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TITLE

Chaperons moleculae in brain tumors-CHAMOBRAT TRIAL: HSP60 and microRNAs related levels in tissue and circulating exosomes in human brain tumors before and after ablative surgery

IL CANDIDATO
Dott.ssa Francesca Graziano

LA COORDINATRICE
Chiar.mo Prof. Felicia Farina

TUTOR

Chiar.mo Prof. Domenico Gerardo Iacopino

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CANDIDATE

Francesca Graziano, MD

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Dedication

To all the women of my matriarchal family that, each one with different points of view and cultural background, have taught me the fundamental principles of living, loving and deeply beliving in something.

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Abstract

Current regimen for high-grade gliomas is maximal safe surgical resection followed by external beam radiotherapy with concurrent temozolamide. Maximal tumor resection, however, must be balanced with preservation of the patient's neurological function. A crucial prognostic factor in oncological neurosurgery is the extent of resection. Several studies have addressed the importance of extent of resection in gliomas surgery. Despite development in the fields of pre operative and intraoperative neuroimaging and neuromonitoring have ameliorated the survival rate and the quality of life for patients affected by high grade gliomas, the clinical outcome of patients with such gliomas remains extremely poor. Among molecular proteins implied in the brain cancerogenesis, Heat Shock Proteins (HSPs) or chaperones, and miRNAs associated with HSP genes may represent a novel and important research field. The aim of the current project is to research the presence, levels, expression and distribution of HSP60 and some miRNAs involved in their regulation, in pathological tissues and in exosomes isolated by blood samples, obtained from patients with gliomas and atypical meningiomas, before and after ablative surgery. Our preliminary results show that exosomal HSP60 and its regulators can be considered tools for improving diagnostic procedures for diagnosis, patient stratification and/or prognosis of disease outcome. We suggest to start to look at the brain tumors through the Chaperone Eye and consider at least some of these tumors to be chaperonopathies by mistake or collaborationism. Looking at tumors through the Chaperone Eye implies that patient examination should include qualitative and quantitative analyses of Hsps, including those in exosomes, before and after surgery and other treatments for monitoring disease evolution and response to treatment. This conduct

will provide new insights on brain tumors that will enhance progress in clinical applications of chaperones and exosomes, including their use as novel biomarkers or, in future, therapeutic agents.

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List of Abbreviations: High grade glioma (HGG), heat shock proteins (Hsps), Glioblastomas multiforme (GBM), extent of resection (EOR), magnetic resonance imaging (MRI), computerized tomography (CT), and ultrasound (US), diffusion tensor imaging (DTI), Diffusion tensor tractography (DTT), transcranial magnetic stimulation (TMS), direct electrical stimulation (DES), 5-Aminolevulinic acid (5-ALA),, Central Nervous System (CNS), peripheral nervous systems (PNS), basement membrane (BM), extracellular matrix (ECM) and epithelial to mesenxhimal transition (EMT), isocitrate dehydrogenase 1/2 (IDH1/2), blood-brain barrier (BBB), matrix metalloproteases (MMPs), temozolomide (TMZ), Karnofski Perfomance Status (KPS), O^6-methylguanine-DNA methyltransfer-ase (MGMT), carmustine (BCNU) and lomustine (CCNU), vincristine (PCV), Fluorescence guided surgery (FGS), protoporphyrin IX (PpIX), Contrast-enhanced US (CEUS), ultrasound contrast agent (UCA) Intraoperative ultrasound (iUS), somatosensory evoked potential (SEP), chaperoning system (CS), ubiquitin-proteasome system (UPS). chaperone-mediated autophagy (CMA), immune system (IU), microRNA (miRNA), Argonaute (AGO), miRNA-protein complex (miRISC), multivesicular bodies (MVB), Transmission Electron Microscopy (TEM), Atomic Force Microscopy (AFM) and Dynamic Light Scattering (DLS).

CHAPTER 1: INTRODUCTION

Gliomas and other neuroepithelial tumors constitute 49% of primary brain tumors, and meningiomas are the next most frequent histologic type (27%)[1, 2]. Gliomas display histological similarities to glial cells, including astrocytes and oligodendrocytes and, consequently they are classified as astrocytoma, oligodendroglioma, or oligoastrocytoma. According to the 2007 World Health Organization (WHO) classification, gliomas can be categorized as low-grade (WHO grade I and II) and high-grade (WHO grade III and IV). More than half of all gliomas are GBM (Glioblastomas multiforme) (WHO grade IV astrocytoma). Genetic studies have identified a number of recurrent chromosomal abnormalities and genetic alterations in malignant gliomas, particularly in GBM. The overall survival is usually only around 12 months and the overall 5-year survival rate remains less than 5 % [1, 2]. Several studies have addressed the importance of EOR in GBM surgery [3-7]. The European Organization for Research and Treatment of Cancer/National Cancer Institute of Canada Phase III trial has established as current regimen for high-grade gliomas (HGG) a maximal safe surgical resection followed by external beam radiotherapy with concurrent temozolamide oral administration therapy. Maximal tumor resection, however, must be balanced with preservation of patient's neurological functions. Multiple pre-operative techniques have been developed to assist in identifying eloquent areas and their relationships to brain lesions, including functional magnetic resonance imaging (MRI), diffusion tensor imaging (DTI) and transcranial magnetic stimulation (TMS). Intraoperative adjuncts, such as neuronavigation, direct electrical stimulation (DES) mapping, intraoperative MRI or computerized tomography, and 5-aminolevulinic acid (5-ALA) fluorescence, have also been used to maximize the safety of aggressive resection around eloquent areas [8-10,11-14].

The dismal clinical outcome of gliomas has made them an urgent subject of cancer research for identification of novel factors associated with their development. Among the proteins implicated in brain carcinogenesis, molecular chaperones also called heat shock proteins, represent a novel and important research field. Hsps are evolutionarily conserved proteins involved in various cellular processes including brain tumors, and their expression variations are tightly associated with the progressive staging and prognosis of gliomas, which provides an opportunity for using these molecules as biomarkers in diagnosis, and assessing prognosis and response to treatment [15-17].

In this research project, we aim to present a comprehensive review of the main pre-operative and intra-operative approaches currently available to manage brain tumors, with particular attention to HGG. In our project we present a laboratory study on HSP proteins (in particular HSP 60) and miRNA HSP60 related, detected on blood samples and on pathological tissue samples of patients affected by brain tumors (mainly GBM and atypical meningiomas). These proteins, also called chaperons moleculae, were studied over time during the pre operative time and during the post op follow up of the patients enrolled in the study in order to understand their variability and their potential correlation with the brain disease. The main goal of this project is to potentially identify novel biomarkers to detect and follow up HGG or meningiomas and to provide new insights in the understanding of brain tumors and in the developing of future therapeutic targets.

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1 Microscopic, functional and clinical anatomy of the brain

The nervous system is composed by the central nervous system and by the peripheral one. The central nervous system is made by the brain and the spinal medulla, located respectively, inside the cranium and inside the vertebral canal. The nervous system is responsible of integration, processing and coordination of sensitive information and motor stimuli. The superior functions, such as intelligence, memory, learning process, emotions are organized in the central nervous system. The peripheral nervous system includes nerves, ganglion structures seated outside the central nervous system and it favors the connection from periphery to the centre. The neurons are elements highly specialized in transferring information between one region to the other one. The nervous tissue is formed by neurons and glial cells. Neurons present specific properties such as excitability and pulse transmission capability. They communicate each other or with effector organs thanks to membrane communication called synapsis. The communication is immediate and unidirectional. Glial cells are supporting and trophic cells and contribute to the revitalization of the nervous cells. Both neurons and glial cells are coming from a common staminal cell from a macroscopic point of view the central nervous system is formed by grey substance and by a white one. The grey one contains neurons cellular bodies and the white one mainly the assons myelinized. Glial cells are divided in astrocytes, oligodendrocites, microglia cells, ependimal cells and Schwann cells. All of them act as scaffolds to neurons and provide them metabolic support. Different brain regions are able to rework the electrical connections when new information are elaborated. This ability is called neuronal plasticity and this allows for neuronal learning.

Neuroplasticity can be defined as a continuous process subserving short, middle and long-term remodeling of the neurono-synaptic organization, in order to optimize the functioning of neural networks – during phylogeny, ontogeny, physiological learning and after brain insult [18]. Neural plasticity is possible solely in a dynamic account of cerebral distribution, in which the nervous system is an ensemble of complex subcircuits that form, reshape and flush information dynamically [19]. Over the years, this traditional static view of thefunctional organization of the brain had changed [20]. In the last 10 years the impressive work research of Duffauet al. has broken with this classical modular and inflexible model by proposing a dynamic and connectomal anatomy underlying neural circuits, that explains a major inter- individual anatomo-functional variability [21] and that allows postlesional cerebral adaptive phenomena able to maintain neurological and cognitive functions [19] opening the window to new therapeutic strategies in brain-damaged patients, especially in the field of neurooncology [22]. Advances in functional neuroimaging have recently provided new insights into the neurobiology of cerebral functions, especially with regard to language, by studying the whole brain during functional tasks, both in healthy subjects and in brain-damaged patients. Using task-based functional MRI, the classical concept of language organization shifted towards distributed models [23]. A step forward the understanding of function brain organization and re- organization after cerebral injury, has been made thanks the knowledge provided by the intra operative brain mapping in awake patients. Intraoperative mapping enables to check whether brain structures that have to be surgically removed for oncological (e.g. involved by a glioma) or epileptological purposes are still essential for neurological, neurocognitive and behavioral functions. Direct electrical stimulation offers a unique opportunity to map directly the human connectome in vivo. In clinical routine, this technique that permits to achieve tumor resection or epileptological area up to individual functional boundaries [24] has extensively been validated as an easy, reliable, reproducible, and safe mapping method, and it is now considered as the gold-standard in glioma surgery [24-25]. Conversely to functional neuroimaging, which provide purely anatomical fiber reconstruction without functional implications, DES is able to detect the structures essential for neural functions, particularly with regard to white matter bundles, by inhibiting a subpathway during a few seconds – with the possibility to validate if the same functional consequences are reproduced when repeat stimulations are applied over the same structure. Of note, by gathering all cortical and axonal sites where the same type of errors has been caused by stimulation, one would build up the subcircuit of the disrupted subfunction.

As a consequence, DES represents a unique tool to detect with a great precision (about 5 mm) and reproducibility, in vivo in humans, the structures that are crucial for neural functions. DES is able to map brain function patient specific and lesion specific, since it can define by direct electrical stimulation, the functions compromised and, whether activated, the areas that replace that specific function.

1.1 Central Nervous system tumors epidemiology and risk factors

Central Nervous System (CNS) cancers are a group of different tumour entities anatomically close to each other but diverse in terms of morphology, site, molecular biology and clinical behaviour and presumably aetiology. In Europe, the World incidence of primary CNS cancers ranges from 4.5 to 11.2 cases per 100,000 men and from 1.6 to 8.5 per 100,000 women. The two most common CNS cancers, high-grade glioma and brain metastasis occur more frequently during adulthood and especially among the elderly. In Europe, the peak of incidenceis 18.5/100,000 in people aged over 65 years [26]. The relative frequency of CNS tumours is however highest during childhood, when they account for 23% of all the cancers diagnosed. In adults the 5-year survival rate for the primary CNS cancers in Europe was 17% for males and 19% for females (1995–2002), with differences across European regions. Survivorship is higher for young European patients – 63% – than for the elderly ones. Statistics on CNS tumours are estimated by grouping all malignancies arising in all the CNS anatomic sites: meninges, brain, spinal cord, cranial nerves and other localisation of CNS. However, rare tumours are more appropriately defined as a combination of topographical and morphological characteristics, as defined by the International Classification of Diseases for Oncology.The Surveillance of Rare Cancers in Europe (RARECARE; www.rarecare.eu) project, is a large collaboration of population-based cancer registries (CRs) across Europe which provides a list of rare cancers on the basis of topography and morphology [2]. Clinical factors, such as difficulties in achieving diagnosis, in clinical decision making and in conducting clinical studies and the lack of standardised treatment mainly affected the definition of the type of cancers of the RARECARE list. Under the threshold proposed by the RARECARE project, incidence lower than 6 per 100,000 per year, CNS malignant tumours are included. However, despite the rarity of entities selected by using this cut-off, there are many sub-entities for each group (i.e. different histologies and WHO grade of glial tumours) with different prognosis and different treatment approaches. The key epidemiologic determinants of glioma risk include advancing age, male sex, and Caucasian race [26-29].

Few environmental or lifestyle exposures, except for ionising radiation, have been found to be consistently associated with glioma risk. Suspected risk factors include lifestyle behaviors (e.g., smoking, alcohol consumption, coffee drinking), infectious agents (e.g., polyomaviruses, cytomegaloviruses, influenza, varicella zoster, Toxoplasma gondii), diet/vitamins (e.g., nitrosamine compounds, vitamin C, vitamin D3), beauty products (e.g., hair dyes and lighteners, hair waving and straightening chemicals), industrial exposures (e.g., rubber manufacturing, petroleum products), mobile phones, electromagnetic fields, allergies/immunity, agricultural/farm animal exposures, handedness, birth weight/height, and various genetic polymorphisms [28-30].

1.2 Histopathological classifications of brain tumors

Glial cells or neuroglia are a heterogeneous cellular compartment of the nervous tissue associated with neurons and have various roles in the central and peripheral nervous systems (CNS and PNS, respectively). These cells are more numerous than neurons, and are functionally different from them since they are involved in the maintenance of neuronal homeostatic balance and myelination, providing structural support and protection for neurons and furthermore, during embryonic development glial cells regulate the differentiation and the neuronal survival. In the CNS, glial cells are variable in number and in type, including two main groups: macroglia and microglia. The first group includes the larger types of glial cells originating in the neural plate represented by astrocytes and oligodendroglia, whereas the second group includes smaller types of cells originating in the mesoderm. In the PNS, glial cells are represented by the Schwann cells that develop from the neural crest cells that migrate away from the neural tube; and satellite cells that appear postnatally [31]. Until the discovery of Neural Stem Cells (NSCs) in the CNS, it was assumed that glial cells were the only ones endowed with the capacity to divide and, consequently, the histological classification of brain tumors was based on glial cells features. In fact, gliomas display histological similarities to glial cells, including astrocytes and oligodendrocytes and, therefore, they are classified as astrocytoma, oligodendroglioma, or oligoastrocytoma. More than half of gliomas are glioblastoma multiforme (GBM; WHO grade IV astrocytoma). The new 2016 World Health Organization Classification of Tumors of the Central Nervous System is both a conceptual and practical advance over its 2007 predecessor [32]. For the first time, the WHO classification of CNS tumors uses 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.

Glioblastomas are divided in the 2016 CNS WHO into (1) glioblastoma, IDH-wildtype (about 90 % of cases), which corresponds most frequently with the clinically defined pri- mary or de novo glioblastoma and predominates in patients over 55 years of age; (2) glioblastoma, IDH-mutant (about 10 % of cases), which corresponds closely to so- called secondary glioblastoma with a history of prior lower grade diffuse glioma and preferentially arises in younger patients and (3) glioblastoma, NOS, a diagnosis that is reserved for those tumors for which full IDH evaluation cannot be performed. In this new classification, the diffuse gliomas include the WHO grade II and grade III astrocytic tumors, the grade II and III oligodendrogliomas, the grade IV glioblastomas, as well as the related diffuse gliomas of childhood. This approach leaves those astrocytomas that have a more circumscribed growth pattern, lack IDH gene family alterations and frequently have *BRAF* alterations (pilocytic astrocytoma, pleomorphic xanthastrocytoma) or *TSC1*/*TSC2* mutations (subependymal giant cell astrocytoma) distinct from the diffuse gliomas. In other words, diffuse astrocytoma and oligodendrogliomas are now nosologically more similar than are diffuse astrocytoma and pilocytic astrocytoma; the family trees have been redrawn [32].

CHAPTER 2: HIGH GRADE GLIOMA

2 Gliomagenesis overview

GBM incidence in the United States was estimated to be 3:100,000 with more than 10,000 cases being diagnosed annually. It constitutes 45.2% of all malignant CNS tumors, 80% of all primary malignant CNS tumors, and approximately 54.4% of all malignant gliomas. Mean age at diagnosis is 64 years and it is 1.5 times more common in men than in women and 2 times more common in whites compared to blacks. The incidence has increased slightly over the past 20 years mostly due to improved radiologic diagnosis and increase in life span of men and women [26] Recent genetic studies have identified a number of recurrent chromosomal abnormalities and genetic alterations in malignant gliomas, particularly in GBM. The main biological processes in gliomagenesis are extracellular matrix (ECM) remodelling and epithelial-mesenchymal transition (EMT)[33]. Tumor-cell growth and invasion are linked to ECM remodelling, involving proteolysis. Physiologically, basement membrane (BM) separates epithelial cells from the ECM preventing their interaction with the microenvironment. Destabilized BM allows contact between epithelial cells and various signalling ECM proteins. Expression of mesenchymal cytoskeletal proteins and deposition of ECM proteins promote the migratory potential of glioma cells by activating integrin and signalling pathways. These changes activate the invasiveness and migratory potential of glioma cells, leading to poor prognosis with evasion from treatment agents and establishment of resistance to therapeutics. One of the major challenges in glioma treatment is the migratory potential of glioma cells breaching into the blood-brain barrier (BBB)-protected areas of the brain. Hence, unravelling the migration mechanisms has always been of great importance in glioma research. The major players contributing to glioma-cells dissemination are ECM degradation through matrix metalloproteases (MMPs), intracellular cytoskeletal rearrangements, and stimuli to chemo attractants. The MMPs mainly aid in degrading the basement membrane along with ECM. During EMT, MMPs secreted by glioma and stromal cells facilitate ECM remodelling, invasiveness through ECM degradation, promoting migration by release of growth factors embedded within the ECM for activation of signal transduction cascades. The levels of MMPs are correlated with histological grading of gliomas.

2.1 Clinical presentation and prognostic outcome

Clinically, patients with GBM may present with headaches, focal neurologic deficits, confusion, memory loss, personality changes, and seizures. As demonstrated extensively, GBM prognosis si related to age, Karnofski Perfomance Status (KPS), gender, hystology type and mainly to the extent of resection and adyuvant therapy.The impact of both extensive resectionand chemotherapy with temozolomide (TMZ)on patient survival has been demonstrated. In particular, following the randomized Phase III trial by Stupp et al., the current standard, postsurgical, first-line treatment for newly diagnosed glioblastoma is based on concurrent radiation therapy and TMZ chemotherapy followed by 6 cycles of adjuvant TMZ therapy, i.e., the Stupp protocol [4]. Temozolomide treatment has been associated with significantly better tolerability and fewer side effects than other drugs previously used for chemotherapy in patients with glioblastoma [6]. The role of O^6-methylguanine-DNA methyltransfer-ase (MGMT) promoter methylation has been studied and associated with a better outcome in patients with glioblastoma undergoing TMZ chemotherapy, and analysis of MGMT promoter methylation status has become standard in patients with newly diagnosed glioblastoma.[6] Sanai et al.proved that in newly diagnosed glioblatomas an EOR above 78% correlates with significant survival advantage. Such benefit further improves when EOR is between 95% and 100%, as documented by postoperative MRI. Yet the use of 5-ALA and fluorescence-guided resection may help to increase tumor EOR and achieve a better outcome [7,8, 10]. Despite the advancements in treatment modalities, GBM prognosis remains poor, with an overall patient survival ranging between 12 and 18 months,a 2-year survival rate of 26.5%, and a 70% recurrence rate within 1 year from initial treatment [26, 29, 34].

2.2 Glioma therapeutic management

Currently, the standard of care for newly diagnosed glioblastoma is derived from a randomized clinical trial published in 2005 and consists of maximal feasible surgical resection followed by radiotherapy with concurrent and adjuvant temozolomide [4]. This treatment regimen, known as the Stupp regimen, has resulted in a median survival of 14.6 months in patients receiving temozolomide therapy alone compared with 12.1 months in patients receiving external beam radiation alone [2-5].The adoption of the Stupp regimen has been credited for improvement in the survival of patients with glioblastoma multiforme from 2005 to 2008 compared with the survival from 2000 to 2003, particularly among younger patients.

The US Food and Drug Administration (FDA) approved the use of temozolomide for the treatment of glioblastoma multiforme in March 2005. The FDA also approved carmustine wafers (initially in 1997) and bevacizumab (in 2009, for glioblastoma multiforme that has progressed after initial treatment) for the treatment of glioblastoma multiforme, but neither of these treatments has demonstrated a significant role in the upfront treatment of this disease· Cytotoxic therapy for GBM has evolved, more due to the approval of temozolomide for newly diagnosed GBM. Active agents include also the nitrosureas: carmustine (BCNU) and lomustine (CCNU), platinum agents, etoposide, irinotecan and PCV combination.

Temozolomide is a newer oral alkylating agent that has excellent penetration into the central nervous system. It is an imidazotetrazine derivative of dacarbazine. It has 96-100% bioavailability and promotes the methylation of the O^6 position on guanine (N7-guanine and N3 adenine). Major toxicities include nausea and myelosuppression (often low platelet counts). Usually, an oral 5-HT3 antagonist is given 30–60 minutes prior to each dose. Dosage is usually started at 75 mg/m^2 daily concurrently with 6 weeks of regional radiotherapy to the surgical cavity and followed by 6 adjuvant cycles with maintenance dose at 150 mg/m^2 daily p.o. for 5 days for the first cycle and if well tolerated then scaled up to 200 $mg\ m^{-2}\ day^{-1}$ for 5 days in 28-day cycles [34]. Concurrent RT and TMZ results in a median overall survival (OS) of 14.6 months and 2-year survival rate of 26.5%. Temozolomide can be effective for recurrent GBM while its efficacy can be increased with metronomic rather than standard schedule as well as with high average daily dose (N100 mg/m^2) [35]. Patients with pro- moter MGMT methylation treated with RT/TMZ have a median survival of 23 months and 5- year OS of 14% versus RT alone (15 months and 5% respectively) [4].The nitrosureas, carmustine (1,2 bis[2-chloreoethyl]-1-nitrosurea BCNU) and lomustine (CCNU), are two alkylating drugs used in the treatment of GBM and are associated with nausea, vomiting, rash, pulmonary fibrosis (rare) and a delayed myelosuppression and typically given in the dosage of 80 $mg\ m^{-2}\ day^{-1}$ i.v. on days 1–3 every 6–8 weeks with radiation [36]. Single agent BCNU is used most often as second line treatment in GBM that has progressed after TMZ. Carmustine biodegradable wafers (Gliadel) are an implantable depot form of BCNU that are placed in the cavity that is formed after re- section of newly diagnosed or recurrent tumor. The wafers release topically BCNU for about 3 weeks. Although they are considered relatively safe they are not used by most centers because of delayed wound healing, intracranial edema, cerebrospinal fluid leakage, intracranial infection and seizures have been reported.

CHAPTER 3: MENINGIOMAS

3 Meningiomagenesis overview

As described in 1922 by H. Cushing, meningiomas are tumors that originate from meningeal cells [37]. The 2007 World Health Organization (WHO) classification of meningiomas reports the benign meningiomas are the most frequent (92% of the total), followed by atypical meningiomas (5-7%) and malignant meningiomas are the less frequent, being 1-3 % of the total. In the 2016 update of the WHO classification of brain tumors, that one regarding meningiomas did not undergo revisions, besides the consideration of brain invasion as an important criterion for the atypical meningiomas characterization (WHO grade II). Indeed, the presence of brain invasion in WHO grade I meningiomas determines tumor recurrency and predicts a mortality rate similar to those meningiomas classified as WHO grade II. Therefore, brain invasion should not be considered just a staging feature but a true grading feature, that can, alone, satisfy the histological criterion for diagnosing an atypical meningioma, WHO grade II [32, 38, 39]. Overall survival rates for patients affected by meningiomas (whether benign or atypical), are 81% at 2 years and 69% at 5 years. For malignant meningiomas the 5 year survival rate is 54.6 %[40]. Age is a prognostic factor, since older patients have poorer prognosis. In patients that undergo a complete benign meningioma resection, a 20.5 % recurrence rate at 5 year is documented [41, 42].

3.1 Meningioma therapeutic management

The benign histology of meningiomas influence the surgical approach since the complete tumor removal determines the definitive cure of the pathology. According to the fourth classification of WHO in 2007, 92% of meningiomas are divided into benign tumors (WHO grade I) which comparatively grows in slowly. However, 1-3% are known as malignant meningiomas which has high recurrence rate, morbidity rate, and mortality rate even after surgical treatment. The treatment principle of meningioma is complete surgical removal (Simpson grade I-III), however, the recurrence rate of malignant meningiomas after complete extirpation is 20-40% by the decade and it increases to 40-60% in partial extirpation patients (Simpson grade IV). Also, the 5-year overall survival rate of malignant meningiomas is 32-64%, so adjuvant radiation therapy or radiosurgery is attempted regardless of the extent of surgery. The treatment principle of a meningioma is surgical removal, and for that reason, surgical status becomes an important factor for the prediction of recurrence.

Prognosis for patients with incomplete mass removal is not good, including recurrence and others. Benign meningiomas are less invasive, conversely, malignant meningiomas often recur. Tumor recurrency may be managed by surgery, repeated meningiomas removal may, itself, determine a poor prognosis due to the morbidity of the surgical injury to the superficial cutaneous tissue and to the surrounding vascular and nervous structures. Therefore, in the malignant meningiomas or in those atypical radiotherapy or radiosurgery is encouraged [41, 42].

CHAPTER 4: SURGICAL BRAIN TUMOR APPROACH

The management of a patient affected by brain tumor is demanding and requires maximal attention. The first step is the patient clinical exam. It's essential to evaluate the patient neurological status and the general clinical performance (Karnofski Perfomance Status, KPS) and the anaesthesiological risk, in order to evaluate the eligibility of the patient for surgery and to predict the post operative outcome. The second step is to depict neuroradiologically the lesion. Pre operative neuroimaging allow for an anatomical and funtional charcterization of the lesion. Indeed it's essential to understand the exact brain tumor location, its relationship with vascular and nervous structures and than to evaluate the lesion from a functional point of view, which it means to evaluate if it involves and compromise eloquent areas.

At the end of the pre operative approach, the proper surgical planning is the next step. Intra operatively, specific tools such us neuronavigation, fluorescence, brain mapping and monitoring etc, allow for a more accurate, complete and safe brain tumor removal.

Following, the pre operative neuroimaging studies and the intra operative managament are discussed in detail.

4 Pre operative neuro-radiological evaluation

The gold standard in detecting a brain glioma is magnetic resonance imaging (MRI). Specific sequences include a volumetric T1-weighted, Gd-enhanced sequences, FLAIR sequences, and T2-weighted sequences [43]. Functional MRI (fMRI) has become a widely available clinical application for pre-surgical evaluation of functional areas prior to brain tumor surgery. It is a non-invasive brain-mapping tool to guide neurosurgical treatment decisions. The fMRI depicts functional networks involved in an investigated function such as a motor or language task. However, especially near the tumor mass, vascular changes can lead to a neurovascular uncoupling instead of the regular coupling and thereby produce false-negative fMRI results, making it unreliable for planning [44]. Although fMRI allow for cortical localization of neurological function, it is unable to delineate subcortical white matter tracts arising from or connecting relevant cortical areas.

In the past decade, pre-operative functional MRI and DTT became part of the clinical routine to decrease the surgical risk of tumors in eloquent brain areas, like motor, language, and visual cortex. In order to reduce the risk to damage the motor, or sensitive, or cognitive pathway, diffusion tensor imaging (DTI) tractography (DTT) is one of the most successful pre-operative examinations [45]. DTI is a MRI technique that measures water diffusion tensor in living tissues. DTI is sensitive to thediffusion of water molecules. In white matter, theprincipal direction of this diffusion corresponds with the main fibre orientation within a given voxel. DTT is a method based on diffusion tensor magnetic resonance imaging. With the use of this information, DTT is capable of depicting subcortical white matter tracts in vivo, which is not possible by conventional imaging. DTT algorithms can be divided into deterministic and probabilistic methods, the reliability of which has been previously validated by dissection studies [44, 45]. Probabilistic tractography provides the possibility to investigate probabilistic connectivity of different brain areas; thus, it can identify the subcortical nuclei based on their cortical connections [46]. By determining individual anatomy and identifying the dislocation of thalamic nuclei, surgery can be planned to target the core of the tumor, which may be safer and more effective than surgical resection planned according to the results of conventional MRI. The pre-surgical DTT of the corticospinal tract and its inclusion in the neuronavigation system lead to a larger EOR and to an improved overall functional status.

4.1 Brain surgery guided by Fluorescence and Neuronavigation

Fluorescence guided surgery (FGS) has revolutionized the neurosurgical treatment of brain tumors over the past decade. The use of 5-ALA (5-Aminolevulinic acid) and FGS in patients with gliomas was described in 1998 [47]. 5-ALA allows intra-operative visualization of the tumor bulk in addition to the surrounding zone of tumor infiltration present in malignant gliomas. 5-ALA-induced fluorescence supports the neurosurgeon, during tumor resection, with real-time information for differentiating tumor from normal tissue in a way that is independent of neuronavigation and brain shift. 5-ALA is a precursor of the Heme synthesis pathway, which leads to the production of protoporphyrin IX (PpIX). This is a molecule that emits fluorescence when excited by appropriately filtered light. Under blue-violet light excitation, PpIX emits light in the red region of the visible spectrum, enabling identification of tumor tissue that would otherwise be difficult to distinguish from adjacent normal brain tissue. Fluorescence-guided surgery with 5-ALA allows the identification of tumor tissue with great accuracy, but only on the surface of the surgical cavity; to categorize an area as 5-ALA positive, it is necessary to expose and evaluate it in blue light [47-49].

In other words, 5-ALA does not allow a complete overview of the tumor; for example, 5-ALA cannot show a residual mass if its surface is not directly exposed [50, 51]. Neuronavigation allows the use pre-operatively acquired images such as those provided by CT, MRI, functional MRI, and DTI to achieve orientation in the surgical field. Neuronavigation is extremely helpful in finding the tumor and the surrounding neurovascular structures, but it is affected by brain shift and brain deformation that progressively degrade the information during surgery [52].

4.2 Intraoperative elettrophisiological mapping and monitoring

The concept of the brain connectomics has revolutionized glioma surgery in eloquent hemispheres thus, only the functional mapping could be considered the primary method for determining tumor resectability [53, 54]. Indeed, if direct electrical stimulation (DES) demonstrates no functional localization within the tumor, or within portions of the tumor, then resection is performed within the context of maximal safe resection.Thus, lesions viewed by some physicians as “inoperable” or “unresectable” based on imaging studies may very well be amenable to resection with the use of DES [53, 54]. Intra-operative localization of eloquent cortex may be achieved through cortical electrical stimulation in awake patients and somatosensory evoked potential (SEP) phase reversal technique in sedated patients. The technique uses discrete cortical electrical stimulation and remains the gold standard because it can be used to localize a variety of eloquent cortical areas (sensory, motor, and language areas) [53]. Its use, however, requires an awake patient. The SEP phase-reversal technique is mainly useful for localizing the central sulcus, usually around the upper limb somatosensory focus. While other non-invasive, pre-surgical modalities such as functional MRI, TMS, magnetoencephalography, and diffusion tractography may become increasingly useful adjuncts for localizing eloquent areas and pre-operatively assessing surgical risk, intra-operative DES is currently the most accurate, and robust method available for identifying functional brain tissue. While tractography is helpful here for defining white matter pathways and guiding the use of subcortical DES, resection limits should be ultimately defined by DES alone [55].

4.3 Intra operative CT, MRI and CEUS

Intraoperative MRI and CT (iMRI and iCT) overcome brain shift and brain deformation and offer high spatial resolution and a wide field of view, but they are expensive as well as time- and space-consuming [56].

Additionally, it is not possible to operate under direct imaging guidance; consequently they cannot be considered real-time intra-operative imaging modalities. Intraoperative ultrasound (iUS) has been used in neurosurgery since the early 1980s, and over the years a lot of applications have been reported. iUS is truly a real-time, dynamic technique that offers a good temporal and spatial resolution [57]. Its high spatial resolution allows accurate tissue differentiation, which has been shown to improve the EOR in glioma surgery. Contrast-enhanced US (CEUS) is an iUS modality that uses an ultrasound contrast agent (UCA) to improve the contrast between tumor, healthy tissue, and artefacts. CEUS can highlight all glial tumors, particularly GBMs, with a specific contrast enhancement, which also allows their characterization and visualization in the surgical volume. It can overcome the neuronavigation limitations and may highlight fluorescent tumor areas hidden by brain collapse [57-59].

CHAPTER 5: THE CHAPERONOLOGY

5 Molecular chaperones: locales of residence, functions, and roles during tumorigenesis

Molecular chaperones are an important class of molecules with various functions that can be expressed intra- and extracellularly. These moleculae are components of the chaperoning system (CS), an organism together with co-chaperones, chaperone co-factors, and chaperone receptors and interactors [64, 65]. They are conserved during evolution and their main function is the maintenance of protein homeostasis by assisting in the folding of client polypeptides, refolding of partially denatured proteins, and degradations of proteins damaged beyond repair [60]. The main partners of the chaperoning system in the maintenance of protein homeostasis (i.e., the canonical functions) are the ubiquitin-proteasome system (UPS) and the chaperone-mediated autophagy (CMA) machinery, whereas the major partner for non-canonical functions is the immune system (IS).

Heat shock proteins (Hsps) are are a group of intracellular proteins induced by different stressors, such as heat shock, hypoxia, ischemia, heavy metal or ethanol exposure, and infections [63, 66]. These proteins can be also called molecular chaperons, although not all molecular chaperons are Hsps. They can be classified according to their molecular weight, as follows: Super heavy, 100, 90, 70, 60, 40, small Hsp (sHsp), including chaperones within the following ranges in kDa: 100 or higher, 81-99, 65-80, 55-64, 35-54, and 34 or lower, respectively [64, 65]. As chaperons moleculae, they are involved in various physiological mechanisms in normal cells, such as DNA replication, gene expression regulation, participation in immune system regulation [68, 69] and cell differentiation [70].

CS and IS are normally in a stable equilibrium in order to maintain a proteins homeostasis. When this interaction is altered for chaperon mutation or chaperon overexpression or specific post-translational modification, pathologic conditions such as chronic inflammatory or autoimmune disorders, or even cancer progression and metastasization, may occur [63, 67].

Few studies have evaluated the relationship between human tumors and Hsps and there is evidence of a positive correlation between Hsps levels and tumour progression. HSP60 is over expressed in some human brain cancers, in particular in glioblastoma, and it is responsible of a cytoprotective effect that involves stabilization of survivin levels and restraint of p53 function [50]. High expression of HSP60 was detected in high-grade gliomas and also in meningiomas [51, 52].

Other chaperons moleculae, such as HSP40 (DnaJ), HSP70, and HSP90 were found elevated in all brain tumours [134-136]. In glioblastoma, as well as in other cancers, a positive correlation has emerged between high expression of Hsps and the triggering of the invasive and metastatic potentials of glioma cells [33, 137]. Indeed, high levels of some Hsps in various types of cancer, usually indicate a protective effect for tumoral cells against therapy-induced apoptosis and lead to a poor prognosis in terms of survival rate and response to therapy, since they may confer a multi-drug resistance [16, 63, 71, 72, 85, 86].

Brain tumor tissue studied by Western Blot and Immunohistochemistry (IHC) showed a marked increase in the constitutive expression of HSP27, HSP90, GRP 78 and 75, and HSP70, and the co-chaperone HSPBP1 [138-142, 143,145-147]. Experimental *in vivo* tumors grown from glioma cell lines express HSP27 and HSP70 at high levels [141, 148]. HSP27, HSP60, HSP70, and HSP90, namely the Hsps overexpressed in glioma cells, can all be released via exosomes [149].

It is likely that extracellular Hsps participate in immune system stimulation or, on the other hand, they may favour tumor escaping from immune reaction [64, 65, 77, 94].

The role of HSP60 on gliomagenesis is still poorly understood, although it has been established that it is differentially expressed in glioblastoma cell lines [149, 150]. Depletion of HSP60 in *in vivo* models of GBM WHO grade IV, leads to the intracranial tumor regression [100]. HSP60 upregulates various pathways that provide survival benefits to malignant cells, bypassing apoptosis and/or senescence [125], and probably the chaperonin regulates the miRNAs pathway too. Increase or decrease in the levels of HSP60 and the pathophysiological significance of these quantitative variations seem to be dependent on the site in which the chaperonin resides [133]. In normal cells, cytosolic HSP60 has a pro-apoptotic role, it binds pro-caspase3 allowing its activation [152]. In tumor cells, HSP60 does not activate the caspase pathway assuring malignant cell survival and, thus, contributes to tumorigenesis [153] playing an anti-apoptotic role [154]. It has been observed that extracellular HSP60 can mediate apoptosis via its ligand (e.g., Toll-like receptor 4), causing a pro-survival effect in glial cells [155]. HSP60 is also involved in microglial activation leading to over proliferation under abnormal conditions [155]. It is possible that glioma cells use exosomes to release HSP60 [156] and other Hsps as a means to modulate the immune system and to release miRNAs molecules, targeting Hsp gene sequences, for example. This would results in a modulation of gene expression in the target cells, determining a modification of the tumour microenvironment and favouring tumour dissemination (Fig. 1).

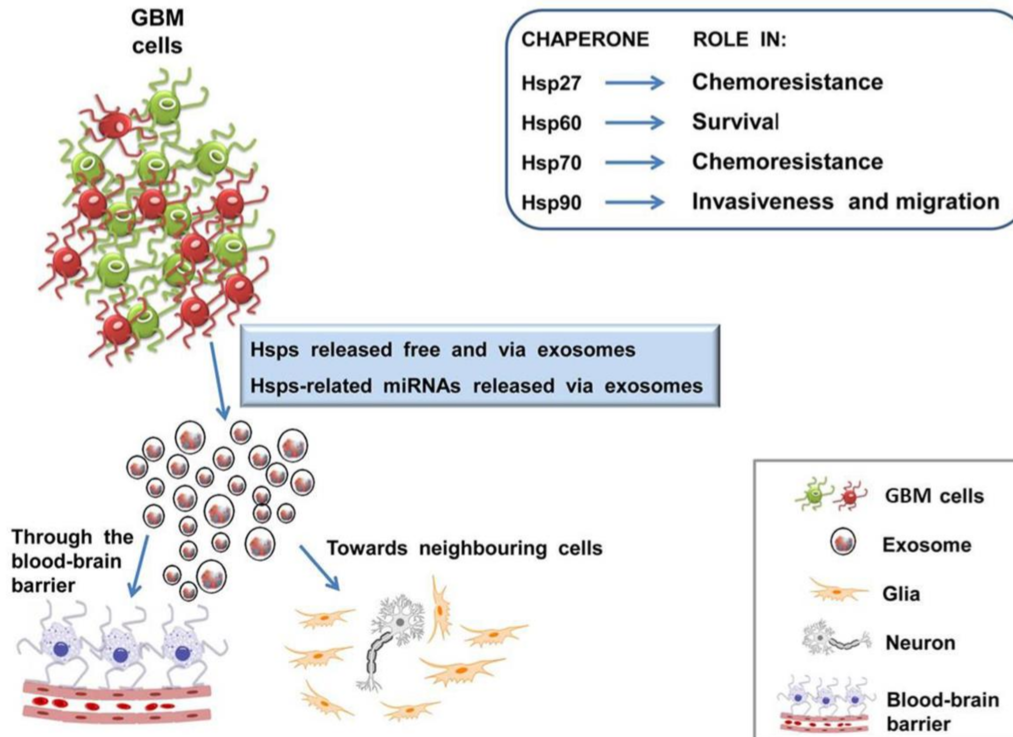

Fig. 1 Chaperones and exosomes from glioblastoma multiforme (GBM) cells are central and conspicuous players in patients and constitute attractive targets for therapeutics along with pertinent conspicuous players in patients and constitute attractive targets for therapeutics along with pertinent miRNAs. The figure presents the elements that provide the foundations for research toward the miRNAs. The figure presents the elements that provide the foundations for research toward the development of new therapies and summarizes the main thesis of this review, as a proposal for future development of new therapies and summarizes the main thesis of this review, as a proposal for future research, since various points are still under scrutiny. GBM cells differentially express various Hsps, which play pivotal roles in chemoresistance, apoptosis escape, invasiveness, and cell migration (shown in the top right inset). Hsps also occur extracellularly, free or in exosomes released by GBM cells carrying these proteins and miRNAs. Exosomes with their cargo would participate in the modulation of the immune system and in the regulation of gene expression in the target cells, and would modify the tumor microenvironment, ultimately favoring tumor dissemination, for instance, through the blood–brain barrier. The challenge consists in manipulating these elements to use them as therapeutic agents.

5.1 miRNAs: intracellular localization, functions, and roles during tumorigenesis

It has been largerly demonstrated that alteration of the gene expression regulation, including chaperons genes, represent an important mechanism favouring tumorigenis and cell metastization.

There is a large number of non-coding nucleic acids (i.e., they do not encode proteins) which have different structures and which act as key regulators of gene expression in many different cellular pathways and locales which have a complex role in patho-physiological mechanisms, that nowadays is more clear [73-76]. Non-coding RNAs are a large group of RNAs that contains subgroups with different functions. They are classified based on the molecular length, as follows: non-coding RNAs with more 200 nucleotides in length are “long” non-coding RNAs (lncRNA), while those with less than 200 nucleotides are the “small” non-coding RNAs (sncRNAs). lncRNAs appear to be epigenetic regulators affecting at various levels protein-coding gene expression. sncRNAs seem to be involved in different key cellular activities, including development and differentiation, transcriptional and post-transcriptional gene silencing, all functions that seem to be determined by the subcellular localization [77]. sncRNAs include various different small RNAs, such as microRNAs (miRNAs), small nucleolar RNAs (snoRNAs), and piwi-interacting RNAs (piRNAs) [78]. miRNAs recognize target RNAs and play a key role in modulating gene expression in a sequence-specific manner [79-80].

Most microRNAs are transcribed by RNA polymerase II under the control of various transcription factors, as long transcripts characterized by a complex hairpin structure (pri-miRNA) and are processed in the nucleus by a RNase III, named Drosha, to generate the microRNAs precursors (pre-miRNA) with a length of 70-100 nucleotides. These precursors are exported from the nucleus to the cytoplasm, in which they undergo further maturation mediated by another RNase III (Dicer), generating a double-stranded RNA (dsRNA) of 22 nucleotides. This dsRNA is subsequently incorporated in a protein complex, known as RISC (RNA-induced silencing complex), which promotes the formation of single-stranded mature molecules. In the RISC protein complex, mature miRNAs are able to pair with complementary sequences present in the 3'-UTR region of mRNAs and to regulate gene expression at the post-transcriptional level. miRNAs may induce mRNA degradation or blockage of translation without mRNA degradation, depending on the complementarity of the bases between the miRNA and its target site [81].

The involvement of miRNA in tumorigenesis was first documented in the majority of B-cell chronic lymphocytic leukaemias (B-CLL). In these tumors miRNAs miR-15a and miR-16a gene sequences were deleted or down-regulated [82].

Since this evidence, many studies pointed out on association between genomic position of miRNAs and the cancer-related genomic regions, accompanied by miRNAs overexpression/downregulation that resulted in transcriptional control and epigenetic changes and in defects in the miRNA biogenesis machinery [83-85].

The mechanism of miRNAs dysregulation in cancer involves amplification or deletion of their genes, abnormal expression of transcriptional factors that regulate miRNAs expression or [82, 84, 86] epigenetic alterations, i.e., aberrant DNA methylation and histone acetylation of miRNAs genes [87] and defects in enzymes involved in the miRNAs maturation steps [88].

The identification of different expression profiles of miRNAs in the neoplastic tissue compared with its normal counterpart supports the hypothesis of their involvement in tumor development and progression, including evasion of apoptosis, and also induction of cell migration, epithelial-mesenchymal transition (EMT), and angiogenesis [89, 90]. miRNAs expression in different tumor-cell types (lung, breast, stomach, prostate, colon, and pancreas) was found distinctive of tumor type, suggesting a definite role of miRNA in human tumorigenesis and indicating that miRNA expression is specific for tumor cell and could be used for tumor classification, diagnosis, and assessing prognosis [91].

The cellular site where miRNA-mediated post-transcriptional regulation occurs, is not clear. It has been shown that Argonaute (AGO) protein-miRNA complex is present in cellular organelles, including endoplasmic reticulum, Golgi apparatus, lysosomes, and endosomes [92-94]. miRNAs have been found in different cellular fractions and in the extracellular environment into which they would be secreted by passive and/or active mechanisms, for example by loading them on the extracellular vesicles, such as exosomes [95]. In the passive mechanism, miRNA secretion would be driven by the natural affinity of the nucleotide sequences with lipid-rafts [96], whereas the active mechanisms (regulated secretion) would be due to translocation into microvesicles that act as signal transporters and mediate distant cell-cell communication [97].

Numerous studies have shown the presence of miRNAs within the exosomes and that the variation of the intracellular amounts of individual miRNAs was reflected within the exosomes [98]. It seems plausible that miRNAs released with the exosomes from cancer cells are able to alter gene expression in surrounding and far away tissues and thereby contribute to the progression of tumors [99]. Currently, the mechanism of miRNA sorting into the exosomes is still poorly understood.

5.2 **Exosomes: nanovectors for extracellular chaperones and miRNAs**

Exosomes are endosomal extracellular small particles of 20-100 nm in diameter that can be secreted by virtually all cell types. Exosomes play an important role in cell-cell communication, exchanging information with the aim to maintain cellular homeostasis [100, 101,102]. The mechanism of exosomes secretion is quite complex. Multivescicular bodies (MVBs) incorporate early endosomes and, after a maturation process, a change in the molecular composition of the vescicels occurs, becoming intraluminal vesicles (ILVs). The vesicles in the MVBs, during this maturation process merge with the lysosomes, which cause degradation of their contents (e.g., in the case of receptors); or constitute a temporary storage compartment; or they may blend with the plasma membrane, releasing exosomes. In brief, MVBs merge with the plasma membrane, resulting in exocytosis of the vesicles contained in MVBs, i.e., exosomes [103-104]. Exosomes are also present in body fluids such as blood; urine; breast milk; saliva; bronchoalveolar lavage; and cerebrospinal, ascitic, and amniotic fluids [101]. Exosomes have specific, cell-type dependent contents. Various functions have been attributed to exosomes, depending on the source cell and its contents. For instance, exosomes are involved in cell-to-cell information transfer [105], inflammation [106], coagulation [107], stem cell activation [108], programmed cell death [109], and in the immune response [106].

It seems plausible that the exosomes' composition depends on the parental cells contents, including lipids; nucleic acids, such as DNA, non-coding RNA (e.g. ribosomal RNA [rRNA], and miRNAs); and proteins. Different sets of proteins are found in exosomes, some strictly involved in vesicle trafficking, such as cell surface receptors; others pertaining to the endocytic pathway (i.e., endosomal sorting complex required for transport, ESCRT, such as Alix; tumor susceptibility gene 101, TSG101; integrin; and tetraspanins); and still other proteins involved in long distance communication, such as cytokines [110], hormones [111], growth and transcription factors [112], and Hsps [113, 114].

It has been demonstrated that HSP60, HSP70, and HSP90 are secreted by cancerous cells via the exosome pathway [16, 113]. Exosomal Hsps may have opposite effects: immunosuppressing or immunostimulating, depending on the interaction between exosomal Hsps and cells of the immune system. For instance, HSP60, HSP70, and HSP90α are actively secreted via the exosomal pathway, and mediate immunomodulatory effects and immune response against cancer cells [114, 115]. Extracellular HSP70 activates macrophages [116] and natural killer cells [117, 118], while HSP90α, when released by invasive cancer cells via exosomes, enhances cancer cell migration [119].

Our research group provided, for the first time, the evidence of identification of HSP60 in exosomes. We explained as well the supposed mechanism by which the chaperonin is loaded onto exosomes, and its location in them [113, 120]. Under normal conditions, HSP60 acts as a molecular chaperone intracellularly but, in several pathological where it is overexpressed, it is released by both, the classical pathway and through exosomes. For example, in heart failure, HSP60 is secreted by cardiomyocytes and its presence in the serum correlates with disease severity and cardiovascular risk [119, 121]. HSP60 is released by adult cardiomyocytes via the exosome pathway in both, the basal state and following mild stress [122]. On the other hand, fibrosarcoma cells release HSP60 through the conventional endoplasmic reticulum-Golgi protein transport pathway [123].

In *in vivo* studies, our research group studied the levels of HSP60 on blood sample of patients affected by bowel cancer, before and after tumor removal. The exosomal HSP60 levels in the plasma of patients before colon cancer surgery were significantly higher than in the exosomes from the same patients after tumor ablation [16]. HSP60 exportation by exosomes, which would undergo post-translational modifications [124], points to roles of this chaperonin in inflammation, immune system modulation, and regulation of tumor microenvironment and growth. Therefore, exosomal HSP60 may contribute to the regulation of gene expression in target cells at distant sites (reviewed in [125]).

Exosomes can be vector not only for HSPs by also for miRNA. Their expression in exosomes, indeed, suggest a regulatory activity on gene expression in recipient and donor cells, and horizontal transfer of genetic information [126,127].

The mechanism by which miRNAs are loaded on to exosomes remains unclear. It is debated whether miRNA molecules are specifically charged onto exosomes, or incorporated passively, as result of mutual affinity between repeated RNA sequences and lipid rafts. As is known, during maturation the miRNA-protein complex (miRISC) is incorporated in late endosomes and subsequently in exosomes, but certain exosomal miRNAs are independent of miRISC [92]. On the other hand, specific sequences present in certain miRNAs may guide their incorporation onto exosomes [128], a possibility supported by the finding that exosomes from diverse sources, including several human body fluids, contain only one copy of a given abundant miRNA because much more copies would be expected from random sampling. This suggests a tightly regulated sorting mechanism that has yet to be clarified. The number of molecules that are loaded within exosomes and whether they can actually determine a modulation of gene expression in the recipient cells are still under study.

In conclusion, it has been shown by various researchers [128-131], that specific mechanisms for miRNAs sorting into secreted vesicles do exist, and that the specifically sorted molecules depend on the translational status in source cells.

It seems plausible that potentially specific miRNAs encapsulated in exosomes can educate the recipient cells, representing an important pathophysiologic event in long distance cell-cell communication. In this regard, it has to be highlighted that miRNAs are preserved in a stable form inside the exosome, in which they are protected from endogenous RNase activity.

CHAPTER 6: AIM OF THE THESIS

This thesis is part of the project, approved by the local ethics Committee of the University Hospital of Palermo and called: "Chaperons moleculae in brain tumors-CHAMOBRAT TRIAL". This project has the goal to research expression of HSP60 and miRNA related levels in tumor and peritumoral cells of primary brain tumors *in vivo* and in circulating exosomes on blood samples before and after ablative surgery.

In order to evaluate the role of HSP60 in brain tumor, in this thesis the experimental design was developed as follow:

1. Evaluation of HSP60 protein level and miRNAs-related in brain tissues;
2. Isolation and characterization of plasmatic exosomes from patients with brain tumor before and after ablative surgery;
3. Evaluation of HSP60 protein level and miRNAs-related in plasmatic exosomes from patients with brain tumor before and after ablative surgery.

Following each phase of the study is described step by step.

Material and Methods

1 Patient selection and enrollment

In our study, after approval by the local ethics Committee, subjects were recruited at the Division of Neurosurgery of the University Hospital of Palermo, in period ranging between December 2015 and October 2018. Written informed consent was obtained from each subject. Following, the Table 1 describes the type of pathology and the follow up for each patient.

In the period from December 2015 and October 2018 a total of 64 patients, affected by different brain lesions were studied. However, among 64 patients only 33 subjects were enrolled in the research and studied over time. Indeed, since our aim was not only to identify HSP60 levels and miRNA related levels on pathological tissue sample, but also to monitor the disease over time and to verify any potential matches between the recurrency of the tumor on the clinico-radiological evaluation and on the molecular analysis, we decided to select only tumors keen on recurrency, such as gliomas and atypical meningiomas. Patients affected by meningioma WHO grade I, were as well enrolled.

Indeed, in these patients, brain surgery provides a complete and definitive cure of the disease. Monitoring HSP60 levels over time in these patients, could be useful to verify if the molecular profile was in agreement with the benign prognosis of the Meningioma WHO grade I.

A total of 33 brain surgeries were performed and blood sample and pathological tissue sample were taken from patient on the day of the surgery. For each patient, the blood samples were collected at different time points, at the time of surgery (Before Surgery, BS), at one week (After Surgery 1, AS1), one month (After Surgery, AS2) and three months after surgery (After Surgery, AS3). A Clinic-Radiological analysis was also performed: most of the patient underwent a neurological assessment every day till the discharge, afterwards, at one and three months. Moreover, a brain MRI post gadolinium at one week and three months from the discharge was performed in order to verify possible matches between the blood sample analysis and the radiological exam.

Pt. No	Name	Age	Sex	Type of Lesion	1°Sample T0 (blood and path tissue)	2 Sample T1 (one week)	3 Sample T2 (one month)	4 Sample T3 (three months)	Notes
NCH01	D. M.	28/04/1948	F	Anaplastic astrocytoma, NOS	02/04/2015	14/04/2015			
NCH02	V. A.	24/02/1946	F	Transitional meningioma (WHO I)	09/07/2015			26/11/2015	23/05/2016 (6 months)
NCH03	C. E.	03/01/1939	F	Neuroblastoma	15/09/2015		11/11/2015 25/11/2015		Dead
NCH04	T. P.	04/07/1956	M	Transitional meningioma with aspects of Atipycal meningioma	13/11/2015		03/05/2016 18/07/2016 26/06/2017		Recurrence 26.06.17 Dead
NCH5	D. M. L.	13/05/1983	F	GBM (WHO IV)	02/12/2015		14/01/2016		16/05/2016
NCH6	T. P.	15/01/1951	F	GBM WHO IV	12/01/2016	20/01/2016			Dead
NCH7	A.G.	22/5/1951	M	Neurofibroma	29/01/2016	05/02/2016		13/05/2016	
NCH8	G. M.	07/04/1962	M	Atipycal meningioma (WHO II)	04/03/2016	16/03/2016		10/06/2016	
NCH9	V. F. C.	23/03/1968	F	Not diagnostic	16/03/2016	29/03/2016	13/05/2016		
NCH10	G. S.	03/01/1953	M	GBM (WHO IV)	24/03/2016	30/03/2016		20/07/2016	
NCH11	S. A.	26/12/1986	F	Transitional Meningioma (WHO I)	30/05/2016	09/06/2016	18/07/2016		09/12/2016

Pt. No	Name	Age	Sex	Type of Lesion	1°Sample T0 (blood and path tissue)	2 Sample T1 (one week)	3 Sample T2 (one month)	4 Sample T3 (three months)	Notes
NCH12	LC. G.	26/10/1961	F	Atipycal Meningioma (WHO II)	10/10/2016	14/10/2016			
NCH13	LC. D.	10/12/1939	M	GBM (WHO IV)	10/11/2016	21/11/2016			
NCH14	G. T.	05/10/1951	M	Meningioma (WHO II)	07/02/2016	13/02/2016			
NCH15	M.R.	04/06/1960	M	GBM (WHO IV)	20/04/2017	27/04/2017	25/05/2017	05/07/2017	
NCH16	G. G.	02/01/1955	M	GBM (WHO IV)	12/07/2017	18/07/2017			07/12/2017 Recurrence October 2018
NCH17	T. A. C.	03/02/1960	F	Transitional Meningioma (WHO I)	25/07/2017	31/07/2017			14/12/2017
NCH18	S. E.	25/07/1955	M	GBM (WHO IV)	18/07/2017		07/08/2017		Recurrence August 2018
NCH19	G. G.	10/04/1942	M	Transitional Meningioma (WHO I)	20/07/2017	27/07/2017			
NCH20	C. G.	14/07/1945	M	Transitional Meningioma (WHO I)	31/07/2017	07/08/2017			
NCH21	F. R.	02/11/1967	F	Transitional Meningioma (WHO I)	05/04/2018	NO	22/05/2018	22/07/2018	
NCH22	S. F.	17/01/1967	M	GBM (WHO IV)	08/05/2018	16/05/2018	21/06/2018	21/08/2018	
NCH23	B. K.	01/01/1981	F	GBM (WHO IV)	22/05/2018	25/05/2018	22/06/2018	22/08/2018	
NCH24	M. D.	13/02/1965	M	GBM (WHO IV)	28/06/2018	03/07/2018	30/07/2018	04/10/2018	
NCH25	Z. M.	16/04/1938	M	Transitional Meningioma (WHO I)	29/06/2018	03/07/2018	30/07/2018	NO	

Pt. No	Name	Age	Sex	Type of lesion	1°Sample T0 (blood and path tissue)	2 Sample T1 (one week)	3 Sample T2 (one month)	4 Sample T3 (three months)	Notes
NCH27	S. E.	25/07/1955	M	GBM (WHO IV)	16/07/2018	20/07/2018	NO		
NCH28	C. P.	19/02/1948	F	Transitional Meningioma (WHO I)	16/09/2018	19/09/2018	22/10/2018		
NCH29	M. A.	16/10/1971	F	Emangioblastoma	19/09/2018	24/09/2018	12/10/2018		
NCH30	M. M.	23/04/1956	F	GBM (WHO IV)	16/10/2018	22/10/2018			
NCH31	G. G.	6/03/1951	M	Transitional Meningioma (WHO I)	16/10/2018	22/10/2018			
NCH32	DF. G.	15/11/1962	M	GBM (WHO IV)	22/10/2018				
NCH33	DL. G.	12/04/1953	M	GBM (WHO IV)	25/10/2018				

Table 1: Table showing patients enrolled in the study and the follow up exams.

2 Specimen collection

As said before, blood was collected from each patients recruited and it was processed for serum isolation. Then the serum samples were stored at − 80°C until processing.The histological specimens were fixed in formalin, embedded in paraffin and cut in 5 μm size sections, which will be used for immunostaining

3 Histological analysis and Immunohistochemistry

For histological analysis, the samples fixed in formalin and embedded in paraffin, were sectioned with a thickness of 5 μm with a cutting microtome. These sections were dewaxed in xylene for 10 minutes and rehydrated by sequential immersion in a descending scale of alcohols and transition in water for five minutes. The sections were then stained with hematoxylin and eosin, mounted with coverslips and finally, observed with an optical microscope (Leica DM 5000 B) connected to a digital camera (Leica DC 300F).

Immunohistochemistry was performed on 5 μm thick sections of paraffin-embedded tissue. The sections were dewaxed in xylene for 30 min at 60°C and rehydrated, at room temperature, by sequential immersion in a graded series of alcohols and transferred into water for five minutes. Subsequently, the sections were immersed for 8min in Sodium Citrate Buffer (pH 6) at 95°C for antigen retrieval and, subsequently, immersed for 8min in acetone at -20°C to prevent the detachment of the sections from the slide. All subsequent reactions were conducted at room temperature. After a wash with PBS (Phosphate Buffered Saline pH7.4) for 5min, the sections were treated for 5min with Peroxidase Quencing Solution (reagent A of Histostain®-Plus 3rd Gen IHC Detection Kit, Invitrogen) to inhibit any endogenous peroxidase activity. Another washing with PBS for 5min was carried out and the sections were treated with a blocking protein (reagent B of Histostain®-Plus 3rd Gen IHC Detection Kit, Invitrogen) for 10min to block non-specific antigenic sites. Subsequently, the sections were incubated overnight, with a primary antibody against human Hsp60 (mouse anti-Hsp60 monoclonal antibody, Sigma, St. Louis, MO, catalogue no. H4149, dilution 1:400). Appropriate positive and negative controls, were run concurrently. After a wash with PBS (Phosphate Buffered Saline pH7.4) for 5min, the sections were incubated with a universal biotinylated secondary antibody (Biotinylated Secondary Antibody reagent C Histostain®-Plus 3rd Gen IHC Detection Kit, Invitrogen) for 10min. After a subsequent washing with PBS for 5min, the sections were incubated with streptavidin-peroxidase complex (Streptavidin-Peroxidase Conjugate reagent D Histostain®-Plus 3rd Gen IHC Detection Kit, Invitrogen) for 10min, and following a further washing in PBS for 5min, the slides were incubated in the dark for 5min with the DAB chromogen (diaminobenzidine) (DAB chromogen reagents E1 and E2 Histostain®-Plus 3rd Gen

IHC Detection Kit, Invitrogen). Nuclear counterstaining was carried out using hematoxylin (Hematoxylin aqueosus formula, N. Cat. S2020, DAKO). Finally, the slides were prepared for observation with coverslips with an aqueous mounting solution. The observation of the sections was performed with an optical microscope (Leica DM 5000 B) connected to a digital camera (Leica DC 300F).

4 MicroRNAs extraction from paraffin embedded tissue and Real Time PCR

Formalin-fixed paraffin-embedded tissues were sectioned at 10 µm, two thick sections were used for RNA isolation and were placed in 1.5 mL microcentrifuge tube DNase/RNase free, to avoid the nucleic acid degradation. Samples were dewaxed with xylene and rehydrated with alcohol scale at room temperature and in agitation. Then pellets were treated with Triazol to lysis tissues, following the manufacturing instruction (miRNeasy Mini Kit®, Qiagen, Hilden, Germany, Cat No: 74104). Briefly, tissue samples were homogenized in QIAzol Lysis Reagent and after addition of chloroform, the homogenate was separated into aqueous and organic phases by centrifugation. RNA partitions to the upper, aqueous phase, while DNA partitions to the interphase and proteins to the lower, organic phase or the interphase. The upper, aqueous phase was extracted, and ethanol was added to provide appropriate binding conditions for all RNA molecules from 18 nucleotides (nt) upwards. The samples were then applied to the RNeasy Mini spin column, where the total RNA binds to the membrane and phenol and other contaminants are efficiently washed away. Then, miRNAs were enriched in a separate fraction through the RNeasy MinElute® Cleanup Kit. Finally, RNA was dissolute in 50µl of RNase/DNase-free H_2O and was determined using Thermo Scientific NanoDrop ND-2000 1-position Spectrophotometer (Thermo Scientific Massachusetts, USA).

The miRNA profiling from our samples had required the following 3 steps: reverse transcription using the miScript II RT Kit (Qiagen, Cat No: 218161), preamplification using the miScript PreAMP PCR Kit (Qiagen) and miScript PreAMP Primer Mix (Qiagen), because the starting materials contain low RNA amounts. Finally, quantification by real-time PCR was performed using the miScript SYBR Green PCR Kit (Qiagen). For the reverse-transcription reaction 20 ng of miRNA-enriched RNA were added to 5X miScript HiSpec Buffer, 10X miScript Nucleics Mix, RNase-free water and miScript Reverse Transcriptase Mix and incubate for 60 min at 37ºC, the for 5 min at 95ºC to inactivate miScript Reverse Transcriptase Mix. The, cDNA generated was preamplificated using the miScript PreAMP PCR Kitand miScript PreAMP Primer Mix prior to miRNA profiling, as described follow. 5 µl of diluted cDNA were added to 5X miScript PreAMP Buffer, HotStarTaq DNA Polymerase, miScript PreAMP Primer Mix (containing the primers: miR-1, miR-206, miR-663, and controls: miR-16, U6 e SNORD45), RNase-free water and miScript

PreAMP Universal Primer, following the manufacturer instruction. Reactions were performed using a PCR Initial activation step (15 min 95ºC) to active HotStarTaq DNA Polymerase and 2-step cycling set up of a denaturation step (30 sec 94ºC) and an annealing/extension step (3 min 60ºC) for 12 cycles. The preaplificated cDNA template was profiling using miScript SYBR® Green PCR Kit (Qiagen) and thermal cycles were performed using the Rotor-gene™ 6000 Real-Time PCR Machine (Qiagen).

5 Blood sample collection and exosomes isolation

Exosomes were isolated from plasma as summarized in Figure 2. Blood samples of 20 patients (drawn one day prior to, and one week after surgical removal of the tumor) were treated with EDTA and centrifuged at 4000 x g for 20 minutes to separate the plasma and then centrifuged at 11000 x g for 30 min to remove cell debris. The supernatant was diluted with PBS then filtered through a 0.2 µm filter (Millex GP, Millipore), followed by 2 step ultracentrifugation at 110,000 x g for 2 hrs to pellet the exosomes. The exosomes were then washed in cold PBS and resuspended in 50 µl of PBS.

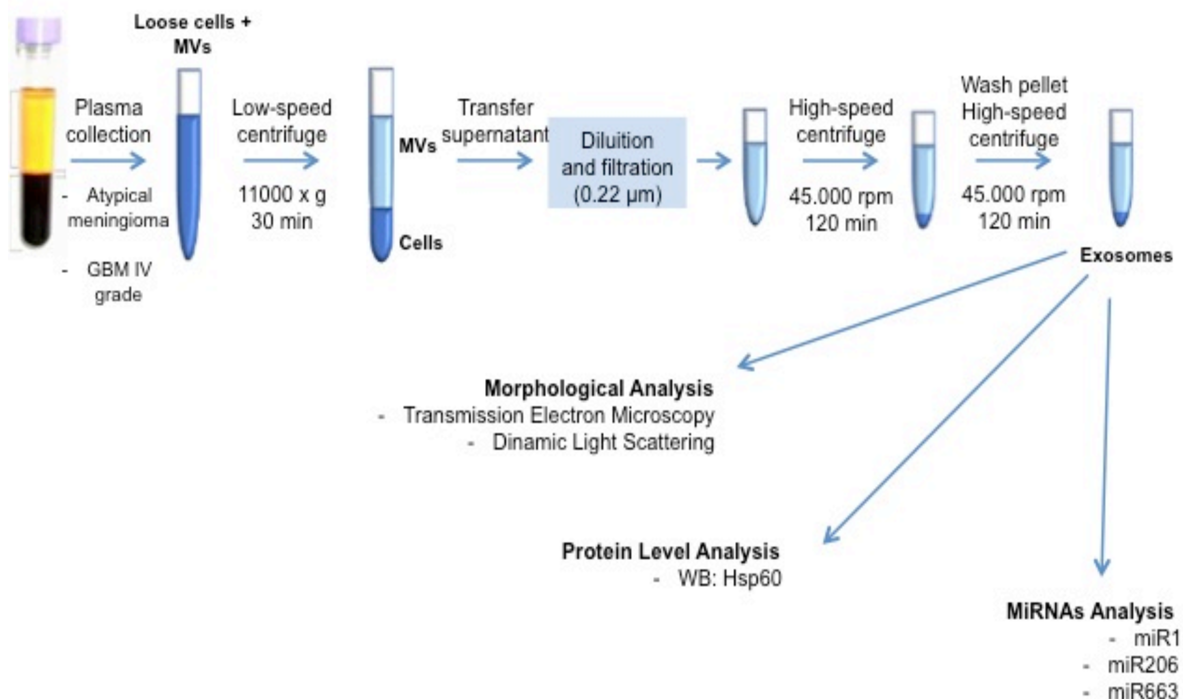

Fig. 2 Procedure of Exosomes extraction

6 Exosomes assessment procedure

To further ascertain the identity of the exosomes obtained from the plasma of the blood samples, exosomal preparations were examined by Transmission Electron Microscopy (TEM), Atomic Force Microscopy (AFM) and Dynamic Light Scattering (DLS). Furthermore, exosomes were tested for markers considered characteristic of exosomes (presence of Alix and Hsc70 proteins).

6.1 Transmission electron microscopy (TEM)

Pellets obtained by ultracentrifugation were resuspended in PBS with the addition of 100µl of freshly made fixative (2.5% glutaraldehyde in PBS) for 30 minutes. After fixation, the preparations were mounted on formvar nickel grids by layering grids over 10 ml drops of exosome preparations for 10 minutes at 24°C. Grid-mounted preparations were prepared for contrast staining by treating them with uranyl acetate (1%) for 5 minutes and with Reynolds' solution for 5 minutes and, finally, rinsing them eight times in distilled water for 2 minutes. After this procedure, the grids were ready for electron microscopy (JEOL JEM 1220 TEM at 120kV).

6.2 Atomic force microscopy (AFM)

Exosome pellets obtained by ultracentrifugation and resuspended in PBS were diluted in water; 50µl aliquots of the diluted samples were deposited on freshly cleaved mica and dried under mild vacuum. Tapping mode AFM images were acquired in air using a Multimode scanning probe microscopeand driven by a Nanoscope V controller (Digital Instruments, Bruker). Single beam uncoated silicon cantilevers (type OMCL-AC160TS, Olympus and TESPA_V2, Bruker) were used. The drive frequency was between 260 and 310kHz; the scan rate was 0.25–0.5Hz

6.3 Dynamic Light Scattering (DLS)

DLS measurements were performed using a Brookhaven Instrument BI200-SM goniometer. The temperature was controlled to within 24° C using a thermostated recirculating bath. The time autocorrelation functions (TCF) were measured by using a Brookhaven BI-9000 correlator and a 100 mW solid-state laser (Quantum-Ventus MPC 6000) tuned at $\lambda = 532$ nm. Measurements were taken at 90° scattering angle. All samples were filtered through 0.2 µm cellulose acetate (Millipore) syringe filters to remove gross contaminants.

7 Western Blotting Analysis

The assessment of exosome quality by identification of Alix, CD81 and Hsc70 proteins was performed by Western Blotting analysis carried out on the exosomes purified by ultracentrifugation. For the Western Blotting analyses, 50μg od exosomes protein were used, and the primary antibody used for Alix (mouse anti-Alix, 1A12 clone, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA, n° catalogue sc-53540) was diluted at 1:500; the one for Hsc70 (mouse anti-Hsc70, B-6 clone, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA, n° catalogue sc-7298) was diluted at 1:500; while the one for CD81 (mouse anti-CD81, B-11 clone, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA, n° catalogue: sc-166029) was diluted 1:1000.

A standard western blotting procedure was followed to separate protein using a 12% polyacrylamide gel (SDS-PAGE); in which equal amounts of protein (50 μg) were added to 10X Laemmli buffer and heated for 5 min at 95 °C. Therefore samples were loaded in each well and transferred onto a nitrocellulose membrane (BioRad, Milan, Italy). The membranes were stained with Ponceau S to verify the quality of transfer and loading similarity. After blocking with 5% albumin bovine serum (Sigma Aldrich), membranes, with the spotted proteins, were analyzed to measure the protein levels, probing with specific primary antibodies for 12 hours followed by incubation with horseradish peroxidase-conjugated secondary antibody if necessary. The final detection was performed using the enhanced chemiluminescence (ECL) detection system, Western Blotting Detection Reagent (Amersham Biosciences, GE Healthcare Life Science, Milan, Italy), according to the manufacturer's instructions. Membranes were then exposed to X-ray film from few second to 5 min and the film was analyzed. Densitometric analysis of the bands was evaluated and quantified using the NIH Image J 1.40 analysis program (National Institutes of Health, Bethesda, MD). Each experiment was performed at least three times.

8 Western Blotting analysis of Hsp60 in exosomes

50μg of proteins from exosome preparations were used for western blotting analysis and a primary antibody for Hsp60 (mouse anti-HSP60, LK1 clone, Sigma-Aldrich) diluted at 1:2.000 was used to detect the presence of Hsp60 in plasma exosomes. The analysis was performed following the procedure described above.

9 microRNAs extraction from plasma exosomes and Real Time PCR

Exosomes obtained by ultracentrifugation were stored at -80°C until needed. Total RNA including small RNA was isolated from exosome samples using the miRNeasy Mini Kit (Qiagen) according to the manufacturer instructions, as described above. Reverse transcription was

performed using the miScript II RT kit and the miScript Reverse Transcriptase Mix according to the manufacturer's instructions. Complementary deoxyribonucleic acid (cDNA) was preamplified using the miScript PreAMP PCR Kit (Qiagen) qRT–PCR analysis was performed using the miScript SYBR Green PCR Kit (Qiagen) and the primers indicated in Table 2. miRNA were selected from the literature based on their previously reported association with Hsp60 expression and brain tumor correlation, miR-1, miR-206, miR-663. miRNA levels were normalized to that of U6, SNORD45 and miR-16 and changes in the transcript level were calculated using the $2^{-\Delta\Delta CT}$ method[2]. The PCR was carried out using the Rotor-gene™ 6000 Real-Time PCR Machine (Qiagen).

Name	Sequence
Hs_miR-1_2	5' UGGAAUGUAAAGAAGUAUGUAU
Hs_miR-206_1	5' UGGAAUGUAAGGAAGUGUGUGG
Hs_miR-663b_2	5' GGUGGCCCGGCCGUGCCUGAGG

Table 2. Primers used for qRT-PCR

Results and Discussion

Nowadays when we are dealing with a brain tumor, the neurosurgical approach includes a pre-operative clinical and neuro-radiological full assessment with gadolinium, and functional neuroimaging. In GBM WHO grade IV, the mainstay of treatment is the "sopramarginal" resection followed by radiation therapy and chemotherapy [10]. A crucial prognostic factor in oncological neurosurgery is the extent of resection (EOR). Several studies have addressed the importance of EOR in GBM surgery. In this effort, tumor visualization is a key factor to maximize the EOR. Numerous solutions exist: neuronavigation, fluorescence, and intraoperative imaging (MRI, CT, ultrasound) [4,7,11,13,48, 52].

Despite the improvement of current approaches for patient's treatment, the prognosis for GBM patients remains poor because of tumor genetic and phenotypic heterogeneity, multiple activation of key oncogenic pathways, acquired therapeutic resistance, and cytoprotective mechanisms in GBM cells.

Nowadays, the approach that takes into account the multifaceted role of molecular chaperones in tumorigensesis is increasingly acknowledged [61]. In the field of biomedical research, the "Chaperonology" studies molecular chaperones and the possible malfunctioning of them, which given rise to a variety of pathological conditions, known as chaperonopathies [62, 64, 65, 67]. As previously said, some chaperones have anti-apoptotic properties and have been found elevated intracellularly in many human cancers [101, 102].

Our research group has studied the chaperone HSP60 in detail and we have demonstrated the overexpression of this protein during human brain carcinogenesis [16, 114, 121, 126, 132]. Hsp60 can interact directly with molecules in various cell compartments and can also be found on the membrane surface of normal and tumour cells. HSP60 regulates proteins involved in apoptosis and cell cycle and when it is dysregulated it can promote cancer progression. HSP60 role in carcinogenesis depends on tumor-type and must be determined accordingly to the context of the tumor under consideration.

The goal of our research was to study the expression of HSP60 and miRNA related levels on primary brain tumors *in vivo* and in liquid biopsy (circulating exosomes) before and after ablative surgery. It has to be acknowledged that patients enrollment is still on going and the results that we are reporting are incomplete. Indeed, molecular results from patients affected by Meningioma WHO Grade I, are still in process, as well the results from the full follow up of other patients affected by

Menigioma Grade II and GBM WHO Grade IV. After the patient enrollment and the radiological investigations, in brief, we can divide our results in the following steps:

First Step: Histological and Immunohistochemical analysis of HSP60 in brain tissues

The evaluation of the immunohistochemical levels of HSP60 was performed on healthy cortical brain tissue derived from autopsy (A) and in tumor biopsies of high grade brain tumors: (B) Atypical Meningioma, (C) Glioblastoma multiform (GBM IV) (Fig 3). As approved by the local ethics Committee of the University Study of Palermo, the healty cortical brain tissue samples were taken from the hystopathological archives of the Forensic Medicine of the University Hospital of Palermo. Since the samples of healty cortical brain tissue derived from autopsy of subjects dead for causes non related to brain disease, they have been considered as the regular group control to asess the HSP60 level and compare it with the level of the same protein on pathological tissue samples, taken from the patients enrolled in the study. HSP60 immunopositivity was visible at cytoplasmic and membrane levels. The percentage of positive cells was calculated in 10 random high power fields (HPF) at a magnification of 400x, and was expressed as means. The immunohistochemical reactions show that HSP60 expression level increase gradually throughout the carcinogenic steps (Fig.3). Statistical analyses were carried out using the GraphPad Prism 4.0 package (GraphPad Inc., San Diego, CA, USA). Standard statistical analyses were employed to calculate the means of percentage positivity and the standard deviations (SD). Immunohistochemistry demonstrated increased levels of HSP60 in tumor samples compared with controls (Atypical meningioma: $p<0.001$ vs control; GBM IV $p<0.05$ vs controls). A strong diffuse cytoplasmic positivity for the HSP60 protein was present in 100% of the tumor specimens examined (bar 100μm).

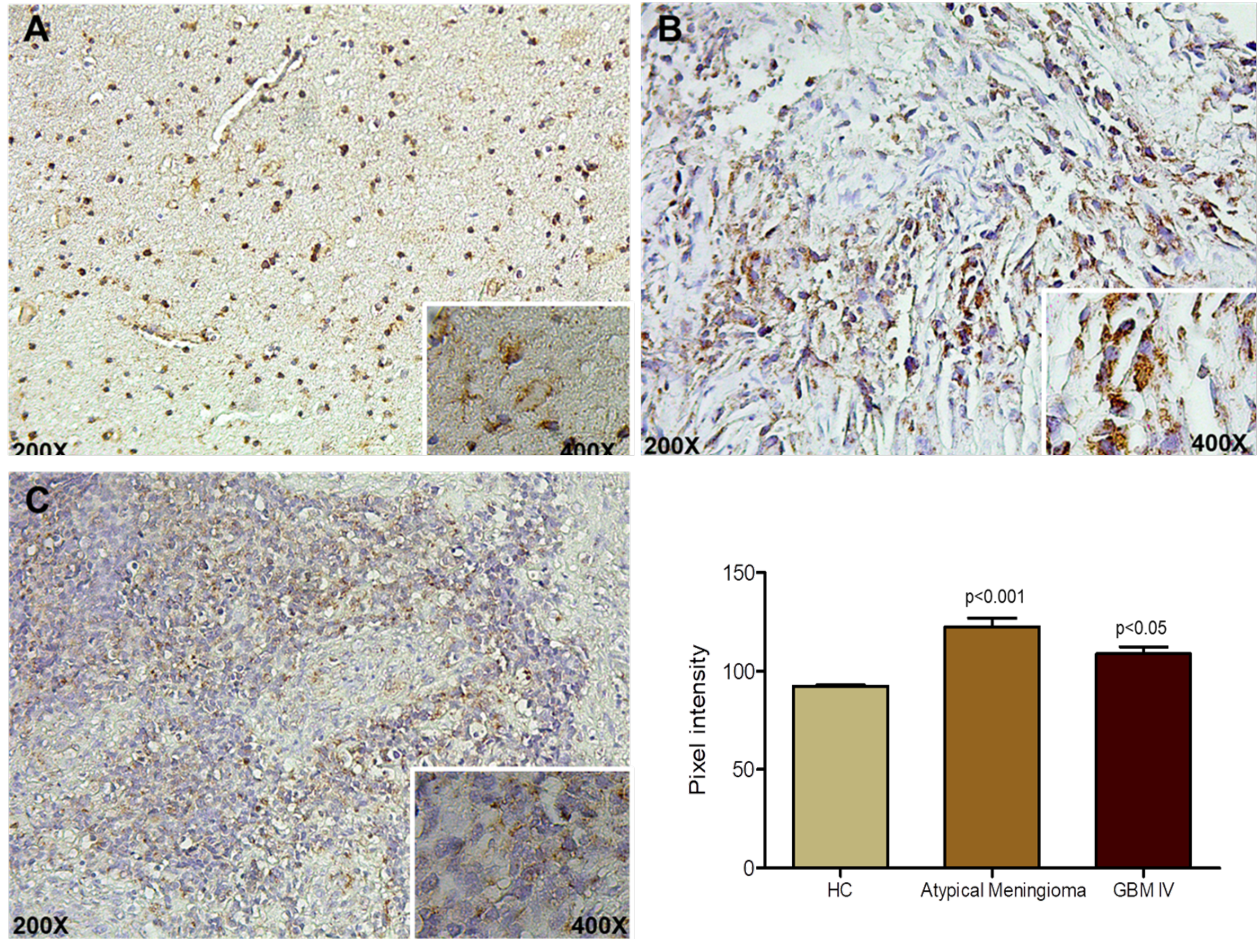

Fig. 3 Immunohistochemical analysis showing the increase of HSP60 expression onmeningioma samples (B) and GBM (C) than in normal tissues (A) used as control.

Second step: HSP60 levels on exosomes from brain tumors

Exosome obtained from plasma of patient with atypical meningioma and GBM, before and after surgery, were analyzed by TEM and AFM, and we found that the size and the morphology of the obtained exosomes were those of typical exosomes (Fig. 4 A and B, representative TEM and AFM images). Furthermore, our exosomes preparations were analysed by Dynamic Light Scattering (DLS), that measures the size of each particle, by direct observation of scattered light and the particle motion (Fig. 4 D and E). We found that our vesicle populations have different size, depending on the time of follow up. In plasma of patient with atypical meningioma we find exosomes with size of 28.027 (S.D ±4.743) before surgery, 80.319 (S.D ±4.143) after 1 week from surgery, and 133.966 (S.D ±10.685).

In GBM plasma the exosomes size has a size distribution mean of 30 nm (S.D. ±1.776-0.754) without difference before and after surgery.

In addition, the results obtained with the measurements of Alix, Hsc70 and CD81 and protein content by WB analysis is also were typical of exosomes.

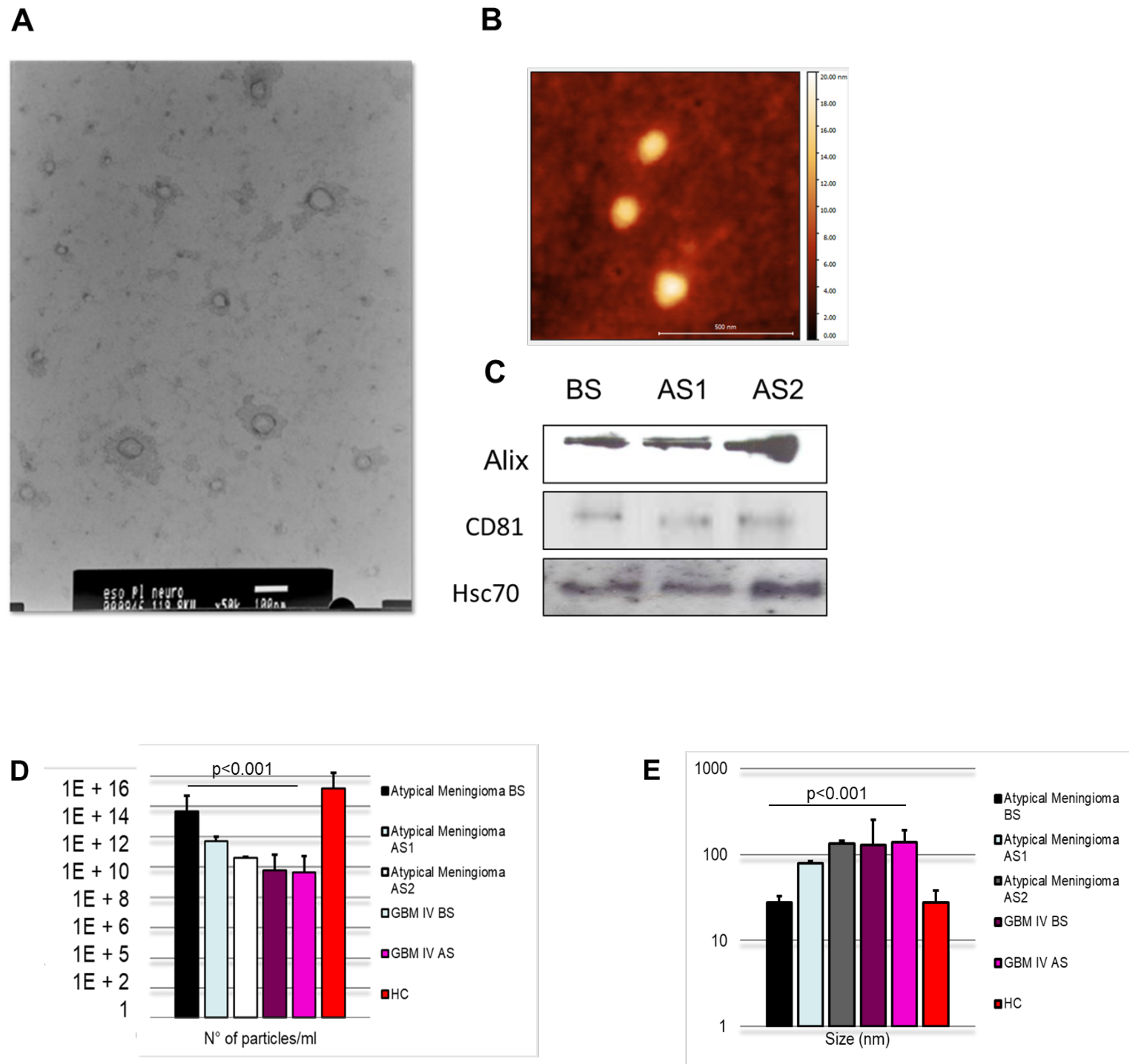

Fig. 4 In A micrography fromTEM (bar 100 nm) and in B AFM image (bar 200 nm) that show exosomes from perpheral blood. In C Western blot analysis for markers considered characteristic of exosomes. D and E panels show the number of exosomes and size respectively, for each preparation.

Part of exosomes isolated from plasma were dedicated to define the presence of HSP60 in patients affected by atypical meningiomas (WHO GII), transitional or meningotelial meningiomas (WHO GI) and glial tumors, mainly glioblastoma (WHO GIV). HSP60 was present in the exosomes, but at different levels in patients before surgery and after surgery (Fig.5). The levels of HSP60 in the exosomes of patients before surgery were significantly higher than in the exosomes from the same patients after surgery (7 days). This molecular data was also confirmed by the brain MRI results that showed the complete tumors removal. However, in few cases, we assisted a tumor recurrency. In particular, the pt NCH04, after two months from surgery, experienced an epileptic seizure. Thus, he underwent a brain CT scan and a brain post contrast MRI scan that depicted a tumor reccurrency (Fig.6). In the same period, patient underwent the follow up blood sample for the molecular analysis. This analysis showed a significant increase of HSP60 in the exosomes, thus confirming, from the molecular point of view, the tumor recurrency. This data may open up new frontiers in the diagnosis and follow up of these patients affected by atypical meningiomas since the HSP60 increase after the tumor removal could be related to the tumor recurrency, as in our data demonstrated.It could be advisable to perform this evaluation on more patients in order to have data statistically significant.

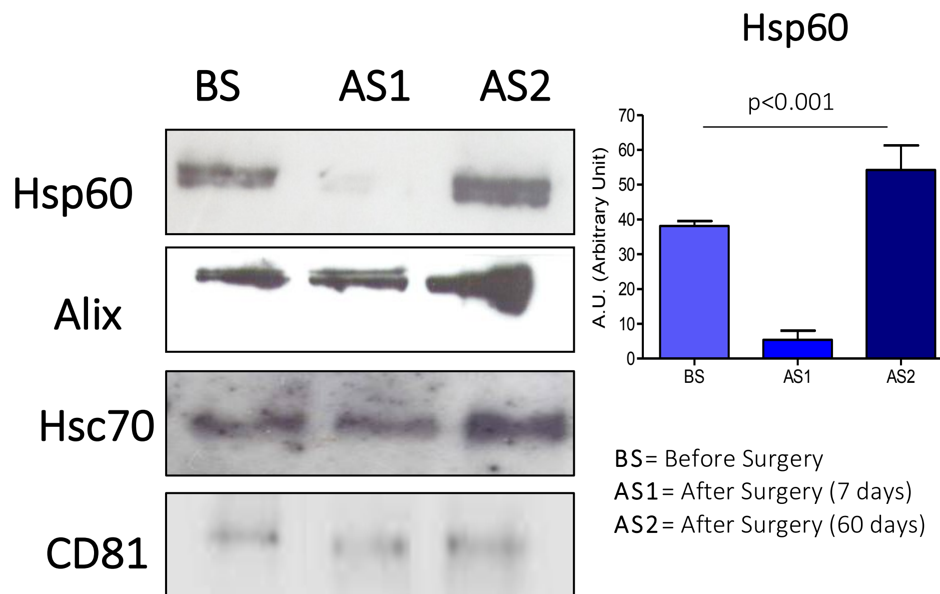

Fig 5 WB analysis: It shows the high level of exosomal HSP60 on blood sample at the time of surgery (BS), its decrease at one week from surgery (AS1) and its increase at two months after surgery (AS2).

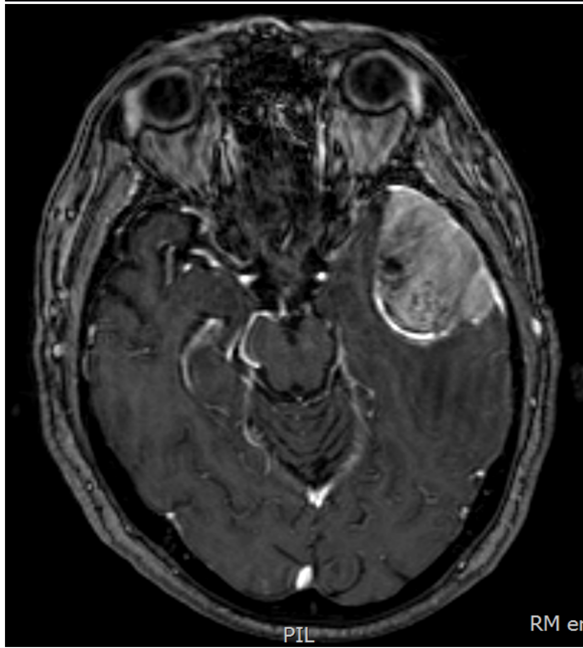

Fig. 6 Brain MRI post gadolinum: axial cut. The frontotemporal meningioma recurrenceis depicted

The analysis of HSP60 level in exosomes from GBM patients doesn't show the any variation at different time from surgery (Fig.7). Indeed, HSP60 levels maintened high at any time of the follow up. The immediate post op brain MRI showed an apparent macroscopic tumour complete removal; however, since GBM is not a "curable" brain disease, after few months the brain MRI showed a tumor recurrency (Fig. 8).

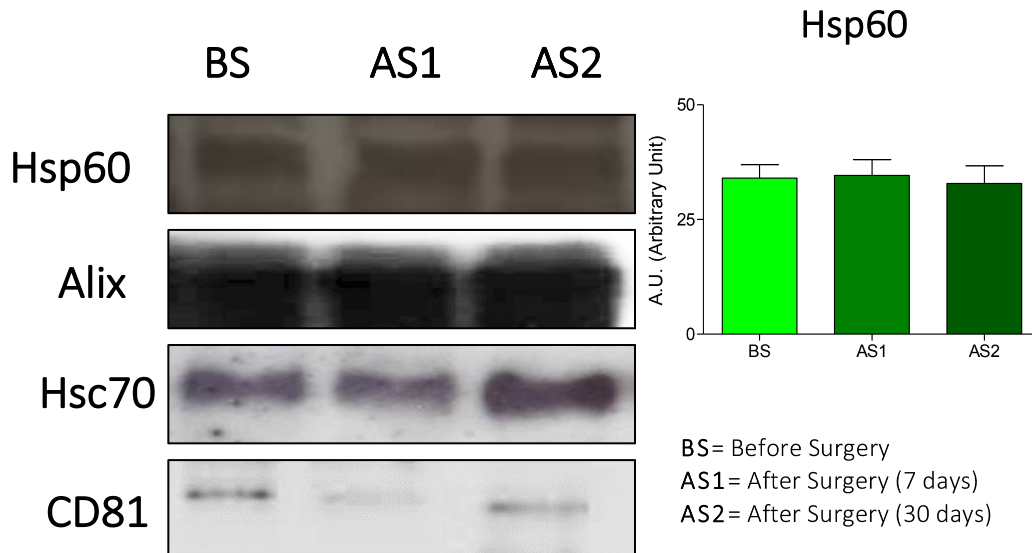

Fig. 7 WB analysis: It shows the level of exosomal HSP60 on exosomes from patients with GBM. The analysis of HSP60 level in GBM patients not shown the any variation at different time from surgery.

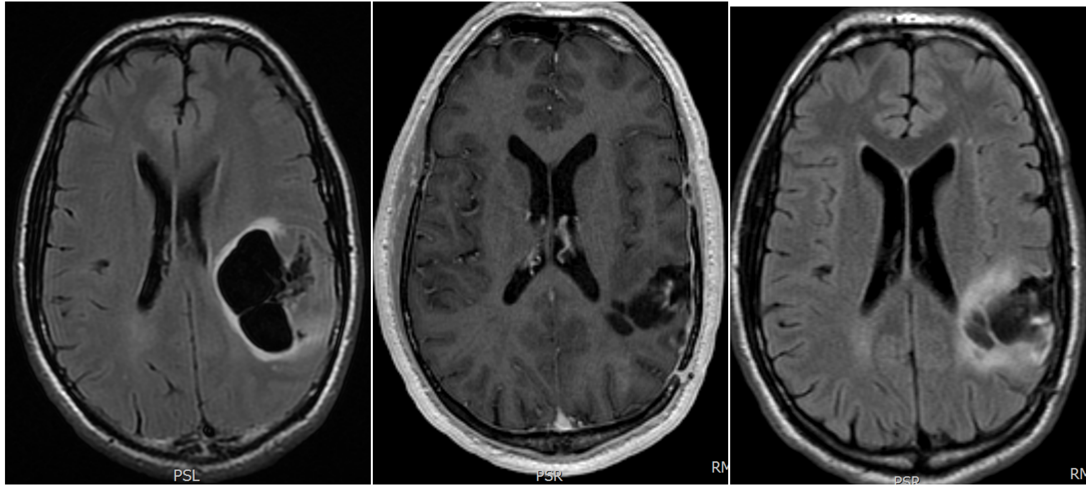

Fig. 8 Brain MRI post gadolinum: axial cut. The temporopaietal brain glioma is depicted (on the left), after surgery (in the middle) and after three months (on the right).

Third step: HSP60 dependant miRNAexpression on exosomes from brain tumors

To determine whether the surgical ablation of brain tumor alters miRNAs levels in circulating exosomes, we selected two miRNAs involved in Hsp60 regulation (miR1 and miR2016) and one known to be dysregulated in brain tumor (miR663). To aim this we employed RealTime PCR in three exosomes samples of Atypical Meningioma and four of GBM, to assess miRNAs changes at two time points: after 7 days and after 30 days surgery. We observed that miR1 is downregulated in exosomes isolated from plasma of patient with atypical meningioma after 7 days from surgery; instead, after 30 days miR1 is upregulated. MiR206 and miR663 are downregulated after 30 days surgery (Fig.9). Moreover, the HSP60 variation correlates positively with the miR1 expression. miR206 and miR663 are downregolated after 30 days from surger. In GBM, exosomal miR1 and miR663 do not shown significant differences between follow-up times; contrarily, miR206 is upregulated after 30 days surgery (Fig.10). We did not find any statistically significant correlations at this stage. It has been observed that miR206 is dawnregolated in pt affected by high grade gliomas than in patients affected by low grade gliomas). Moreover, the higher miR206 expression seems to correlate with higher servival rate.This data shoul be correlated to the gliomas IDH mutant

in order wto verify any mathes considering that also the IDH mutation is related to a better prognosis.

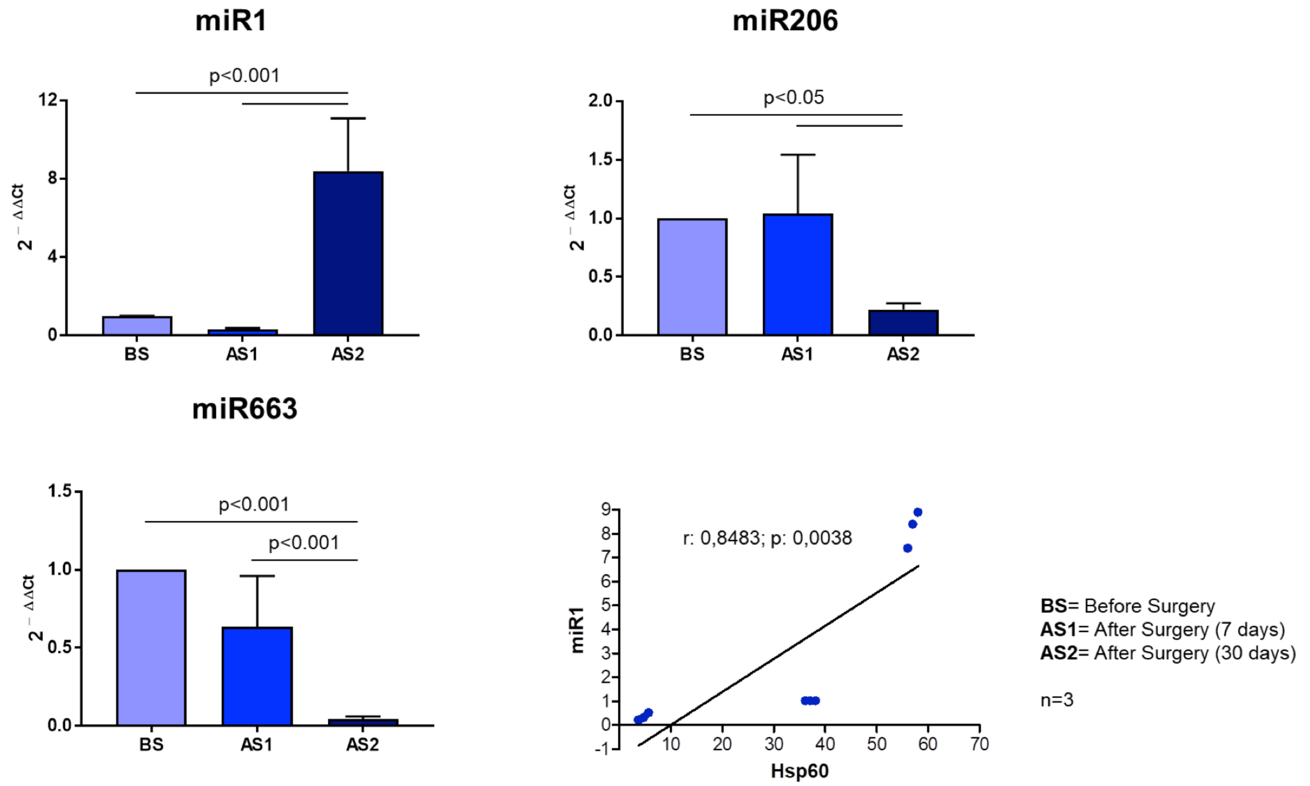

Fig. 9qPCR data showing miRNA HSP60 related levels analysis on atypical meningiomas

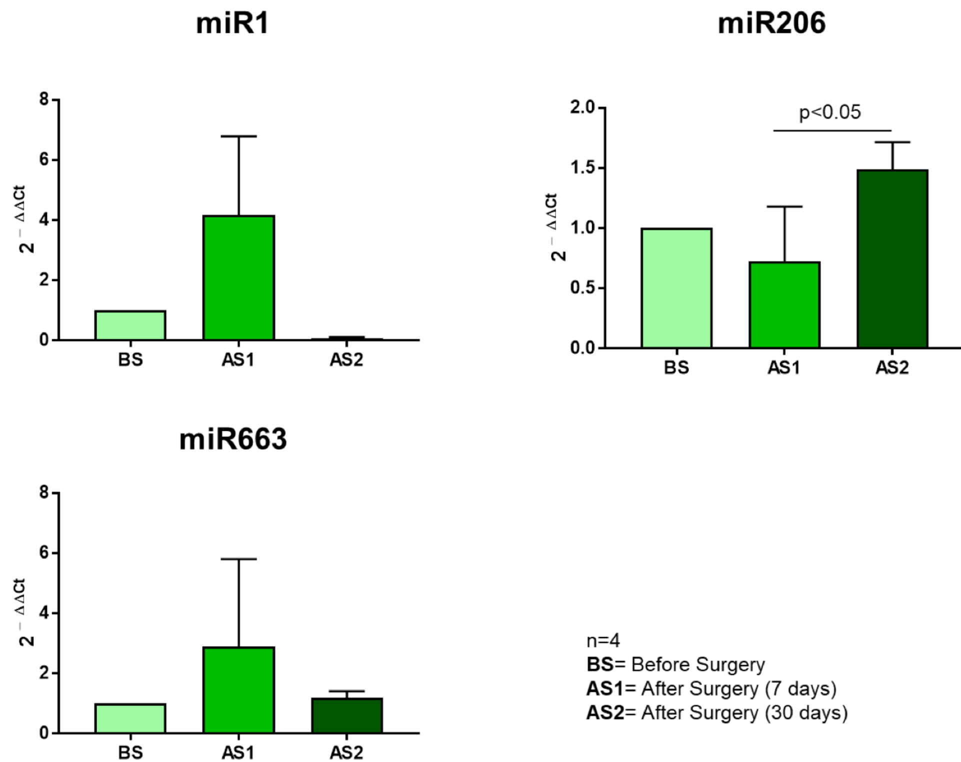

Fig. 10qPCR data showing miRNA HSP60 related levels analysis on high grade gliomas

Fourth step: Histological analysis of MiRNAs levels in tumor brain tissues

We isolated miRNAs from formalin-fixed paraffin-embedded tissues (four 10 μm thick sections) using Triazol and miRNeasy Mini Kit®. qRT-PCR evaluation of tissue levels of miR1 and miR206 in high grade tumor brain and HC was assessed according to the Livak method $2^{-\Delta\Delta CT}$ (Fig 11).We observed a downregulation of miR1 (A) and miR206 (B) in atypical meningioma tissue and conversly an upregulation in GBM IV.

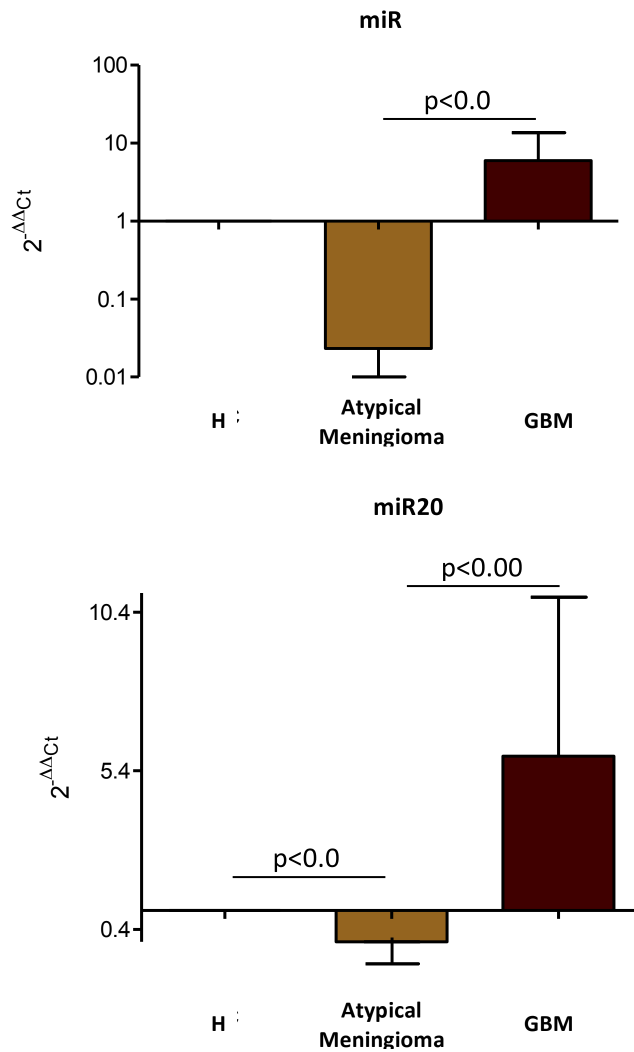

Fig. 11qRT-PCR evaluation: A downregulation of miR1 (A) and miR206 (B) in atypical meningioma tissue and conversly an upregulation in GBM IV is depicted.

Conclusions and Future Perspectives

Chaperonology is a scientific discipline that studies molecular chaperones and the pathological conditions in which chaperones may become pathological factors, known as chaperonopathies [61]. Chaperone therapy, or chaperonotherapy, involves the use of chaperones in the treatment of chaperonopathies [62, 63]. As demonstrated in literature, chaperons moleculae are implied in different physiological and pathological mechanisms and their overexpression or down regulation may be correlated with human brain tumor iniziation and progression. Among chaperons moleculae implied in brain cancirogenesis, our research group has studied in particular HSP60 and miRNA related proteins in patients affected by meningiomas and GBM WHO IV grade.

In patients affected by brain atypical meningioma WHO Grade II, we observed that HSP60 levels were elevated in circulating exosomes isolated from plasma before surgery and the levels were decreased after 7 days from surgery. The radiological post operative exam, showed a complete tumor removal and from the clinical point of view the patients were free from epileptic seizures, headache and other neurological defict. At that point the molecular, radiological and clinical evalutations, all togheter agreed for a complete cure from the brain disease. However, considering the biological behaviour of the atypical meningiomas, we followed up these patients over time, with clinical and radiological evaluations and as well with molecular analysis through the blood exam. In these patients, after 30 days, exosomal HSP60 levels were increased again and, in one case, patient experienced epileptic seizures and the brain MRI exam showed a large tumor recurrency thus patient underwent again a brain tumor removal. The HSP60 downregulation after 7 days surgery could be related with the tumor ablation and the upregulation after 30 days could be linked with the tumor recurrence. This is consistent as well with miRNAs level data.

In patients affected by GBM WHO Grade IV, conversely, in circulating exosomes we not observed any significant variations in HSP60 levels and in the expression of miRNAs considered. This behaviour could be linked to the infiltrating nature of GBM IV and to the well known impossibility to provide a complete tumor removal. Even though, this data needs to be supported by a larger population analysis, the results so far provided by our study seems reliable. It's acknowledged that brain GBM is not just a "tumor"; the infiltrative power of this entity is so high that neoplastic glial cells may disseminate even far away from the site where, from the neuroradiological exam, is supposted to born and grow the lesion. GBM grade IV tumors should be considered as a true brain disease that involve all the brain, and the radiological exams could not show the "Caput Medusa's" like cells infiltration. The devolepments in the neurosurgical armamentarium have been extremely important to favour a macroscopically "supramarginal", as

nowadays encouraged, tumor removal, while preserving brain functions and so reducing morbidity rates. However, mortality rate, doesn't change, since, as the biomolecular analysis may demonstrate, the tumoral glial cells are still alive, spread somewhere in the brain, this is why HSP60 and miRNA related levels, remain high in the liquid biopsy after the surgery. The consideration of the results of the molecualar analysis in these patients, not only confirm the GBM grade IV is a true brain disease, but could also have major implications in patient management.

Our study points out, also, on an intuition of our research group, that in brain tumor cells, HSP60, which was classically considered a mitochondrial protein, is secreted outside the cell by exosomes. According to different studies, the protein can be secreted into the extracellular matrix and in human peripheral blood it can be detected and it can determine immunosuppressive or immunostimulating effects. This is an intuiton that have important implications in the clinical follow up of our patients. Indeed, since these proteins can be detected on blood circulating exosomes, through a so called "liquid biopsy", it could be possible, in future, to enoroll in the normal pre operative patient blood examination, the molecular HSP60 analysis, that could be easily made even in small peripheral Hospitals.

The discovery that molecular chaperones can be determinant factors in the process of tumorigenesis and the increasing understanding and characterization of exosomes, particularly in what refers to their release by tumor cells and contents, including chaperones and miRNA, provide elements to develop novel treatment strategies and means.

Exosomal HSP60 and/or its regulators, have potential advantages as brain tumor biomarkers and in other cancer, as well. The challenge for the future will be to completely clarify the role of exosomal HSP60 in cancer and standardize quantitative tests measuring this chaperonin and regulators, with high sensitivity and relatively easy application, using routine blood samples collection. In addition, these non-invasive tests could provide information useful not only to assess the clinical status of the patient but also to determine whether or not surgery is indicated, and to evaluate the response to tumor ablation and/or to the administration of anticancer drugs.

There is currently sufficient information to consider molecular chaperones-Hsps, as HSP60, as promising biomarkers for early diagnosis and follow up of brain tumors and also as potential therapeutic targets in those cases in which the chaperone favours carcinogenesis and, therefore, the chaperone has to be blocked or eliminated (negative chaperonotherapy). Contrariwise, in those cases in which the chaperone fails to stop carcinogenesis due to deficient function, positive chaperonotherapy would be indicated, namely the defective chaperone should be boosted to restore its function or replaced (using gene therapy or direct administration of normal chaperone).

In conclusion, chaperones and their regulators, could represent potential first new biomarkers for primary brain tumor diagnosis, for improving diagnostic procedures, for patient stratification, for prognosis of disease outcome and for follow up over time tumor stability or recurrence. The addition of such novel approaches to the conventional treatment strategies and the characterization of the mechanisms involved in the Hsps influencing ECM and EMT will certainly aid in glioma management. It is possible that some Hsps are more specific for a tumor type while others might be so for other types. It follows that elucidation of Hsp-tumor specificity is a promising research line to standardize study protocols focusing on specific cases (personalized medicine) [137,138, 140].

Finally, it would be beneficial to start to look at the CNS tumors through the Chaperone Eye and consider at least some of these tumors to be chaperonopathies by mistake or collaborationism. Therefore, looking at tumors through the Chaperone Eye implies that patient examination should include qualitative and quantitative analyses of Hsps, including those in exosomes, before and after surgery and other treatments for monitoring disease evolution and response to treatment. This conduct will provide new insights on brain tumors that will enhance progress in clinical applications of chaperones and exosomes, including their use as therapeutic agents.

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