	> 0.72
Recurrence					
Primary	2 (28.6%)	5 (71.4%)	-	-	
Recurrent	1 (14.3%)	6 (85.7%%)	-	-	< 0.00
WHO 2016 Tumor grade					
I	70 (77.8%)	19 (21.1%)	1 (1.1%)	-	< 0.00
II	3 (9.1%)	26 (78.8%)	4 (12.1%)	-	
III	-	-	1 (100.0%)	-	
IV	-	1 (5.9%)	3 (17.6%)	13 (76.5%)	
Sex					
Female	30 (38.9%)	23 (29.8%)	3 (3.9%)	8 (10.4%)	
Male	43 (67.1%)	23 (35.9%)	6 (9.2%)	5 (7.8%)	> 0.37
Age					
1 – 10	8 (50.0%)	3 (18.8%)	-	5 (31.2%%)	< 0.05
11 – 20	10 (71.4%)	0 (0.0%)	-	1 (7.1%)	
21 – 45	27 (45.0%)	25 (41.7%)	5 (8.3%)	3 (5.0%)	
> 45	28 (54.9%)	15 (29.4%)	4 (7.8%)	4 (7.8%)	

Both primary and recurrent tumors showed higher proliferation indexes (figure 4)

Figure: 4 Recurrent tumors vs Ki-67 index



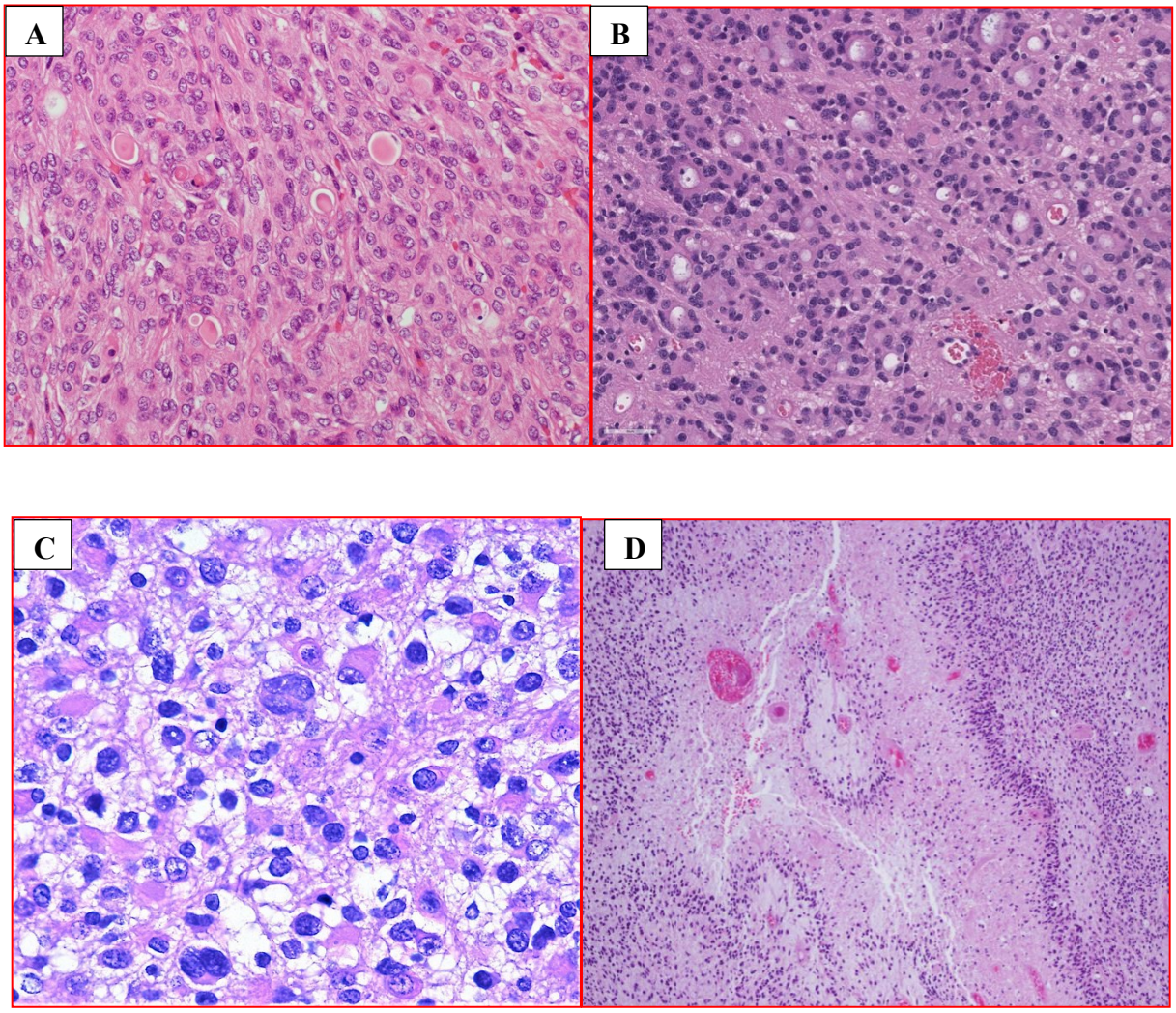
Recurrent meningiomas showed higher proliferation indexes as compared to primary tumors, however both primary and recurrent tumors had a Ki-67 of above 5%.

Table: 6. The correlation between 2016 WHO grading systems and Ki-67 index

		2016 WHO grading
Ki-67 index	Pearson's Correlation	0.87
	Kendall's tau_b	0.75
	P value	0.00
	Total	141

Strong positive correlations were seen in both Pearson's and Kendall's tau_b for 2016 WHO grading and Ki-67 index by 0.87 and 0.75 respectively with strong statistical significance.

Figure: 5 Sample images of different CNS tumors at different grades



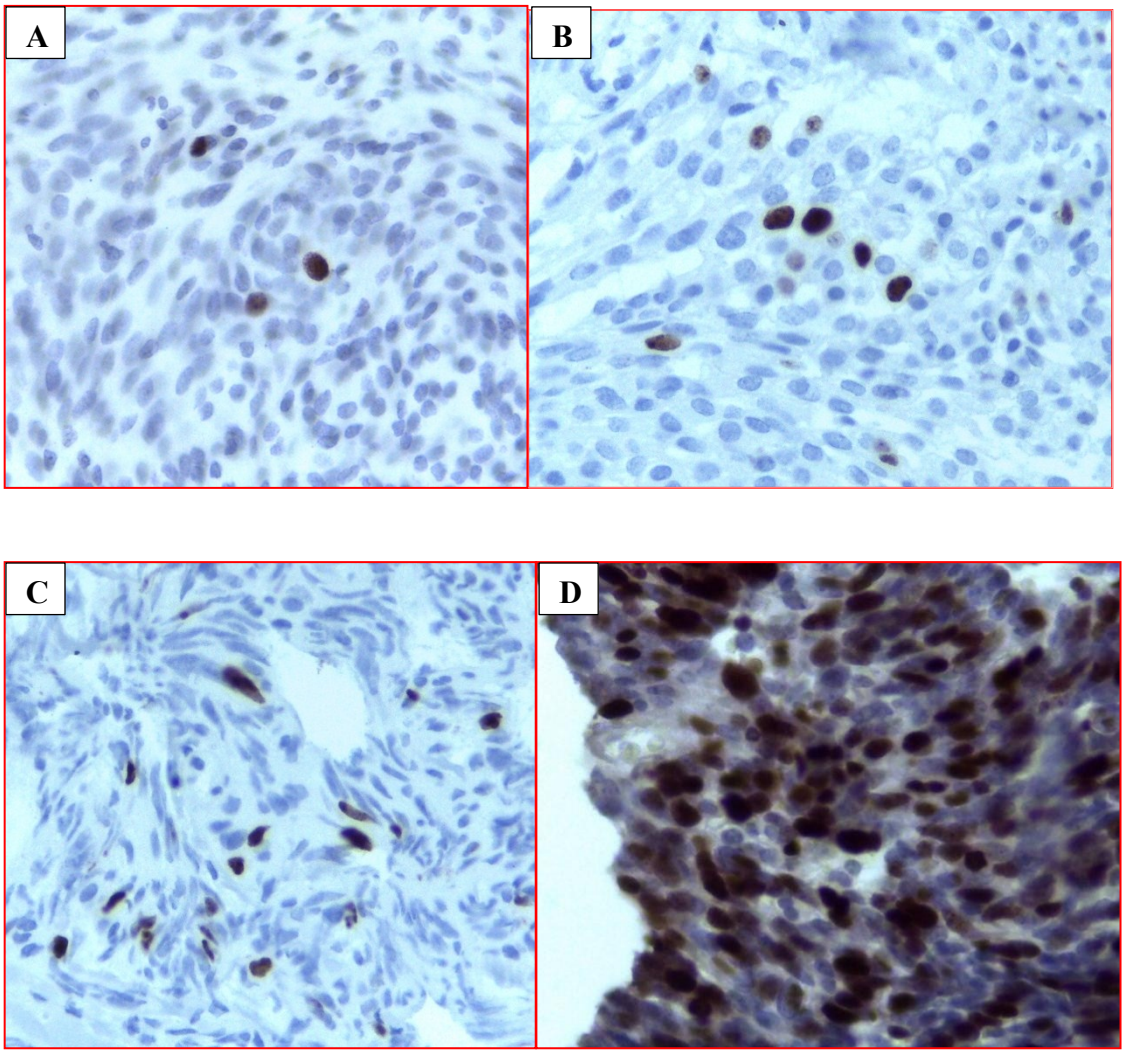
A: Figure showing intracellular lumina with eosinophilic secretions referred to as "pseudopsammoma bodies" seen in Secretory Meningiomas, corresponding to WHO grade I (x40)

B: Figure showing a perivascular pseudo and true rosettes as seen in Ependymomas, corresponding to WHO grade II (x40)

C: Figure showing a diffusely infiltrating tumor cells with oval to elongated astrocytic nuclei and varying appearance of tumor cytoplasm and fibrillary glial processes with increased mitotic activity, cellularity and nuclear atypia, as seen in Anaplastic astrocytoma NOS, corresponding to WHO grade III (x40)

D: Figure showing palisading necrosis as seen in Glioblastoma NOS, corresponding to WHO grade IV (x10)

Figure: 6. Ki-67 index in different grades (TMA core sections, x40 magnification)



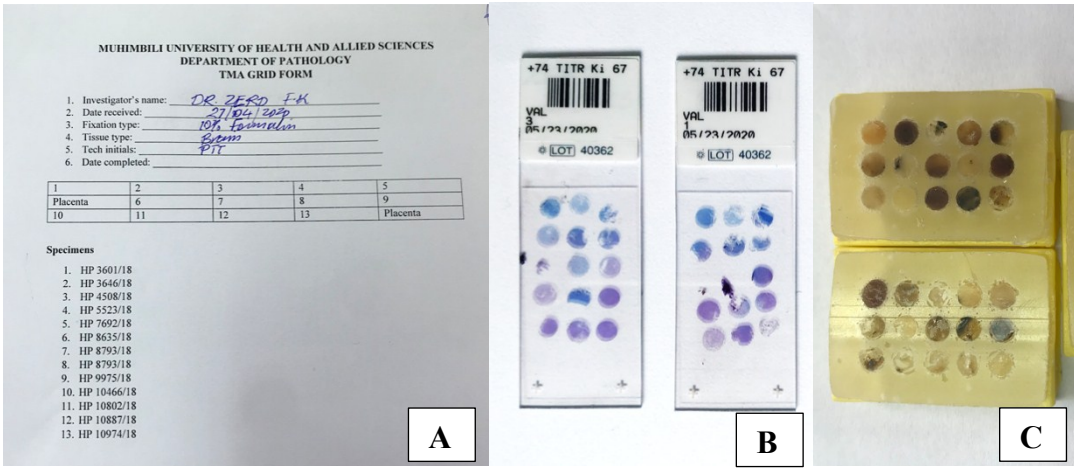
A: Section with proliferation index of 3% corresponding to grade I

B: Section with proliferation index of 7% corresponding to grade II

C: Section with proliferation index of 14% corresponding to grade III

D: Section with proliferation index of 90% corresponding to grade IV

Figure: 7 TMA grid form, TMA slides and TMA blocks



A- Grid form showing distribution of cases in a TMA block with placenta as navigation and orientation but also was used as control.

B- TMA slides after Ki-67 staining ready for interpretation

C- TMA blocks

CHAPTER FOUR

4. Discussion

The central nervous system tumors are the most widely classified of all the other tumors of major systems of the body, with around 86 major tumor types apart from their variants. The CNS tumors are always a cause of concern among histopathologists due to its wide variation in the morphology and also the difficulties faced in grading these tumors accurately. Grading of the CNS tumors in this study was done according to the WHO 2016 criteria (53).

Histological grading of CNS tumors can be strenuous despite criteria given by WHO more often due to finite tumor material submitted. The grading is based on the cellularity, mitotic count, microvascular proliferations, and necrosis. The number of mitosis is of foremost importance but can be hard to identify in the H&E stained sections. Ki -67 is a novel non-histone nuclear protein that is expressed in all active phases of the cell cycle and thus labelling with the monoclonal antibody against this antigen readily identifies cells that are actively proliferating and hence identification is simplified (14,30), it is present in the cells of all phases of cell cycle namely G1, S, G2 and M phase except G0 phase where the cells are in quiescent or resting stage. Thus, determination of Ki- 67 is an excellent factor correlating cellular growth. MIB-1 labelling index is the number of Ki -67 labelled tumor nuclei expressed as a percentage of the total number of tumor nuclei counted (29). Studies has shown that Ki-67 expression is significantly higher in malignant tissues with poorly differentiated tumor cells, as compared with tissue with well differentiated tumors (31)

Outstandingly this study has demonstrated the distribution of CNS tumor in our setup and in relationship with different demographics, and it has evidently showed that Ki-67 LI has a key role in grading of CNS tumors with overall strong positive correlation, also it has shown a significant relationship with tumor recurrence where 71.4% of primary tumors had a Ki LI of >5% and 85.7% of recurred tumors had a Ki LI >5%. Our study has also successfully used TMA technique to significantly reduce cost in Ki-67 IHC.

There are very few studies which were done on Ki-67 for all CNS tumors, majority of studies are done on specific tumor group of CNS on utility of Ki-67 labelling index. No study has been done in Tanzania on the use of Ki-67 labelling index on CNS tumors.

Our study showed a male preponderance with male to female ratio of 1.2:1, this was shown also by two separate studies performed on two continents, Mckinney et al, and Fan et al found similar results suggesting a male to female ratio of 1.5:1 (54,55). However a study on primary brain tumors showed that the prevalence in females was higher than that in males (56) an another study in Mexico showed a male to female ratio of 1:1.3 (57). The slightly higher male preponderance in our setup is not very clear and it could be due to higher male predominance in astrocytomas and ependymomas which are known to have male predominance and, in our study, has been some significant contributing tumors.

Mean age for males was 37 years and for females was 38 years, the commonest affected age group was young adults followed by adults similar to a study done by Mehta et al where tumors were more common in age group of 31 – 40 years followed by 41 – 50 years (58). This was also seen in a study done by Thotakura et al where by the commonest affected age group was 4^{th} to 5^{th} decade but there was slight male preponderance of male to female ratio 1.84:1 (28). In our study children contributed to about 11.3% of all cases, and this was similar in a study done in Rwanda where children contributed to about 15.45% of all tumors of the central nervous system (59). The higher mean age for all sexes is attributed to majority of tumors in our study being meningiomas and astrocytic tumors where presentation is common among young adults and adults, however there was also a significant contribution to common pediatrics tumors in our study which has contributed to a slight peak in childhood tumors.

Most tumors were Supratentorial with the frontal lobe being the commonest site of these tumors, this was also observed in another study where frontal lobe was the commonest site of primary brain tumors (58). Several other studies reported Supratentorial region being the most common affected with the frontal lobe being the most common site for primary brain tumors (57,60,61). In another study by Almutrafi et al, showed that the cerebral meninges were the most common

site followed by the frontal lobe. This could be attributed to the predominance of meningioma over other neoplasms in their study (62). The supratentorial location in our tumors has been contributed by the vast majority of our tumors being known to affect the supratentorial region with astroctytomas and meningiomas affecting the frontal lobe more often.

Meningioma was the most common primary CNS tumour seen in our study with a male to female ratio of 1:2.1 and peak age on 4th decade, this was similar to a study done by Ikeri et al on meningiomas with male-to-female ratio of 1:3.8; with a peak in the 4th decade (63). In favor to our study meningioma was most common tumor as noted by Surawicz et al (64) in USA and Lee et al (65) in Korea also found that most common tumor was meningioma. Most of meningiomas was of grade I and this was similar to a study done by Telugu et al where by grade I meningiomas composed of 86.6% of all meningiomas (66). Meningiomas are the most reported brain tumors with overall lifetime risk of developing meningioma of about 1% with more than 75% of meningiomas being grade I (25). Several findings have suggested hormonal association especially estrogen and meningiomas making prevalence higher in pre-menopausal women similarly in our study where meningiomas were seen more in women of <50 years of age.

Astrocytomas were the second most common primary intracerebral tumors in our study (32/141). Although low-grade astrocytomas have a tendency to grow slowly, they may progress to higher grade types as anaplastic astrocytomas or glioblastomas (3). Overall astrocytomas were more common in males with a male to female ratio of 1.3:1, this was also seen in a study done by Shivaprasad et al whereby astrocytomas showed a male preponderance of male to female ratio of 1.7:1 (47). Our study has showed that lower grade astrocytomas; pilocytic astrocytomas M:F (6:5) and diffuse astrocytomas M:F (5:2) were slightly prevalent in males but a study done by Tihan et al, showed that pilocytic astrocytoma M:F (5:6) were slightly higher in females whereas diffuse astrocytomas M:F (11:4) were higher in males (35). The slight higher prevalence in males of these tumors in our study is attributed to overall distribution where there were more males as compared to females, however pilocytic astrocytomas have been shown to have no significant sex predilection by WHO 2016 (25).

Majority of supratentorial tumors were of grade I and II, and this was also similar to Infratentorial tumors where grade I and II tumors were prevalent, however grade IV tumors were more prevalent in supratentorial region and this is most likely due to the fact that majority were tumors from the supratentorial region. Higher proliferation was seen more in males than in females however this results was statistically not significant, Darweesh M et al also demonstrated a higher Ki-67 LI in males than in females with non-significant statistical tests (67).

In this study Ki-67 has outstandingly demonstrated a significant proportion of grade I tumors with aggressive behaviors of about 22.2% of which if they were to be detected earlier their management would differ and hence prevention of recurrence of this tumors and therefore improvement of patient overall survival, also our study has demonstrated 7 recurrent tumors where all of them were meningiomas grade I by histology alone, majority of the recurrent and primary tumors had a Ki-67 of >5% with a mean Ki-67 LI of 7.9%, no recurrent tumors were seen in Ki-67 LI of >10% and this is due to shorter recurrence time, aggressive treatment options and shorter survival intervals. A study by Rao et al showed that recurrent brain tumors had a mean Ki-67 LI of 8.6% and this was statistically significant on multivariate analysis(46).

Our study has demonstrated a strong positive correlation between Ki-67 and grading after Pearson's correlation and Kendall's tau_b respectively, and these results were statistically strongly significant. Many other studies have demonstrated a positive correlation between Ki-67 and grading of CNS tumors (39,66). In this study only a single core of tumor was used for TMA, therefore with regards to tumor heterogenicity, it is possible to postulate that these small cores may not be representative of their donor tumors, however some studies showed that a single core is representative whilst others suggest two to three cores are representative(68,69). Our study also involved only 141 cases out of 212 cases were studied due to missing of important details that could not be retrieved, and this information composed of clinical information mainly providing the anatomical site of tumors and missing tissue blocks for Ki-67 staining.

4.1 Conclusion

Ki-67 LI has more potential of detecting grade I tumors with aggressive behaviors and therefore subjecting these patients to a more appropriate treatment hence significantly reducing the risks of recurrence and improving the overall patient survival. Furthermore, the study showed that in Tanzania the most common CNS tumors are meningiomas followed by astrocytomas. The most common affected region was supratentorial with frontal lobe leading as the most affected anatomical site. This is to our knowledge the first study in Tanzania to analyze all central nervous system tumor types involving patients of all age groups consequently forming a baseline 'data and a reference for further epidemiological and clinico-pathological studies in the country and the region at large.

This is also the first study in Tanzania to study the central nervous system tumours, involving patients of all age groups over a period of 10 years, therefore it is a strong indication of the epidemiology of CNS tumours in our country.

4.2 Recommendation

Ki-67 LI should be ordered along with CNS biopsies and be mandatory in all grade I tumors in our setup as it has been able to identify aggressive tumors despite presenting as lower grades histologically, therefore aids in preventing tumor recurrences and improving patient overall survival by detecting tumors that needs aggressive therapy.

This study should also initiate more studies on association between proliferation index and overall patient survival in our setup.

5. DISSEMINATION PLAN

The results of this study were presented to the Department of Pathology at Muhimbili University of Health and Allied Sciences and submitted to the Research and Publication committee of Muhimbili University of Health and Allied Sciences and also to Muhimbili National Hospital Management as well as to the heads of respective departments where data have been collected. With the help from supervisor, a manuscript for publishing the study was prepared and handled to the Office of the Director of Postgraduate Studies before the award of Masters of Medicine in Anatomical Pathology.

REFFERENCES

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APPENDICES

APPENDIX I – Data collection sheet

1. Patient's Registration

1. ID NO ____
2. HP NO _____

Variables from patient files

2. Age at diagnosis _____years

3. Sex 1. Male 2. Female

4. Site of biopsy

1. Cerebellum
2. Cerebrum
3. Sella
4. Pineal
5. Ventricle (in or adjacent to)
6. Dura based
7. Unspecified (i.e. brain, intracranial, etc)

Variables from the examination of microscopic slides

5. Histology Results Histological findings

1. WHO Classification _____
2. WHO Grading _____

6. Immunohistochemistry by Ki-67 assay (Ki-67 LI)

1. <5%

2. 5-9%

3. ≥10-20%

4. ≥20

APPENDIX II – Procedure for hematoxylin and eosin staining

1. Dewax sections and bring down to water.
2. Stain in Harris hematoxylin for 10 minutes.
3. Rinse in running tape water.
4. Differentiate in 1% acid alcohol for 5-10 seconds.
5. Rinse in tap water
6. Blue the sections in tap water for 10 minutes.
7. Counter stain in 1% aqueous eosin for 3 minutes.
8. Rinse in tap water.
9. Dehydrate the sections through ascending grades of alcohols.
10. Clear in two changes of xylene (3 minutes in each)
11. Mount with DPX or mounting machine

APPENDIX III – Procedure for immunohistochemistry

1. Cut on Dako slides and bake for 30 minutes on hot plate.
2. Dewax sections in xylene and rehydrate to tape water.
3. Ring sections with PAP pen and add 2-3 drops of peroxidase blocking solution, leave it for 15 minutes.
4. Place slides in tap water.
5. Heat antigen retrieval solution or citrate buffer in pressure Cooker until it starts boiling.
6. Place slides in antigen retrieval solution in pressure cooker and close the lid.
7. Time for 2 minutes at full pressure
8. Remove slides from pressure cooker and place in water. Do not allow slides to dry from this step onwards.
9. Add Tris Buffer Saline (TBS) for 3 minutes.
10. Drain off TBS and cover sections with primary antibody at appropriate dilution and incubate for 60 minutes.
11. Wash gently TBS on slide for 5 minutes
12. Drain off TBS from slides and cover sections with 2-3drops of Horse raddish Peroxidase (HRP) for 30 minutes, the reagent should be at room temperature before use.
13. Wash gently for 5 minutes with TBS (make up DAB reagent by adding one drop of DAB to 1 ml of substrate buffer)
14. Drain TBS from slides and apply working DAB reagent to the sections and incubate for 5 minutes.
15. Wash slides well in water
16. Counter stain with hematoxylin for 30 seconds, dip in 1% acid alcohol 2 dips.
17. Blue in tap water for 2 minutes.
18. Dehydrate, clear in xylene and mount with DPX.

APPENDIX IV – Procedure for tissue microarray (TMA)

1. Select relevant formalin fixed paraffin embedded material (tissue samples) including corresponding hematoxylin stained tissue section.
2. Mark relevant area on the tissue section. It is recommended to have a freshly cut hematoxylin stained section corresponding to the paraffin block.
3. Design the template used for TMA production.
4. Randomize the samples within the template to avoid artifacts caused by technical problems such as coverage of the antibody over the whole TMA section and sectioning problems.
5. Add additional orientation markers to the template for orientation.
6. Organize the tissues according to the template.
7. Continue with microtomy and IHC

APPENDIX V – Ethical clearance letter

MUHIMBILI UNIVERSITY OF HEALTH AND ALLIED SCIENCES
OFFICE OF THE DIRECTOR OF POSTGRADUATE STUDIES

P.O. Box 65001
DAR ES SALAAM
TANZANIA
Web: www.muhas.ac.tz

Tel G/Line: +255-22-2150302/6 Ext. 1015
Direct Line: +255-22-2151378
Telefax: +255-22-2150465
E-mail: dpgs@muhas.ac.tz

Ref. No. DA.287/298/01A/ 12th July, 2019

Dr. Francis Zerd
MMed. Anatomical Pathology
MUHAS.

RE: APPROVAL OF ETHICAL CLEARANCE FOR A STUDY TITLED: "THE USEFULNESS OF Ki-67 IMMUNO EXPRESSION IN THE CLASSFICATION AND GRADING OF CENTRAL NERVOUS SYSTEM (CNS) TUMOURS IN TANZANIA"

Reference is made to the above heading.

I am pleased to inform you that, the Chairman has, on behalf of the Senate, approved ethical clearance for the above-mentioned study. Hence you may proceed with the planned study.

The ethical clearance is valid for one year only, from **10h July, 2019 to 9th July, 2020.** In case you do not complete data analysis and dissertation report writing by **9th July, 2020,** you will have to apply for renewal of ethical clearance prior to the expiry date.

Dr. Emmanuel Balandya
ACTING: DIRECTOR OF POSTGRADUATE STUDIES

cc: Director of Research and Publications
cc: Dean, School of Medicine, MUHAS

APPENDIX VI – Permission to collect data at Muhimbili National Hospital

MUHIMBILI NATIONAL HOSPITAL

Cables: "MUHIMBILI"
Telephones: +255-22-2151367-9
FAX: +255-22-2150534
Web: www.mnh.or.tz

Postal Address:
P.O. Box 65000
DAR ES SALAAM
Tanzania

In reply please quote:

MNH/TRCU/IRB/Permission/ 2019/124 **03rd July, 2019**

Head of Department
Central Pathology Laboratory
Muhimbili National Hospital

RE: PERMISSION TO COLLECT DATA AT MNH.

Name of Student	**Dr. Francis Zerd**
Title	**"THE USEFULNESS OF KI-67 IMMUNO EXPRESSION IN THE CLASSIFICATION AND GRADING F CENTRAL NERVOUS SYSTEM (CNS) TUMORS IN TANZANIA".**
Institution	Muhimbili University of Health and Allied Sciences
Supervisor	**Dr. Edda Vuhahula** **Dr. Leah Mnanago**
Period	22th July 2019 to 30th December, 2019

Approval has been granted to the above mentioned student to collect data at MNH.

Named MNH based supervisor must ensure that the student abide to the ethical principles and other conditions of the research approval.

Sincerely,

Dr. Faraja Chiwanga
Head of Teaching, Research and Consultancy Unit

c. c DCSS
c.c **Dr. Leah Mnango**
c. c **Dr. Francis Zerd**

HEAD, TEACHING, RESEARCH & CONSULTANCY UNIT
P. O. Box 65000
DAR-ES-SALAAM
MUHIMBILI NATIONAL HOSPITAL

All Correspondence to Addressed to the Executive Director