Title	***Vacuolar ATPase and drug resistance of high grade gliomas: a study to investigate possible therapeutic roles for proton pump inhibitors.***
Acronim	GLIODRUG-V
Protocol Version	V. 1.0
Protocol Date	07-July-2019
Kind of Study	Prospective observational study on biological material
Document Status	Final
Promotor	University of Milano-Bicocca, Dipartiment of Medicine and Surgery
Principal Investigator	Prof. Carlo Giorgio Giussani, UO Neurochirurgia; ASST Monza, Monza, Italia, carlo.giussani@unimib.it

Principal Investigator	
Carlo Giorgio Giussani Associate Professor Department: University of Milano-Bicocca, Department of Medicine and Surgery Institution: ASST Monza - Ospedale San Gerardo City, Nation: Monza, Italia	
Firma	Data

Vacuolar ATPase and drug resistance of high grade gliomas: a study to investigate possible therapeutic roles for proton pump inhibitors.

TABLE OF CONTENTS

1. Introduction

Gliomas are primary brain tumors originating from glial cells. They are classified according to the 2016 WHO classification into three grades from II to IV[1].
Grade III and IV gliomas are considered aggressive primary brain tumors with a poor prognosis which depends on several factors. In particular, the most aggressive and malignant of the primary brain tumors are the so called Glioblastoma Multiforme (GBM), that are classified as WHO grade IV gliomas[1]. GBMs accounts for 69% of all gliomas and for 12-15% of all primary brain tumors[2]. Patients with GBM have a poor median overall survival (OS) of 14-16 months with gross total surgical resection and adjuvant chemo-radiation therapy. Nowadays, there are no curative treatments and tumor recurrence is expected. The use of alkylating agents has improved the prognosis of HGGs but after the introduction of Temozolomide (TMZ) in 2005 as standard first line treatment, no other improvement has been made[3]. Moreover, so far, there is not a standardized second line treatment after tumor recurrence and several drugs or treatment strategies are available[4].

GBMs have been found to have a small population of cancer stem cells (CSC) that contribute to tumor spreading, invasion, proliferation and maintenance. In particular, CSCs seem to be responsible for cancer resistance to adjuvant treatments and such resistance is responsible for tumor recurrence during the neuro-oncological follow-up[5,6].
Features of drug resistance can also be transmitted cell to cell by the cross-linking network sustained by microvescicles (MV) like exosomes and large oncosomes (LO) that transport a varied population of proteins, lipids, DNA, and RNA species[7] that enhance and influence mechanisms involved in drug resistance[8–10]. Among the molecules that can be transported by this MV network there is H^+ Vacuolar ATPase (V-ATPase), a multisubunits proton pump that is mainly involved in the acidification of endosomes and lysosomes in eukaryotic cells. It is made of a membrane-embedded V0 sector, which regulates protons permeability, and an enzymatic V1 ATPase sector[11–13].

In case of tumors, the role of V-ATPase seems to be crucial in order to maintain a normal intracellular pH in a metabolic setting based on anaerobic glycolytic reactions[13]. In fact, due to loss of functions of mitochondria during tumorigenesis, metabolism of neoplastic cells is based on anaerobic glycolysis which can easily afford ATP through the consumption of glucose but that, on the other hand, leads to production of lactates. The excess of lactates would determine a reduction of the intracellular pH and, consequently, activation of apoptotic reactions leading to the death of neoplastic cells[14]. In this setting, the excess of protons is counterbalanced by the extrusion of H^+ from the intracellular to the extracellular environment through the activation and overexpression of V-ATPase. For this reason, many tumors show a V-ATPase overexpression,whose inhibition is related to a dysregulation of cytosolic pH that ultimately leads to the activation of pro-apoptotic proteins, alteration of metabolic chain reactions and of endosomal vesicles[14,15].
Other than providing those functions, V-ATPase plays a crucial role in chemoresistance of solid tumors. In fact, excess of protons in the extracellular space determines a reduction of pH that can reduce the penetration and the efficacy of chemotherapeutic agents[15,16,16]. Moreover, in few studies on breast cancer, B cell leukemia and melanoma, inhibition of V-ATPase have proved to increase the drug efficacy *in vitro*[15]. In addition, it is supposed that V-ATPase can act as a pump for extrusion of chemotherapy drugs from the intra-cellular to the extra-cellular compartment[15,16].

In 2015 Di Cristofori and colleagues demonstrated how V-ATPase is overexpressed in HGGs while it is poorly expressed in LGGs. From a clinical point of view, it was demonstrated how expression of V-ATPase is related to the patient's prognosis independently from other clinical risk factors. Moreover, it has been demonstrated that blocking the V-ATPAse with specific drugs, in *in vitro* patient-derived GBM cultures, increased the efficacy of TMZ if compared with TMZ alone[17].

Highly tumorigenic glioma stem cells secrete in their microenvironment many micro- and macrovescicles that are loaded with factors able to influence the behavior of cells in the tumoral niche and cells of the immune system. In particular, GBMs secrete LOs loaded with the homeobox genes POU3F2 and HOXA10 and with the V-ATPase subunit V1G1[9]. Interestingly, such LOs were found to be able to reprogram both neoplastic and non-neoplastic cells toward an oncogenic state through the activation of proliferative and motility pathways[9]. So far, it seems that configuration or expression of specific subunits might influence the tumor formation or the tumor aggressiveness, probably via Homeobox genes[9]. In fact, another study by Bertolini and Colleagues, reported that neurospheres with high levels of V-ATPase expressed high levels of homeobox genes (HOXA7, HOXA10, SHOX2, and POU3F2) In this setting, V-ATPAse can be considered a key player involved in multiple functions that are necessary to afford CSCs survival and tumor progression. Virtually, V-ATPase is considered a possible target to sabotage the neoplastic metabolic machinery. In addition, inhibition of V-ATPase is considered a possible strategy to increase efficacy of antineoplastic drugs. This aspect of V-ATPase has not been studied in gliomas[14].

With these premises, the aim of this study is to understand the possible role for proton pump inhibitors (PPI) as adjunctive treatments for high grade gliomas.

2. Experimental Design

2.1 Study Rationale

GBMs are still considered tumors with few available treatment options that are able only to achieve a temporary local control of the disease. In case of a GBM, tumor recurrence is generally expected within 12 months and it is due to the presence of marginal tumoral cells with pro-oncogenic molecular phenotypes that are resistant to actual chemotherapies and to radiation therapy[5,18,19]. In particular, according to the study of Persano and Colleagues, in case of GBM it is possible to distinguish three neoplastic layers within the tumor that show different molecular patterns: the central core; the intermediate layer and the peripheral layer[5]. Nowadays, surgery still represent the first treatment option in case of suspected GBM and it aims to remove the contrast enhancing lesion seen at the pre-operative brain MRI. In particular, the peripheral layer is made of low replicating cells and it can be considered normal when tissue sampling is made far from the tumor cavity[5]. In fact, Clavreul et al. in 2015 demonstrated that peripheral GBM layer contains glioblastoma-associated stromal cell (GASC) that can show different carcinogenic properties and that are probably responsible for tumor recurrence[20–22]. These findings can be considered in line with previous studies that showed some invasive tumor cells, various types of reactive cells, and angiogenesis with different immunophenotypes in peritumoral brain edema[23].

Nevertheless, some research teams are trying to understand if surgical removal of peritumoral FLAIR hyperintensity is able to reduce the tumor recurrence rate prolonging the OS[24].

Metabolism of GBMs is mainly anaerobial and it is sustained by glycolysis[25]. Anaerobial glycolysis is a simple metabolic reaction that leads to the transformation of glucose in ATP and lactates. Glucose is delivered to the tumor through neoangiogenetic processes[26]. Production of a significant amount of lactates determines a decrease of intracellular pH that is counterbalanced by V-ATPase activity through the extrusion of H+ ions from the intracellular to the extracellular environment. In vitro inhibition of V-ATPAse has proved to increase CSC apoptosis due to decrease of intracellular pH[17].
Moreover, increased V-ATPase activity determines an extrusion of H+ ions in the extracellular environment that can positively affect different pro-tumoral activities. In fact, a decrease of extracellular pH leads to activation of proteases able to destroy the extracellular matrix. Such activity enhances tumor spreading. Moreover, a low extracellular pH can reduce the efficacy of antineoplastic agents since a low pH might affect the structural integrity of drugs and their ability to pass through the plasmatic membrane. Finally, V-ATPase can act as an active pump able to excrete antineoplastic agents[15].
For this reason, PPIs are considered new anti-cancer drugs able to increase tumoral cell death, reduce tumor invasion and increase chemotherapy efficacy[14].
Moreover, GBMs with high V-ATPAse expression has proved to be able to transmit highly malignant features through a network of MVs and to activate proliferation of GASC *in vitro* through the transmission of G1 subunit of V-ATPAse[9,27].
In this view, our work is intended to study: 1) the effects of PPIs on CSC and GASCs cultures as in vitro add-on treatments; 2) the MVs load in terms of miRNAs and DNA (ssDNA, exoDNA[28]) during the neuro-oncological follow-up in order to understand how it changes after surgery and adjuvant treatments; 3) the possible roles of V-ATPase as a clinical marker to be used to check tumor response to adjuvant treatments.

2.2 Objectives

- **Primary objective:** to determine and investigate the role of V-ATPase in drug resistance and the role of proton pump inhibitors as add-on treatment for high grade gliomas *"in vitro"* on CSC cultures obtained both from tumor and peritumoral margins.
- **Secondary objective:** to determine whether MVs content in human blood is similar to the MVs content in CSCs *"in vitro"* in order to study the mechanisms of tumor recurrence and in to study possible translational aspects of our research.
- **Tertiary objective:** to determine whether mechanisms of drug resistance can be transmitted via the cross-linking network sustained by exosomes and how this network is regulated in relation to the expression of V-ATPase.

2.3 Type of Study

This is a prospective observational cohort study on biological materials.

2.4 Study Population

Planned number of subjects undergoing surgical removal of intra-axial brain tumor at the Neurosurgical Division of Ospedale San Gerardo: almost 45 per year. Thirty patients are expected to have a glioma of which 25 patients are expected to be diagnosed with a GBM and 5 with a LGG. We plan to enroll 25 patients/year.

2.5 Inclusion Criteria

Patients => 18 years old;

Patients with an intra-axial brain tumor suspect for glioma;

Patients able to sign a consent form for research purpose;

Patients with planned craniotomy for brain tumor resection.

2.6 Exclusion Criteria

Patients < 18 years old;

Patients with known brain tumors different than gliomas;

Patients unable to sign a consent form for research purpose;

Patients undergoing brain tumor biopsy;

Patients with poor intra-operative or small surgical sample for histopathological diagnosis;

Histology diagnostic for tumors different than gliomas.

2.7 Experimental Design

Our study will use glioma tissues, after all sampling for diagnosis will be collected. Moreover, we will take blood samples from patients undergoing surgery for HGGs and during the oncological follow-up. Tumor samples will be harvested in order to obtain parallel tumor cultures to test *in vitro* tumor sensitivity to different chemotherapies, with and without inhibition of V-ATPase. Moreover, we will analyze the expression of V-ATPase (especially subunit V1G1) in vescicles released by cancer cells, in order to understand how V-ATPase can influence drug resistance and tumor progression. HGG-released vescicles will be also isolated in the circulation from patients' blood in order to analyze tumor responses to oncological treatments and the genetic and epigenetic status of the tumor, including amplification of oncogenes (i.e. *c-Myc*), and the content of specific coding and non-coding RNA, DNA and retrotransposon elements, through the use of next generation sequencing (NGS)[28].

Duration for enrollment: 24months;

Duration for follow-up analysis: 12 months for each patient.

Maximum length of the study: 36 months including patients' follow-up

2.8 Withdrawal Criteria

The subject may withdraw at will at any time. The patient may be withdrawn from the trial at the discretion of the investigator for safety concerns. Patients who will meet the exclusion criteria after a prior inclusion in this study will be withdrawn. A patient withdrawn analysis will be made to clarify the related rate and possible impact on the study results.

2.9 Participating Units

Clincal data and collection of biological samples

ASST-Monza Ospedale San Gerardo

- U.O. di Neurochirurgia: Prof. C. Giussani; Dr. A. Di Cristofori; Dr. A. Trezza

Stem cell cultures and extraction of MVs

Università degli Studi di Milano Bicocca – Dipartimento di Medicina e Chirurgia

Laboratorio di Genetica Medica: Dr.ssa Angela Bentivegna

3 Materials and Methods

This study will evaluate patient data and biological tissues collected prospectively and anonymized prior to the analysis. The study protocol follows the ethical guidelines of the 1975 Declaration of Helsinki (as revised in Brazil 2013).

3.1 Clinical Variables collected

3.1.1. Anagraphic Data

Patient ID (anonymously assigned by the center in consecutive order); Date of Birth; Gender

3.1.2. Anamnestic Data

Karnopfsky Performance Status; ASA score; Previous Surgery

3.1.5 Pre-operative and post-operative radiological data

Tumor volume on pre- and post-operative brain MRI; extent of resection

3.1.6 Surgical Data

Date of surgery; location of the tumor; post-operative complications (e.g. hematoma, seizures)

3.1.7 Histopathological findings

Formal histological diagnosis with tumor grade and molecular classification according WHO 2016 classification of the tumors of the central nervous system.

3.1.8 Follow-up

Complications during adjuvant chemo-radiation therapy; treatment response; chemotherapy administered with number of cycles and dosage. Dose of radiations delivered. Time to tumor progression and overall survival.

3.2 Study supplies and products.

Patients will be treated according to local hospital procedure. No additional costs (materials, salaries, other) due to the study will be charged to the hospital. Study products will be those commonly used in the hospital according to the local rules.

3.3 Data handling

Data collection will be performed using an electronic database system, in accordance with the European Statement 679/2016/UE. The submitted data will be checked at San Gerardo Hospital (Monza) by clinicians (Neurosurgery Division) and by the the Medical Genetics Laboratory (Dr. Angela Bentivegna), of School of Medicine and Surgery, University of Milano-Bicocca. Once examined, the record will be accepted into the dataset for analysis.

3.4 Surgical methodology

Patients will be operated for intra-axial brain tumors. Part of the tumor specimen, will be sent for cell culture; while the majority of the tumor specimen will be used for a formal histological diagnosis. In order to achieve a gross total resection and confirm it histologically, a sample of marginal tissue of the surgical bed will be sent for cell culture and histology. Marginal tissue will be collected from non eloquent brain areas. Intraoperative ultrasound will be used to check presence or absence of high cellularity of the sample.

Patients with brain tumors other than gliomas at histological diagnosis will be excluded from the study. Sample of these tumors will be eliminated.

If the tissue removed from the patient enrolled in this study will not allow to perform the sample for the study without affecting the optimal histological diagnosis, the patient will be excluded from the study and no sampling will be executed.

3.5 Tissue and blood samples preservation, storage and management

3.5.1. Sampling and analysis

Part 1 – stem cell cultures sampling and processing

Biological samples from patients undergoing surgery for intra-axial brain tumor will be taken and processed. Tumor margins will be collected as well, in order to check tumor resection with histopathological findings and in order to investigate the role of CSCs in the brain adjacent to tumor in determining the tumor recurrence due to resistance to treatments. Both tumor area and its marginal tissue will be taken during the surgery and collected for laboratory procedures and histological analysis. Blood samples will be collected 1-3 days before surgery and 1-3 days after surgery and will be processed for serum collection and stored for further analysis on MVs.
The fresh tissue (tumor and marginal tissues) will be treated in order to obtain single cell suspensions and neurospheres. Then, each patient-derived primary cell cultures will be expanded and subsequently divided into 2 parts: 1) one destined to CSCs cultures for *in vitro* tests; 2) one for biological storage, for further retrospective analyses. The storage of biological samples will be at the Medical Genetics Laboratory (Dr. Angela Bentivegna), of School of Medicine and Surgery, University of Milano-Bicocca. All the materials for

the study will be provided by the same laboratory, so there are no additional costs for the Neurosurgical Division and for Hospital San Gerardo.

Part 2 - in vitro study of drug resistance

Neurospheres from marginal tissues will be obtained by co-culturing of marginal tissue with MVs taken from CSCs cultures medium, in order to better define the role of V-ATPase in drug resistance and the role of MVs in transferring drug resistant phenotypes from a CSCs culture to another.
CSCs cultures will be treated with antineoplastic drugs with and without PPI. In this way, it will be possible to understand the *in vitro* response to antineoplastic agents and PPI.

Anti-neoplastic agents taken into account for this study are: Temozolomide, Vincristine, Carboplatin, Etoposide, Irinotecan (CPT-11), Bevacizumab, BCNU, CCNU, Procarbazine, Irinotecan + Carboplatin, Procarbazine + Vincristine + CCNU (PCV). All these agents have been used for treatment of HGGs as first or second line therapy.

PPIs used for this study are: concanamycin, Bafilomicine-A1, Archazolide-A. All the procedures will be performed for research use at the Medical Genetics Laboratory (Dr. Angela Bentivegna), of School of Medicine and Surgery, University of Milano-Bicocca..

Part 3 – serial analysis of blood samples

During the first year of neuro-oncological follow-up, patients will undergo to collection of blood samples pre- and post-operatively, three months, six months, nine months and one year after surgery. Each sample will be processed in order to study the MVs content of subunits of V-ATPase (especially subunit V1G1) and the genetic and epigenetic content (DNA, mRNA and miRNA). The results will be matched with the brain MRIs performed as part of the neuro-oncological follow-up. The blood volume will be of about 5-10 mLs and it will be taken during the normal blood sampling performed during the neuro-oncological follow-up for checking chemotherapy toxicities. No adjunctive invasive procedures will be performed on patients.

During this part of the study, it will be possible to understand the relations between tumor volume and MVs concentrations in the bloodstream in order to understand the role of surgery on the existing vesicular network between the brain and the rest of the body. Moreover, it will be possible to study the effects of adjuvant treatments on the MVs contents during the time of response to treatment and at tumor recurrence. Finally, analysis of DNA, miRNA and mRNA contained in the MVs will allow us to explore the presence of new markers able to predict tumor recurrence and to understand in vivo any possible changes of the neoplastic cells during the treatment.
These results will be compared with the results obtained *in vitro* in order to understand if the experimental model can reflect the mechanisms active in the patient.
This part of the research project will allow us to transfer the project from the bench to the bedside and it will be the starting point for future projects.

MVs isolation will be performed as reported below.

For all patients 20 mL fasting blood sample will be collected in a sterile vacuum tube. The collection will be done in the same time of routine preoperative blood samples or planned follow-up controls, since no others invasive procedure should be required from the patients since their enrollment in the study. The blood sample will be stored at 4°C and handled within 6 hours. Then, 5 mL will be centrifuged at 3000 rpm for 10 minutes at 4°C, the plasma phase will be collected and aliquoted in 1.5 mL sterile tubes and stored at -80°C until use or shipment. Then, we will isolate peripheral blood mononucleate cells (PBMC) through Ficoll gradient from the remaining 15 mL. After isolation, live PBMC will be frozen in standard media 20% Fetal Bovin Serum (FBS) 10% Dymethil Sulfoxide (DMSO) and stored in liquid nitrogen until use. All these parts of the experiment will be performed at the Medical Genetics Laboratory (Dr. Angela Bentivegna), of School of Medicine and Surgery, University of Milano-Bicocca.

3.5.2 Storage and transport

The biological material will be shipped and stored to the Medical Genetics Laboratory (Dr. Angela Bentivegna), of School of Medicine and Surgery, University of Milano-Bicocca the same day of sample collection. No additional costs are required for biological sampling transport since the laboratory is close to the hospital. The biological material will be stored for a maximum period of 10 years.

3.6 Analysis of tumor recurrence

Tumor recurrence will be evaluated according the RANO criteria. Patients will undergo routine neuro-oncological and radiological assessment in order to check the brain and the surgical bed during adjuvant treatments.

3.7 Neuro-Oncological Follow-up

All patients will undergo to a neuro-oncological follow-up. Each patient will be treated with a standard first line treatment according to the Stupp protocol with concomitant chemo-radiation therapy and subsequent adjuvant chemotherapy. Every 3 months, each patient will undergo a brain MRI.
During every neuro-oncological evaluation, each patient will have a blood sample taken in order to check the blood count. During this standard procedure, an additional sample of blood will be taken for this study in order to extract circulating exosomes and DNA.
Brain MRIs will be examined to calculate tumor volumes and residual tumor volumes and to diagnose tumor recurrence.

3.8 Statistical analysis.

Data will be expressed as median and interquartile range (IQR), number and relative percentage, or hazard ratio (HR) with related 95% confidence intervals (CI). Normal distribution of continuous variables will be assessed by Kolmogorov-Smirnov test. To compare baseline characteristics of the groups in the univariate analysis, continuous variables will be analysed using the Mann-Whitney test or ANOVA according to the type of distribution. Categorical variables will be analyzed by Fisher exact test or Chi-Square Test as

appropriate. A 1:1 propensity-score-analysis, with a caliper of 0.1SD, may be conducted for significant variables ($p<0.05$) at univariate to reduce the retrospective bias of selection.
Multivariate analysis will be performed by logistic regression analysis or Cox regression as appropriate. DFS, OS and TSS will be evaluated by Kaplan-Meier method. Comparison between groups will be performed with the log-rank test. A Receiver Operating Characteristic (ROC) curve analysis will be employed as appropriate. The optimal cut-off value will be established based on the Youden index. All statistics will be 2-tailed and statistical significance will be accepted when $p<0.05$.

3.9 Data management.

Data will be stored in an electronic database. Each investigator may have access to the whole dataset.
The subject will be identified by an alphanumeric code linked to the name of the patient that the enrolling investigator can see. Appropriate measures such as encryption or deletion will be enforced to protect the identity of human subjects in all presentations and publications as required by local/regional/national requirements. During the patients entering in the database, data quality will be ensured. At least one report analysis will be performed each year.
Major protocol deviations will lead to exclusion of data from the analysis, while data will not be excluded because of minor protocol deviations. The list of major protocol deviations will be detailed and documented in the clean file document prior to database release.

The personal data subject of the study will be processed in compliance with the European Regulation on the Protection of Personal Data (GDPR), of Legislative Decree 196/2003 and subsequent amendments and additions, and of any other Italian law applicable to the protection of personal data (hence referred to as "applicable data protection law").
With regard to personal data made available to the Promoter in a pseudo-anonymized form, the Institute and the Promoter are considered joint owners, each for their own area of competence, and both will act in compliance with the applicable data protection law.
Furthermore, the Institute and the Promoter will cooperate to take the necessary measures to comply with the applicable data protection law, and will also have to implement appropriate technical and organizational measures to guarantee the GDPR compliance requirements.
The Institute is responsible for providing its Principal Investigators and Research Personnel with all necessary information concerning the methods through which the Promoter will eventually collect and manage their personal data before such information is provided.
The Promoter ensures the correctness of the processing of personal data as provided by the GDPR. If the user becomes aware of a data breach of personal data, the user himself must promptly notify the other users of this event, and in any case no later than 24 hours from the knowledge of the event. In this case, the user will have to cooperate fully with the other users to remedy the data breach, fulfilling the mandatory notification within the set times and managing any damage caused.
The data breach of personal data is defined by the art. 33 and 34 of the GDPR.
The roles and responsibilities related to the project will be explained in a further and separate act between the Promoter and the institute.

4 CRF

4.1 Rules for completing CRFs

The investigator staff must ensure that all information derived from source documentation is consistent with the source information. By uploading the complete dataset, the Investigator confirms that the information is complete and correct.

4.2 Corrections to CRFs

Corrections to the data on the CRFs can only be made by communicating to the Data Manager the alphanumeric code linked to the name of the patient and asking to modify the data.

5. Ethics

The register will be conducted in accordance with the Declaration of Helsinki and according to local and regional ethical standards.

5.1 Critical documents

Before the Investigator starts the study (i.e., obtains informed consent from the first subject), the following documents must be available:

• Regulatory approval and/or notification as required

• Signed and dated agreement on the final protocol

• Signed and dated agreement on any substantial amendment(s), if applicable

• Approval/favourable opinion from IEC clearly identifying the documents reviewed: the

protocol, any substantial amendments, subject information/informed consent form and any other written information to be provided to the subject, subject recruitment procedures.

6. Significance and Innovation

This work is meant to increase our knowledge on glioma progression and on drug resistance of high grade gliomas and the role played by V-ATPase. First, this study is meant to better investigate the role of V-ATPase on drug resistance and the possible roles that V-ATPase inhibitors could play as add-on treatments for HGGs. This would be one of the first dedicated study in literature and it could set the theorical bases for an *in vivo* study.

Secondarily, this study is meant to investigate the molecular changes that tumors may develop through the analysis of MVs and circulating DNA during the normal neuro-oncological follow-up. The aim is to find molecular settings that might underpin the resistance to adjuvant treatments or that might be involved in developing drug resistance during the neuro-oncological follow-up and that could be related with development of tumor recurrence.

This study might allow in future to understand new strategies to increase the efficacy of adjuvant treatments against HGGs. These treatments will aim to dismantle the tumoral cell metabolism blocking the mechanisms that regulate the intracellular pH.

7. Responsibilities

The Investigator is accountable for the conduct of the study. If any tasks are delegated, the Investigator should maintain a list of appropriately qualified persons to whom he/she has delegated specified significant study-related duties.

The medical care given to, and medical decisions made on behalf of, subjects should always be the responsibility of a qualified physician.

The Investigator will take all necessary technical and organizational safety measures to prevent accidental or wrongful destruction, loss or deterioration of data. The Investigator will prevent any unauthorized access to data or any other processing of data against applicable law.

8. Reports and publications

The information obtained during the conduct of this study is considered confidential and belongs to the investigator group for the purpose of scientific publications. No confidential information shall be disclosed to others. Such information shall not be used except in the performance of this study group.

All the investigators may decide after a consensus, to share the data with other groups or trials occasionally. In this case, the agreement of the whole study group is needed.

9. Retention of clinical documentation

Subject notes must be kept for the maximum period permitted by the hospital, institution or private practice.

The Investigator must agree to archive the documentation pertaining to the register in an archive after completion or discontinuation of the study if not otherwise notified.

Clinical study documentation must be retained until at least 2 years.

10. References

1. Louis DN, Perry A, Reifenberger G, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathologica*. 2016;131(6):803-820. doi:10.1007/s00401-016-1545-1

2. Ostrom QT, Cote DJ, Ascha M, Kruchko C, Barnholtz-Sloan JS. Adult Glioma Incidence and Survival by Race or Ethnicity in the United States From 2000 to 2014. *JAMA Oncology*. 2018;4(9):1254. doi:10.1001/jamaoncol.2018.1789

3. Ricard D, Idbaih A, Ducray F, Lahutte M, Hoang-Xuan K, Delattre J-Y. Primary brain tumours in adults. *The Lancet*. 2012;379(9830):1984-1996. doi:10.1016/S0140-6736(11)61346-9

4. Martínez-Garcia M, Álvarez-Linera J, Carrato C, et al. SEOM clinical guidelines for diagnosis and treatment of glioblastoma (2017). *Clinical and Translational Oncology*. 2018;20(1):22-28. doi:10.1007/s12094-017-1763-6

5. Persano L, Rampazzo E, Della Puppa A, Pistollato F, Basso G. The three-layer concentric model of glioblastoma: cancer stem cells, microenvironmental regulation, and therapeutic implications. *ScientificWorldJournal*. 2011;11:1829-1841. doi:10.1100/2011/736480

6. D'Alessio A, Proietti G, Sica G, Scicchitano BM. Pathological and Molecular Features of Glioblastoma and Its Peritumoral Tissue. *Cancers*. 2019;11(4):469. doi:10.3390/cancers11040469

7. Basu B, Ghosh MK. Extracellular Vesicles in Glioma: From Diagnosis to Therapy. *BioEssays*. June 2019:1800245. doi:10.1002/bies.201800245

8. Tumilson CA, Lea RW, Alder JE, Shaw L. Circulating MicroRNA Biomarkers for Glioma and Predicting Response to Therapy. *Molecular Neurobiology*. 2014;50(2):545-558. doi:10.1007/s12035-014-8679-8

9. Bertolini I, Terrasi A, Martelli C, et al. A GBM-like V-ATPase signature directs cell-cell tumor signaling and reprogramming via large oncosomes. *EBioMedicine*. February 2019. doi:10.1016/j.ebiom.2019.01.051

10. Figueroa J, Phillips LM, Shahar T, et al. Exosomes from Glioma-Associated Mesenchymal Stem Cells Increase the Tumorigenicity of Glioma Stem-like Cells via Transfer of miR-1587. *Cancer Res*. 2017;77(21):5808-5819. doi:10.1158/0008-5472.CAN-16-2524

11. Forgac M. A new twist to V-ATPases and cancer. *Oncotarget*. 2018;9(61):31793-31794. doi:10.18632/oncotarget.25883

12. McGuire C, Cotter K, Stransky L, Forgac M. Regulation of V-ATPase assembly and function of V-ATPases in tumor cell invasiveness. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*. 2016;1857(8):1213-1218. doi:10.1016/j.bbabio.2016.02.010

13. Stransky L, Cotter K, Forgac M. The Function of V-ATPases in Cancer. *Physiological Reviews*. 2016;96(3):1071-1091. doi:10.1152/physrev.00035.2015

14. Pérez-Sayáns M, Somoza-Martín JM, Barros-Angueira F, Rey JMG, García-García A. V-ATPase inhibitors and implication in cancer treatment. *Cancer Treatment Reviews*. 2009;35(8):707-713. doi:10.1016/j.ctrv.2009.08.003

15. Luciani F, Spada M, De Milito A, et al. Effect of Proton Pump Inhibitor Pretreatment on Resistance of Solid Tumors to Cytotoxic Drugs. *JNCI Journal of the National Cancer Institute*. 2004;96(22):1702-1713. doi:10.1093/jnci/djh305

16. Rofstad EK, Mathiesen B, Kindem K, Galappathi K. Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice. *Cancer Res*. 2006;66(13):6699-6707. doi:10.1158/0008-5472.CAN-06-0983

17. Di Cristofori A, Ferrero S, Bertolini I, et al. The vacuolar H+ ATPase is a novel therapeutic target for glioblastoma. *Oncotarget*. 2015;6(19):17514-17531. doi:10.18632/oncotarget.4239

18. Balasubramaniyan V, Vaillant B, Wang S, et al. Aberrant mesenchymal differentiation of glioma stem-like cells: implications for therapeutic targeting. *Oncotarget*. 2015;6(31):31007-31017. doi:10.18632/oncotarget.5219

19. Zhou L, Wang Z, Hu C, et al. Integrated Metabolomics and Lipidomics Analyses Reveal Metabolic Reprogramming in Human Glioma with IDH1 Mutation. *Journal of Proteome Research*. January 2019. doi:10.1021/acs.jproteome.8b00663

20. Lemée J-M, Clavreul A, Menei P. Intratumoral heterogeneity in glioblastoma: don't forget the peritumoral brain zone. *Neuro-oncology*. 2015;17(10):1322-1332. doi:10.1093/neuonc/nov119

21. Aubry M, de Tayrac M, Etcheverry A, et al. From the core to beyond the margin: a genomic picture of glioblastoma intratumor heterogeneity. *Oncotarget*. 2015;6(14). doi:10.18632/oncotarget.3297

22. Clavreul A, Etcheverry A, Tétaud C, et al. Identification of two glioblastoma-associated stromal cell subtypes with different carcinogenic properties in histologically normal surgical margins. *Journal of Neuro-Oncology*. 2015;122(1):1-10. doi:10.1007/s11060-014-1683-z

23. Histopathological findings in the peritumoral edema area of human glioma. *Histology and Histopathology*. 2015;(30):1101-1109. doi:10.14670/HH-11-607

24. Pessina F, Navarria P, Cozzi L, et al. Maximize surgical resection beyond contrast-enhancing boundaries in newly diagnosed glioblastoma multiforme: is it useful and safe? A single institution retrospective experience. *J Neurooncol*. 2017;135(1):129-139. doi:10.1007/s11060-017-2559-9

25. Pistollato F, Abbadi S, Rampazzo E, et al. Hypoxia and succinate antagonize 2-deoxyglucose effects on glioblastoma. *Biochemical Pharmacology*. 2010;80(10):1517-1527. doi:10.1016/j.bcp.2010.08.003

26. Basanta D, Simon M, Hatzikirou H, Deutsch A. Evolutionary game theory elucidates the role of glycolysis in glioma progression and invasion: *Game theory and the role of glycolysis*. *Cell Proliferation*. 2008;41(6):980-987. doi:10.1111/j.1365-2184.2008.00563.x

27. Bertolini I, Terrasi A, Cristofori AD, Bosari S, Vaira V. Abstract 2889: V-ATPase control of EV signaling in glioma stem cells. *Cancer Research*. 2017;77(13 Supplement):2889-2889. doi:10.1158/1538-7445.AM2017-2889

28. Nilsson RJA, Balaj L, Hulleman E, et al. Blood platelets contain tumor-derived RNA biomarkers. *Blood*. 2011;118(13):3680-3683. doi:10.1182/blood-2011-03-344408