Università degli Studi di Cagliari

DOTTORATO DI RICERCA

in

INGEGNERIA BIOMEDICA

Ciclo XXVIII

TITOLO TESI

BIOENGINEERING-ENHANCED NEUROSURGICAL SOLUTIONS

Settore scientifico disciplinari di afferenza

MED/27

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Esame finale anno accademico 2014 – 2015

To my patients, because improving their care
is the greatest trigger to keep on putting
so many efforts in basic and clinical research

Outline

Abstract (En)

The advancements in basic sciences and the availability of sophisticated technological aids have led over the last few years to the rise of innovative surgical strategies, the identification of better prognostic/predictive biomolecular factors, and the development of novel drugs all meant to profoundly impact the outcome of neurosurgical patients. This thesis touches upon the window of opportunity to exploit bioengineering techniques in three subspecialties of this vast discipline: neuro-oncology, radiosurgery and neuro-traumatology. After a thorough identification of some unresolved clinical problems and the limits of current management strategies in those areas, some technical solutions are proposed and defined from either experimental hypothesis or clinical research investigations.

The neuro-oncology section presents the exciting topic of nanodrugs for adjuvant chemotherapy in high-grade gliomas, the most aggressive primary brain tumours. The use of hyaluronic acid nanoshells is proposed to encapsulate prodrugs and exploit the mechanisms of interaction between glioma cells and hyaluronic acid, a natural component of extracellular matrix. The theoretical advantages of this approach are discussed with details regarding the possible scalability of this technique to increase the efficacy and biodegradability of other molecules suitable as contrast media for neuro-imaging and radiotracers for nuclear medicine investigations.

The radiosurgery section in fact continues the previous one, highlighting the rationale for further implementation of radiosurgical protocols thanks to nanoshell-encapsulated radioenhancers and multi-imaging fusion protocols. Experimental data on the optimization of radiosurgical plans for artero-venous malformations close to the motor strip or basal ganglia are presented, demonstrating the dramatic reduction in radiation dose to the pyramidal tract, and supporting the anticipated benefits in terms of radioprotection and avoidance of post-radiosurgical deficits.

Finally the neurotrauma section presents the clinical results from a prospective study on an innovative device for non-invasive monitoring of intracranial pressure, a tool that given the high reliability demonstrated in this research might find a role in preclinical or neurointensive care settings and reduce the need for serial neuroimaging in traumatic brain injured patients.

The last chapter concludes this thesis duly outlining some forecasts and supporting literature for the widespread application of bioengineering enhanced solutions in neurosurgical theatres, wards or outpatient clinics.

Abstract (It)

I recenti progressi tecnologici hanno prodotto un'evoluzione della pratica neurochirurgica permettendo di perfezionare innovative tecniche chirurgiche, di identificare nuovi fattori prognostici e di sviluppare nuove terapie farmacologiche a beneficio dei nostri pazienti. La presente tesi ha come scopo principale quello di descrivere le opportunità attualmente offerte dall'applicazione di tecniche derivate dall'ingegneria biomedica in tre distinte sottospecialità neurochirurgiche: la neuro-oncologia, la radiochirurgia e la neuro-traumatologia. Partendo da una accurata definizione di alcune pressanti problematiche pertinenti a queste sottospecialità, vengono qui proposte alcune ipotesi sperimentali e investigazioni cliniche volte a superare i limiti delle attuali strategie diagnostiche e terapeutiche.

La sezione neuro-oncologica è interamente incentrata sullo sviluppo di nanofarmaci per trattamenti chemioterapici in pazienti affetti dai più aggressivi tumori primitivi del sistema nervoso centrale: i gliomi di alto grado. Nello specifico, l'impiego di nanosfere di acido ialuronico viene proposto come elegante strategia per ottimizzare il trasporto di chemioterapici basata sulla selettiva interazione tra cellule gliali e acido ialuronico, uno dei principali componenti della matrice extracellulare cerebrale. In tal senso, vengono analizzati sia il razionale per proporre tale approccio terapeutico, che le possibilità di estendere la medesima strategia ad altre molecole appartenenti alla famiglia dei mezzi di contrasto superparamagnetici impiegati in risonanza magnetica ad alto campo, o ancora a radiotraccianti utilizzati per indagini di medicina nucleare.

La sezione radiochirurgica infatti prosegue evidenziando le opportunità di ottimizzazione di protocolli radiochirurgici grazie all'impiego di radioenhancers incapsulati in tali nanosfere, o mediante la definizione di protocolli di fusione di immagini preoperatorie. In tal senso, vengono descritti i dati sperimentali relativi a piani di cura per malformazioni arterovenose adiacenti ad aree cerebrali eloquenti, dimostrando la significativa riduzione della dose radiante al tratto piramidale ed i relativi benefici in termini di radioprotezione e prevenzione di deficit postradiochiurgici.

Infine la sezione relativa alla neuro-traumatologia offre i risultati sperimentali ottenuti con un'innovativa strategia per la misurazione non invasiva della pressione intracranica, che alla luce dell'accuratezza dimostrata potrà presto trovare spazio sia in ambito preclinico che ospedaliero nel trattamento dei pazienti ricoverati per trauma cranico grave.

Nel capitolo conclusivo vengono infine offerte alcune previsioni, supportate dalla relativa letteratura scientifica, relative al crescente impiego che l'ingegneria biomedica avrà nei prossimi anni nella pratica neurochirurgica.

Acknowledgements

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My sincere gratitude goes also to Prof. Roberto Israel Foroni, from the Minimally Invasive Robotic Surgery Laboratory of the Department of Neurosurgery at the University of Verona (Italy); Prof. Sara Eyal, from the Institute for Drug Research at the Hebrew University of Jerusalem (Israel); and Prof. Andrea Soddu, from the Brain and Mind Institute at the University of Western Ontario (Canada). Being hosted for fruitful and enlightening research periods in their laboratories provided a fundamental contribution to the realization of some of the research projects described in this thesis. Indeed, they have been extremely valuable in helping me to focus my scientific interest towards meaningful research questions, to implement the related experimental process, and conduct insightful post-data analysis and interpretation.

I want also to thank Dr. Antonio Nicolato, from the Radiosurgery and Stereotactic Neurosurgery Unit of the Department of Neurosurgery at the University of Verona (Italy), and Dr. Lara Prisco, from the Department of Anaesthesia at the University of Cambridge (UK). The deep breadth of knowledge in their areas of research and clinical practice represented an invaluable resource during the preparation of the experimental sections of this thesis.

Finally, my huge appreciation goes to all those Institutions such as the Canadian Embassy in Italy (Rome, Italy), the Kathleen Foreman Casali Foundation (Trieste, Italy), the Friends of the Hebrew University of Jerusalem (Milan, Italy), and the Keystone Future of Science Fund (Keystone, USA) that have helped to fund my research. Their grants and support have made possible all that follows.

Chapter I:

What Neurosurgery Can Learn from Biomedical Engineering

Most Relevant Publication:
Ganau M, Foroni RI, Soddu A, Ambu R. Biomedical Engineering and Nanoneurosurgery: From the Laboratory to the Operating Room. In Handbook of Clinical Nanomedicine: Nanoparticles, Imaging, Therapy and Clinical Applications (Editors: Bawa R, Audette GF and Rubinstein I) Pan Stanford Publishing, Chapter 49: pp 1433-40; 2016

1.1 Challenges in Neurosurgery

Neurosurgery involves the repair, resection, replacement, or improvement of the Central and Peripheral Nervous Systems (CNS, PNS) and their functions in numerous ways. Recently, many remarkable discoveries in neuroscience have deepened the understanding of vexing diseases affecting both of the CNS and PNS; and this trend, paralleled also by exponential advances in basic sciences and biomedical engineering, allowed for new translational projects theorized and implemented by few, enlightened, multidisciplinary research teams. Curiously, until a few decades ago, when such a cross-sectional approach was not contemplated, most surgeons tended to be generally unaware of innovative technologies ready for out-of-laboratory use; whereas the vast majority of scientists often ignored the surgeons' needs and therefore which new tools, materials and techniques to propose them.

Nowadays, thanks to the fruitful and dynamic interaction between surgeons, engineers, and scientists some of our most pressing problems are in the process of being finally solved. As a result of this never-ending scientific journey, the entire specialty of neurosurgery, which has always progressed at the intersection between technology and medicine, is now heading toward a previously unimagined frontiers of diagnosis and treatment. In some areas of our specialty, this paradigmatic change has already occurred or will become more evident in the next few years when it is expected to bring about changes in every aspect revolving around our patients: from the design of hospitals' wards and operating rooms, till to the functions and potentialities of new drugs and scalpels.

In this thesis we will initially focus on exploring the applications of biomedical engineering and allied sciences, such as nanotechnology, in the various neurosurgical subspecialties; before moving to the next sections devoted to the experimental research conducted during the course of this PhD in the areas of drug delivery, neuroimaging, and non-invasive monitoring of intracranial pressure.

1.2 State of the Art in Neuro-Oncology

Primary brain tumours originate in the central nervous system and, although representing serious pathological conditions which are often life threatening, normally do not spread to

surrounding tissues. They occur in people of all ages, but are statistically more frequent in children and older adults; data from the International Agency for Research on Cancer and various other national registries (including the *International Central Brain Tumor Registry of the United States* and the *Cancer Research UK*) show a crude incidence of 15-30 new cases per 100.000 inhabitants in industrialized countries, with 70,000 new cases diagnosed worldwide every year.

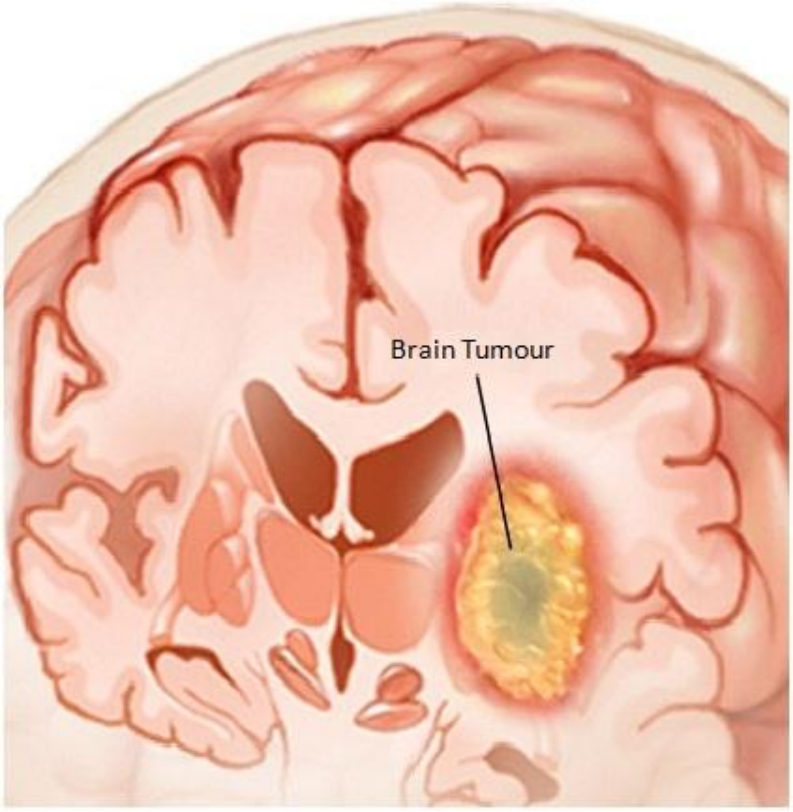

Fig. 1.1: *Artistic representation of a deep sited parieto-temporal brain tumour*

The most common primary CNS tumours are meningiomas and gliomas, together accounting for more than 60% of all primary lesions. Of note, gliomas represent 80% of the malignant ones. CNS tumours are characterized by a wide clinical and histological heterogeneity; this is particularly true for gliomas, because 35-40% of them have epigenetic modifications as the underlying mechanism driving malignancy (*Kondo Y, et al. 2014*). As a result, scientists and clinicians all over the world are still unable to predict the clinical evolution of each single patient diagnosed with CNS neoplastic lesions, and the neuro-oncology community has tried to put all possible efforts in the identification of better diagnostic and therapeutic strategies aimed at improving the prognosis of those patients.

From a strictly surgical perspective, maximum but safe resection, followed in case of malignant lesions by adjuvant radiotherapy and chemotherapy, has shown the best clinical results in terms of overall and relapse-free survival. Therefore the surgical and medical treatment of deep-seated or functionally critically located neoplastic lesions have become the ultimate frontier of clinical practice. In fact, whenever tumours or other space occupying lesions are located in critical brain areas, as those responsible for speech or motor functions, aggressive surgical excision might not be efficiently and safely feasible, requiring instead a tailored management strategy. This led to the introduction, into daily clinical practice, of innovative techniques for optimized tumour visualization, such as spectroscopy and intraoperative magnetic resonance imaging (MRI). Also, for more than a decade, intra-operative neuronavigation, along with cortical and subcortical electrostimulation (IOM) or awake surgical techniques, have provided the operating neurosurgeon with a continuous feedback to estimate the extent of resection (*Ganau M, et al. 2015*).

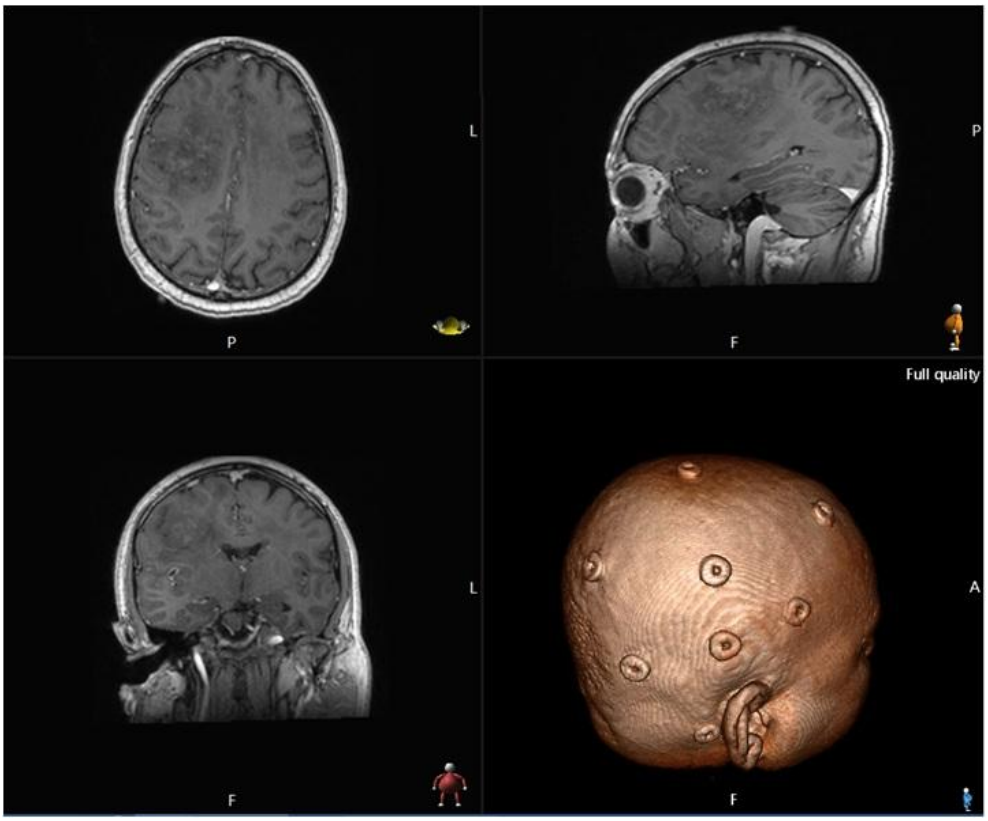

Fig. 1.2: *Preoperative MRI with registration fiducials for intraoperative neuronavigation*

Nonetheless margins for improvement are still considerable, given that the rates of complete resection in resectable tumours are disappointingly low and range from 20% to 60% despite the use of neuronavigation (*Schucht P, et al. 2015*). Also, recent studies show that patients with large

tumours are more prone to worsening in executive functions soon after operation, despite the use of IOM (*Talacchi A, et al. 2010*)

Furthermore, beside the current limits in surgical practice, also chemotherapy regimens for many malignant tumours are failing to deliver the expected results in terms of extended progression free survival or overall survival, and in almost all histotypes quality of life (QoL) has being significantly affected by their toxicity.

As a result of this introductive overview, the current prognosis for most brain tumours has improved over the last 20 years, but unfortunately is still dismal. Many limits of current management strategies are basically due to the poor understanding of the many biological variables responsible for the phenotypic behavior of brain tumours. To this regard, several nanodevices for single cell proteomic analysis are in advanced stage of investigation with potential to shed light on the fingerprint of brain tumours (*Ganau M, et al. 2014*).

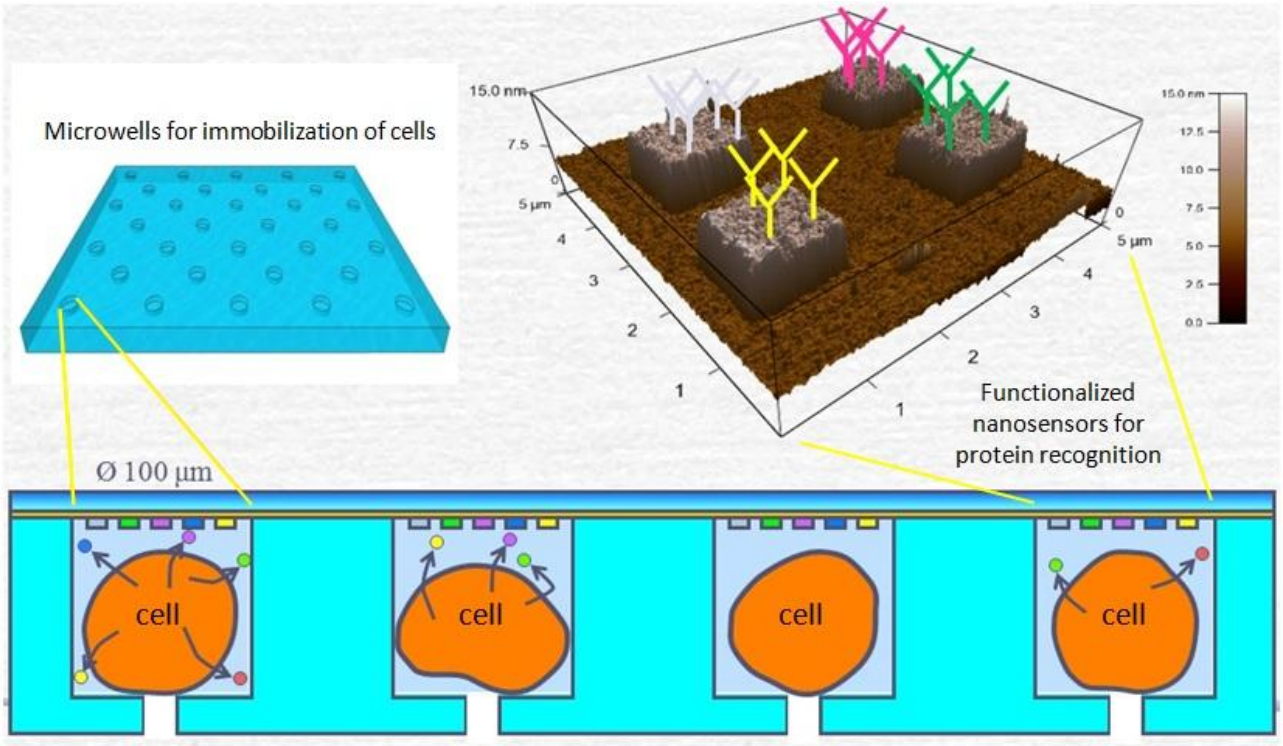

Fig. 1.3: *Microwells and Immunofunctionalized nanosensors for proteomic analysis of gliomas*

Such biomolecular markers are potentially of great value to neurosurgeons, radiation oncologists, and neurooncologists in optimizing brain tumour treatment. They all come with great expectations, and might prove to be extraordinary tools providing new insights on the biological understanding of this class of tumours upon which to base enhanced therapeutic strategies.

1.3 State of the Art in Vascular Neurosurgery

Rupture of a vascular malformation may lead to subarachnoid haemorrhage (SAH) or intracerebral haemorrhage (ICH). An intracranial aneurysm is the most common cause of SAH, whereas an arteriovenous malformation (AVM) is more likely to be associated with an ICH. AVMs are congenital abnormal connections between arteries and veins, bypassing the capillary system.

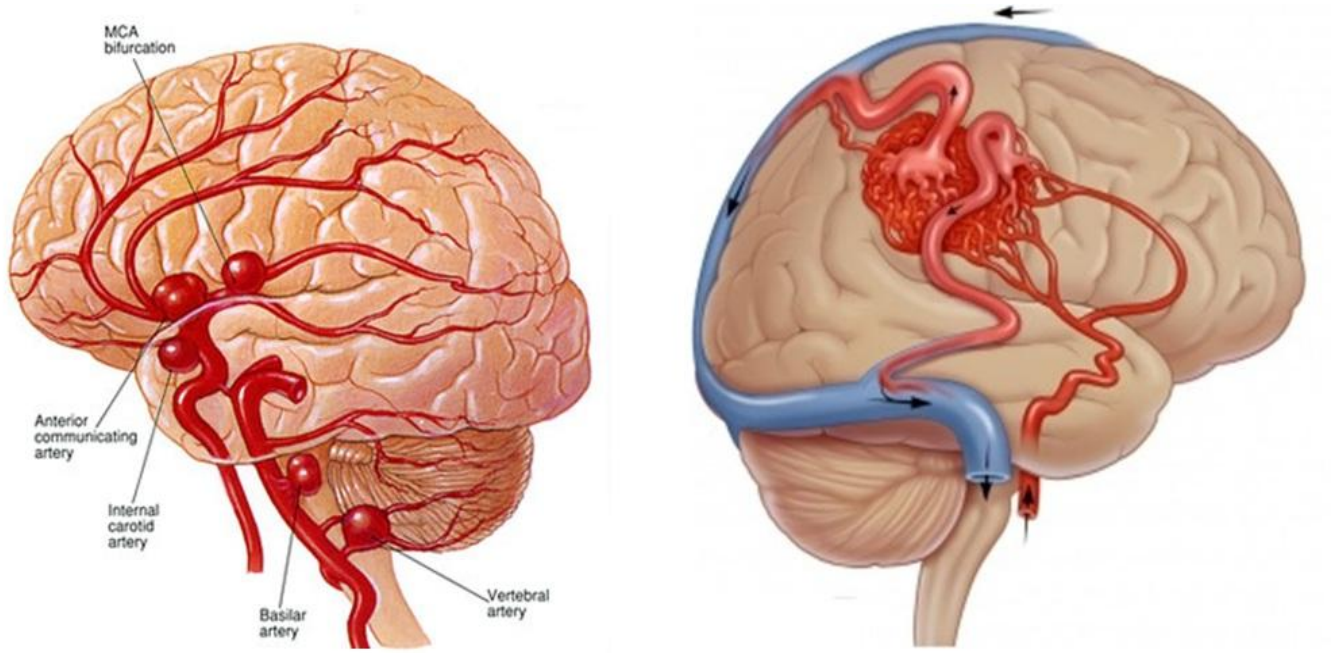

Fig. 1.4: *Artistic representation of cerebral aneurysms (left image) and AVMs (right image)*

Clinical registries show that the prevalence of unruptured intracranial aneurysms might be estimated as 1 in every 20-30 adults, with approximately 1/4 of these aneurysms at risk to rupture in a lifetime; on the other hand, AVMs have approximately 1/5 to 1/7 the incidence of intracranial aneurysms, and in roughly 80% of cases they are asymptomatic (*National Institute for Neurological Disorders and Stroke*). In case of bleeding, both aneurysms and AVMs may cause life-threatening acute cerebrovascular events, which typically occur in working-age people. For decades the mainstay of treatment for those vascular malformation had been clipping or microsurgical excision, and back then neurosurgeons could have hardly imagined that the treatment of those neurovascular pathologies was deemed over the years to become less surgical and to include radiosurgical and endovascular techniques (*Allain JC, et al. 2013*).

Regarding the treatment of aneurysms, the endless evolution of vascular neurosurgery is best represented by the concept of endovascular remodeling, so that nowadays reconstructing cerebral arteries diseased with aneurysms is becoming more appealing than clipping or coiling the aneurismal dome itself (*Graziano F, et al. 2014*). Nonetheless, stents are also in continuous evolution and soon they will serve as more than just scaffolds (*Barath, K, et al 2004; Salmasi S, et al. 2014*). In fact, their nanofunctionalization is offering to neurovascular specialists surgical opportunities that were out of reach at the microsurgical level: specifically, the production of biodegradable and bioactive coated stents are the most emerging trends.

On one hand, encouraging data from cardiac interventional radiography are showing that biodegradable dual-drug-eluting stent can offer new opportunities for a sequential and sustained release of anti-platelet (i.e. acetylsalicylic acid) and anti-smooth muscle cells (i.e. paclitaxelin) drugs to prevent thrombosis or luminal occlusion (the main long-term complication of any endovascular treatment). On the other hand bioactive coatings restoring an antithrombotic surface are already offering alternatives to systemic anticoagulation, and could in future be functionalized to attract magnetized cells to repair the blood vessel. By modifying the stent coatings (i.e. using ion beams to create biomimetic surface textures) it would be possible to promote endothelial cells proliferation, activate resident stem cells from the arterial walls, and induce a faster vessel repair (*Myerson JW, et al. 2014; Lee CH, et al. 2014*).

Another area of significant innovation is represented by the introduction of laser tissue soldering (LTS) of biological tissues where the innovative functionalization of current energy absorbers is offering a substantial breakthrough for vascular anastomosis (i.e. intra-extracranial bypasses). Various studies on near-infrared absorbing gold nanorods or chromophores-loaded nanoshells, have demonstrated that with optimally chosen settings of irradiation time, and carefully selected coating and scaffold properties of energy-absorbing immunoneutral nanoparticles, improved LTS procedures can become easier to perform, while preserving in the long-term the vessels' mechanical properties and patency (*Esposito G, et al. 2012*).

Regarding cerebral AVMs the current state of the art is toward a multistage/multimodality management including endovascular embolization plus surgical excision or endovascular embolization plus radiosurgical treatment. This approach has now allowed to increase the number of complex AVMs amenable for complete treatment, and has ultimately reduced the cumulative risk of bleeding of such a multistage/multimodality management compared to the one typical of each single therapeutic option.

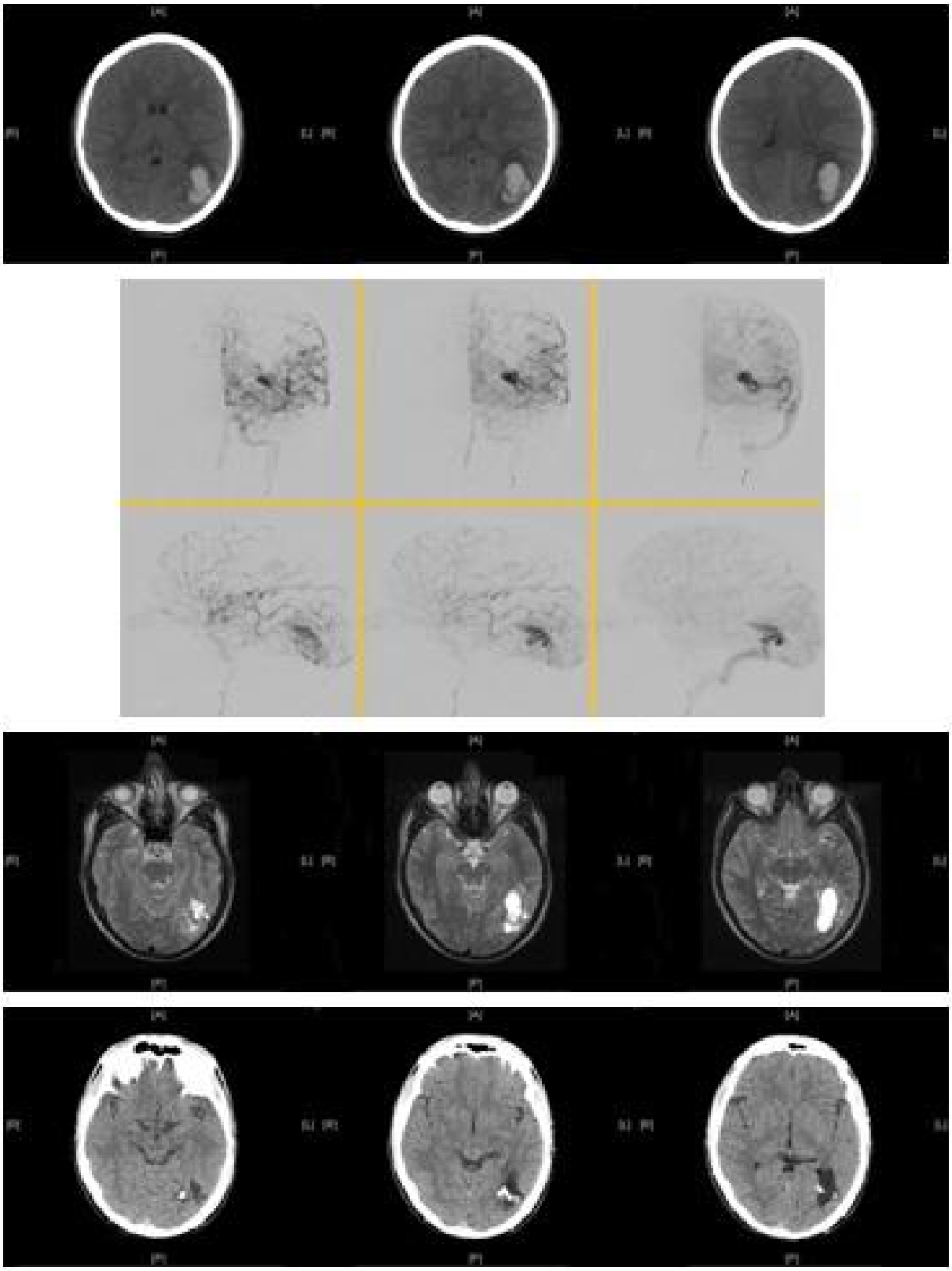

Fig. 1.5: *Left temporo-occipital AVM presenting with intraparenchymal bleeding (CT on upper series). The feeders from left posterior cerebral artery and left middle cerebral artery were treated with angiographic embolization (DSA on second series).The T2 weighted MRI and CT controls at 1 month and 1 year from treatment (last two series of images) show the good exclusion of the AVM, but unfortunately demonstrate a large residual poroencephalic cavity.*

Despite these advances in the management of intracranial vascular pathologies, the number of patients with severe disabilities following their surgical, endovascular or radiosurgical treatment is still high, calling for a further refinement of current techniques. As witnessed in the last decades this might only come from a better understanding of the underlying pathophysiology and the translation of new methodologies into the clinical arena.

1.4 State of the Art in Neuro-Traumatology

Severe traumatic brain injury (TBI) patients represent a substantial proportion of traumatisms requiring admission to Intensive Care Units (ICU), and can be defined as a disruption in the normal function of the brain caused by a blunt impact to the head, or by a penetrating head injury. Among the approximately 30 million injury-related Emergency Department hospitalizations occurring each year in the United States, 16% include TBI as a primary or secondary diagnosis. Of note, the related mortality rate may be astonishingly high, with some clinical series reporting a 50% mortality, resulting in over 50,000 deaths/year (*Center for Disease Control* and *Brain Trauma Foundation*). Severe TBI is classically associated with loss of consciousness for at least 30 minutes usually with transitory or permanent memory impairment. The related neurologic deficits vary in intensity, length and clinical manifestation depending on the severity of injuries; as such the degree of recovery is highly variable: survivors may have motor deficits, abnormal speech, loss of thinking ability or emotional problems. Initial assessment of TBI patients require evaluation of the extent of impairment of cognitive functions, usually through international scales such as the Glasgow Coma Scale (GCS), and neuroradiologic investigations to highlight the nature of the brain damage.

A Computed Tomography (CT) scan performed upon admission to the Emergency Department may indentify traumatic lesions involving bony structures, such as calvarial and skull base fractures, and/or intracranial compartments with localized haemorrhagic lesions, such as extradural, subdural or intraparenchymal haematomas, and/or more diffuse parenchymal lesions, such as basal contusions or post-traumatic SAH.

Unfortunately all those post-traumatic lesions which are considered as the consequence of the primary injury may evolve over time due to the occurrence of other pathological mechanisms of secondary injury, mostly mediated by the inflammatory cascade; as such, any lesion involving the intracranial compartments may cause mass effect on the surrounding brain structures due to brain oedema or shift of the midline structures (i.e. ventricular system).

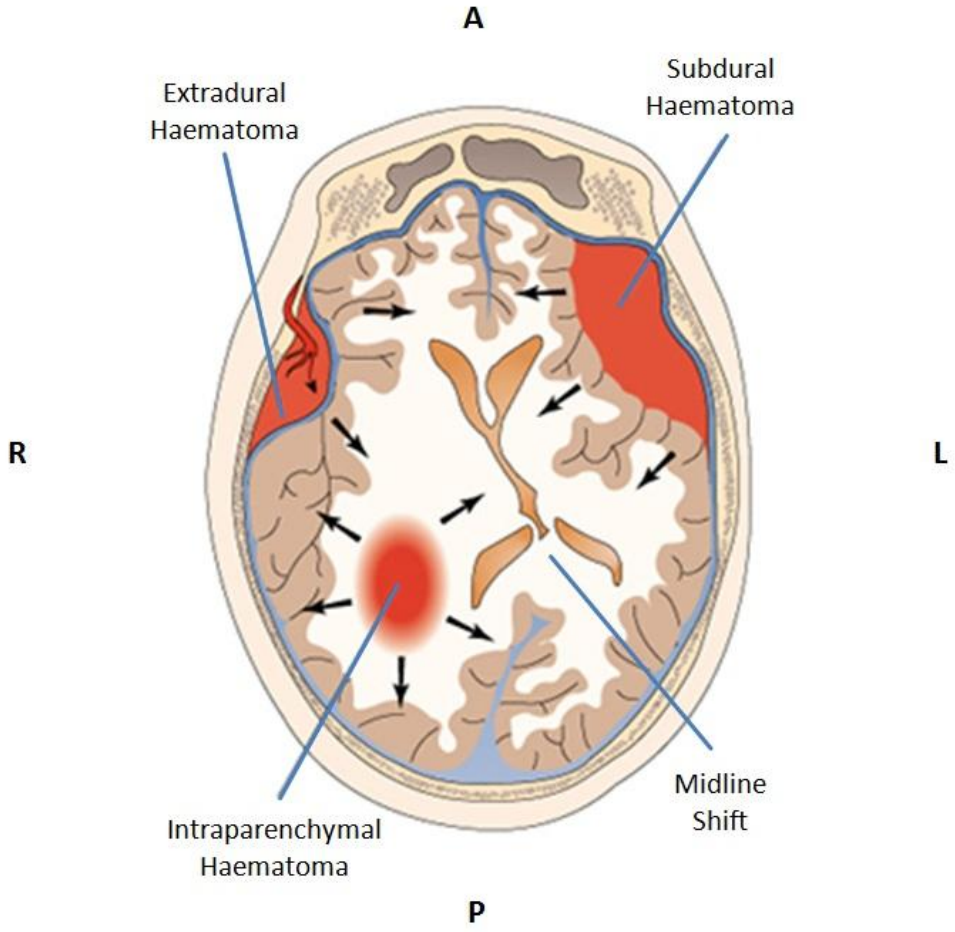

Fig. 1.6: *Artistic representation of intracranial haemorrhages and subsequent mass effect due to TBI (A: anterior; P: posterior, R: right, L: left)*

To tackle the pathologic events related to TBI, monitoring in neurocritical care has evolved from being primarily focused on intracranial pressure (ICP) and cerebral perfusion pressure to include a wide array of monitoring modalities, providing insight into oxygenation, perfusion, electrophysiology and metabolism of the brain.

Today, multimodality neuromonitoring is not only meant to identify second insults or intracranial complications (such as delayed hemorrhages, increase in ICP, etc.), but also to guide the clinicians along the difficult choices of therapeutic management. Nonetheless those approaches are mainly invasive (i.e.,: ventricular catheters, microdialysis catheters, subdural electrocorticography, etc.) and for this reason have a number of possible complications, spanning from periprocedural bleeding to high infection rate, which are still limiting their benefits.

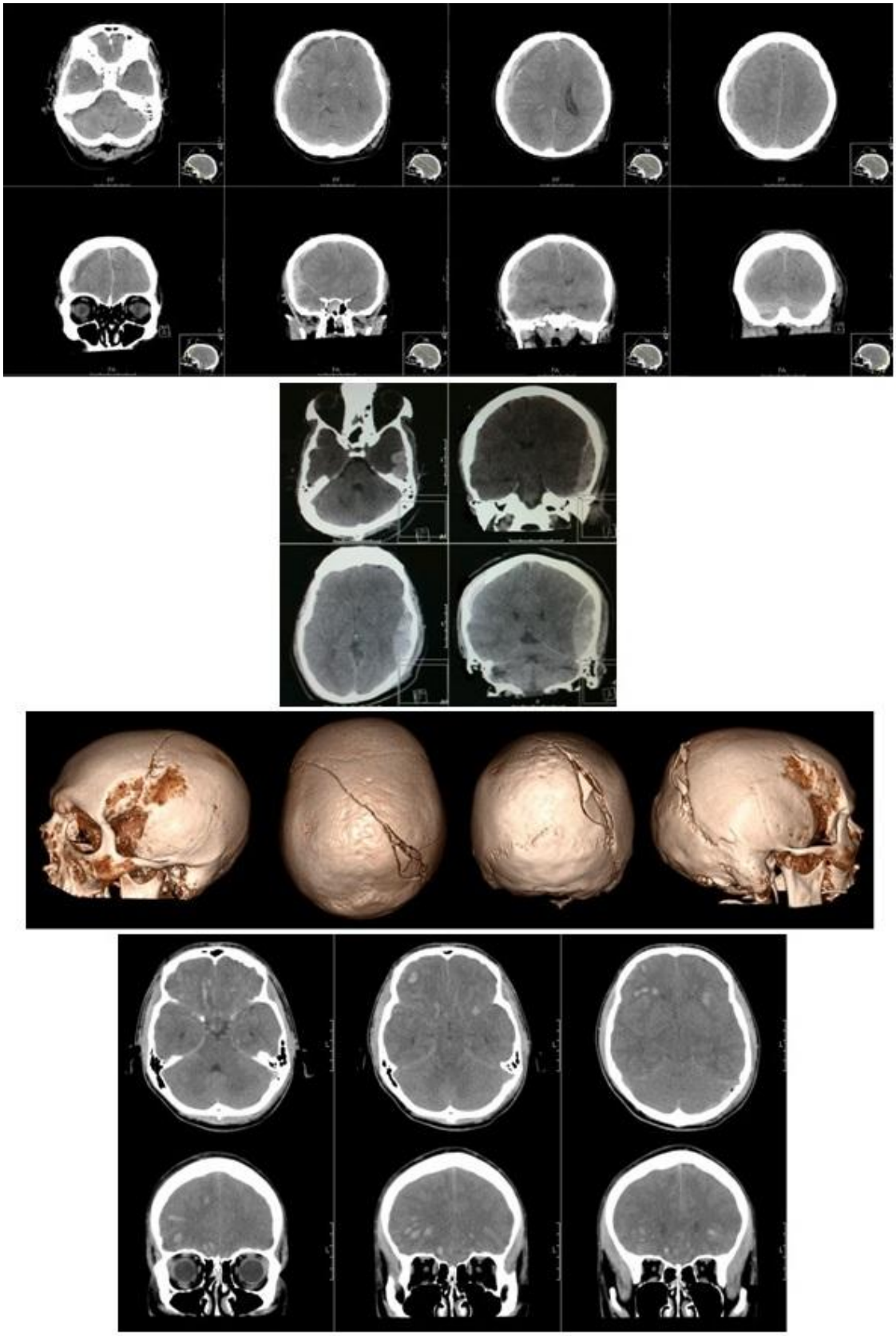

Fig. 1.7: *CT scans showing different types of primary injuries in patients with TBI. Note the right acute subdural haematoma (upper series), left temporal extradural haematoma (second series), the 3D reconstraction of a left occipital depressed skull fracture extended controlaterally (third series), and the diffuse bilateral frontobasal contusions (bottom series).*

Whereas these approaches are still the gold standard, we are witnessing a strong trend toward minimal invasiveness, and this is continuously reshaping monitoring protocols for TBI worldwide. Non-invasive clinical methods to monitor cerebral metabolism and cerebral electrical activity have been widely advocated to be a valid alternative to invasive monitoring modalities. For instance, protocols for continuous monitoring of peripheral blood glucose variability could allow to identify and prevent secondary injury and systemic inflammatory response syndrome in TBI patients; and the use of Event-Related Potentials (in particular Mismatch Negativity and other long-latency components, such as N100 and P300), has been proven reliable in assessing and predicting the recovery of consciousness in unresponsive patients whose behavioral assessment would otherwise be impossible in an ICU setting.

Presently, the biggest area of research is devoted to designing and testing devices able to provide an estimated measure of ICP by a non-invasive way. Some of these methodologies have already reached clinical stage of development, such as the sonographic measurement of optic nerve sheath diameter (ONSD), but still lack accuracy, this therefore represents the biggest challenge of today's clinical research in neurotrauma.

1.5 Identifying Current and Future Needs in Neurosurgery

The exploitation of biomedical engineering-related technologies is expected to deliver enhanced diagnostic and therapeutic solutions in each of the above mentioned subfields of neurosurgery. In particular, the advancements in basic sciences and the subsequent availability of sophisticated technological aids to surgery are expected to lead in the coming years to the identification of better prognostic/predictive biomolecular factors, the development of novel drugs, and the rise of innovative surgical strategies, all meant to profoundly impact the outcome of neurosurgical patients.

For instance, basic research in neuro-oncology is now shifting into investigating the complex cellular and molecular processes responsible for the recurrence of the disease, and clinicians are waiting to see those discoveries translated from bench to bedside. New concepts of tumour origin and proliferation have led to envisaging the next generation of chemotherapy agents, which might activate immunologic memory serving as vaccines directed towards gliomas' specific antigens (*Block O, et al. 2015*). These strategies might ultimately lead to the development of drugs

acting as veritable Trojan horses, and meant to specifically target cancer stem cells and circulating tumour cells (*Ganau M, et al. 2015*).

The continuous pursuit of minimal invasiveness will also extend the indications for radiosurgery which, combining the effectiveness of the conformal 3D dose distributions with a reduced toxicity of radiation, is already making a significant contribution to the management of patients harboring brain lesions, either neoplastic, vascular or functional ones. In fact, updated techniques for radiological planning and robotic neurosurgery are already in advanced stages of laboratory validation and are meant to increase even further the accuracy of surgical or radiosurgical treatments.

Also, the scientific contribution of several clinical trials, aimed at better defining the radiobiological parameters of several brain tumours, is already leading to some attempts to create tumor control probability models meant to revolutionize in the near future today's protocols. To this regard, more accurate imaging techniques might in future play a role in achieving a better surgical planning and postoperative follow up: key to this will be the designing of enhanced contrast agents for pre- and intra-operative identification of vascular and neoplastic lesions.

For instance, significant efforts are now put in the characterization of compounds that have a high affinity in binding to neoplastic cells, which might certainly lead to an optimized definition of tumours' boundaries or to an easier differential diagnosis with postoperative changes (i.e. recurrence, radionecrosis, etc). Similar to this approach, strategies for improved radiosurgical treatment include the designing of effective radioenhancers which are awaited to increase the toxicity of radiation while sparing the healthy parenchyma surrounding the target area.

Finally, also neuro-traumatologists have well highlighted the need for new methods of non-invasive monitoring of TBI patients, aimed at the early identification and limitation of biological cascade responsible for secondary injuries. The research strategies currently exploited to reach this goal are based on the continuous refinement of algorithms based on the analysis of multiple parameters influencing the pressure-volume relationship between ICP, volume of cerebrospinal fluid (CSF), blood, and brain tissue, and cerebral perfusion pressure (CPP) known as the Monro-Kellie doctrine.

The needs highlighted in all those neurosurgical subfields confirm that the development of novel therapies will only come from an unprecedented integration of neuroscience, bioengineering, molecular biology, and physiology, enabling real personalization and adjustment of treatment, critical in such challenging pathologies desperately needing concrete improvements in patient outcomes. The next chapters will focus on our attempts to contribute to these endeavours.

Chapter II:

Functionalizing Nanodrugs for Treatement of Brain Tumours

Research presented at:
International School on Smart Nanomaterials 2015, Rostov-on-Don, Russia

Most Relevant Publications:
Ganau M, Tackling gliomas with nanoformulated antineoplastic drugs: suitability of hyaluronic acid nanoparticles. Clin. Transl. Oncol. 16(2): 220-3; 2014

Ganau L, Paris M, Ligarotti GK, Ganau M. Management of Gliomas: Overview of the Latest Technological Advancements and Related Behavioral Drawbacks. Behav Neurol. 2015: 862634; 2015

Recognition:
Canada-Italy Innovation Award 2015

OVERVIEW AND RESEARCH QUESTION

2.1 Background on Gliomas

Gliomas represent the most frequent class of malignant primitive tumours of the central nervous system (CNS). According to their aggressiveness, the World Health Organization (WHO) classifies them into Grade 1 and 2, or low-grade gliomas (LGG), and Grades 3 and 4, or high-grade gliomas (HGG).

Although relatively rare (incidence of 5/100,000 person/years in Europe and North America), HGG are associated with disproportionately high morbidity and mortality regardless the application of state of the art treatment strategies; in fact, their outcome remains poor, with a median survival of only 14.6 months (*Ohgaki H, et al. 2009*). Also LGG, despite the relatively slow growth, do not present a good outcome, as approximately 70% of Grade 2 gliomas are known to evolve to anaplasia, leading to neurological disability and ultimately to death within 5-10 years (*Sanai N, et al. 2011*).

WHO Class	Phenotype	Grading					Survival
		Differentiation	*Nuclear Atypia*	*Mitotic Activity*	*Microvascular Proliferation*	*Necrosis*	*(Years)*
Astrocytoma							
Grade 2	Fibrillary of gemistocytic astrocytes	Well differentiated	Occasional	Absent	Absent	Absent	6-8
Grade 3	Same as above	Regional or diffuse anaplasia	Present	Present	Absent	Absent	3
Grade 4	Pleomorphic astrocytes	Poor differentiation	Prominent	Prominent	Prominent	Present	1-2
Oligodendroglioma							
Grade 2	Monomorphic cells with uniform round nuclei	Well differentiated	Occasional	Prominent	Not Prominent	Absent	12
Grade 3	Same as above	Regional or diffuse anaplasia	Prominent	Prominent	Prominent	Possible	3-10
Mixed Oligoastrocytoma							
Grade 2	Neoplastic glial cells with both astrocytic and oligodendroglial phenotypes	Well differentiated	Occasional	Occasional	Absent	Absent	6
Grade 3	Same as above	Regional or diffuse anaplasia	Prominent	Prominent	Possible	Absent	3
Grade 4	Same as above	Diffuse anaplasia	Prominent	Prominent	Present	Present	1-2

Legend: *WHO*: World Health Organization

Table 2.1: *Pathological characteristics of LGG and HGG*

As for most malignant brain tumours, the mainstay for treatment of HGG is maximal resection (ideally, gross total resection: > 95% of the lesion) followed within 30 days from surgery by radiation therapy with concurrent or adjuvant chemotherapy (*Talacchi A, et al. 2010*). In fact, clinical evidence that a proactive and aggressive treatment plan improve the outcome of glioma patients when compared to biopsy alone, prompted maximum but safe resection to become the ultimate goal of the neurosurgical treatment (*Talibi SS, et al. 2014*). Nonetheless, the improvement of patients' outcome following gross total resection of HGG mainly relies on extended progression-free survival, rather than on improved QoL or overall survival. Specifically, the impact of surgery on progression-free survival and overall survival has been debated for decades, because of the contradictory results published prior to the diffusion of routine post-operative MRI and volumetric analysis of the extent of resection, which ultimately confirmed the importance of radical removal to extend survival (*Schucht P, et al. 2015*).

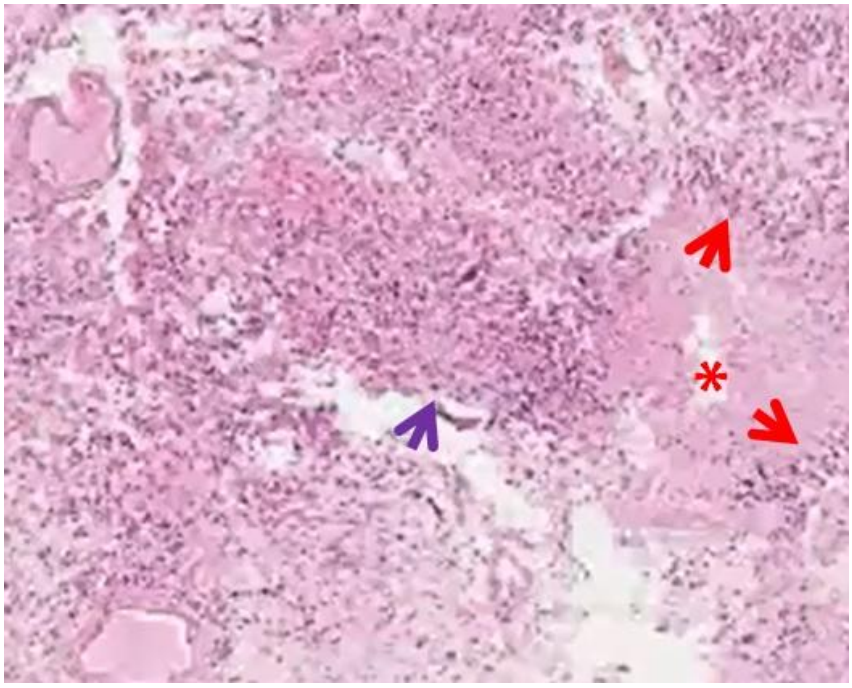

Fig. 2.1: *Microphotograph showing the histological pattern of HGG (Hematoxylin-Eosin, 100x): note the pseudopalisading cells (red arrows), necrosis (asterix) and hypercellularity (blue arrow)*

Concerning LGG, some recent studies have shown similar results confirming that LGG managed with biopsy or subtotal resection followed by a wait and see approach are a higher risk of malignant transformation compared to those treated with more extensive tumour removal (*Duffau H, et al. 2009; Soffietti R et al. 2010*).

Other studies suggested that the extent of removal does not influence the outcome, whereas the best improvements generally come from the postoperative gain of previously impaired functions. To this regard, Talacchi et al. concluded that although the worsening in executive functions soon after operation leaves the overall cognitive burden initially unchanged, it is often transitory and capable of improvement prospectively (*Talacchi A, et al. 2010*).

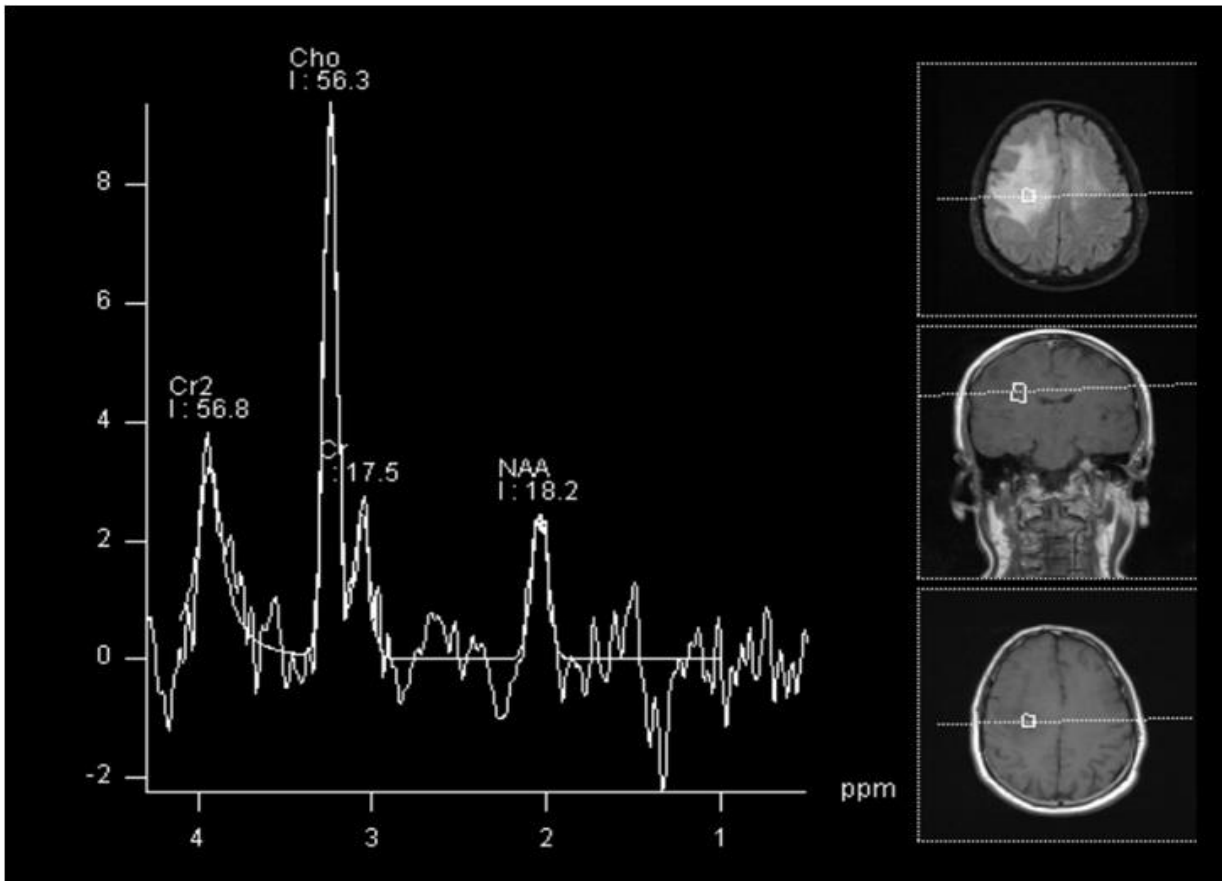

Fig. 2.2: *MRI spectroscopy of LGG: compared with a normal brain, the signal of Choline (Cho) is elevated due to increased membrane synthesis in rapidly dividing tumour cells; N-acetyl-aspartate (NAA), a putative neuronal marker, is decreased due to neuronal loss or dysfunction; the Creatine (Cr) peak is related to the increased tissue energy metabolism.*

In the process to enhance the efficiency of surgical resection, adjuvant radiotherapy has established itself as one of the most valuable tools, especially because conformational treatments now allow preservation of surrounding healthy brain parenchyma. A recent study meant to establish the role of adjuvant radiotherapy on 14,461 patients affected by HGG found a statistically significant interaction between overall survival and histological grade, whereas no significant interactions were observed between radiotherapy and extent of resection (*Rusthoven CG, et al. 2014*).

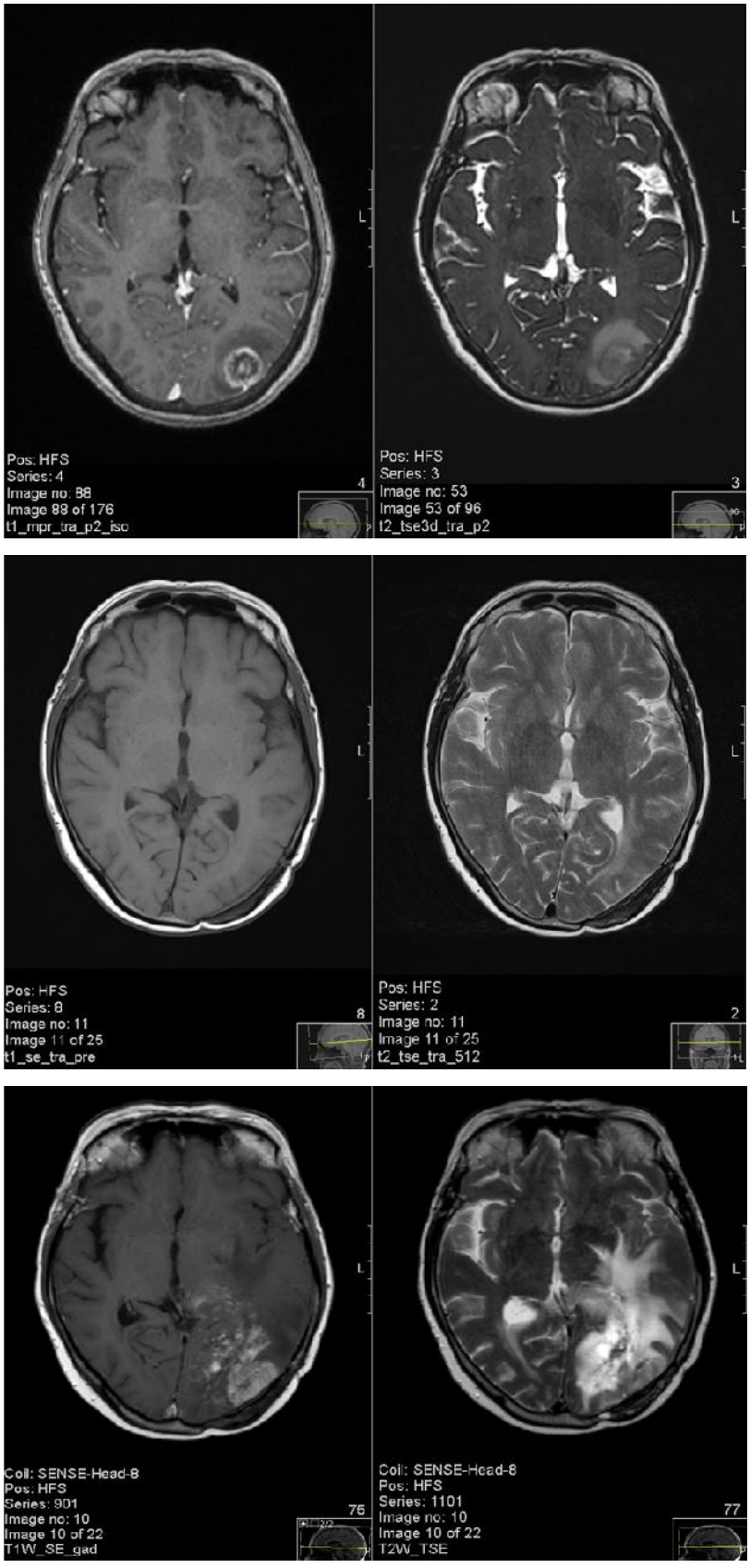

Fig. 2.3: *Recurrence of left parieto-occipital HGG (T1 and T2 weighted MRI sequences, left and right respectively): preop scan (top), post radiotherapy follow up (middle), recurrence of the lesion 18 months after initial surgical excision (bottom).*

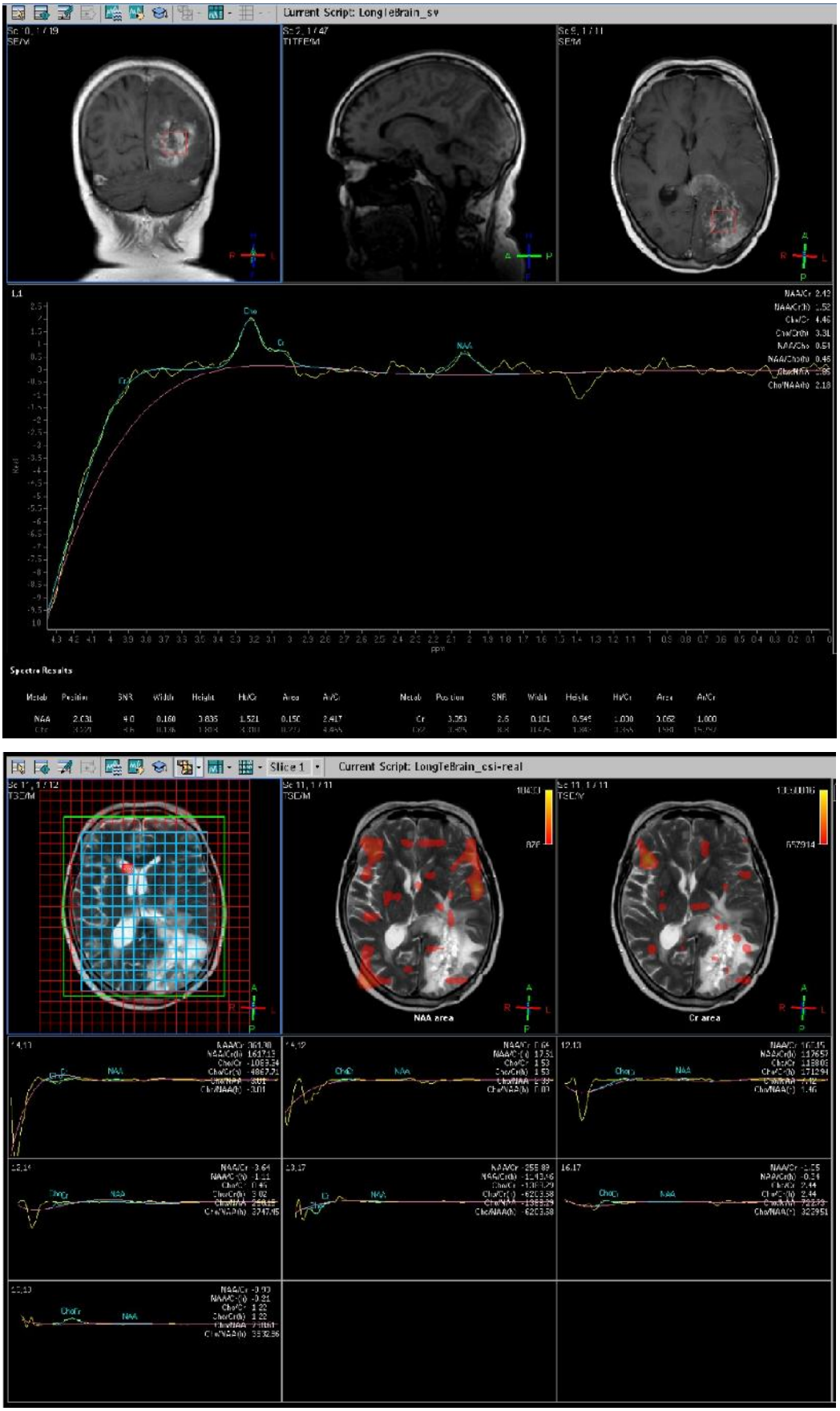

Fig. 2.4: *Single and multivoxel MRI Spectroscopy from patient in Fig. 2.3 ruling out radionecrosis and confirming the neoplastic nature of the recurrent left parieto-occipital HGG.*

The effect of radiotherapy is further increased by adjuvant chemotherapy with the alkylating/DNA methylating agent temozolomide. This treatment strategy known as Stupp protocol is nowadays the gold standard regimen for Grade 4 gliomas, as it has demonstrated to provide patients with a significant increase in 2-year survival from 10.4% to 26.5% (*Stupp R, et al. 2005*).

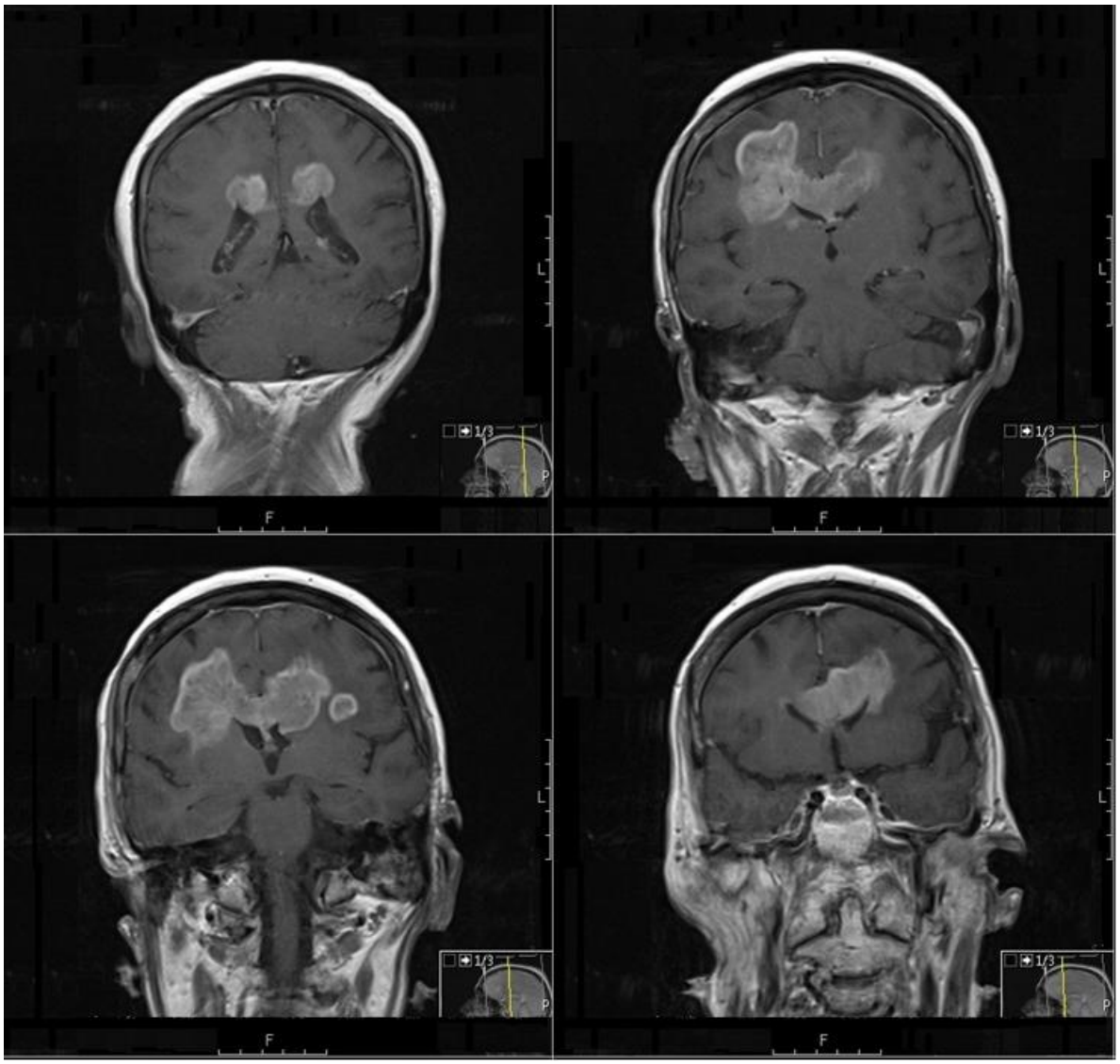

Fig. 2.5: *Spreading of HGG: note the "butterfly" growth pattern across the corpus callosum with infiltration of the controlateral hemisphere, suggesting that only radiotherapy and chemotherapy are viable options for the management of this patient.*

Whilst the adjuvant radio- and chemotherapy options have become established treatment modalities for HGG, their role in LGG is highly debated. Several studies have been conducted over the years on this topic, and recently some long term survival analyses are becoming available. For instance, Nitta et al. aiming to test the hypothesis that adjuvant therapy might not be necessary for LGG cases in which total radiological resection is achieved, enrolled in a longitudinal study a total

of 153 patients treated for LGG between 2000 and 2010 (*Nitta M, et al. 2015*). The multivariate analysis conducted on the data retrieved from those patients did not identify Ki-67/MIB-1 proliferation index (based on monoclonal antibodies directed against different epitopes present during all active phases of the cell cycle like G1, S, G2, and mitosis, but is absent from resting cells in G0 phase) or radiotherapy as prognostic factors, but it did identify chemotherapy as a prognostic factor for progression free survival, and extent of resection for both progression free survival and overall survival of LGG.

These findings support the current accepted practice of using more aggressive treatment with radiotherapy only in LGG patients with a poor prognosis, such as those with diffuse tumours (in particular astrocytomas rather than oligodendrogliomas), and those with partial resection.

Of note, some advancements have also been accomplished in the field of radiation therapy. For instance, the observation that local control and median survival can be improved through the radiation dose escalation, has gradually introduced stereotactic radiosurgery (SRS) in the therapeutic panel for HGG.

Although limited data are available concerning salvage SRS, a 2012 retrospective study of 77 recurrent HGG patients showed that the median post-treatment survival doubled for those receiving Gamma Knife SRS compared to patients treated with second-surgery alone, and advocated SRS as an alternative to open surgery for HGG at the time of recurrences, because of the significantly lower complication rate (*Skeie BS, et al. 2012*).

The availability of sophisticated technological aids to neurosurgical management of gliomas is now pushing towards an optimization of all the treatment options described above, with the ultimate goal to improve the survival and QoL of affected patients. In particular, new solutions exploiting biodegradable materials are particularly sought to bypass the blood brain barrier (BBB) which is still the main limiting factor to the introduction of new drugs for second line chemotherapy, or to reduce the toxicity of radiotherapy and radiosurgery to healthy brain while increasing their lethal action toward the tumoural mass.

2.2 Understanding the Blood Brain Barrier

More than 97% of CNS drugs fail to go into clinical trials due to poor penetration into the brain; the impenetrability of the BBB is in fact the major roadblock in developing new therapies and represents the main reason for this high rate of failure (*Pardridge WM, et al. 2007*). The brain

microvasculature is the gate for all metabolic nutrients that supply the CNS: the human brain consumes about 15-20 W power daily, accounting for 15-20% of the overall basal metabolic rate, and 15-20% of the oxygen and glucose transported in the blood stream.

What tightly regulates transport into the brain is the biomechanical and biochemical barrier between the capillaries and the cellular compartment (which include not only neurons and astrocytes, but also microglia and pericytes) accounting for 3% and 80% respectively of the total brain volume. The surface area of the microvasculature responsible for the dynamic interaction between the brain and the blood stream has been esteemed to range between 100 and 200 cm^2/g tissue, corresponding to a total surface area of 15-25 m^2. Some further figures explain the complexity of this system: capillaries may be as small as 7-10 μm in diameter, while the average intercapillary distance is about 40 μm and most neurons result to be within 10-20 μm of the nearest capillary (*Wong AD, et al. 2013*).

The interface known as BBB is very complex: on one hand endothelial cells constitute the walls of capillaries and are connected by tight junctions (formed by claudins, a family of more than 20 isoforms, as well as occludins) and adherence junctions (formed by vascular endothelial cadherins, catenins and vinculins), creating a barrier with an extremely high electrical resistivity; on the other hand the outer surface of capillaries is itself surrounded by pericytes and astrocytes. Finally, the basement membrane surrounding endothelial cells and pericytes is comprised of fibronectin, laminin and collagen type IV; and has a thickness of about 100 nm (*Dejana E, 2004*). All together those structures convey in what has recently been described as the neurovascular unit: a concept introduced to recognize the importance of functional interaction between neurons and non-neuronal cells for the brain's well being (*Hawkins BT, et al. 2005*).

Adherence and tight junctions are structurally and functionally linked, as they regulate paracellular transport; whereas transcellular transport is regulated by specialized transporters, pumps and receptors.

As such, the BBB is permeable to the passage of H_2O and lipid-soluble molecules by passive diffusion, and to glucose and small aminoacids that are crucial to neural function by selective transport; on the other hand, it prevents the entry of lipophilic molecules and potential neurotoxins by way of an active transport mechanism mediated by P-glycoprotein (P.gP). Highlighting the importance of transport across BBB, it has been estimated that 10-15% of all proteins in the neurovascular unit are transporters (*Wong AD, et al. 2013*).

Although the structure above described is homogeneously distributed in almost the entirety of the CNS, few specialized regions of the brain lack a proper BBB: specifically, the circumventricular organs located at the surface of the III and IV ventricles (such as the vascular

organ of lamina terminalis, the subcommissural organ, the median eminence, intermediate lobe and posterior lobe of the pituitary gland, the subfornical organ, the pineal gland and the area postrema) allow a direct communication between the brain and the vascular system.

This permeability is functional to the role of periventricular organs, and allows neurons and glia at these sites to sense changes in the concentration of various molecules, such as hormones, neurotransmitters and cytokines into the circulation.

Furthermore it is well known that the BBB can exhibit a significant plasticity in response to abnormal physiological conditions (i.e. to increase the influx of nutrients when needed), and that several pathologies (such as neuroinflammatory diseases as well as primary and secondary tumours) or specific drugs (i.e. cyclosporine A, mannitol, etc) can lead to its disruption and subsequently to an increased permeability (*Ito H, et al. 2003; Rapoport SI, 2001*).

This spontaneous or temporarily induced permeability has been extensively exploited to overcome the BBB, and still represent the main strategy to bypass the limitations imposed by paracellular and transcellular transport. Nonetheless the use of physical and chemical method to disrupt the BBB comes at a cost in terms of toxicity, especially after repeated exposure, therefore alternative methods are intensively sought (*Hinow P, et al. 2005*).

In light of the above, the specific aim of this section is to investigate the opportunity to develop innovative nanocarriers with potential to overcome the BBB, tackle glioma cells, and deliver their chemotherapy payload where it is most needed. Specifically, experimental evidence from the existing literature will be provided to support this theoretical hypothesis, and to envisage the possibility to intervene in the various stages of the invasive process, or to exploit those nanocarriers as both diagnostic and therapeutic tools.

EXPERIMENTAL WORK

2.3 Rationale for Proposing Hyaluronic Acid Nanoshells

The development of drug delivery systems able to induce accumulation of a prodrug or its metabolites in aggressive tumours is providing new approaches to achieve enhanced antitumor activity while reducing systemic toxicity. Only recently, the integration of core concepts from the

field of biotechnology, nanotechnology and pharmacodynamics provided us with new insights on the possibility to identify anti-neoplastic targets and evaluate the theoretical feasibility of producing drug-incorporated nanoparticles.

Indeed, gliomas could represent the perfect proof of concept for nanoformulated drugs due to the intrinsic characteristics of those tumours: mainly, the high aggressiveness, poor prognosis, and high degree of chemo-resistance. A thorough analysis of the current therapeutic armamentarium available for primary brain tumours, and the possible targets of bioengineered nanoparticles, is perfectly suited to better understand the opportunities provided by such innovative therapeutic approaches and maximize their possible research and clinical values.

As previously explained a common concerns that arise while facing pharmacological design of drugs intended for CNS malignancies include not only delivery to tumour cells and surrounding tissue, but also penetration through the BBB as tight junctions and Pg.P prevent entry of most anticancer agents into the brain (*Muldoon LL, et al. 2007*).

Thus, the major issues for antineoplastic formulations are: 1) the choice of drug administration (systemic or local) and consequently the residence in the systemic blood circulation or in the brain tissue adjacent to topic injection, 2) the drug loading efficiency and 3) the burst release effect through the extracellular matrix (ECM).

To date the systemic side effects are the most important factors influencing patient's compliance to treatment and clinical outcomes. In fact beside general toxicity, such as nausea, cutaneous rash and vomiting, every agent currently in use is responsible for specific side effects (i.e. myelosuppression for Temozolomide, leukoencephalopathy for Carmustine, etc.), which represent the main dose limiting functions. Accordingly, the development of a drug delivery system able to enhance accumulation of a prodrug or its metabolites within gliomas, while reducing its accumulation elsewhere, would provide a promising approach to achieve enhanced antitumor activity along with reduced collateral damages (*Villano JL, et al. 2009*).

Since HA has immunoneutrality, we and others recently proposed it as a biocompatible and biodegradable material for tissue engineering and development of drug delivery systems (*Luo Y. et al. 1999; Freed LE, et al. 1994; Ganau M, et al 2012*). The possibility to develop anticancer agents using biodegradable microspheres and nanopolymers was proposed for a wide range of antibiotic and chemotherapeutic agents, basically with the aim to obtain prolonged plasma half-life, improved solubility and pharmacokinetic features of the resulting conjugate (*Emerich DF et al. 2000; Avgoustakis K, et al. 2002*). However, only recently formulations of several drugs or prodrugs conjugated to polymeric coated HA nanoparticles of Poly(ε-coprolactone), Polylactide, Poly(lactic-co-glycolic acid), Poly(ethylene)-glycol, Polycarylates and Chitosan were found effective as smart

delivery systems both in vitro and in vivo (*Kundu P, et al. 2012; Madan J, et al. 2012; Tripodo G, et al. 2015; Xin H, et al. 2012*). For instance the conjugation of HA and Poly(ε-coprolactone) has been exploited for the preparation of drug delivery systems for either oral and parenteral administration of drugs as Docetaxel for breast cancers (*Youm I, et al. 2014*); whereas a HA/Polylactide derivative gel was used as coating material for titanium prosthesis for the continuous release of antibiotics such as Vancomycin and Tobramycin and the prevention of postsurgical infections (*Pitarresi G, et al. 2013*).

The translation to neuro-oncology of those successes in the development of innovative drugs appears feasible for several reasons as HA conjugates could leverage on numerous advantages over existing formulations. On one hand, their ability to overcome the BBB and to produce biologic effects on the CNS is mainly based on receptor-mediated endocytosis in the brain capillary endothelial cells (*Wohlfart S, et al. 2012*). On the other, their propensity for tumour targeting takes advantage of the peculiar interaction between glioma cells and the ECM, as in the case of matrix metalloproteinases (MMPs) triggered release (*Sarkar N, et al. 2008; Gu G, et al. 2013*).

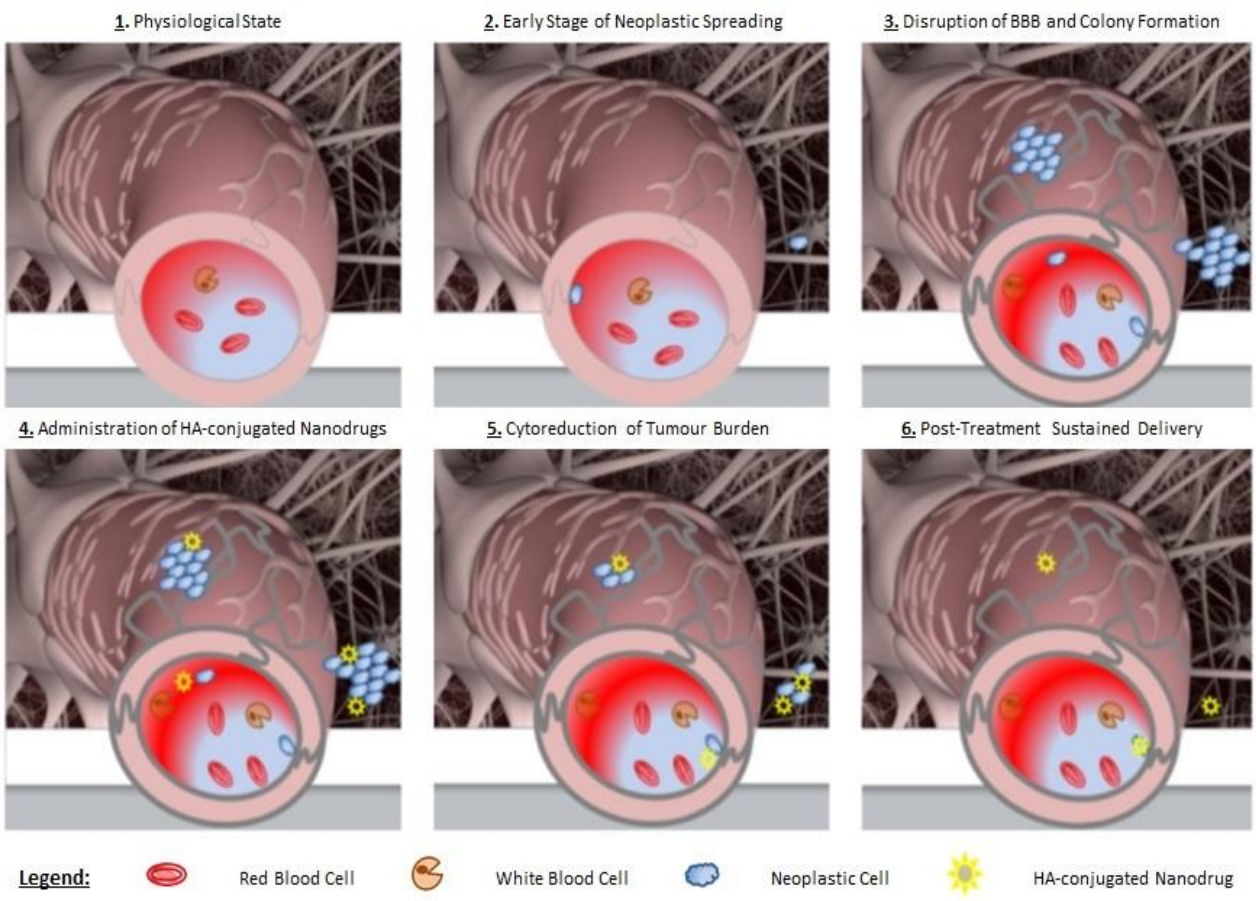

Fig. 2.6: *Artistic representation of the stages of neoplastic invasion into the ECM and proposed action of HA-conjugated nanodrugs*

HA is a natural linear polysaccharide constituted by repeating units of N-acetyl-D-glucosamine and D-glucuronic acid with the monosaccharides linked together by alternating β-1,3 and β-1,4 glycosidic bonds.

The carboxyl groups of HA are predominantly ionized at pH 7.4 and therefore in physiological conditions, HA appear as a polyanion, known as hyaluronan (*Fraser JR, et al. 1997*). HA is found in a wide molecular weight ranging from 20kDa of HA oligomers (o-HA), till to the high-molecular weight (HMW) of bulk HA (~1.5MDa). In solution, the chains of HA adopt a random coil conformation, and its high hydrophilic nature leads to multiple hydrogen bonds with H_2O, explaining the viscous and elastic characteristics of the connective tissues in which this polysaccharide is abundant.

Beside chemical conjugation it has been proved that HA can also be linked to other prodrugs or to proper delivery systems by weak interactions such as those involved in the formation of ion pairs (*Nitta SK, et al. 2013; Oyarzun-Ampuero FA, et al. 2013*), expanding the number of possible candidates for conjugation with or encapsulation within those nanocarriers.

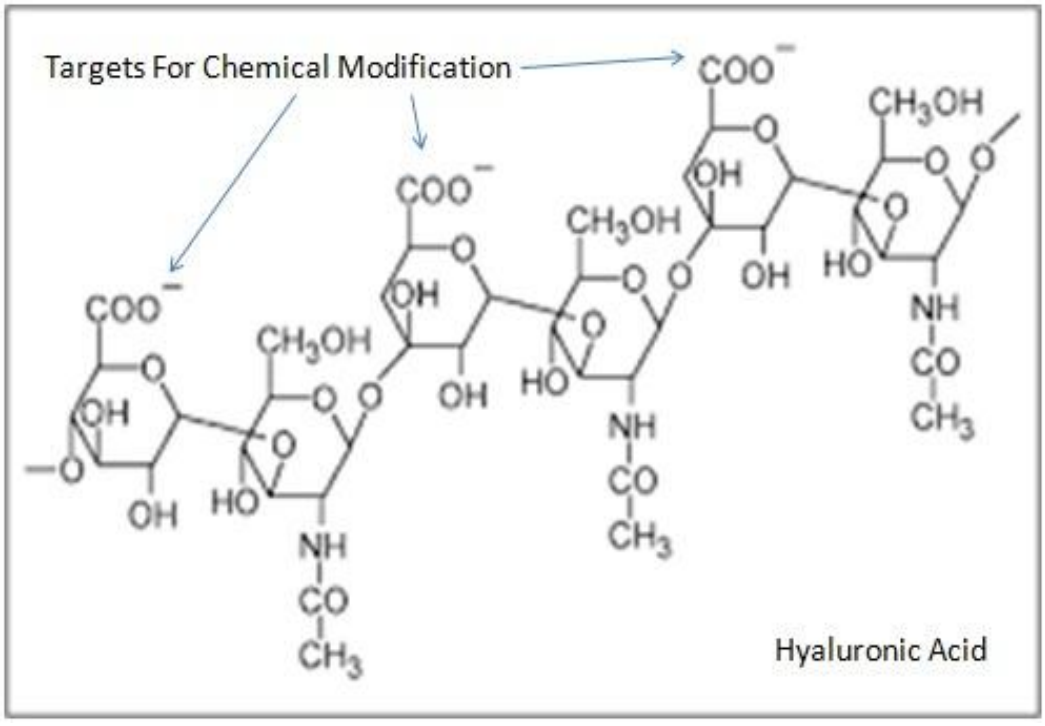

Fig. 2.7: *Chemical Formula of HA and targets for its modification*

Indeed, these characteristics not only allow to overcome many of the concerns related to drug tolerance and side-effects, but also those related to the risk of cytotoxicity and genotoxicity of

other nanomaterials which have strongly affected the clinical testing of drugs with a previously unremarkable laboratory track (*Greish K, et al. 2012*).

Herein the specific reasons supporting the use of HA nanoshells as a therapeutic approach for the management of gliomas are proposed and discussed.

2.3 a): Extracellular Matrix and Hyaluronidases

During brain development the ECM modulates the migration of glial and neuronal precursor cells, guides axonal growth cones, synapse formation and cell proliferation. In normal reparative processes, as well as in primary brain tumours, the ECM is present in increased amounts and undergoes remodelling.

One of the main components of ECM is HA; it is noteworthy that interactions between astroglial cells and HA play crucial roles in cell adhesion, growth, and migration during inflammation, wound healing, and neuro-oncogenesis (*Knudson CB, et al. 1993; Rooney P, et al. 1995*). HA is increased approximately four-fold in primary brain tumours, reaching levels comparable to those present during CNS development (*Delpech B, et al. 1993*).

The primary cell surface receptor for HA is CD44, a transmembrane glycoprotein belonging to the superfamily of immunoglobulin receptors, implicated in a various range of physiological and pathological processes, such as cell-matrix interaction and cell migration.

Since the invasive properties of malignant gliomas are due to their adhesion to ECM components, the overexpression of CD44 and of another ubiquitous receptor for HA, the Receptor for HA-Mediated Motility (RHAMM), in gliomas may be relevant to their highly invasive behaviour within the brain.

Noteworthy, a gradient of expression was identified amongst gliomas, with HGG presenting more RHAMM than does lower grade lesions or normal human astrocytes (*Akiyama Y, et al. 2001*). Furthermore, the subsequent penetration of the brain parenchyma by glioma cells is related to the cleavage of the ECM, and glioma cells have developed multiple mechanisms to achieve this goal.

Whilst CD44 is essential for HA uptake and also for intracellular transportation into lysosomes, specific enzymes called hyaluronidases (HYALs) are the basis for endogenous or exogenous HA degradation.

Many isoformes of HYALs exist in the human genome: HYAL1-4, PH20, and HYALP1; although the precise mechanisms of HYALs expression and secretion in glioma cells are not fully understood, they certainly correlate with the invasive behaviour of such tumours (*Junker N, et al. 2003*).

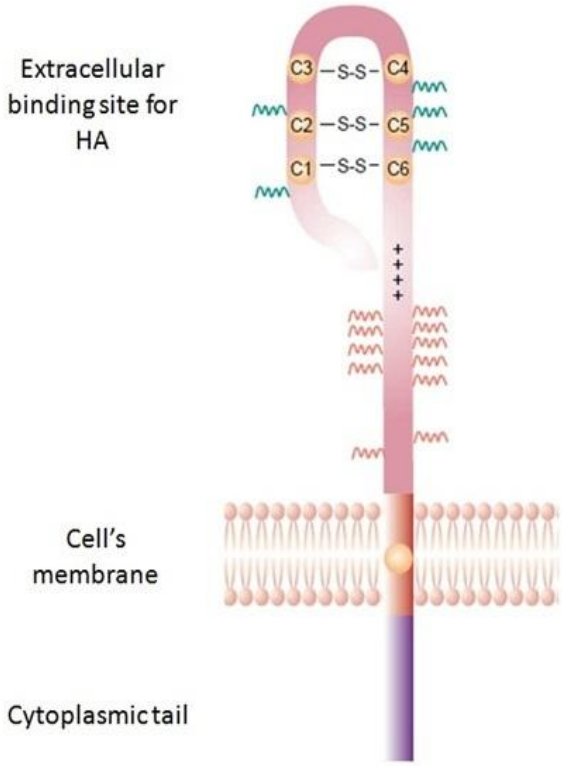

Fig. 2.8: *Artistic representation of a CD44 transmembrane receptor*

Another pathway of ECM invasion is represented by MMPs, a large family of endopeptides (currently consisting of 25 members), which are broadly subclassified as: membrane type MMPs, collagenases, gelatinases, stromelysins, etc (*Rao JS. 2003*). MMPs are produced in cells as proenzymes and require proteolytic cleavage for activation, they are overexpressed in numerous malignancies, and appear critical for invasion and metastases.

Among MMPs, the two most strongly implicated in glioma invasion seems to be the gelatinases MMP-2 and MMP-9, which are upregulated by genetic, hypoxic and stromal stimuli (*Csoka AB, et al. 2001*).

As such, chemotherapeutic strategies for glioma patients can take advantage of bioconjugates between HA and anticancer agents because of many reasons: as showed above the overexpression of CD44 and RHAMM in glioma cells represents the basis to hypothesize the selective targeting of HA-nanoparticles toward primary brain tumours, and both exogenous and/or endogenous release of the prodrug carried may be envisaged.

Furthermore, the different response of glioma cells to endogenous and exogenously added HA appears remarkable: administration of small o-HA, that compete for endogenous HA polymer interactions, results in attenuation of HA induced signalling (*Gilg AG, et al. 2008*). This behaviour antagonizes the malignant properties of glioma cells in vitro and in vivo, for this reason o-HA

released upon dissolution of the HA nanoshells within the ECM holds the potential for a biological "weapon" to inhibit glioma invasion, eventually enhancing apoptosis and downregulating key cell survival mechanisms.

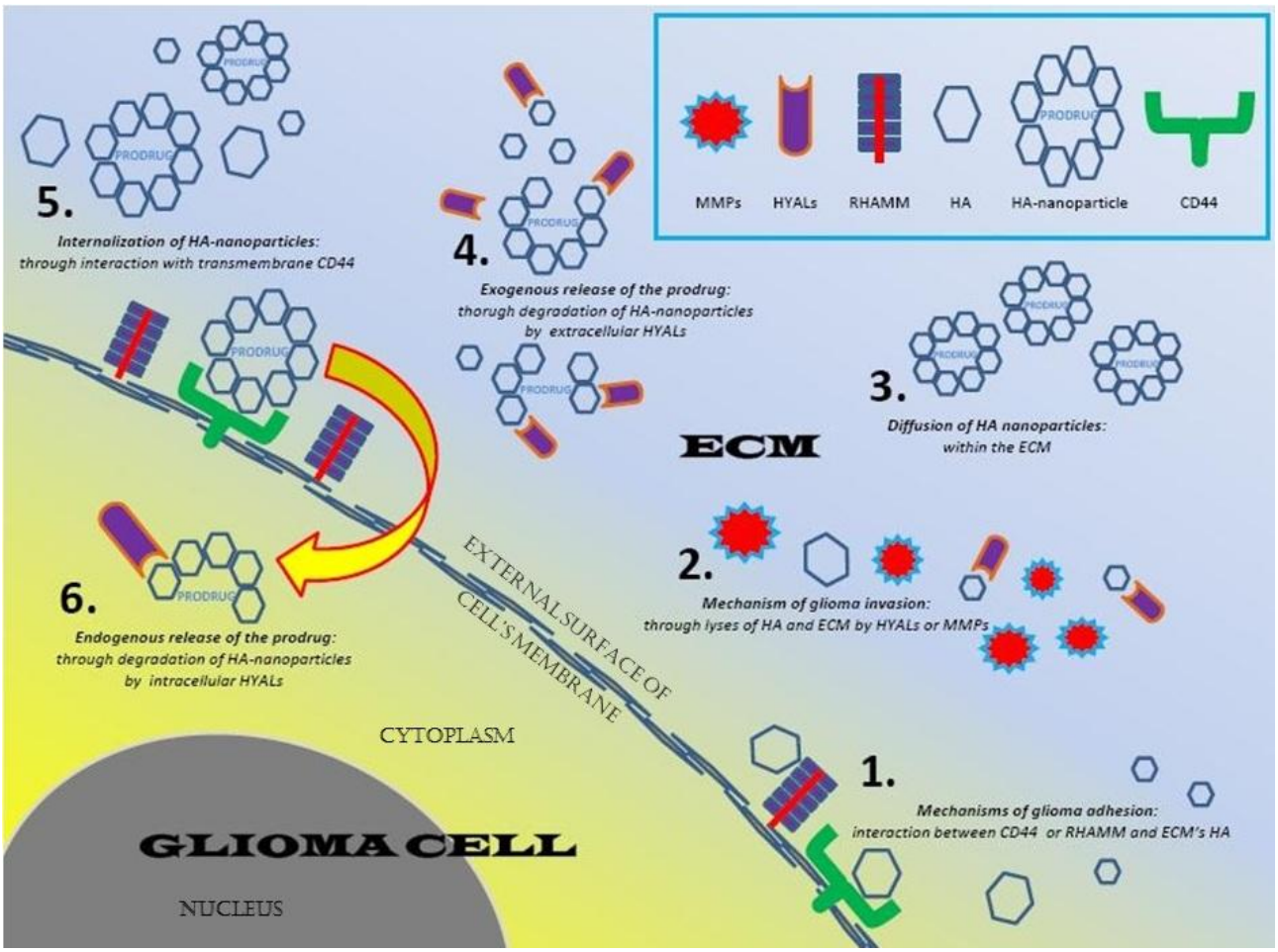

Fig. 2.9: *Nanoshells of HA and their mechanism of action*

Beyond HGG, one of the aspects that could further extend the theranostic use of HA-nanoparticles in neuro-oncology could be their ability to track resident and circulating tumour cells (CTC) as well as resident and circulating tumour neurospheres (TNS) (*Cheng B, et al. 2014*). In fact, CTCs are recognized as the main factor responsible for formation of secondary brain metastasis spreading from solid cancers, whereas TNSs are microaggregates of cancer stem/progenitor cells recently linked also with cell renewal and tumour recurrency in HGG (*Liu G, et al. 2006*). Since, as previously described, the possibility to target HA induced signal interactions in malignant gliomas is almost certain, envisaging the capability to target even CD44+ TNSs (*Anido J, et al. 2010*), would provide further evidence of the groundbreaking potentials of such prodrug carriers.

2.3 b): Fabrication Strategy

The technical limitations of encapsulating prodrugs within HA nanoshell were initially raised especially for the high-molecular weight of bulk HA. Nevertheless, HA degradation to a low-molecular weight o-HA seems an easy way to overcome this initial problem, and the possibility to complex a chosen drug with the anionic domain of HA to form the nanospherical aggregates was recently tested with great success.

In fact the production of cisplatin-incorporated HA nanoformulated drugs can be obtained as described by Jeong et al. by spontaneous aggregation of nanoparticles of HA and cisplatin, due to the formation of strong ion complex (*Jeong YI, et al. 2008*).

The need to experimentally evaluate the pharmacokinetics and pharmacodynamics aspects of these nanoformulated drugs, which are related to the specific drug/prodrug tested, their molecular weight and the kind of preparation/administration chosen, appears therefore justified by this premises.

- To obtain LMW HA, the enzyme hyaluronidase (HAse) (10 units/mg of HA) is added to a solution of 2.0 g of HMW HA (1500 kDa) in pH 6.5 PBS buffer (4 mg/mL). The degradation is carried out at 37 °C, 190 rpm stirring for 1 h, followed by 95 °C for 20 min.
- The solution is dialyzed against water for 4 days at room temperature, then filtered through a 0.2 μm cellulose acetate membrane and lyophilized. This process allows to obtain 1.12 g of LMW HA (56% of the initial 2.0g of bulk HA).
- The choice of polymer/drug ratio depends on the prodrug tested: previous tests dissolving in 10ml of deionized water 100mg of LMW HA and various amount of cisplatin (Chemical Formula $H_6C_{l2}N_2Pt$, Molar Mass 300.01 g/mol) showed that the best loading efficiency is obtained at a HA/cisplatin rate of 100/5 and 100/10.

The aggregation of HA and cisplatin is obtained through simple ion complexes; the size of the nanoshells varies from 100 to 200 nm depending on the polymer/drug ratio.

According to the relevant literature it is well known that the MW of HA influence the size of the nanoshells, so that the higher the MW the larger their diameter; also the lower the ratio the lower the zeta potential of the nanoshells.

Having confirmed that complexing a given compound with HA to realize nanoshells is feasible and not particularly difficult, we have identified possible prodrugs with Molar Mass and chemical characteristics suitable for future preclinical tests.

2.4 Externalities in Other Areas of Neuro-Oncology

HA nanoparticles as carriers for antineoplastic drugs hold the potential for an innovative, high throughput therapeutic approach in neuro-oncological chemotherapy protocols, but their use is also fostering further advancements at the forefront of parallel research fields in neuro-imaging, nuclear medicine and radiotherapy. In fact their use as innovative nanocarriers promise to provide better stability, tolerance and biodistribution also for contrast agents, radiotracers and radiosensitizers.

Among antineoplastic candidates previously considered unsuitable because of their systemic toxicity or hydrophobicity we attempted to identify drugs with good potential in terms of cytotoxicity/cytostability but also with MW and other biochemical characteristics suitable for incorporation into HA-nanoshells.

2.4 a): Chemotherapy

As stated above the MW has been the main limiting factor, knowing that the type of binding to HA and the size of the prodrug/drug considered directly affect the expected size of the final compound. Our analysis of current databases led to identify one potential candidate: Cinnamaldehyde (Chemical Formula C_9H_8O, Molar Mass 132.16 g/mol).

Cinnamaldehyde (CA) is an active monomer isolated from the stem bark of *Cinnamomum cassia*, a traditional oriental medicinal herb, which is known to possess marked antitumor effects in vitro and in vivo. Its analogues are structurally characterized by the presence of cinnamoyl moiety, and due to the presence of highly reactive α,β-unsaturated carbonyl pharmacophore in their structures these molecules are apt to react with some enzymes and/or receptors as electrophiles, inducing diverse therapeutically relevant pharmacological functions. Naturally occurring molecules, such as trans-cinnamaldehyde, 2-benzoyloxycinnamaldehyde, and 2-hydroxycinnamaldehyde are representatives of this group, and have attracted significant interest for their bioactivities, especially the anti-inflammatory and anti-cancer properties (*Chen BJ, et al. 2016*). The former are attributed to CA ability to block nuclear factor-κB activation in immune cells; whereas the latter have been shown both in terms of cytostatic and cytotoxic effects in a number of human cancer cells including breast, colorectal, head and neck cancers as well as gliomas.

CA demonstrated to inhibit proliferation and to induce apopotosis, the first property is due to the interaction with cell-matrix adhesion, in fact CA has proven to induce an upregulation of the expression of E-cadherin and a downregulation of the expression of MMP-2 and MMP-9; whereas the second property on the apoptotic pathway seems due to the inhibition of the transcription activity of PI3K/AKT (*Li J, et al. 2016*). Furthermore tests conducted on p53-wild and p53-mutant human head and neck cancer cells reveled that CA induces apoptosis regardless of p53 status (*Ahn SG, et al. 2015*).

Finally the apoptotic activity of CA has been confirmed also in human U373 glioblastoma cells characterized by various levels of resistance to different pro-apoptotic stimuli, in those essays CA showed to overcome the intrinsic resistance of U373 cancer cells to programmed death and to determine a cytostatic effect (*Bruyère C, et al. 2011*).

Indeed, compounds possessing both anti-cancer as well as anti-inflammatory properties like CA may therefore provide an attractive therapeutic tool for cancer therapy; for what specifically concerns gliomas this is especially relevant when it comes to consider them along other immunotherapy protocols which are consider another possible effective tool in the battle to prevent their recurrence (*Roth-Walter F, et al. 2014*).

2.4 b): NeuroImaging

Conventional or high-Tesla MRI could benefit from new constrat agents capable to early detect microaggregates of neoplastic cells, this in fact represent a fundamental step to progress in the early diagnosis of tumours and follow up of patients to rule out recurrence of the disease. Implementing this approach is pivotal to reach the sensitivity required to track CTCs or detect metastasis in preclinical phase of development.

Achieving this goal would in fact provide several interesting insights into the neuro-oncogenesis and in turn lead to the development of screening protocols and ultra-early therapeutic approaches. To this regard, HA seems to be a good platform to complex Manganese oxide nanoparticles (Chemical Formula: Mn_3O_4, Molar Mass 228.812 g/mol) with potential for selective accumulation within HGGs.

The use of or targeted tumor MRI in vivo has been so far mostly tested with Poly(ethylene)-glycol. Luo et al. conjugated Mn_3O_4 with Poly(ethylene)-glycol creating nanoparticles of a mean diameter of 8.0 nm and characterized by a good water-dispersibility, colloidal stability, cytocompatibility and hemocompatibility (*Luo Y, et al. 2015*).

Chen et al. further investigated the applicability of MnO-Poly(ethylene)-glycol nanoparticles conjugated with fluorescent dye Cyanine5.5 as a dual-modal imaging nanoprobe for MRI and near infrared fluorescence. The dual potential imaging role of those nanoparticles was tested conducting experiments on the detection of brain gliomas in mice, showing both in vivo and ex vivo a preferential accumulation of those nanoprobes in the tumoural region (*Chen JS, et al. 2015*).

Finally, encouraging results in terms of cytotoxicity come from tests conducted on engineered nanoparticles synthesized by the encapsulation of Poly(ethylene)-glycol phospholipid shell around the MnO core. By quantifying the induction of reactive oxygen species in human glioblastoma and neuroblastoma cell lines, Choi et al. demonstrated that their cytotoxicity is not significant and confirmed their high potential as an innovative diagnostic tool (*Choi JS, et al. 2015*).

2.4 c): Nuclear Medicine

Due to its extraordinary high sensitivity (down to picomolar level) and quantitative nature, radionuclide-based imaging is considered a standard modality for molecular imaging, although burdened by the poor resolution (≈ 5 mm) of both Proton Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) (*Toy R, et al. 2014*). Indeed, in selected neuro-oncological cases, the use of PET scans can determine a change in treatment management in up to 50% of the cases, and these performances have further supported the optimization of known tracers or identification of new ones (*Lopci E, et al. 2015*).

Among established tracers proposed for gliomas ^{18}F-Fluorodeoxyglucose (Chemical Formula: $C_6H_{11}FO_5$, Molar Mass: 181.1495 g/mol), despite its satisfactory performance in LGG and inflammation, has lost its initial appeal in favour of ^{11}C-Methionine (Chemical Formula: $C_5H_{11}NO_2S$, Molar Mass: 149.21 g/mol) which has almost no tracer uptake in normal brain tissue, and shows a significantly increased uptake in HGG (sensitivity and specificity of 89% and 100%, respectively), with an optimal tumour-to-background ratio (*Yamane T, et al. 2010; Huang MC, et al. 2005*; *Chung JK, et al. 2002*). Among more recently proposed tracers ^{13}N-ammonia demonstrated to be superior to ^{18}F-Fluorodeoxyglucose in the diagnosis of LGG and HGG for better tumor to normal gray matter contrast, nonetheless its highest concentrations are usually found in the heart and liver, whereas pancreas, brain, spleen and stomach only follow in terms of biodistribution (*Shi X, et al. 2013*).

The above premises justify a possible attempt to encapsulate those tracers within HA nanoshells in order to leverage on the elective interaction with CD44 and specifically increase the vehiculation within the CNS, while reducing the accumulation elsewhere, and favouring their

release in the ECM adjacent to glioma cells. The main limitation to this approach is that the decay of those tracers is comprised between few minutes and 1 hour, although previous experiments with ^{18}F-Fluorodeoxyglucose radiolabeled long-circulating Poly(ethylene)-glycol-coated liposomes showed that they could remain in blood circulation at near constant levels for at least 90 minutes (*Marik J, et al. 2007*). For this, the radionuclides selected so far for conjugation with nanoparticles (^{64}Cu, ^{76}Br, ^{89}Zr, ^{124}I) are all characterized by longer half-life (*Liu Y, et al. 2012*). As such, only further investigation will allow to understand the real value of incorporation or labeling of nanoparticles with radionuclide for diagnostic or theranostic purposes.

2.4 d): Radiotherapy/Radiosurgery

Noteworthy, HA-nanoshells can have also a role as radiosensitizers: agents administered in conjunction with radiation therapy and intended to increase the lethal effects of radiation while limiting the injury caused to adjacent healthy tissue. Several compounds have been proposed as radiosensitizers for brain tumours, and some of them are in advanced stage of validation.

Molecule	*Stage of Development (Ref)*	*Mechanism of Action*
Bromodeoxyuridine	**Phase III Clin Trial** (Prados et al, 2004)	It is a halogenated pyrimidine selectively incorporated into DNA of cancer cells and sensitizes them to radiation damage
Estramustine	**Preclinical Study** (Vallbo et al, 2002)	This estradiol-based antimicrotubule agent selectively accumulates in glioma cells inducing their apopstosis
Lonidamide	**Phase II Clin Trial** (Stewart et al, 1993)	It exerts a powerful inhibitory effect on oxygen consumption and aerobic glycolysis, leading to lactic acid accumulation in neoplastic cells
Motexafin Gadolinium	**Phase II Clin Trial** (McHaffie et al, 2011)	This metalloporphyrin demonstrates selective tumor localization, and acts generating reactive oxygen species
Legend: *Ref*: Reference, *Clin Trial*: Clinical Trial		

Table 2.2: *Radiosensitizers for brain tumours and their stage of development*

For instance, HA-complexed radiosensitizers might potentially expand the opportunities for adjuvant radiotherapy or radiosurgery with agents such as Bromodeoxyuridine (Chemical Formula $C_9H_{11}BrN_2O_5$, Molar Mass 307.10 g/mol), increasing its incorporation into actively replicating glioma cells during de novo DNA synthesis in the first; or Lonidamine (Chemical Formula

$C_{15}H_{10}Cl_2N_2O_2$, Molar Mass 321.158 g/mol) allowing its safer vehiculation within the CNS, while reducing the related severe side effects (which include organ toxicity and myelosuppression).

Bromodeoxyuridine is known to impair the proliferative capacity of primary tumor-initiating human glioma cells and may therefore represent a means of targeting cancer stem cells; in fact this compound significantly suppresses the progression of gliomas even in the absence of radiation therapy (*Levkoff LH, et al. 2008*); gven those premises its applicability might theoretically further increase by the conjugation within HA nanoshells.

Assanhou et al. proposed the co-delivery of Paclitaxel and Lonidamine using a dual-functionalized liposome (D-α-tocopheryl-Poly(ethylene)-glycol succinate and HA), and demonstrated a satisfactory loading of both the chemotherapic and radiosensitizer and appropriate release in the intracellular environment (*Assanhou AG, et al. 2015*). Noteworthy, the release of Lonidamide was effective in inducing a suppression of the intracellular adenosine triphosphate (ATP) production by interfering with the mitochondrial function for enhanced P-gP inhibition, thus supporting the hopes for its possible role as a regulator of the multidrug resistance process.

HA are thus opening new diagnostic and therapeutic perspectives, and mathematical simulations are now being held with the purpose of tackling gliomas' specific antigens to create compounds for diagnostic or therapeutic scopes that will act as veritable Trojan horses; and as previously anticipated, their success will mostly depend on the ability to target infiltrating stem cells and CTCs, both considered as the major responsible for cell renewal and tumour recurrency.

DISCUSSION AND FUTURE DEVELOPMENTS

2.5 Tenets of Hyaluronic Acid Nanoshells

While conventional drugs and contrast agents are rapidly and relatively homogeneously distributed within the body to both cancer and healthy tissues, the use of nanocarriers allows for a much effective targeting because their functionalization can lead to an optimal biophysical and biochemical interaction for site specific distribution.

Nonetheless, attempts to improve chemotherapy homing or contrast agents and radioenhancers accumulation into brain tumours has so far relied on the enhanced permeability and

retention effect (EPR), the property by which molecules like liposomes, nanoparticles, and macromolecular drugs tend to accumulate in tumour tissue much more avidly than they do in normal tissues (*Maeda H, 2001*). Essentially, the passive extravasation of molecules as large as 200 nm from the microcirculation to the ECM of the tumour site is favoured by the fact that unlike healthy vasculature, the neoformed vessels characterizing the tumour microenvironment present a discontinuous vascular endothelium. The rate of tumour neoangiogenesis results in fact in the formation of gaps ranging between 100 and 1000 nm in width between pericytes and endothelial cells (*Hashizume H, et al. 2000*). Unfortunately, though, EPR is inconsistent in the early stages of oncogenesis, when therapeutic intervention should be more effective considering the submillimetric diameter of the lesions, but also throughout the tumour during its later stages of progression.

This consideration has two implications: firstly, it is not possible to rely on EPR either for diagnostic or therapeutic purposes if we are to tackle parenchymal lesions in their early stages of growth or if we eventually aim to detect CTCs and cancer stem cells; secondly, only active mechanisms (i.e. based on ligand-receptor interaction) can allow to target the tumour microenvironments and deliver nanoparticles within it if we hope to detect and early treat recurrences close to the surgical site despite complete resection.

Furthermore, whereas nanocarriers can be considered for multiple scopes within the field of neuro-oncology, special considerations should be taken into account when it comes to each specific scope: smaller particles are certainly more prone to passive diffusion, while larger particles are more likely to be characterized by longer blood residence time and higher binding activity. This latter is also influenced by the length and flexibility of the polymer used which directly determines the active fractional area.

In general, a diameter between 60 and 100 nm together with a tailored polymeric coating such as the one advocated for our HA nanoshells seem appropriate to maximize the blood residence time, while allowing to leverage on both passive and active transport through the BBB (*Nagayasu A, et al. 1999*).

In this regard, the presence of CD44 has been demonstrated on endothelial cells and this could suggest a receptor-mediated transport across the BBB (*Savani RC, et al. 2001*). Indeed, experimental evidence has already showed that HA conjugates enhance the delivery of prodrugs within the CNS significantly more than when treatment for disruption of the BBB are used (the uptake is 12 folds higher than the basal one when the prodrug is conjugated with HA, versus 8 folds when cyclosporine is concomitantly administered with the prodrug), also the fact that no further increase is obtained by using PgP inhibitor cyclosporine confirms that HA conjugates intrinsically possess the ability to bypass PgP (*Mittapalli RK, et al. 2013*).

Nonetheless, the spherical and symmetrical shape poses several disadvantages in terms of margination and active fractional area, for instance targeted nanorods have been observed to localize at the target site seven times more than their spherical counterparts (*Kohlar P, et al. 2013*).

As such, the development of multimodality agents, although attractive, has to face enormous challenges both during the conception and laboratory implementation. Despite the striking adaptability to multiple diagnostic and therapeutic scenarios, “one size fits all” does not seems the right approach to the design of HA nanocarriers, and their functionalization should significantly be modified depending on whether those nanoparticles are eventually meant to seek the microvasculature, a deep sited lesion or a blood circulating target.

Chapter III:

Optimizing Treatment Protocols for Stereotactic Radiosurgery

Research presented at:
The International Leksell Gamma Knife Society 2016, Amsterdam, The Netherlands

Most Relevant Publications:
Ganau M, Foroni RI, Gerosa M, Zivelonghi E, Longhi M, Nicolato A. Radiosurgical options in neuro-oncology: a review on current tenets and future opportunities. Part I: therapeutic strategies. Tumori. 100(4): 459-65; 2014

Ganau M, Foroni RI, Gerosa M, Ricciardi GK, Longhi M, Nicolato A.
Radiosurgical options in neuro-oncology: a review on current tenets and future opportunities. Part II: adjuvant radiobiological tools. Tumori. 101(1): 57-63; 2015

OVERVIEW AND RESEARCH QUESTION

3.1 Principles of Stereotactic Radiosurgery

Lars Leksell the pioneer of stereotactic radiosurgery (SRS) defined this therapeutic option as "a technique of closed skull destruction of a predetermined intracranial target by single-fraction, high dose of ionizing radiation using a precision stereotactic apparatus" (*Leksell L. 1951*). In contrast with spatially less accurate radiotherapy techniques, SRS has the capacity to maximize/optimize the dose exposure on the target volume, while minimizing the irradiation of surrounding critical structures, thereby reducing collateral damage. Those characteristics led SRS to be considered not only as a potential adjuvant to surgical treatment but in some cases also as a valuable alternative option.

Radiosurgery includes a wide range of reliable, minimally-invasive options that can be used as either primary or adjuvant therapy in the management of both malignant and benign pathologies, including also vascular or functional ones. SRS, in the absolute majority of cases, differs from conventional "ab externo" radiotherapy (RT) basically because a single large fraction radiation is used, with very few exceptions, instead than multiple daily fractions. Moreover, RT relies on the difference in susceptibility between tumor tissue and normal brain whereas SRS delivers an ablative dose to the target margin with a higher dose delivered centrally. In this regard, the accuracy of SRS presents a much smaller standard error (less than 0.3mm), and the dose gradient is extremely steep so that radiation outside of the target volume is minimized.

Currently SRS techniques may utilize different types of penetrating radiation. They include the cyclotron- or synchrotron-generated particles, such as protons or heavy-charged-particles, and photon devices such as modified linear accelerators (LINAC) or Gamma Knife (GK):

- ***Gamma Knife:*** Its core is represented by 192 individual ^{60}Co sources aligned with a collimation system, able to delivery each of the radiation beams to a very precise focal point: as a consequence even very small targets can be treated by a high radiation dosage, whereas peripheral dose levels remain low (*Mamalui-Hunter M, et al. 2013*). GK allows for very complex intracranial SRS treatments, in which accuracy is guaranteed by possible fusion of different imaging source (CT, MRI, Angiography, etc). Moreover, critical structures such as optic pathways and cranial nerves may be shielded through the application of beam blocking patterns that minimize the contribution of putatively dangerous shots.

- ***Linear accelerator radiosurgery:*** Recognized as an effective alternative to GK radiosurgery, particularly in case of larger target volumes, the LINAC option is characterized by an easy patient set-up, and a wide possibility of reaching any irradiation position within the human body (*Rowshanfarzad P, et al. 2013*). Few basic parameters such as, patient couch angle, gantry arc angle, isocenter position, and collimator size are able to provide significant flexibility in the conformal 3D dose distribution for a single isocenter. By using multiple isocenters, and exploiting strategies to increase LINAC accuracy it is also possible to maximize the buffer exposure of the target, and of the adjacent critical structures (*Faught AM, et al. 2013*).
- ***CyberKnife:*** CyberKnife (CK) combines a lightweight 6-MeV LINAC designed for radiosurgery and mounted onto a highly maneuverable robotic arm which can position and point the LINAC (*De Salles AA, et al. 2008*). In the market for 15 years now, CK SRS has shown its advantages including easier fractionation, no need for general anesthesia even in young patients, and flexibility to treat lesions throughout the body. On the other hand the absence of a stereotactic frame and the mobility of the radiation source entail a slightly superior error margin if compared to GK SRS (*Fürweger C, et al. 2010*). In fact whenever the patient moves, internal controls detect the change and stop radiation, before correcting the trajectory of the beam, and start irradiating all over again. Moreover, CK operative programs are essentially based on CT scan imaging recognition; therefore, for peculiar types of targets this might involve a less sophisticated imaging.
- ***Proton Beam:*** High-energy protons represent an extremely important alternative option to photon and electron beams. In fact, proton beams offer the advantage to improve tumor control especially for the treatment of small volumes, where it is necessary to obtain a localized dose distribution in small deep-sited locations, while relative sparing of the surrounding normal tissue. Furthermore, there is no theoretical limit to the size of the target lesion, and charged-particle radiosurgery can be precisely contoured to treat complex shapes. The drawbacks of this techniques include some compromise in spatial accuracy (because instead of a frame attached to the skull, the patient wears an immobilizing plastic mask or bite block in the mouth to achieve fixation) and the need for beam-modifying devices that must be custom-made for each patient, thereby increasing the time and cost of treatments (*DeLaney TF, 2011*). Currently only 20 hadrontherapy facilities are distributed worldwide (half of them being situated in

Europe), and physicist and physicians are still refining the quality of proton treatments from both a dosimetric point of view, and from the transport beam line control.

Deoxyribonucleic acid (DNA) is the principal target for the biological effects of radiation, and chromosomes are the principal target for radiation-induced lethality as ionizing radiations induce single- or double-strand DNA breaks. On a cellular level, cells die while attempting to divide because of damaged chromosomes, while on a tissue level the type of damage depends on the cells' turnover rate, so that for cancer cells in active replication it may become quickly evident. Nonetheless, in addition to inducing mitotic cell death, radiation can also lead to cell death via the apoptotic pathway. Therefore, early or acute effects on a population of cells result from the death of a large number of cells and occur within a few days or weeks of irradiation in tissues with high turnover rates. Late effects appear after a delay of months and years and occur predominantly in slowly proliferating tissues (*Oh BC, et al. 2007*).

The response to radiosurgery may represent more of a continuum than a truly unique response, however conventionally the linear-quadratic model is the one of choice to estimate the survival curve of a given population of cells treated with a prescribed radiation dose and for each individual population of cells this is described by the α/β ratio which governs the shape of the survival curve.

One key principle to understand SRS is that the histological response depends both on the radiation dose and the time elapsed after irradiation. As a general rule, at lower doses of radiation $\leq$ 20 Gy delivered as a single fraction, vascular changes such as thrombosis, fibrinoid necrosis of vessel walls, hyaline degeneration, and hemorrhage predominate in the CNS. These changes generally occur after prolonged latency and are not apparent until 12 to 14 months after treatment. Whereas, at doses $\geq$ 25 Gy, white matter changes, ranging from demyelination to myelomalacia, predominately at 12 months or longer after treatment (*Hopewell JW, et al. 1989; Schultheiss TE, et al. 1995*).

For the biological reasons described above, beside standard single dose radiosurgical treatment (sSRS), fractionation or multisession protocols are emerging as valuable options for treating large lesions in critical brain areas, thus reducing the adverse effects on surrounding structures. Fractionated radiosurgery (fSRS) is particularly advocated for the treatment of primary lesions, such as HGGs and LGGs, as well as large or multiple metastases and vascular malformations; and has also proven to be effective in fragile classes of patients as the pediatric one (*Maranzano E, et al. 2011; Minniti G, et al. 2011; Wegner RE, et al. 2013; Sperduto PW, et al. 2013; Hoffman LM, et al. 2013*).

Study (Year)	*Early Phase Response*		*Late Phase response*	
	Molecular Pathway	**Biologic Response**	**Molecular Pathway**	**Biologic Response**
2-72h time frame				
Prabhakarpandian et al (2001)	Expression of ***ICAM-1***	Induction of ***inflammatory reaction*** involving the migration of ***polymorphonuclear leukocytes*** and other blood formed elements into the irradiated area		
Gaber et al (2003)	Expression of ***ICAM-1***			
Sharp et al (2003)	Expression of ***ICAM-1***, ***ECAMs***, and ***E-Selectin***			
1-6 months time frame				
Hong et al (1995)			Increased ***mRNA*** levels of ***TNFα*** and ***IL1***	Activation of ***tumor-associated macrophages***, induction of ***cell adhesion***, acute and chronic release of ***inflammatory cytokines***, and ***cell death*** into the irradiated area
Chiang et al (1997)			Increased ***mRNA*** levels of ***TNFα***	
Daigle et al (2001)			Increased ***mRNA*** levels of ***TNFα***	
Tsai et al (2007)			Increased levels of ***iNOS***	
Wang et al (2013)			Expression of ***HIF-1***	

Legend: *ECAM*: Endothelial Adhesion Molecule, HIF: Hypoxia Inducible Factor, *ICAM:* Intracellular Adhesion Molecule, *IL*: Interleukin, *iNOS*: Inducible Nitric Oxide Synthase, *mRNA*: Messenger Ribonucleic Acid, *TNF*: Tumor Necrosis Factor

Table 3.1: *Early and late biological effects of radiation*

The rationale behind fSRS is to increase the total dose while improving outcome; however some questions are still open to debate: for instance, the optimal number of fSRS sessions, the marginal isodose (%) and the marginal dose have not been established yet, moreover the exact long-term incidence of adverse effects on the surrounding brain is still to be determined (*Soffietti R, et al. 2013*).

Alongside the common uses of SRS as primary (single treatment strategy) or adjuvant (ancillary treatment for lesion primarily undergoing surgery or chemotherapy) therapeutic option, salvage SRS (for lesions already treated with all existing therapeutic modalities) is becoming increasingly relevant. Among the various available management modalities, salvage SRS emerged along the last decade as the most commonly viable strategy for recurrent or progressive brain tumors previously treated, as they represent a peculiar challenge mainly due to the intrinsic characteristics of those patients and their primary pathology. For instance, many clinical studies of class III evidence have demonstrated satisfying results concerning the local brain control and survival of patients with relapsed brain disease (*Klironomos G, et al. 2013*). Due to the increased life-expectancy in the near future, the number of patients with recurrent brain tumors will certainly

grow, acting as an impetus to redefine treatment strategies in this direction; all the knowledge acquired in this process will certainly find application also in the management of other intracranial vascular or functional lesions.

3.2 Stereotactic Radiosurgery for Cerebral AVMs

SRS is an effective surgical procedure for patients with properly selected intracranial AVMs and is now being used in the management of lesions previously thought to be unsuitable for any other form of therapy (*Sirin S, et al. 2006*).

The ideal goals of radiosurgery in these patients may be briefly summarized as follows: 1) complete obliteration of the AVMs nidus, sparing the neighboring brain tissue 2) to reduce the risk of intracerebral hemorrhage, 3) and neurological preservation.

Structurally and functionally different from normal vessels, those within the nidus of the AVM are of irregular caliber and have segments of thickened walls alternating with thin-walled segments. Although often thickened, the walls of feeding arteries have generally a normal ultrastructure, on the other hand the walls of veins exhibit some artery like characteristics with a fibrohyalinized internal elastic lamina and a unica media consisting of collagen intermingled with smooth muscle cells and elastic fibers (*Wong JH, et al. 2000*).

AVMs undergo a continuous vascular remodeling, partly due to the increased flow and shear stress, and partly due to proper neovascularization processes induced by increased expression of proangiogenic growth factors, including Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF), Transforming Growth Factor alfa (TGFα), and involving upregulation of Nitric Oxid Synthase (*Sonstein WJ, et al. 1996; Kilic T, et al. 2000*).

Endothelial cells are highly sensitive to radiation, which induces a high rate of early endothelial cell death, followed by proliferation of surviving endothelial and smooth muscle cells with progressive luminal narrowing and intraventricular thrombosis.

Cell adhesions molecules, such as E-selectin and Intercellular Adhesion Molecule-1, and prothrombotic factors such as von Willenbrand Factor (vWF) are known to play an important role in those post-SRS changes as their interaction with platelets initiates the intravascular thrombotic process (*Elisevich K, et al. 1994*).

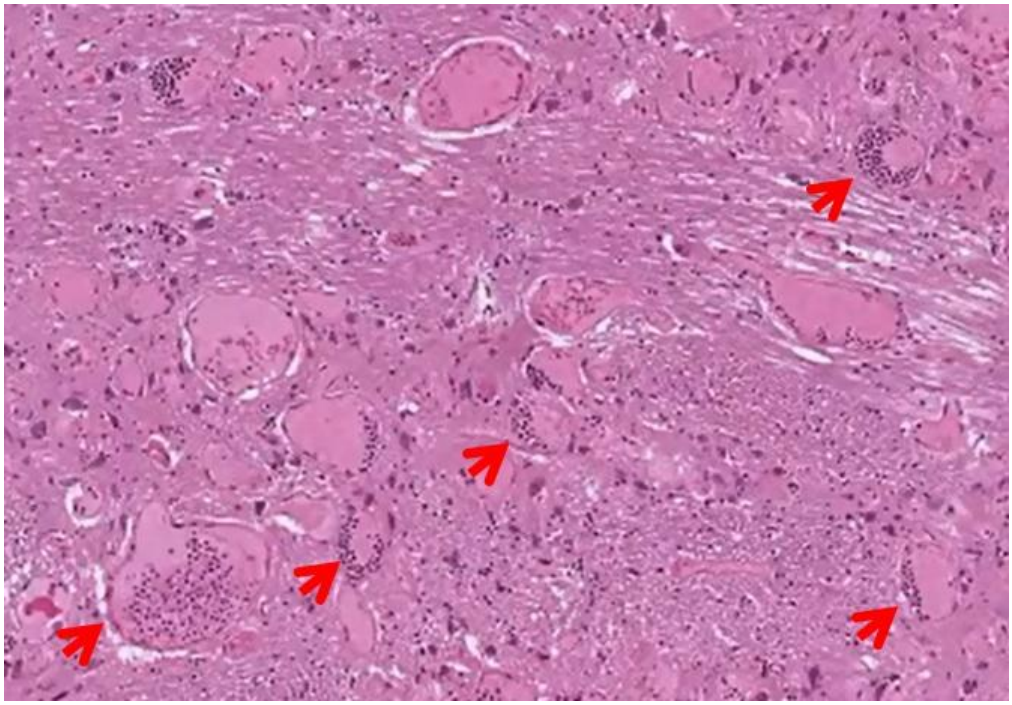

Fig. 3.1: *Microphotograph showing the histological pattern of cerebral AVM (Hematoxylin-Eosin, 200x): note the abundance of abnormal vessels (red arrows) some of which are showing intraluminal thrombosis*

The treatment dose is chosen according to appropriate algorithms, balancing the expected obliteration rate (dose response curve) and the corresponding risk level (dose-complication curve). For small volume AVMs (< 5cc) obliteration rates ranging from 70% to 95% have been documented 3 years after a single procedure. In larger AVMs (volume 5-15cc) results are satisfactory though less spectacular, wth correspondingly higher complication rates (*Flickinger JC, et al. 1996; Pan DH, et al. 2000*).

Furthermore when the original radiosurgical procedure leads to an incomplete response, within the due time, a second treatment may lead to obliteration with an acceptable risk. For all these reasons staged volume SRS has been advocated either for larger or for critically located AVMs (*Sirin S, et al. 2006*).

It is still debated whether radiosurgery leads to a substantial reduction in hemorrhage risk, or not, before complete obliteration occurs Recently Maruyama et al studied this question and concluded that hemorrhage were decreasing over time. Their analysis also showed that hemorrhage was possible, although rare, after seemingly complete angiographic obliteration, due to residual microscopic nidus or perhaps the collapse of the frail granulation tissue of the AVM scar (*Maruyama K, et al. 2005*).

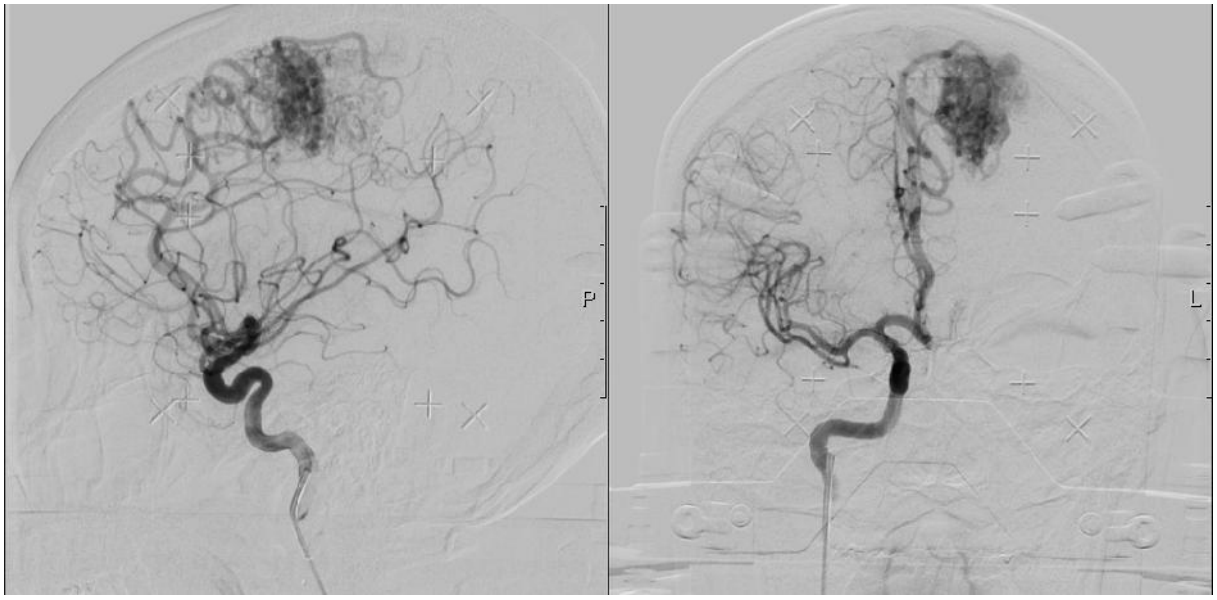

Fig. 3.2: *Angiography performed upon positioning of the stereotactic frame for SRS planning*

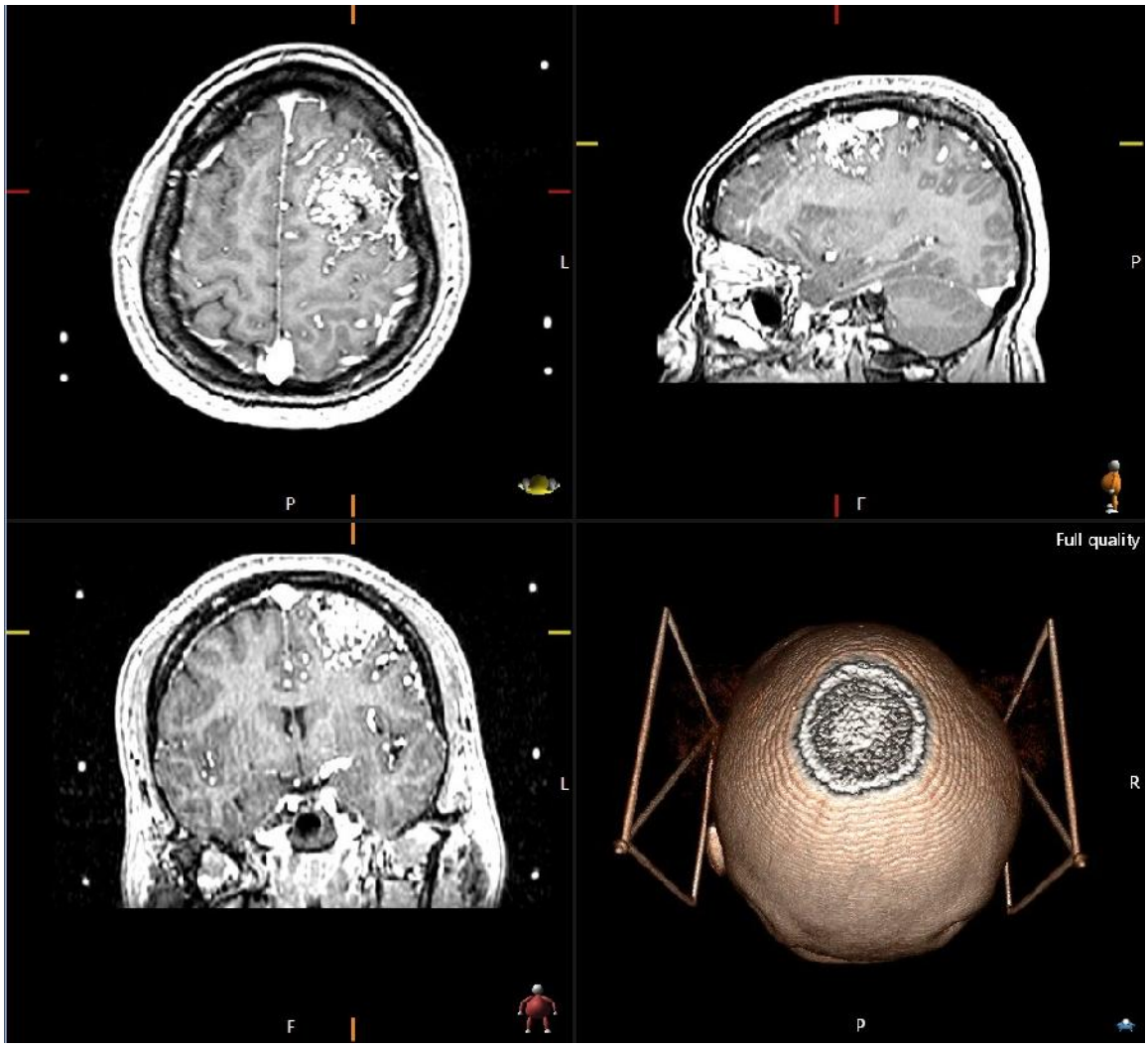

Fig. 3.3: *MRI performed upon positioning of the stereotactic frame for SRS planning*

3.3 Preventing Radionecrosis

Delayed radiation-induced complications remain a significant problem in some patients treated with SRS. The majority of these adverse events remain difficult to predict, but generally occur within 3 years of radiosurgical treatment.

Radiation necrosis may be defined clinically radiologically and pathologically: this clinical entity in fact presents specific imaging characteristics on MRI and PET scans, and often requires conclusive histopathological confirmation on biopsy specimens (*Ganau M, et al. 2012*).

The MRI findings considered typical of radionecrosis include: increased T2 changes, likely representing BBB disruption and accompanying cerebral oedema, central hypointensity in T1 sequences, and contrast enhancement pattern on T1 sequences. (*Korytko T, et al. 2006*).

White matter necrosis, demyelination and vascular changes are prominent in CNS radiation injury, however its primum movens seems to be the disruption of BBB with subsequent increased vascular permeability. This event is likely caused by tissue hypoxia and eventually mediated by increases in VEGF expression by nearby astrocytes, as supported by experimental evidence in vitro and in vivo (*Rumpel H, et al. 1995; Plateel M, et al. 1995*).

The overexpression of VEGF parallels the increased expression of Hypoxia Inducible Factor-1 (HIF-1), a nuclear protein responsible for the transcription of hypoxia-responsive genes (*Marti HJ, et al. 2000*). Further immunohistochemical studies confirmed the increased expression in the BBB of a transmembrane protein, Glucose transport-1 (Glut-1), which may represent an adaptive response in meeting the cellular energy demand in such hypoxic environment (*Nordal RA, et al. 2004*).

Typically, necrosis developing as a result of radiation exposure occurs as a focal process near the previously treated site; radionecrosis presents as a coagulative process predominantly affecting the white matter and causing dramatic perilesional oedema. The pathological events leading to radionecrosis are considered to be centered on small vessels injured by radiation-induced secondary damage, and include vascular hyalinization and thrombus deposition leading to vascular occlusion.

The first line treatment for such condition include steroids, and non-steroidal anti-inflammatory drugs; others therapies include anticoagulants, hyperbaric oxygen, nonetheless failure to respond to conservative management may lead to surgical resection of the lesion causing mass effect on the surrounding brain parenchyma.

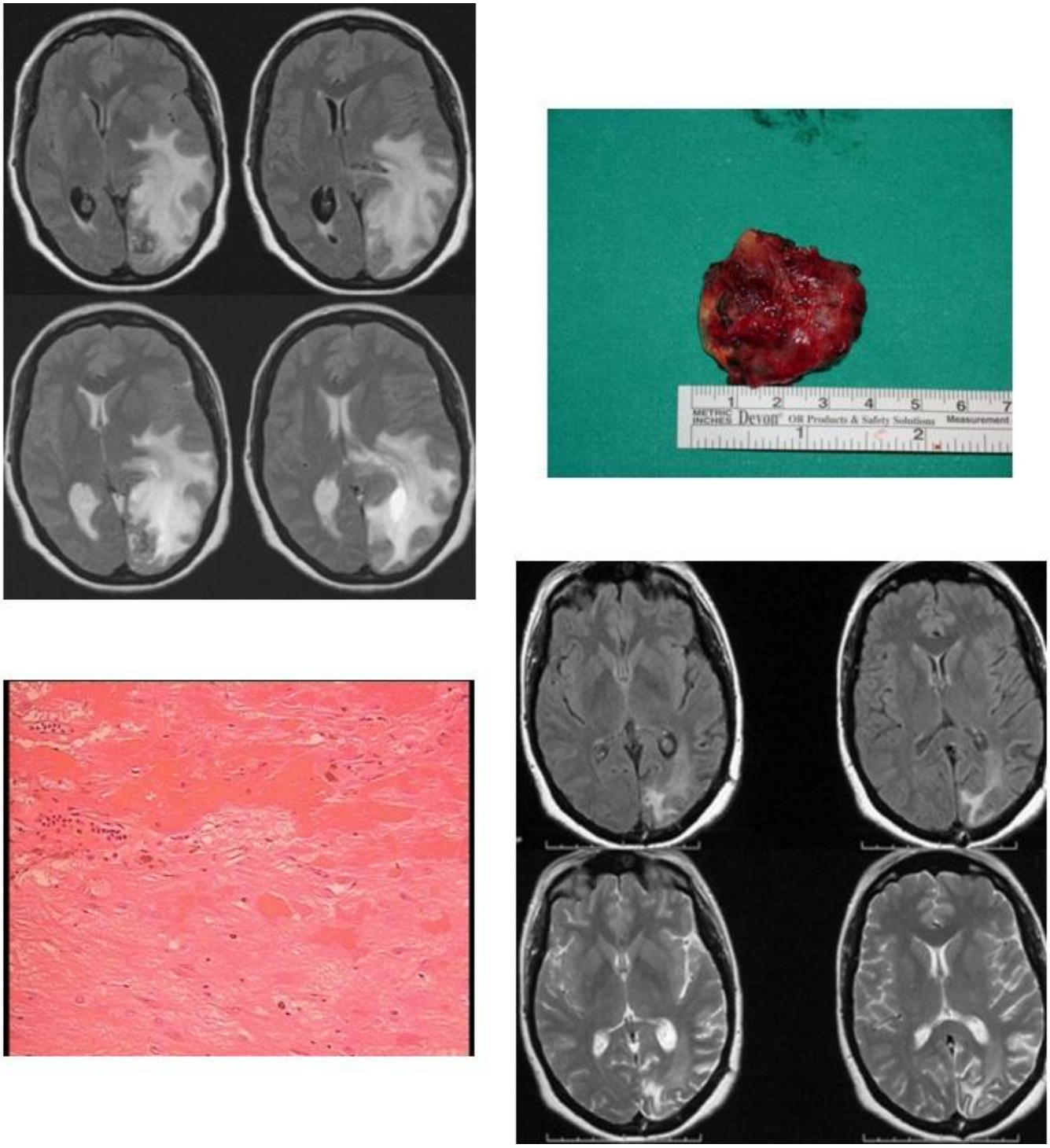

Fig. 3.4: *T1 and T2 MRI head of patient with left occipital radionecrosis 96 months post GK SRS for retrotrigonal AVM, note the radionecrotic nodule surrounded by a dramatic oedema causing compression of the occipital horm of the lateral ventricle and midline shift (above left); a 2.6 cm round shaped radionecrotic nodule is excised with microsurgical technique (above right); microphotograph showing the histological pattern of the radionecrotic nodule (Hematoxylin-Eosin, 200x), note the amorphous material and reactive gliosis (below left); postop T1 and T2 MRI Head at 3 months from surgical excision showing an almost complete resolution of the parenchymal oedema.*

Interestingly the incidence of radionecrosis seems to be higher in patient treated with SRS for vascular lesions in fact as many as 30% of patients present post-SRS imaging changes in the brain surrounding the previously treated AVM. Fortunately, these effects are asymptomatic in two-thirds of affected patients, nonetheless in approximately 9% of them the radionecrosis might become clinically evident and require further medical or surgical treatment (*Finitsis S, et al. 2005; Flickinger JC, et al. 1996*).

The risk of radionecrosis certainly depends on the treatment volume and, to a lesser extent, the dose administered; however the poor understanding of the molecular and cellular responses involved in its pathogenesis has so far prevented from identifying possible risk factors, or events triggering its delayed occurrence, and consequently the design of effective neuroprotective strategies. This said, the availability of more sophisticated techniques has recently created a valuable opportunity to improve SRS protocols so that larger lesions can potentially be treated, while reducing the latency period to obliteration and preserving the adjacent healthy parenchyma.

In light of the above, the specific aim of this section is to investigate how the precision and safety of SRS GK treatments can be further increased by introducing advanced fusion protocols in the planning phase. Specifically, experimental evidence from our clinical series at the University of Verona will serve to support the hypothesis that those neuroimaging fusion protocol could reduce the unintended radiation on cortical tracts adjacent to deep sited AVMs, and prevent post-SRS neurological deficits and radiation induced necrosis.

EXPERIMENTAL WORK

3.4 Fusion Protocols to Preserve Eloquent Areas

In-depth study of the anatomy of any intracranial pathology is a critical component of neurosurgical planning: from this initial diagnostic step every advantage and disadvantage of available management options, from a microsurgical approach to a radiosurgical or endovascular one, or again a combination of all of them, is thoroughly evaluated before a final decision is taken.

As every imaging modality has its pros and cons, imaging fusion protocols have recently gained significant attention as they provide the clinician with a post-processing set of images that

allow to simultaneously leverage on the qualities of each single imaging source merged. Actually, image fusion is the process of using rotation and translation to bring a second image set into alignment with the first image set. This allows the potential concurrent use of multiple image sets to define the target and stereotactic space. While a single MRI sequence alone can be used for delineation of the target, there may be significant advantages to using additional conventional imaging sets (including other MRI sequences and CT scans) or advanced imaging sets (such as catheter-based DSA, positon emission tomography (PET) or DTI-based fiber tracking) in order to more accurately define the surrounding critical structures. Stereotactic space is usually defined by detection of fiducials on the stereotactic head frame (for framed SRS) or mask system (for frameless SRS).

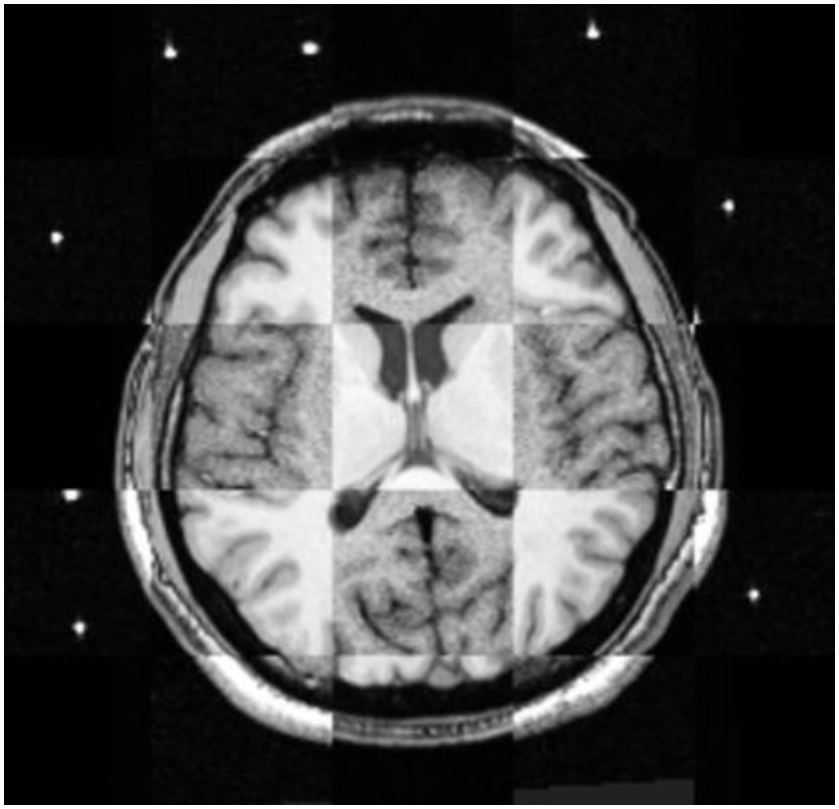

Fig. 3.5: *Overlapping of stereotactically acquired MRI/CT imaging sets*

Noteworthy, while CT scans, despite their poorer resolution of the target, are not susceptible to geometric distortion, MRI sequences do face this problem. Improvements in neuroradiology and experimental virtual simulations for surgery/SRS are partly overcoming the risk of error introduced by inaccuracies of the fusion process, as well as the risk of image changes along the entire fusion workflow process, that if not properly accounted for can mislead the treating clinician (*Sboarina A, et al. 2010*).

When a neoplastic or vascular lesion is deep sited or is close to eloquent brain areas, or its relevant anatomical region is perfused by two or more vascular territories or demonstrate a complex venous drainage each selective imaging modality may provide an incomplete anatomical picture.

Since its introduction, digital subtraction angiography (DSA) has been considered the gold standard in diagnostic imaging for neurovascular disease (*Jalali A, et al. 2015*). Nonetheless several studies have recently demonstrated that bone-subtraction CT Angiography (CTA) is as accurate as DSA in detecting cerebral aneurysms after SAH. In fact, CTA provides similar information about aneurysm configuration and measures, and reduces the average effective radiation dose for vascular diagnostics by 65%. For this diagnostic equivalence in association with dose reduction, some centers are considering to replace DSA with bone-subtraction CTA in the standard diagnostic work-up of spontaneous SAH (*Aulbach P, et al. 2015*).

On the other hand, only MRI can provide meaningful information regarding the surrounding brain parenchyma, and diffuse tensor imaging (DTI) sequences can add further insights on the white matter tracts in proximity to the vascular lesion. In fact, hemorrhagic lesions can disrupt and displace perilesional white matter tracts with the latter occurring in unpredictable directions. To this regard, it has been well described that observed anisotropy decreases in the perilesional segments and this is consistent with neural injury following hemorrhagic insults (*Faraji AH, et al. 2015*).

Those considerations require the use of tractography to accurately define tracts' orientation to optimize surgical entry point, minimize morbidity, and enhance neurological outcomes. As modern post-processing techniques have made the fusion of multiple independently acquired imaging (DSA, CTA, MRI, etc) even more informative to the cerebrovascular neurosurgeon or neurointerventionalist, it seems appropriate to include them in the armamentarium of a comprehensive cerebrovascular neurosurgeon.

3.4 a): AVM in Close Proximity to Corticospinal Tract

Even if GK SRS is considered a non-invasive procedure for deep-sited cerebral AVMs, the rate of radiation related complications for SRS is not negligible, especially for lesions located in critical sites (incidence: 3-18%).

This is often a consequence of: 1) a difficult distinction between eloquent and non-eloquent areas of white matter (which is still based on knowledge of anatomy), 2) presence of anatomical variants, 3) pathological distortion of conventional landmarks.

Herein we report the protocol developed in the Neurosurgical Department at the University of Verona for GK SRS treatment of every AVM in close proximity to corticospinal tract (CST).

All cases treated at our GK centre, with both the 4C model and starting from 2008 even with the Perfexion model, underwent a 3 Tesla MRI scan in a non stereotactic fashion one day before the radiosurgical treatment, herein we report the first 15 cases for which a clinical follow up > 5 years is available.

Pt	Gender /Age	Clinical Presentation	Previous Treatements	Localization	S&M Grade	Neurol. Deficits	Volume (cc)	IE (%)	DS (Gy)
Demographics		Medical History		Type of AVM and Clinical Status			Treatment details		
1	F/37	Haemorrhage	Partial Embolization	Thalamo-Mesenceph	3	Nil	1.0	50.0	16.0
2	F/20	Haemorrhage	None	Basal Ganglia	3	Nil	1.5	50.0	16.0
3	M/24	Haemorrhage	None	Thalamo-Mesenceph	3	Nil	8.3	50.0	14.0
4	F/15	Haemorrhage	Surgical	Subcortical	2	Hemi-paresis	2.4	50.0	16.0
5	F/64	Haemorrhage	None	Thalamo-Mesenceph	4	Nil	38.7	50.0	10.0
6	F/34	Seizures	None	Subcortical	2	Nil	4.3	50.0	20.0
7	F/17	Haemorrhage	None	Thalamo-Mesenceph	3	Nil	8.8	50.0	15.0
8	M/18	Cephalalgia	Partial Embolization	Subcortical	3	Nil	21.4	50.0	14.0
9	M/50	Haemorrhage	None	Subcortical	2	Nil	2.6	50.0	17.0
10	M/34	Cephalalgia	None	Basal Ganglia	2	Nil	0.35	60.0	21.0
11	M/14	Haemorrhage	None	Basal Ganglia	3	Hemi-paresis	4.8	50.0	20.0
12	F/25	Haemorrhage	None	Basal Ganglia	3	Nil	6.6	50.0	15.0
13	F/21	Haemorrhage	Partial Embolization	Basal Ganglia	3	Hemi-paresis	11.1	50.0	13.0
14	F/36	Seizures	None	Subcortical	2	Nil	5.9	50.0	17.0
15	F/34	Haemorrhage	Partial Embolization	Basal Ganglia	2	Hemi-paresis	1.8	50.0	13.0

Legend: *Pt*: patient, *S&M*: Spetzler-Martin grade, *IE*: isodose, *DS*: prescribed dose, *M*: male, *F*: female

Table 3.2: *Clinical and radiological data pertaining to the patients included in the study*

The patients herein described presented with symptomatic cerebral AVMs in close proximity to the CST (6 cases located in basal ganglia, 5 in motor cortex, 4 in thalamo-mesencephalic region).

The Spetzler-Martin (S&M) Grade of those AVMs ranged from 2 to 4, although the most common S&M Grade encountered was 3, representing 59% of the lesions treated. Patient

characteristics were: young age (mean 24 years), generally with haemorrhagic onset (69% of cases) and usually without neurological deficits at admission (73% of cases). AVM volume ranged from 1.0 to 38.7 cc.

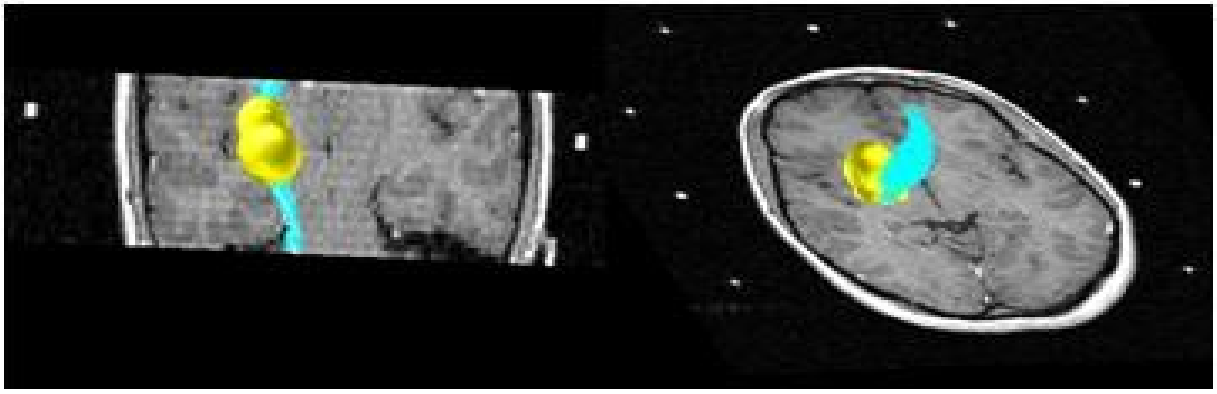

Fig. 3.6: *Postprocessing reconstruction of a basal ganglia AVM (yellow) in close proximity to the CST (light blue)*

3.4 b): Treatment Planning and Clinical Outcome

To preoperatively define the relationship of deep-sited AVMs to the perilesional corticospinal tracts (CSTs) and obtain qualitative and quantitative data for radiosurgical planning we created a stereotactic protocol providing high-definition anatomy of both the vascular lesion and fiber tracts.

The fusion protocol used in those cases can be summarized as follows:

- the acquired images were processed to calculate voxel based fractional anisotropy and to define fiber tracts bilaterally with a 3 Region of Interest (ROI) approach, including the cerebral cortex, the posterior limb of the internal capsule and the cerebral peduncle.
- the day of GK treatment the 3D corticospinal tractography was co-registered and embedded into the 3D conventional MRI volumetric study performed upon positioning of the stereotactic frame.
- the accuracy of the co-registration process was assessed during planning for SRS treatment using the Gamma Plan software, and the position of the CST was always confirmed with the abovementioned 3 ROI.
- to resolve regions of fiber crossing in those patients with subcortical AVMs different variants of the Spherical Deconvolution algorithm were exploited (the constrained and the Richardson Lewis methods).

- two SRS plans were obtained for each patient: one taking into account the 3D conventional MRI only, and the other including the 3T DTI into the fusion protocol.

The greatest advantage of the fusion protocol was to allow for a better definition of the isodose line, perfectly shaping the treatment area of the AVMs, while sparing the adjacent white matter.

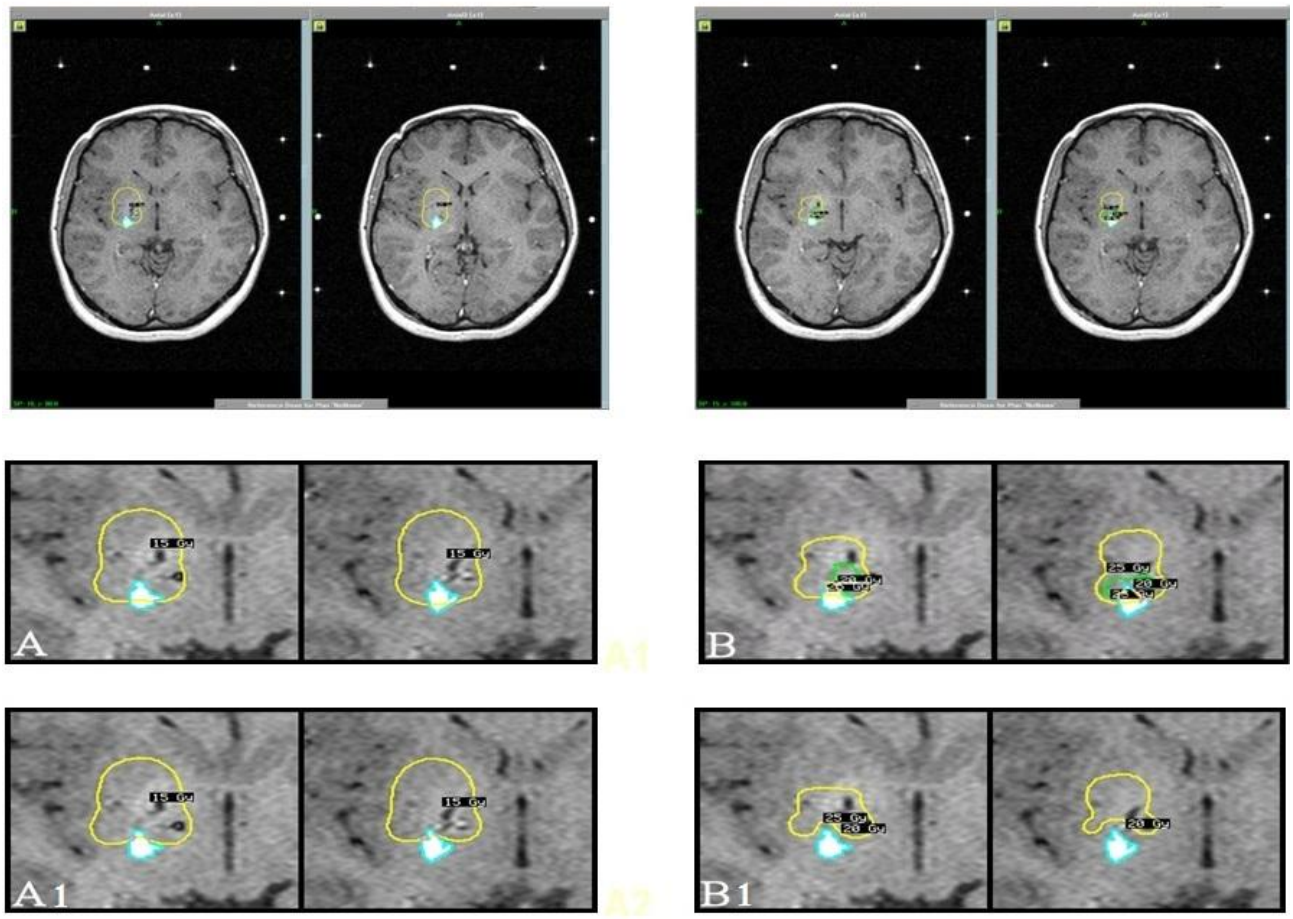

Fig. 3.7: *Exemplificative case showing how remodelling the isocenters in A1 and B1according to insights provided by this fusion protocol allows to significantly reduce the CST radiation exposure even without modifying the prescription dose*

DTI based knowledge of CST gave us the chance to modify the radiosurgical planning and reduce the radiation exposure of CST, realizing a patient tailored treatment. By applying this fusion protocol we managed in all patients to keep the mean maximum dose to the reconstructed CST ≤ 20.5 Gy. Furthermore, dose-volume histograms of the CST revealed that in our cohort the mean volume of the CST receiving 20 Gy was 85 mm^3 while the mean volume receiving 25 Gy was only 23 mm^3. At a long clinical follow up (>5 years in all cases) no new motor deficits have been

observed in this cohort, whereas the AVMs showed the expected radiological involution within 3 years from treatment.

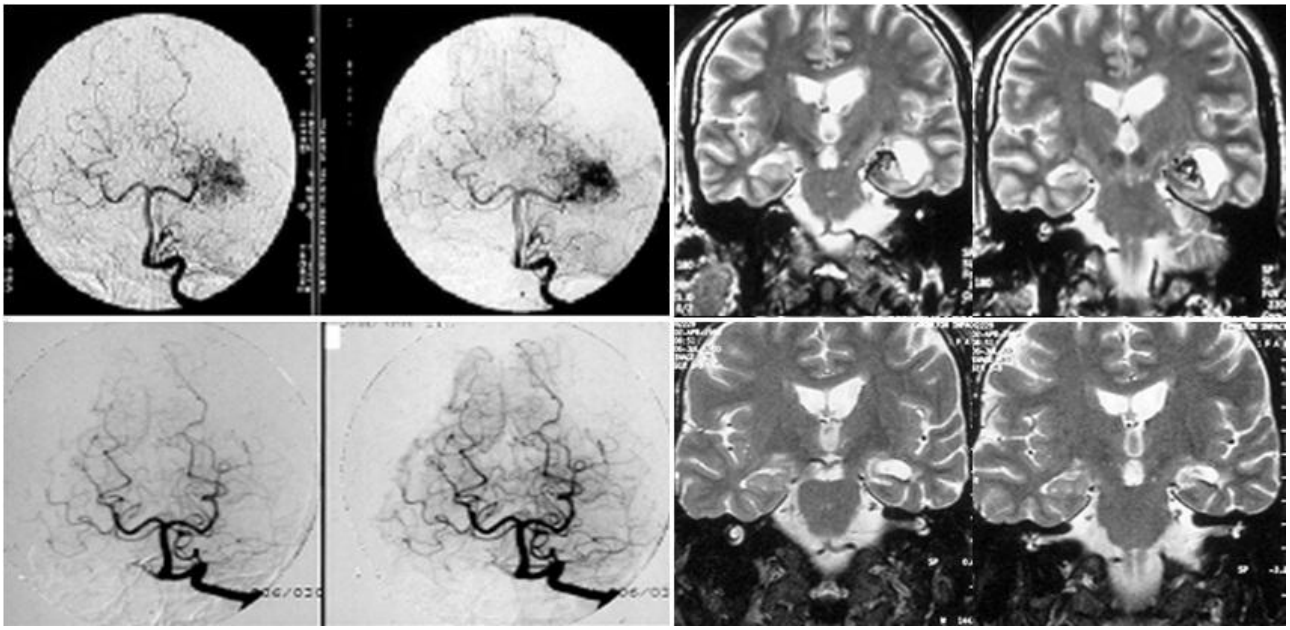

Fig. 3.8: *Left silvian AVM. Angiographic pictures before (left top) and after (left bottom) Gamma Knife treatment. T2 weighted sequences before (right top) and after (left bottom) obliteration clearly show that AVM disappearance is not associated to any macroscopic damage to the surrounding brain tissue.*

Given the increasing availability of the relevant image acquisition and processing technologies, this novel strategy of combining multiple independently acquired cerebral imaging to create a more accurate representation of the anatomical distortion associated with deep sited AVMs has therefore demonstrated to be enough feasible and accurate to be proposed as a valuable adjunct not only in cerebrovascular cases but also in every other stereotactic procedure.

DISCUSSION AND FUTURE DEVELOPMENTS

3.5 Balancing Safety and Efficacy

Integration of 3D tractography of CST within GK surgery is feasible and effective in AVM treatment, and in the near future will show its usefulness even for infiltrating lesions such as LGG

or metastasis. The theoretical power of this approach in many clinical situations is such that 3D reconstructions could be integrated with neurosurgical navigation systems, with the purpose of pre-surgical planning and intraoperative navigation.

DTI produces high-quality white matter images, with high spatial resolution and exquisite anatomical contrast not available from other MRI modalities, providing information about the course, the displacement, or interruption of white matter tracts around a tumor, as well as a widening of fiber bundles due to edema or tumor infiltration.

Indeed implementation of fiber tracking, extended also to optic radiation and fasciculus arcuatus, will enhance the safety of much more treatments; and a growing number of patients will benefit from the ongoing development of this technique.

This study confirmed that although a CST could be "disrupted" and/or "displaced" by the vascular malformation, a qualitative and quantitative analysis (focusing on the anatomical appearance and anisotropy pattern) is always possible and useful. The anisotropy analysis of the perilesional and nonperilesional segments of the CST close to a given lesion, and the comparison with the controlateral one, allows determination of the number of fibers in close proximity to the AVM studied and consequently optimization of the SRS treatment.

DTI is widely used to extract fibre directions, but fails in regions containing multiple fibre orientations (such as in the 5 cases of subcortical AVM included in our series). One of the most successful approaches to solve the fiber crossing issue is spherical deconvolution, which can dramatically improve tractographic results without excessive increase in computational timing and power (*Tournier JD, et al. 2007*).

Spherical deconvolution is useful in improving tractographic reconstruction of the lateral portion of the CST, corresponding to the somatotopic representation of hand, face, tongue, and voluntary swallowing muscles (*Calamante F, et al. 2011*). This portion of the CST is hardly detectable by DTI-based approaches only and the approach used proved robust to noise whilst preserving angular resolution.

One limitation has been however identified: the presence of haemosiderin in those patients with previous bleedings, resulting in artifacts limiting the quality of the MRI and therefore affecting the accuracy of the DTI reconstruction of the CST. As the haemorrhagic presentation is the commonest for patients harboring cerebral AVM, this needs to be seriously taken into account.

Certainly the prospective validation of this fusison protocol will improve the:

- Assessment of the influence of haemorrhagic presentation on fiber tracks visualization, and of the correlation with neurologic symptoms,
- Definition of optimal trackability threshold of fractional anisotropy,

- Definition of the threshold dose of fiber tracts,
- Evaluation of long term fiber reorganization,
- Development of models predicting treatment related complications.

Interestingly, no cases of post-SRS radiological changes have been recorded to far, out of an expected risk ranging between 30% and 10%. The leading hypothesis is that the initial pathological condition favoring radionecrosis is the local coexistence of SRS induced vascular damage and innate tendency to pathological vascularization.

This could be theoretically prevented by a fine modification of the treatment plan ensuring that the radiation is delivery only to the target with a careful sparing of the surrounding healthy parenchyma.

As such, we can assume that the optimazed conformational irradiation allowed by the fusion protocol described could increase the chances to provide a safer treatment to patients harboring intracerebral AVMs. Furthermore, the immediate externality of this study is the translation of the approach described to SRS and surgical planning for any other lesion deep sited or close to eloquent areas.

Chapter IV:

Non-Invasive Monitoring of the Intracranial Pressure

Research presented at:
World Conference of International Brain Injury Association 2016, The Hague, The Netherlands

Most Relevant Publication:

Ganau M, Mirtuono P, Prisco L, Lombardo A, Weinberg G, Papyan S, Ricciardi GK, Foroni RI, Gerosa M, Pinna G. Intracranial pressure monitoring versus a non-invasive recording method in a neurointensive care setting. J. Crit. Care 6 (28): e47; 2013

Recognition:
Keystone Symposia Future of Science Fund Scholarship 2016

OVERVIEW AND RESEARCH QUESTION

4.1 Traumatic Brain Injury and Secondary Damage

TBI is characterized by both a primary and a secondary insult; the first is immediately consequent to the trauma, the second is a consequence of both intracranial and systemic impairment, which are highly correlated with mortality (*Prisco L, et al.. 2012*).

Whereas primary injuries cannot be reversed, the goals of medical and surgical treatment are directed toward the principles of full damage control and are meant to prevent any further secondary injuries to the brain. These start minutes to hours after the TBI and usually last several days. As such, the physical and biological events occurring at cellular and compartmental levels as a result of TBI are extremely complex, and justify the broad spectrum of possible outcomes. The first component of post-traumatic events comprises the activation of resident glial cells, microglia, and astrocytes, and the infiltration of blood leukocytes; the second component regards the secretion of immune mediators, including pro-inflammatory and anti-inflammatory cytokines, as well as chemokines, which specifically drive the accumulation of parenchymal and peripheral immune cells in the injured brain region (*Woodcock T, et al. 2013*).

Cascade of Secondary Events		Mechanisms	Mediators	Monitoring
Early phase	Oedema	Brain swelling	CO_2	↑ICP
	Metabolic Changes	Altered energy demand with cellular hypoxia	ATP synthase	↓pH, ↑glycemia ↑lactate/piruvate ratio
	Axonal Injury	Mechanical shear/stretch with axonal degeneration	Glutamate, Ca^{++}, Cyt c	Serum/CSF levels of ↑APP, ↑MBP
Late Phase	Inflammation	Vasogenic and cytotoxic events	IL1, IL6, IL8, IL10, TNFα, IFNγ	Serum/CSF levels of IL2, IL6, IL10, TNFα
	Vasospasm	Dysregulation of vascular reactivity	HIF-1	↓CBF, ↓CPP, ↑PRx
Terminal Events	Neuronal and Glial Cell Death	Activation of factors triggering apoptosis and necrosis	TNFα, Bcl-2/Bcl-xl/Bax, CASP3, CASP 7,	Serum/CSF levels of ↑S100, ↑NSE, ↑GFAP,

Legend: ***ATP***: adenosine triphosphate, ***Cyt-c***: cytochrome complex, ***CSF***: cerebro-spinal fluid, ***APP***: amyloid precursor protein, ***MBP***: myelin basic protein, ***IL***: Interleukin family, ***TNF***: tumour necrosis factor, ***IFN***: interferon, ***HIF***: hypoxia inducible factor, ***CBF***: cerebral blood flow, ***CPP***: cerebral perfusion pressure, ***PRx***: cerebrovascular reactivity, ***NSE***: neuron specific enolase, ***Bcl-2/Bcl-xl/Bax***: apoptosis regulator proteins of B cell lymphoma family, ***CASP***: cysteine-aspartic proteases, ***GFAP***: glial fibrillary acidic protein

Table 4.1: *Overview of mechanisms responsible for secondary inuries in TBI.*

This cascade of secondary events leads to potentially irreversible damage to neurons that were unharmed in the primary injury. The early phases are characterized by changes in the blood flow to the brain, which might be caused by hypovolemic shock and hypotension, often affecting polytraumatized patients, by the presence of space occupying lesions (such as subdural, extradural or intraparenchymal haematomas) causing brain herniations, or again by the occurence of diffuse cerebral oedema. Whatever the case, the resulting cerebral hypoxia induces numerous intracellular mechanisms attempting to overcome the increased metabolic demand. Inflammation is the direct consequence of this metabolic inbalance, it can often lead to episodes of vasospasm exacerbating the decrease in cerebral blood flow (CBF), and therefore the CPP, rapidly inducing the activation of apoptotic pathways causing neuronal and glial cell death (*Liang D, et al. 2013*).

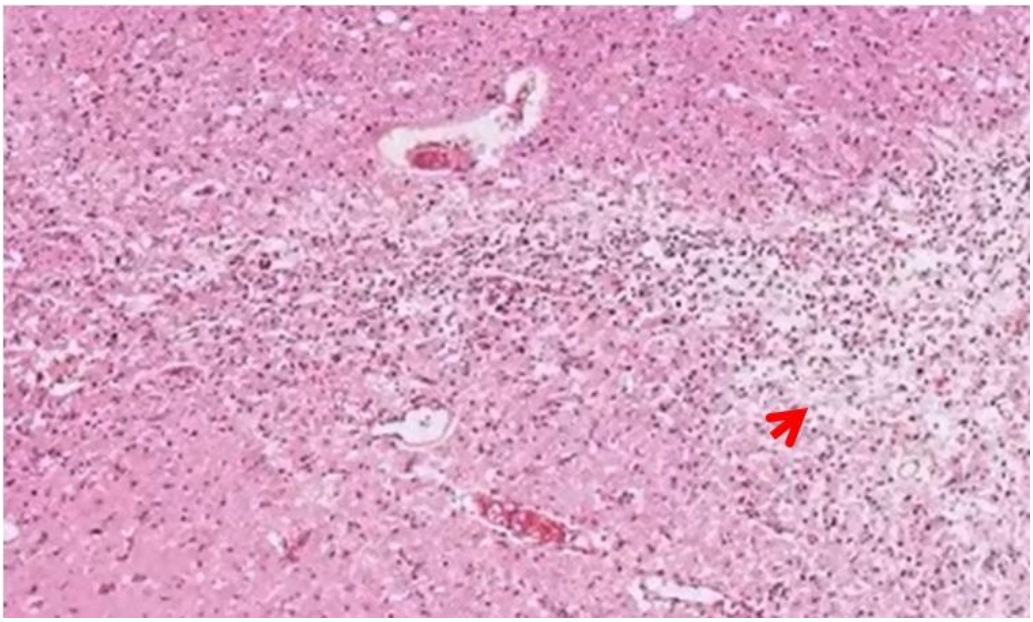

Fig. 4.1: *Microphotograph showing the histological pattern of TBI (Hematoxylin-Eosin, 100x): note the oedema disrupting the parenchymal architecture and the early stages of demyelinisation (red arrows)*

The predictive role of systemic secondary insults has been already confirmed in many studies, but only a few of them considered more than three factors all together. Hypocapnia is the most studied one, followed by hypotension, acidosis, hypoxia and hyperglycemia (*Jeremitsky E, et al. 2003*). Severe hypocapnia and hypercapnia reflect aggressive early management of breathing and both worsen outcome by impairing CBF in a significant way. Unsurprisingly, there is a strong correlation between early hypotension, hypoxia and mortality (*Davis DP, et al. 2006; Chesnut RM, et al. 1993; Manley G, et al. 2001; Chi JH, et al. 2006*). Acidosis and hyperglycemia also play an important role in predicting the outcome as both represent the response to increased metabolic

demand; often this excessive neuroendocrine response to stress mediated by counter-regulatory hormones can ultimately activate irreversible mechanisms of neuronal damage (*Rutherford EJ, et al. 1992; Young B, et al. 1989; Vogelzang M, et al. 2006; Laird AM, et al. 2004*)**.**

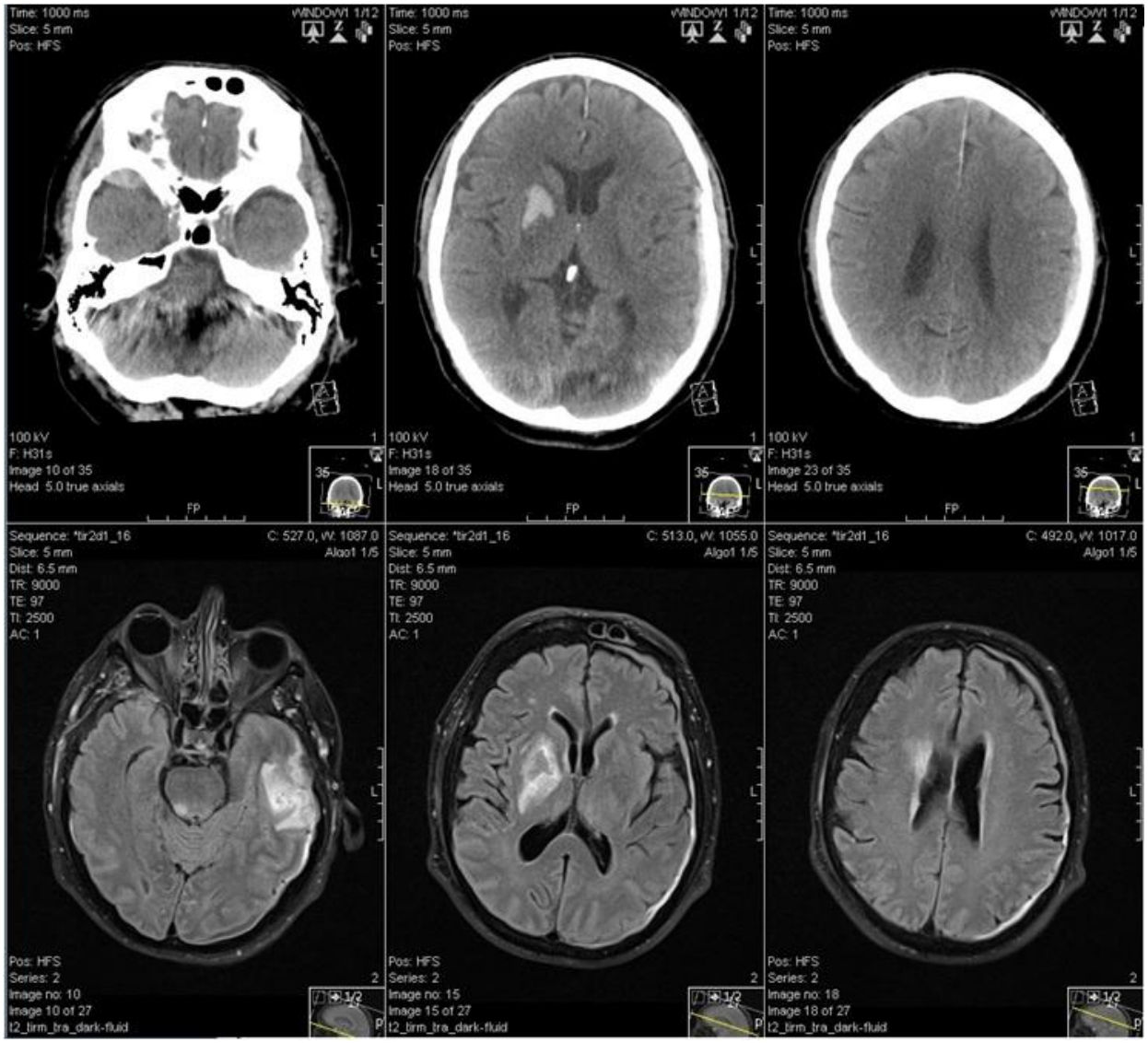

Fig. 4.2: *CT (above) and MRI (below) scans of a TBI patient: note the diffuse contusions in right basal ganglia, right portion of and brainstem and left temporal lobe; as well as the right temporal pole acute extradural haematoma and left convexity shallow acute subdural haematoma.*

Preventing the event briefly mentioned above is therefore the main target of TBI management: to this extent, the increase in ICP, which in the absence of an haemorrhagic space occupying lesion is typically caused by rapidly occurring cerebral oedema, represents the most important single source of information to monitor the cascade of secondary insults.

Based on the Monroe and Kelly doctrine on pressure-volume relationship, at the beginning of 19th century the first attempts were made to use spinal measurements of CSF pressure to

indirectly calculate ICP. Normal ICP varies with age and body pressure but is generally considered to range between 5 to 15 mmHg in healthy adults in the supine position, and between 3 to 7 and 1.5 to 6 in children and infants respectively (*Gilland O, et al. 1974*). Later on, during the mid of last century, the first reports were published on the use of invasive continuous ICP monitoring, leading to a significant progress in the diagnosis of multiple neurological conditions: namely hydrocephalus, subarachnoid haemorrhage, and intracranial haematomas (*Guillaume K, et al. 1951*).

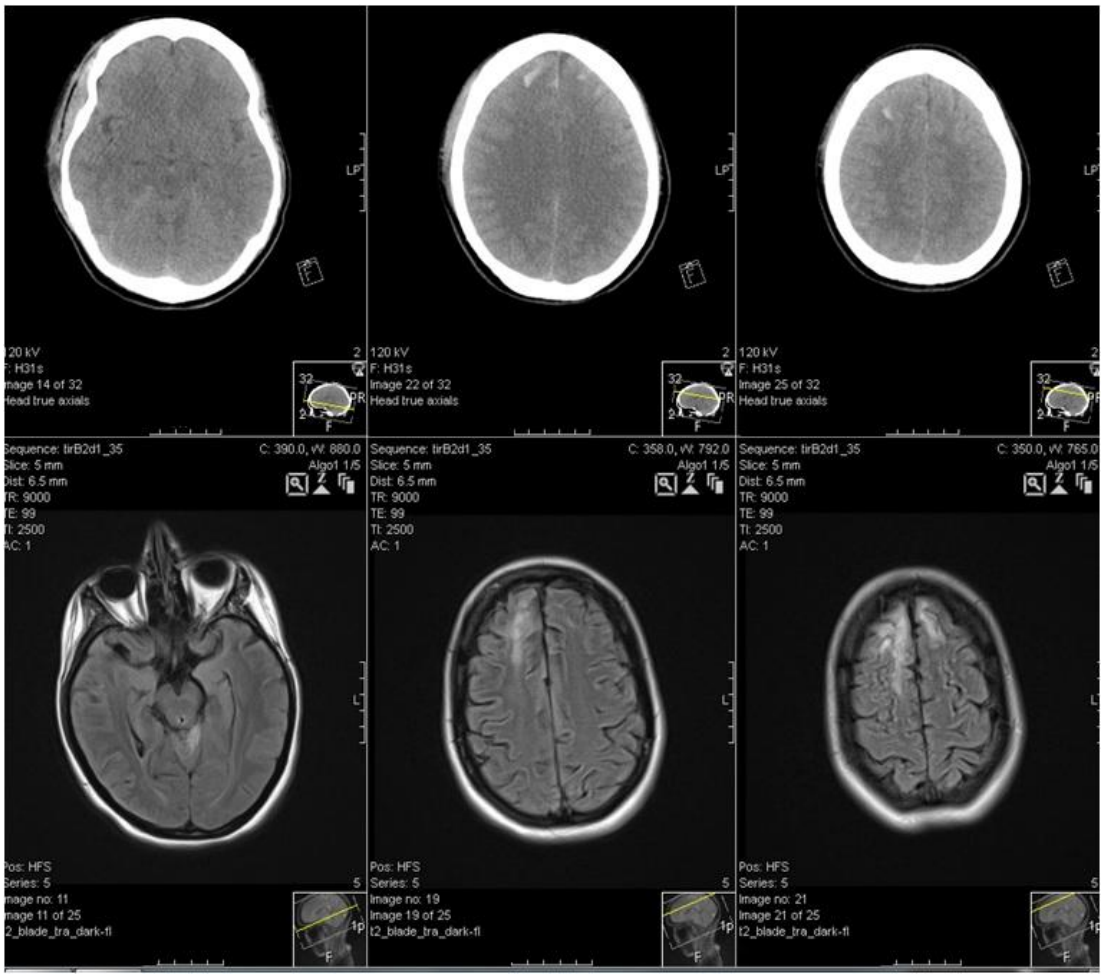

Fig. 4.3: *Early CT (above) and 1 month follow up T2 weighted MRI (below) scans of a TBI patient: note the initial diffuse brain oedema, and the evolution over time of the bilateral frontal subcortical petechial haemorrhages.*

Although few data regarding the monitoring of ICP are available from randomized controlled trials, such monitoring is currently recommended by clinical practice guidelines of the most important international societies (i.e. *Brain Trauma Foundation; UK National Institute for Health and Care Excellence; American Association of Neurosurgical Surgeons*). ICP monitoring is indicated in all TBI with GCS between 3 and 8 and an abnormal CT scan (showing haematomas,

contusions, brain swelling, brain herniation or compression of basal cisterns), whereas in patients with GCS ≤8 but a normal CT scan ICP monitoring is indicated if two or more of the following conditions are present: age >40 years, systolic blood pressure under 90 mmHg, uni- or bilateral motor posturing (*Bratton SL, et al. 2007*).

ICP is used to guide the medical and surgical management of TBI patients, and in order to achieve a better understanding of the secondary events is often coupled to other methods to monitor cerebral oxygen metabolism. In fact, in the setting of severe TBI, given the complexity of cerebral autoregulation, measurements of the cerebral metabolic rate of oxygen and oxygen extraction fraction are often necessary to make the distinction between ischaemia and hyperaemia on one hand, and metabolically coupled hypoperfusion or hyperperfusion on the other. To facilitate the assessment of CBF autoregulation the pressure reactivity index (PRx) was introduced, and starting from 1997 computer-aided approaches became available to calculate and continuously monitor it at the bedside (*Steiner LA, et al. 2003; Zweifel C, et al. 2008; Lee JK, et al. 2009*).

First-tier therapies used to control ICP range from the use of hypertonic saline solutions to treat brain oedema, or the evacuation of the haemorrhagic post-traumatic space occupying lesion or eventually the removal of a piece of the skull in the form of a large decompressive craniectomy (DC) to prevent brainstem compression caused by malignant swelling of the brain parenchyma.

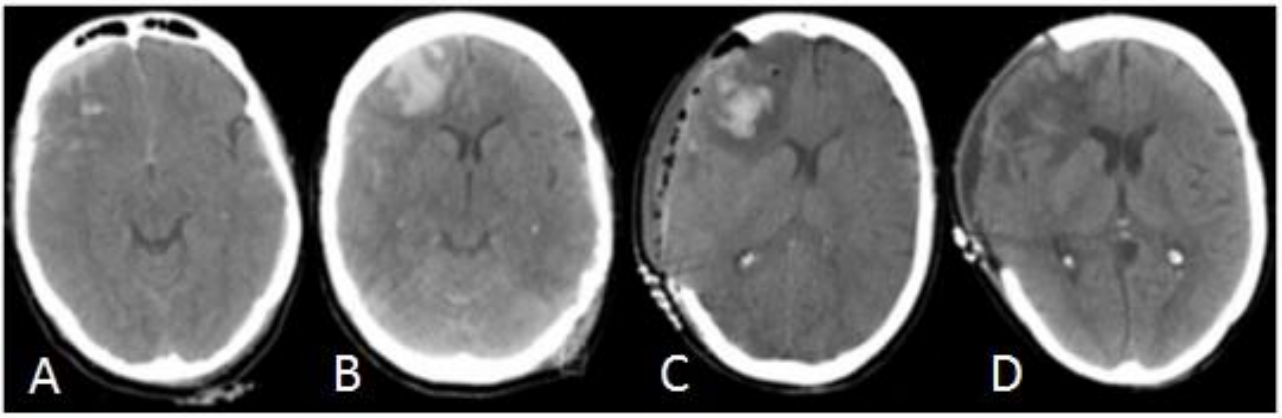

Fig. 4.4: *CT scans showing the progressive evolution of a right frontal subdural haematoma with underlying frontopolar contusion causing midline shift (A and B). The right hemispheric decompressive craniectomy allowed the patient to tolerate cerebral oedema and prevented uncal herniation from compressing the brainstem (C and D).*

The use of DC has been advocated in patients with severe diffuse TBI and increased ICP refractory to first-tier therapies. The DECRA (Decompressive Craniectomy in Patients with Severe Traumatic Brain Injury) study compared patients who underwent early DC for diffuse TBI with patients who received standard medical therapy: 70% in the craniectomy group had an unfavourable

outcome versus 51% in the standard care group. Based on these results, the authors concluded that, as compared with standard care, DC decreased the mean ICP and the duration of both ventilatory support and the Intensive Care Unit stay, but was associated with a significantly worse outcome at 6 months, as measured by the score on the Extended Glasgow Outcome Scale (*Cooper DJ, et al. 2011*).

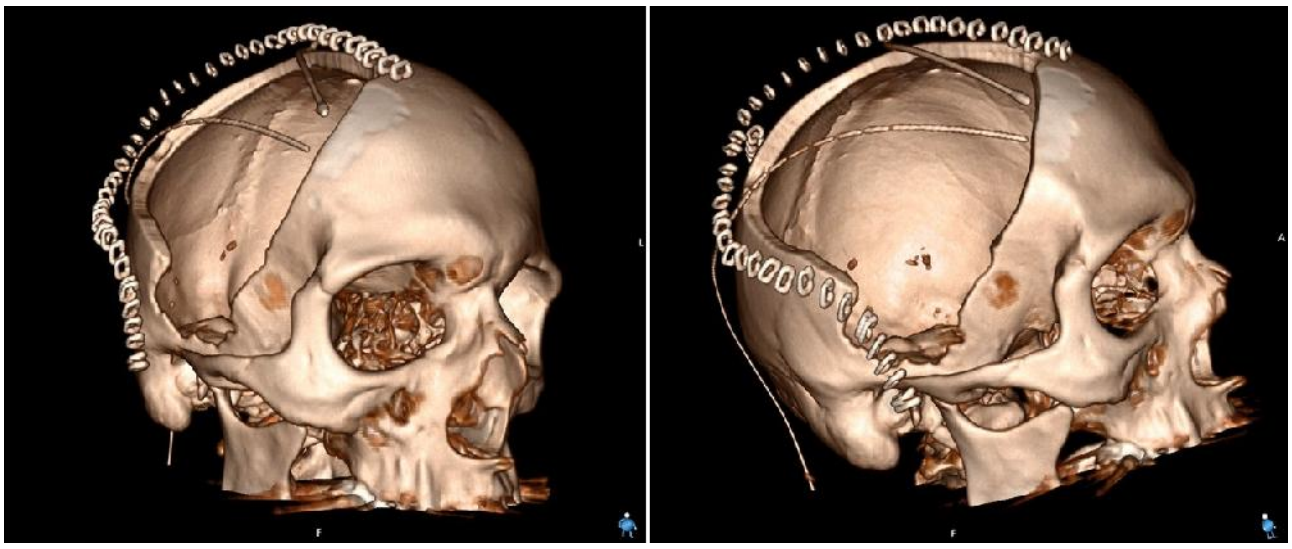

Fig. 4.5: *3D CT scans showing the extent of calvarial defect resulting from a right hemispheric DC. Note the ICP bolt and transducer left in place to guide the medical treatment.*

Nonetheless, the DECRA trial has received a great deal of criticism because of problems with randomization and inferability of those recommendations in the clinical practice, and to date the role of DC when ICP continues to increase ≥20 mmHg remains to be established. The international scientific community's glimmers of hopes to address this issue are now put on the RESCUEicp (Randomised Evaluation of Surgery with Craniectomy for Uncontrollable Elevation of Intra-Cranial Pressure) study, which recently concluded its randomization and enrolment phase.

4.2 Clinical Considerations on Intracranial Pressure

Given its accuracy and reliability, in current clinical practice the continuous invasive ICP monitoring (I-ICPM), using an intracranial catheter connected to or integrated with a pressure transducer, has become an important component of most of the patients requiring neurointensive

care management. Depending on the technique, I-ICPM can be undertaken in different intracranial anatomical localizations: intraventricular, intraparenchymal, epidural, subdural and subarachnoidal.

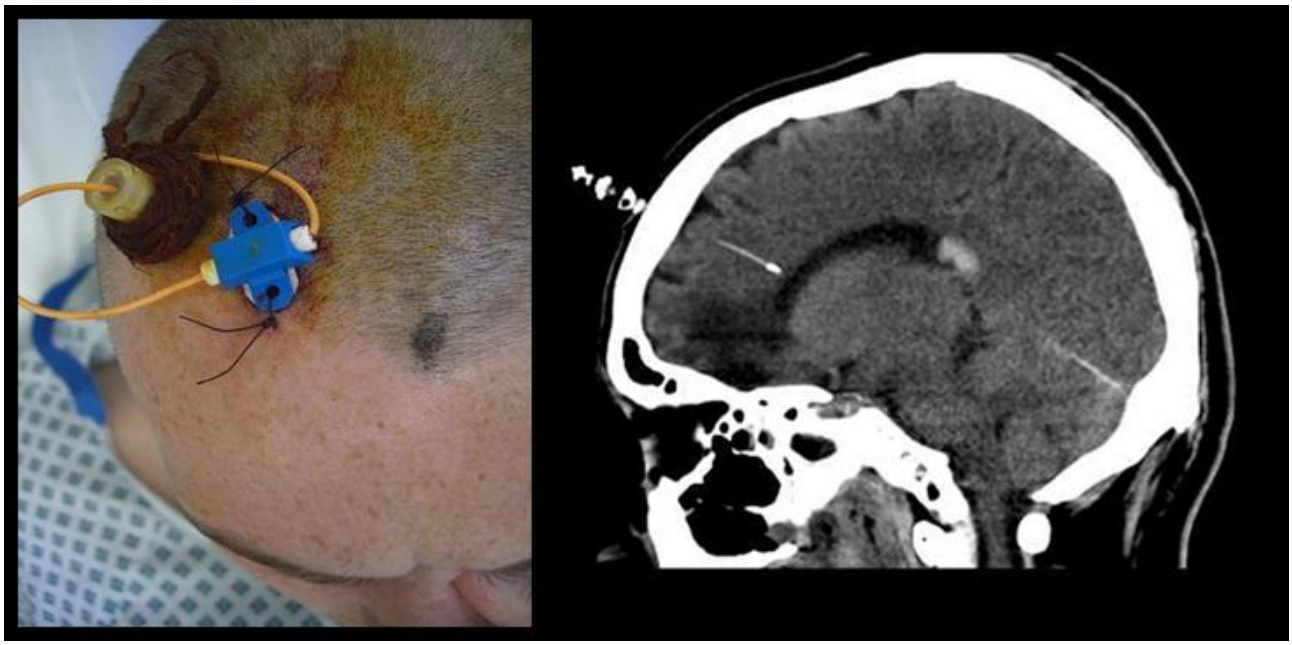

Fig. 4.6: *Right frontal parenchymal bolt for gold standard I-ICPM, note the tip of transducer in close proximity to the corpus callosum.*

Each of those methods for I-ICPM has specific pros and cons, for instance intraventricular bolts can have a twofold indication as they can be used to monitor and treat the ICP all at once, whereas the intraparenchymal bolts allow for a subcortical sensing of ICP and given the shorter intracranial trajectory are considered slightly safer. I-ICPM however comes at a cost: the most common complications of invasive methods are due to the elevated infection and haemorrhagic risk. According to different authors the infection rate correlated to the use of those methods for I-ICPM can be as high as 27% (*Davor D, et al. 2007*).

On the other hand, the rate of haemorrhagic complication is highly variable depending on whether a routine CT scan is performed after the procedure: interestingly when this postoperative check is routinely performed the incidence of haemorrhagic lesions has been described in as many as 41% of cases, although only about 10% resulted in larger than 15 mL (*Gardner PA, et al. 2009*).

A multimodality monitoring armamentarium is certainly warranted not only to provide clinicians with information characterized by a high degree of complementarity and redundancy, but also to protect our patients from the use of invasive technologies (*Ganau M, et al. 2013*).

In the last two decades, numerous efforts have been directed toward the optimization of multimodal brain monitoring to optimize the clinical management of those patients. In this regard, the idea of a non-invasive method of measuring ICP is captivating, but currently available

alternatives to I-ICPM are still lacking precision to be safely considered as reliable as gold standard methods.

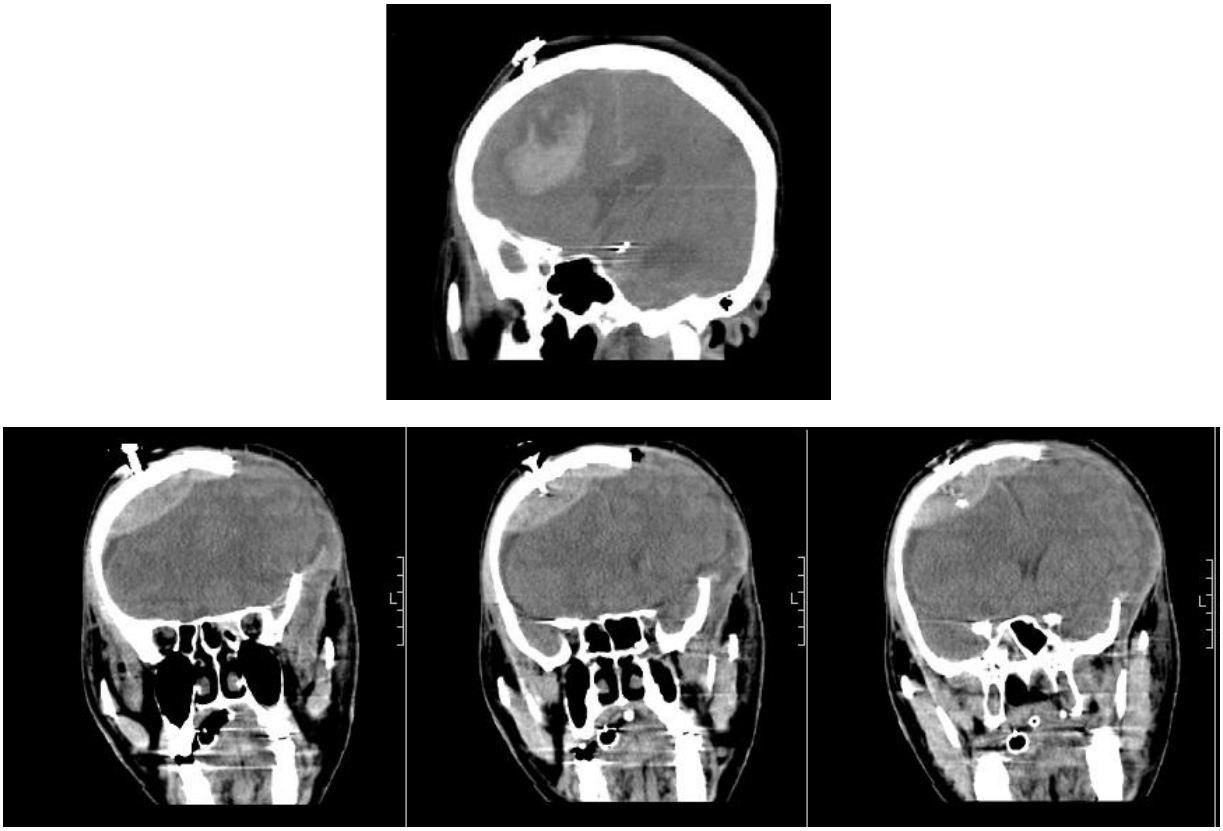

Fig. 4.7: *CT scans showing haemorrhagic complications following the insertion of gold standard intraparenchymal I-ICP. Note the large right frontal intracerebral haemorrhage (above) and the right frontal extradural haematoma (below).*

To date, non invasive ICP monitoring (NI-ICPM) methods can be classified into two categories: those based on morphological data, assessed with MRI, CT, ultrasound, and fundoscopy, and physiological ones, assessed with transcranial and ophthalmic Doppler, tympanometry, ONSD, near-infrared spectroscopy (NIRS), electroencephalography, visual-evoked potentials, and otoacoustic emissions assessment (*Raboel PH, et al. 2012; Czosnyka M, et al. 2014*). Presently, none of the non-invasive techniques seem suitable as a stand-alone substitute for I-ICPM, mostly because of intra- and interobserver variance. Here we describe the methods which proved to correlate the most with gold standard methods:

- ***Transcranial Doppler Ultrasonography:*** applied to measure blood flow velocity in the middle cerebral artery and to calculate the pulstatility index which provides values close to I-ICPM with correlation coefficients ranging between 0.43 and 0.93 (*Bellner J, et al. 2004*),

- ***ONSD:*** between the sheath of the optic nerve and the optic nerve is a small (0.1-0.2 mm) space communicating with the intracranial subarachnoid compartment, being therefore sensible to changes in ICP, so that whenever ICP increases the sheath expands. Its sensitivity and specificity range between 74-95% and 79-100%, respectively (*Geeraerts T, et al. 2008*),
- ***Tympanic membrane displacement:*** the technique takes advantage of the communication of the CSF and the perilymph via the perilymph duct, in fact the stimulation of the stapedial reflex causes a movement of the tympanic membrane, which is shown to correlate to ICP although more accurate in healthy subjects rather than in those with pathologically elevated ICP (*Gwer S, et al. 2013*),
- ***NIRS:*** used to derive the levels of oxygen saturation by estimating deoxygenated and oxygenated haemoglobin in the brain, NIRS may be used as a non-invasive substitute for calculation of PRx (*Lee JK, et al. 2009*). It has demonmay be used as a non-invasive marker of increased ICP slow waves, and therefore reduced CSF compensatory reserve (*Weerakkody RA, et al. 2010*).

Despite still conflicting evidence that it could be realistically associated with better outcome, I-ICPM in NeuroIntensive Care Units (NICU) represents an invaluable tool for the prevention, management, and understanding of secondary injuries after traumatic, neoplastic or cerebrovascular accidents.

In light of the above we attempted to test and optimize a new device based on an innovative algorithm for the monitoring of ICP, aiming to compare its accuracy and reliability in an hospital setting with the state-of-the-art I-ICPM.

EXPERIMENTAL WORK

4.3 Accuracy Benchmarking

Preliminary laboratory studies conducted on animal models led to the development of advanced signal-processing algorithms based on acoustic trans-cranial stimuli for estimation of ICP.

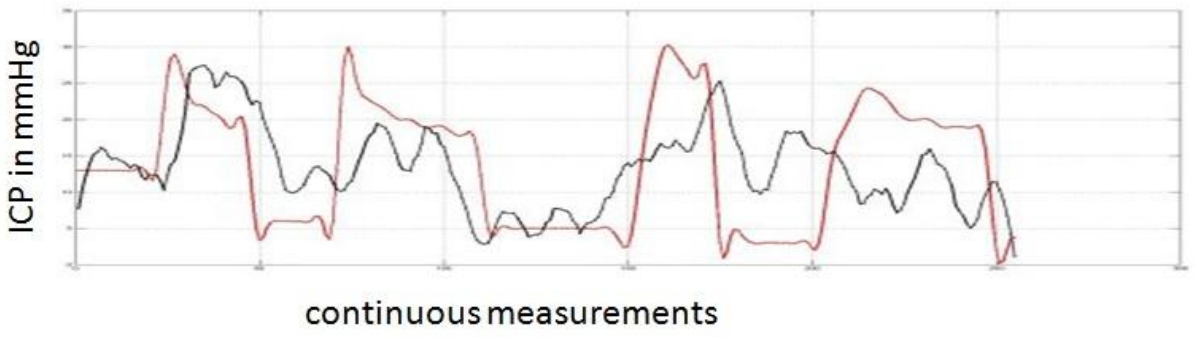

Fig. 4.8: *Comparison between I-ICPM (in red) and NI-ICPM (in black) in an animal model*

Given the satisfactory results on preclinical models, these algorithms were eventually incorporated in a new device for NI-ICPM designed for use in clinical practice (*HS-1000, Head Sense Ltd, Netanya - Israel*).

This device exploits an advanced signal analysis method of an acoustic signal, a short beep sound 10db for ~6 seconds, emitted from a small transmitter incorporated into a earplug placed in the patient's ipsilateral ear, and picked by an acoustic sensor incorporated in the other earplug placed in the contralateral ear. The algorithms developed by the producing company take into account several parameters, including acoustic bioimpedance, blood pressure and breathing rate; the device then transforms them into a digital signal and provides an ICP estimate displayed on a tablet monitor both in the form of a numerical value expressed in mmHg and of a graphic wave. As the acoustic signal is repeated every 6 seconds, the continuous recording following every impulse provides a total of 10 data points/minute.

Fig. 4.9: *NI-ICP monitoring algorithm and visual interface on a dedicated tablet*

In 2013 the device obtained a CE Mark approval and following its further optimization a clinical trial was designed to compare its accuracy to gold standard I-ICPM methods (intraparenchymal or intraventricular ones).

The Italian trial started at the Department of Neurosurgery in Verona once IRB approval was granted, and the device was tested on all patients with survival expectancy >72 hours undergoing I-ICPM for TBI or spontaneous ICH at our NICU.

Only patients with local infection in the ear, pregnant/lactating women and children were excluded from this study. A statistical analysis was performed on all testing session of continuous monitoring which lasted 1.5-6 hours.

Throughout all parallel ICP monitoring sessions, each patient was positioned in a supine position, and the head was placed in a 30-degree angle.

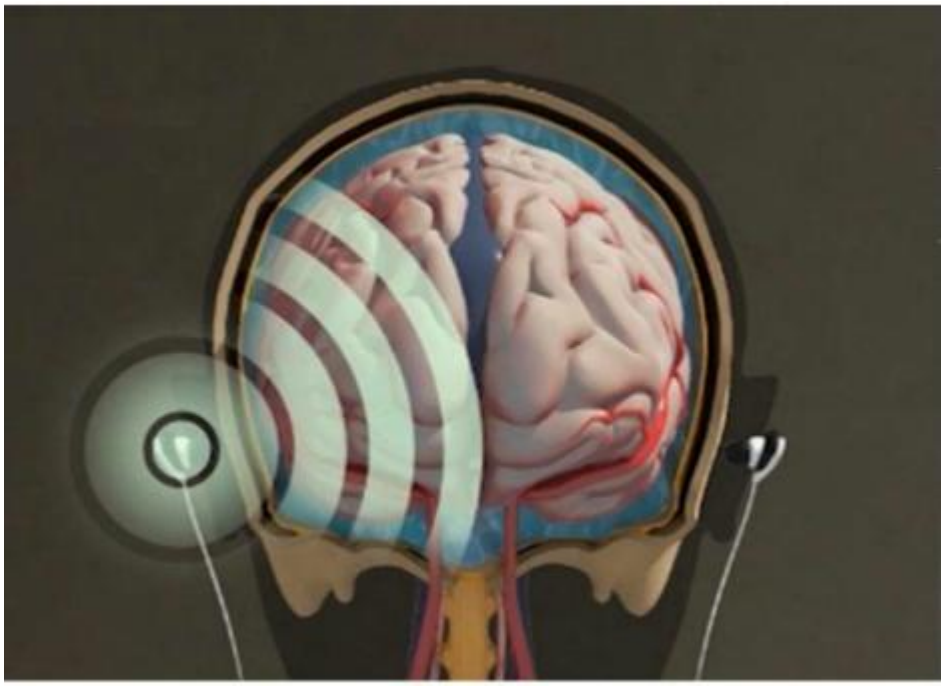

Fig. 4.10: *Artistic representation of the propagation of the acoustic stimuli across patient's head*

4.3 a): Methods for Comparative Analysis

In each monitoring session, parallel comparison of ICP values from I-ICPM and NI-ICPM were executed in the following sequence: the ICP values of the I-ICPM and NI-ICPM were both presented as mean ICP in every 6 seconds. For each data point, the monitor of the NI-ICPM device was programmed to take a snapshot photo of the standard bedside monitor screen at the end of signal transmission, and to save it into its database.

Accordingly, this process allowed for simultaneous collection of data from both I-ICP and NI-ICP devices, providing a maximum of ~10 independent comparison per minute. The length of ICP recording sessions depended on the patient's clinical condition, and the type of I-ICPM (30 minutes for EVD, till to 2 hours for parenchymal microsensor).

The parameters considered in the analysis of data simultaneously collected by the invasive and non-invasive method were the following ones:

- ***3 mmHg variance:*** in this statistical test we calculated the difference in mmHg between the I-ICPM and the NI-ICPM values, counting how often the difference between the two methods was ≤ 3 mmHg, and divided it with the total number of ICP samples. In other words, this parameter describes the amount (in %) of ICP values that were with a maximum of 3 mmHg difference with the gold standard I-ICPM.
- ***5 mmHg variance:*** as any I-ICPM method also presents a certain degree of inaccuracy, (that can be estimated as an internal error factor of 1-2 mmHg) it may be very difficult to follow the I-ICPM trend to such a narrow agreement, therefore we have considered also how often the difference between I-ICPM and NI-ICPM was ≤ 5 mmHg, and divided it with the total number of ICP samples. In other words, this parameter describes the amount (in %) of ICP values that were with a maximum of 5mmHg difference with the gold standard I-ICPM. Of note, for the analysis of both the 3 and 5 mmHg variance the statistical test was conducted on each individual ICP measurement, although the clinical practice recommends monitoring the ICP for at least 15 minutes to define whether it's normal or elevated.
- ***Robust Parameter:*** On post-processing analysis all ICP values expressed in mmHg were classified according to pre-specified cut-off points defined as: A) 0-5 mmHg, B) 5-15 mmHg, C) 15-20 mmHg, D) 20-25 mmHg and E) ≥ 25 mmHg. For each range we defined a certain severity index method. This cut-off points make sense because a difference between I-ICPM and NI-ICPM between 3 and 5 mmHg might not have any clinical meaning for cut-off points A, B or E, but can certainly have importance in the decision making process at cut-off points C and D because they are universally considered as the threshold for aggressive medical and surgical management of raised ICP.
- ***Cohen's Kappa (κ) coefficient:*** to statistically measure the inter-rater agreement between the two categorical values we opted for the calculation of κ, as it measures the agreement between two raters who each classify *N* items into *C* mutually exclusive categories. Cohen's κ is generally thought to be a more robust measure than simple

percent agreement calculation since κ takes into account the agreement occurring by chance. κ values range between 0-1 and the strength of agreement can be subclassified according to the following table providing a reference to the cut-off points of the results:

Cohen's Kappa (κ)	Strength of Agreement
< 0.00	Poor
0.00 – 0.20	Slight
0.21 – 0.40	Fair
0.41 – 0.60	Moderate
0.61 – 0.80	Substantial
0.81 – 1.00	Almost Perfect

Table 4.2: *Cut-off points for statistical analysis with Cohen's κ*

The κ statistical test was selected as the most suitable option to measure the agreement rate between two continuous parameters such as the ICP values recorded by the I-ICPM and the NI-ICPM devices. Since both of them provide for each patient hundreds of sample points, with each point different than the previous one, κ was the best statistical method to analyze the accuracy of the NI-ICPM in general and not for every single sample point.

- ***Pearson Correlation:*** the linear correlation between I-ICPM and NI-ICPM was tested with the Pearson r coefficient, calculating its 95% interval of confidence (CI) and *p* value.
- ***Receiving Operating Characteristics (ROC) Analysis:*** in order to assess the sensitivity of signal detection for the NI-ICPM device a ROC analysis was performed considering as the threshold of 15 mmHg as the cut-off point.
- ***Bland–Altman:*** finally all data obtained from both I-ICPM and NI-ICPM have been compared with the Bland Altman plot, which allows to compare two clinical measurements where one is known to be characterized by a certain degree of error. This makes the Bland Altman test suitable in medical statistics to compare a new measurement technique or method with a gold standard.

4.4 Clinical Validation

A total of 2795 continuous parallel recordings were analyzed for a total of 39967 data points from both I-ICPM and NI-ICPM.

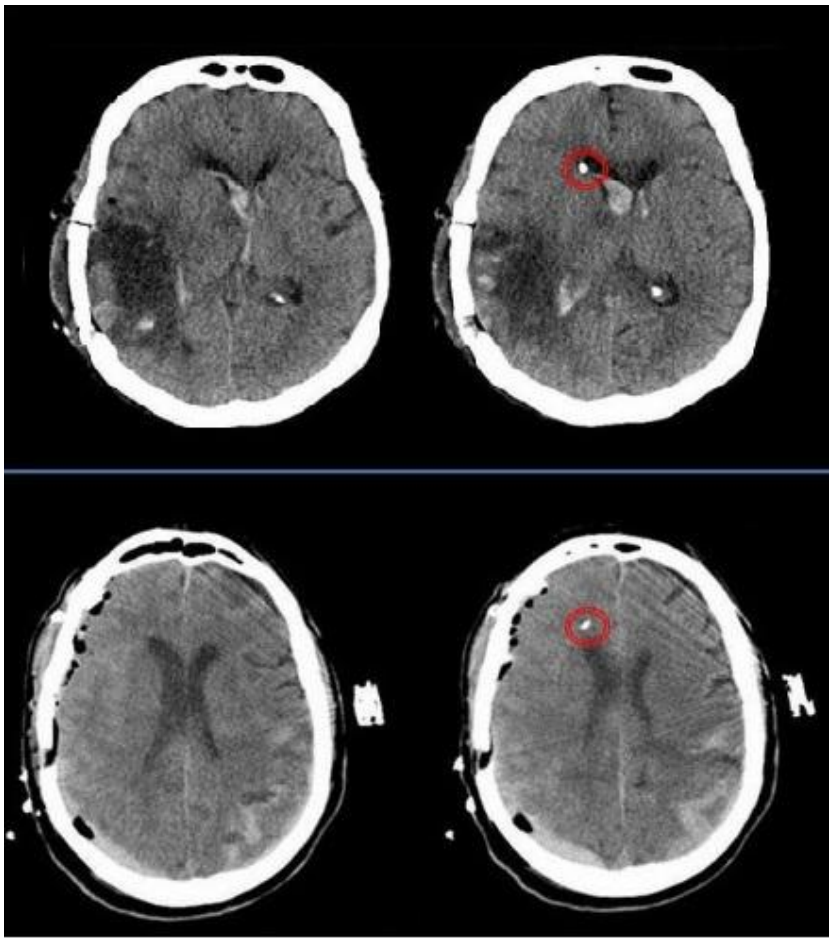

Fig. 4.11: *CT scans performed on patients with enrolled in the present study. note the the tip of the I-ICPM transducer within the red circle.*

The accuracy of the NI-ICPM device tested is confirmed by a differential pressure <5 mmHg and <3 mmHg in 95% and 70% of total data points respectively.

The correlation between NI-ICPM and I-ICPM values was confirmed by multiple tests: Blant Altman: 94.27%, Cohen's κ score: 0.601, and Pearson r: 0.49 with 95% CI: 0.46 - 0.51, and *p* value < 0.0001.

The device proved very accurate for cut-off points A) 0-5 mmHg and B) 5-15 mmHg, whereas it tended to overestimate ICP above 15 mmHg. Not surprisingly the ROC Curve analysis

performed for all measurements considering ≥ 15 mmHg as a cut-off point showed an area under ROC curve of 0.853.

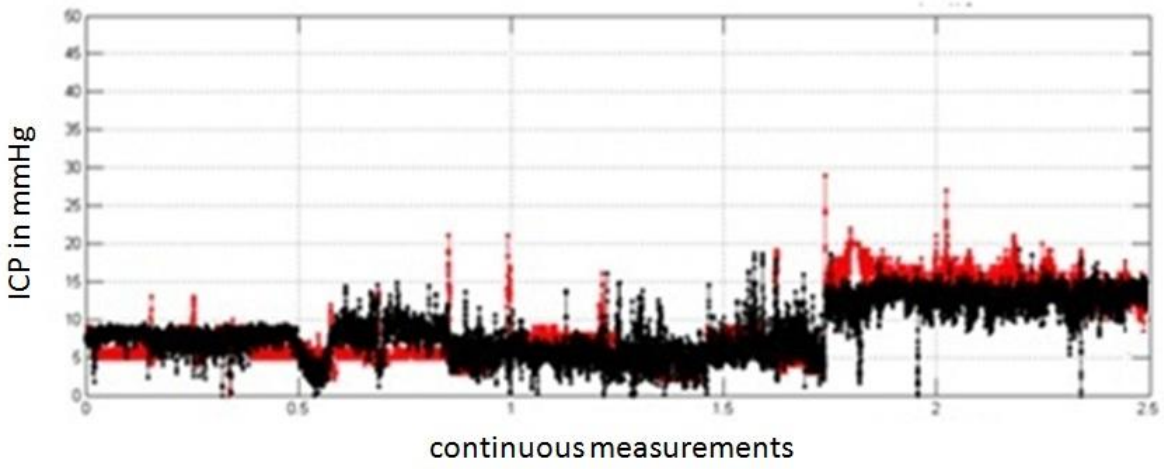

Fig. 4.12: *Graphic Statistics: comparison between I-ICPM (in black) and NI-ICPM (in red) measurements over time*

Overall it is possible to conclude that given the potential utility in quantitative ICP monitoring of the NI-ICPM device tested further algorithm optimization and clinical validation are certainly warranted. Nonetheless the shift from experimental setting to daily bedside use seems now closer than ever.

DISCUSSION AND FUTURE DEVELOPMENTS

4.5 Applications in Hospital and Extra-Hospital Settings

Beside the non-invasiveness the device herein tested has a main advantage over NI-ICPM, because it does not require any calibration at the beginning of the recording process to guarantee accuracy of monitoring; as well as over other methods for NI-ICPM, as it is not affected by intra- or interobserver variability.

A further advantage is the ability to provide clinicians with both a real-time numerical ICP value in mmHg, as well as its trend described by a waveform. The analysis of waveform of the ICP is in fact as important as its sole value and trend because it is useful to calculate the PRx and can be used to estimate optimal CPP levels for individual patients (*Czosnyka M, et al. 2014*).

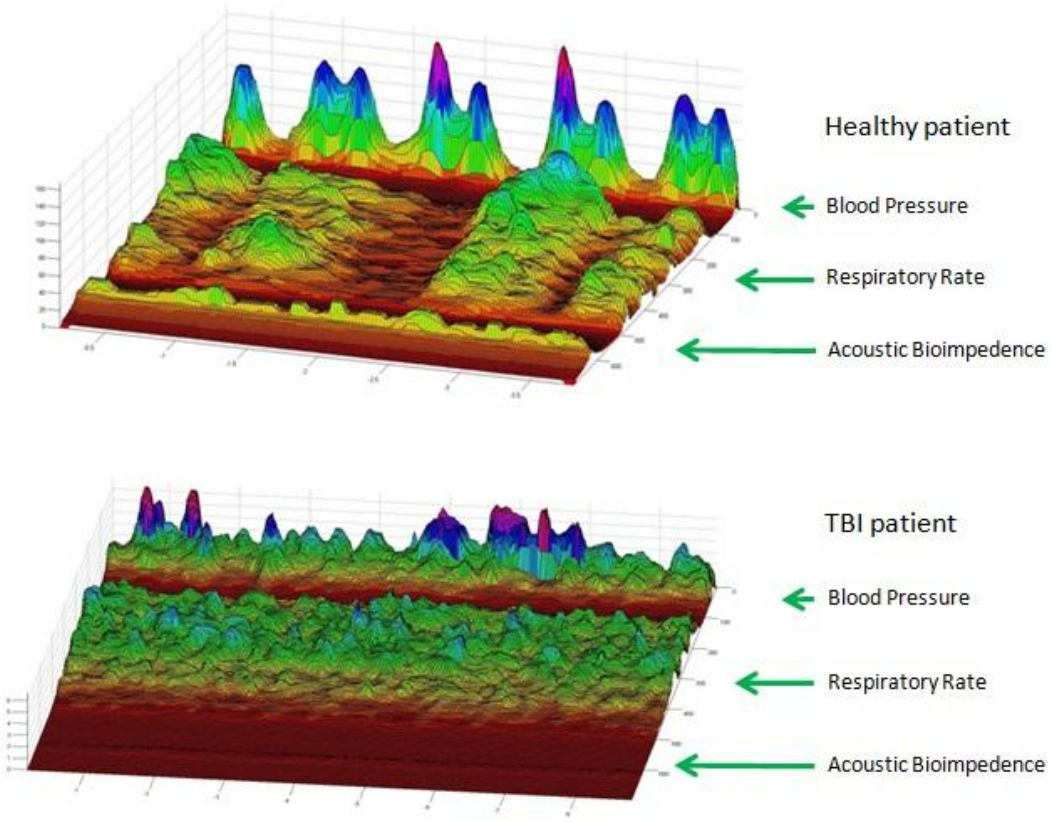

Fig. 4.13: *Graphical reconstruction of the signal and correlation with respiratory rate and blood pressure*

Management of TBI patients however is not the only area of use that can be envisaged: there are actually several other medical conditions where the N-ICPM device tested could find an immediate adoption, overcoming the current problems encountered by gold standard methods for I-ICPM. Briefly we can summarize here a series of clinical scenarios that could benefit from our studies:

- ***Stroke:*** There is a growing interest for the multimodality monitoring of stroke patients, and data relative to ICP could be of paramount importance to better understand the different stages of clinical deterioration in patient affected by ischaemic events and their possible transformation into proper ICH.
- ***Hydrocephalus:*** the abnormal accumulation of CSF in the ventricles may lead to increase in ICP in both the supra- or infratentorial compartments; monitoring ICP in

patient requiring shunting procedure could allow to avoid invasive measurements through lumbar puncture or intraparenchymal bolt, whereas in patient with suspected malfunction of the shunt would allow to avoid tapping of the shunt reservoir.

- ***Idiopathic intracranial hypertension (IIH):*** patients without radiological evidence of hydrocephalus but complaining of persistent headache can often require an I-ICPM. This become mandatory whenever visual disturbances occur and the clinicians need to rule out indications for CSF shunting or more invasive bitemporal decompression or eventually direct unroofing of optic nerves. As those episodes can occur quite often in patients with IIH, the possibility to repeat anytime NI-ICPM could expedite the decision making process.
- ***Syndromic and non-syndromic Craniosynostosis:*** although the risk varies depending on the specific diagnosis a significant proportion of patients with single suture craniosynostosis may develop raised ICP (4-14%), and the incidence is known to be much higher (47-67%) in patients with multiple involved sutures (*Renier D, et al. 1982; Gault DT, et al. 1992*). The relationship between raised ICP and cognitive outcomes is still debated, nonetheless monitoring ICP became an established step in the diagnostic work up of these patients and raised ICP represents to date a clear indication for surgical treatment (*Marchac D, et al. 1994*). Given the fragility of those patients obtaining this information in a non-invasive way would provide remarkable clinical advantages.
- ***Brain tumour****:* whenever a wait and see approach is adopted for patients with neoplastic space occupying lesions, follow up with addition information regarding the trend of ICP could be a valuable help in the assessment of the patient in the outpatients clinic.
- ***Elective intracranial surgery****:* There is a current medical need for ICP monitoring for patient recovering from any elective intracranial surgery. Retrospective data obtained from recovery rooms charts lead to estimate that as many as 17% of patient could possibly experience elevated ICP postoperatively (*Sakabe T, et al. 2010*).
- ***Miscellaneous****:* Additional causes for an increase in ICP include inflammatory or infective conditions (such as meningitis, encephalopathies, intracranial abscesses, Reye's syndrome or other encephalitis, etc).

Other considerations should also prompt further research efforts in making NI-ICPM a reliable alternative to I-ICPM, especially for the pre-hospital triage of patient with suspected raised ICP. The user-friendliness of the device herein tested makes it suitable for use by paramedics and could be used to guide decisions regarding transport and hospitalization of sick patients.

Finally, in the paediatric population NI-ICPM could allow to avoid the general anaesthesia or sedation otherwise compulsory whenever ICP monitoring becomes pivotal in the decision making process for the management of hydrocephalus or cranyosynostosis (*Sæhle T. et al, 2015*). Also, NI-ICPM could meet the need for monitoring of all those patients whose risk related to I-ICPM is deemed too high: examples include patient on cardiopulmonary bypass, and premature infants at risk for developing catastrophic intraventricular or intraparenchymal haemorrhages.

In conclusion, a commitment to the development of methods for NI-ICPM will certainly ensure that the information needed to drive therapeutic decisions are obtained or retrieved with the least harmful technique. The continuous research efforts in this area are showing that providing less costly and more accurate strategies is possible; and only by pursuing this endeavor the scientific community will effectively achieve the goal of providing more tailored and personalized treatment approaches to our patients.

Chapter V:

Conclusions and Outlook

Most Relevant Publication:
Ganau M, Foroni RI, Ambu R. Surgery in the realms of nanometers. In Commercializing Nanomedicine: Industrial Applications, Patents and Ethics (Editors: Escoffier L, Ganau M, and Wong J) Pan Stanford Publishing, Chapter 3: pp 59-75; 2015

5.1 The Future of Neurosurgical Practice

The advent of biomedical engineering is allowing neurosurgery to overcome many of the limits of its current clinical practice, and the previous sections offered some excellent examples of this revolution. However, many other game changing innovations can be forecasted from a careful analysis of the most recent trends in scientific literature, and the state of the art of neurosurgery will be continuously reshaped by their influence. Therefore, at the end of this thesis it is appropriate to outline some of the most striking advancements that in the next few years will be applied either broadly to all aspects of neurosurgery, or specifically to niche areas of its subspecialties.

Present	*Near Term*	*Mid to Long Term*
Drug-eluting stents for endovascular treatment	Ion-beam functionalization of bioactive endovascular stents	Nanorobots for screening of intracranial aneurysms
Coated-shunt catheters for CSF diversion	NEMS-functionalized microelectrode recording	Nanowires for brain-computer interfaces
Liposomal drugs for chemotherapy	Nanoshells and drug-delivery systems for chemotherapy	Nanorobots for detection and selective killing of cancer cells
Nanoparticles-coated spinal implants	NEMS-functionalized spinal implants	Nanoscaffolds for nerve regeneration in spinal cord injury
Nanoparticles with platelet-like functions	Super-paramagnetic oxide contrast agents for MRI	Quantum dots labeling of intracranial and spinal tumors

Vascular, Pediatric, Functional, Oncological, and Spinal Surgery; Haemostatic, and Contrast Agents

Table 5.1: *Present application and forecast for application of innovative technologies in neurosurgery.*

Neurosurgery as a whole will benefit from progress in diagnostics, visual and haemostatic aids. Clinicians are nowadays at a crossroad where therapeutic choices are being made not only considering conventional histological diagnosis but also the latest insights from molecular biology (*Odreman F, et al. 2005*).

A significant body of evidence demonstrates that even genetically identical cells can exhibit significant functional heterogeneity, accordingly molecular diagnostics is rapidly moving beyond genomics to proteomics, with the aim to identify those post-translational modifications expressed under pathologic conditions (*Krutzik PO, et al. 2004*). The proteome and secretome by definition are dynamics and change both in physiologic and pathologic conditions; the ultimate goal of determining them is to characterize the flow of information within the cells, through the intercellular protein circuitry that regulates the extracellular microenvironment.

Indeed, the study of proteomics and molecular biomarkers already allows to identify direct or indirect predictive factors, and soon it will hopefully determine which affected pathway could

become a selective therapeutic target for pharmacological or gene therapy. This applies to degenerative pathologies as well as tumours; in fact, given the cellular and molecular complexity of the CNS and PNS microenvironment, which suggests the importance to understand intracellular and intercellular signalling, several progresses have been made in recent years to achieve a precise, high throughput and low cost analysis of glial cells with potential capability for real-time pathological screening of multiple and lateral sclerosis, subtyping of brain tumors, and identification of circulating tumor cells (*Ganau M, et al. 2014*).

What will also apply to many neurosurgical specialties is the further optimization of current intraoperative visual aids such as intraoperative ultrasound and related contrast agents, or microsurgical filters associated with endovascular tracers. Indeed, the advent of those techniques is already exploited to provide guidance for identification of neoplastic remnants or visualization of cerebral vasculature during aneurysm clipping. One example is the fact that altered tissue metabolism is already offering a valuable ploy: HGG in fact may be intraoperatively detected thanks to the orally or endovenously administered drug 5-aminolevulinic acid (5–ALA) which lead to fluorescence of tumour cells during surgery, allowing identification and resection of tissue that is otherwise indistinguishable from the brain parenchyma (*Yamada S, et al. 2015*).

Also, better haemostatic materials will offer another useful technical breaktrough. In fact, achieving a satisfactory haemostastis can represent a challenging moment even for experienced neurosurgeons in any surgical procedure. Noteworthy cases of accidental damage to large vessels' wall (i.e. dural sinuses) or diffuse oozing from a surgical cavity (i.e. vascular tumors) are amongst the most common causes of perioperative complications (*Ganau M. et al, 2012; Graziano F. et al, 2015; Ganau M, et al. 2015*). To address this problem scientists have developed nanoparticles that exhibit platelet-like functions (PLNs) including site-directed margination, site-specific adhesion, and site-specific aggregation. Those PLNs are designed to mimic both key mechanical and biochemical attributes of platelets: in vivo studies in mouse models demonstrated that they accumulate at the wound site and induce ~65% reduction in bleeding time, being proposed as injectable synthetic hemostats for vascularly targeted payload delivery in both cranial and spinal surgery (*Anselmo AC. et al, 2014*)

Finally, the prevention of excessive scar formation and postoperative fibrosis will become easier and straightforward. These inappropriate events can occasionally occur after any surgical procedure, altering the cicatrization process, disturbing the postoperative course, and rendering reoperations more difficult and risky. Both cranial and spinal interventions are much affected by this adverse event: the literature describes this phenomenon as accompanying up to 20% of all neurosurgical procedures. The scar tissue that forms postoperatively adheres to the dura mater,

penetrates into the spinal canal, and can cause narrowing symptoms, neurological deficits, and pain. The incidence and spread of this excessive scar or epidural fibrosis has favored minimally invasive surgical technique (i.e. by incorporating endoscopic or microscopic access) to minimize the operative field and the use of isolating substances (i.e. autogenous or heterogeneous materials) administered intraoperatively (*Andrychowski J, et al. 2013*). Aiming at reducing the immunological response and the local inflammatory process, laboratory tests have recently been conducted with the local use of membranes presenting a biodegradable nanofibrous net of poly(L-lactide-co-caprolactone) manufactured by an electrospinning process showing that local cicatrization can be accelerated using innovative materials and that the scar formation and epidural fibrosis can be limited or modified locally by their double action in preventing local inflammation processes (*Andrychowski J, et al. 2013*).

Furthermore, it is worth mentioning how each subspecialty will selectively benefit from the advent of biomedical engineering from functional and paediatric neurosurgery to spinal surgery.

Functional neurosurgery which encompasses the management of several neurological diseases from movement disorders, such as Parkinson's disease and dystonia, to pharmacology resistant epilepsy, will witness major improvements due to advanced drugs and optimization of current devices. For instance, epilepsy is still one of the most clinically burdensome neurological disorders with a high percentage of patients (35-40%) resistant to pharmacotherapy despite the large number of available drugs, and is constantly benefiting from laboratory discoveries (*Rosillo-de la Torre A, et al. 2014*).

In fact, proven therapeutic strategies to control pharmacoresistant epilepsy now include epilepsy surgery and neuromodulation, and although not all patients are candidates for these therapies yet, bioengineering-based approaches are already attempting to widen their applicability. Beyond drug delivery strategy which shares biological properties similar to those used in neuro-oncology or in neuro-HIV to bypass the BBB and interact with firing epileptic foci, another appealing area of research is the development of implantable neural interfaces based on semiconductor nanowires (*Ganau M, et al, 2012; Vidu R, et al. 2014*).

Used as interface material in contact with neurons they allow to both deliver electrical stimulation and detect neuronal electrical activity; such approach is therefore opening new areas of research, including the optimization of spatial accuracy of neurophysiological intraoperative recording, enhanced microelectrode arrays for deep brain stimulation (DBS), and brain machine interfaces for neural prosthesis inducing artificial synapses in neuromorphic circuits based on nanoscale memory devices (*Vidu R, et al. 2014*).

Paediatric neurosurgery for instance is witnessing dramatic advancements thanks to the functionalization of shunt catheters for cerebrospinal fluid diversion, which are daily used in the treatment of acute and chronic hydrocephalus. The efficacy of ventricular catheters coated with polypropylene-grafted polyethylene glycol copolymers and silver nanoparticle-embedded polypropylene-grafted polyethylene glycol copolymers in preventing catheter-related infection and reducing inflammatory reaction in the periventricular parenchyma has been largely demonstrated in vitro, in animal models, in the neonatal and the pediatric population (*Thomas R, et al. 2012*). Though evidence from randomized clinical trials is still missing, this is the rationale behind the BASICS trial currently recruiting 1,200 patients in 17 regional neurosurgical units in the UK and Ireland (*Jenkinson MD, et al. 2014*).

Also spinal surgery is witnessing a fast-paced evolution due to the collaboration with material scientists. This is becoming particularly evident in the definition of novel strategies for biomechanically efficient spinal stabilization and spinal cord injury repair. Several biomaterials (including nanoscaffolds, demineralized bone matrix, and ceramics) with propensity to incorporate mesenchymal stem cells, recombinant human bone morphogenetic protein, and endogenous/exogenous growth factors are already showing ideal characteristics to achieve enhanced arthrodesis following spinal fixation. The next step in their upgrading is the ongoing incorporation of micro- and nanoelectromechanical systems (MEMS and NEMS) into current implants for dynamic stabilization. To this regard successful studies conducted in animal models to assess the quality of arthrodesis, are now being translated in clinical practice with the aim to reduce the risk of pullout, osteolysis, and revision surgery (*Benzel E, et al. 2004*).

Regarding the area of spinal cord injury, whereas cell- and biomolecule-based delivery strategies, as well as scaffold-based therapeutic strategies have failed when used alone, a combinatorial approach of all of them has preliminary showed to be effective in laboratory models. Translation from labs to bedside has been limited by the fact that many biomaterials investigated so far were plagued by wide range of adverse effects (especially in early stages of research), or by issues of widespread off-label use.

5.2 Bioethics of Innovative Technologies

The appealing promise of new technologies to redesign the realm of neurosurgery is based on the assumption that the related scientific discoveries will help providing new effective tools to

prevent diseases, promote health and alleviate human suffering. Nonetheless, predicting future degenerative diseases or extending life also bear several unprecedented scenarios. Futhermore, the principal feature of innovative technologies like nanomedicine or biomedical engineering, which makes the case for discontinuity with previous biotechnological waves, is the broader field of possible practical and theoretical applications, so that by entering this new era of neurosurgery we are accessing a funnel, from whom every initial step might be exponentially amplified down the road.

Whereas ethics has provided a rational approach to a wide span of moral controversies, in recent decades bioethicists have specialized themselves to specifically address dilemmas in life sciences. Dilemmas in innovation technology are common, and they are gradually expanding due to the widespread diffusion of nanotechnology- and biomedical engineering-driven solutions to healthcare needs (*York E, 2015; Collins FE, 2002*).

In light of the status quo depicted above some topics deserving particular attention regard the choice of the most appropriate diagnostic and therapeutic strategies, the understanding of their risks and benefits, and the description of the decision making process to patients and their families to obtain a proper informed consent. On one hand, some studies are demonstrating that patients more satisfied with respect to decisional involvement seem able to better cope with their disease, and show a significantly better self-perceived QoL (*Lucchiari C, et al. 2010*). On the other hand, clinical evidence available from recent translational research is still poor, inconclusive or fragmented; and doctors seeking answers in the most up-to-date scientific literature often have troubles in orientating themselves among preliminary results, experts' opinions and controversies. As a result, management choices may differed widely even in a relatively homogeneous group of specialists (*Mathiesen T, 2013*). This therefore highlights the importance to reconsider how specific treatment decisions are taken, especially in an era more and more oriented toward the goal of personalized medicine.

Providing sensible information regarding new investigations, surgical strategies, or therapeutic drugs and disclosing all the pros and cons of each single treatment option is technically and emotionally demanding, time consuming, and may pose special challenges to the process of requesting and obtaining a fully informed consent. This aspect is even more important when it comes to enrol patients in experimental trials for two reasons: the inner characteristics of neurosurgical patients and the nature of innovation.

Neurosurgical pathologies per se often oblige patients to deal with anxiety-provoking perspectives regarding disease prognosis, surgical risks and treatment-related side effects. For instance, Triebel et al. in a case control study demonstrated that the capacity to consent to treatment

tested with standardized psychometric questionnaires is impaired soon after diagnosis of a cerebral tumour in more than 50% of patients compared to healthy controls (*Triebel KL, et al. 2009*) Similar findings were published by Marson et al., specifically they showed that the enrollment of glioma patients in prospective clinical trials may raise ethical issues, as a 23% to 38% of patients with HGG show after diagnosis impairments in research consent capacity (*Marson DC, et al. 2010*). Furthermore, we must always bear in mind that the clinical status of neurosurgical patients, especially in neuro-oncological or neuro-vascular cases, is subject to sudden deterioration: as reported by Sizoo et al. from a neuro-oncological cohort more than half of patients become incompetent relatively early to make decisions due to delirium, cognitive deficits and/or decreasing consciousness obliging doctors and caregivers to shift the goal of therapy from primarily life-prolongation to primarily sustaining the QoL (*Sizoo EM, et al. 2012*).

Given the nature of many innovative solutions proposed, only a careful reasoning paired to the rise of such an emerging field of science and technology will help identifying the central ethical principles and precepts which must be prioritized in shaping the use of those discoveries and determining the right course of action to benefit from them. For this, in basic sciences the point to undertake an ethical investigation is to timely address its unpredictable future developments in timely fashion, whereas in the clinical arena pragmatically recognizing that second opinions and multidisciplinary meetings must play a pivotal role in rationalizing management strategies to ensure that each patient receive the best course of treatment.

In fact, from novel diagnostic devices to performance-enhancing drugs, new medical dilemmas are impacting our goals, values and aspirations as a society. Being well aware that the fundamental questions behind each aspect of those new drugs and medical devices present ethical quandaries for the decision makers highlights the importance of engaging the patients and at large the general public in an open discussion. Despite their complex and often controversial nature, an essential step in this endeavor is certainly represented by a thorough assessment of the potential risk posed by nanoproducts, such as the nanodrugs or radioenhancers discussed in this thesis, to human health. The traditional hazard-driven approach of monocausal toxicological perspective used in material science and translated to the chemical industry might not be appropriate because of the intrinsic peculiarities of nanomaterials and their physical chemical and biological properties at the nanoscale. Since fears about nanomedicine take many forms, mapping out nanorisks, especially those related to its possible cellular and genetic toxicity, call for a strict law regulation on translational research at national and international levels.

Yet, part of the problem has been that many aspects, belonging to the sphere of nanoscale devices or drugs, have appeared so impenetrable to the eyes of the public opinion. Therefore,

realizing that till now the actual purposes of some scientific breakthrough applied to the medical field have been rarely explained in a fashion really understandable to the general public, the upcoming educational challenge for scientists and clinicians will be to favor a new comprehensive approach, focused on sharing the specific pros and cons of the long-term, large-scale diffusion of nanomedicine (from chip-on-a-lab assays to nanodrugs or nanomaterials) with patients in order to obtain a full awareness, along with a proper informed consent, before their use in the clinical practice. As such, building trust requires now more than ever plans for a strategic and intense outreach: those are the basis for an easier transition from an asymmetric to a more balanced transmission of relevant information between science or medical professionals and patients (*Ganau M, et al. 2015*).

To contribute to better understanding those issues, during this PhD thesis we have developed a nanoethical framework which highlights 6 fundamental steps requiring adequate communication with the general public from conception to clinical application of a bioengineering-enhanced or nanotechnologically-enhanced product; those steps focus on: 1) identifying the clinical need(s) and the context in which the new technological solution will be implemented, 2) highlighting the scope(s) to develop, propose and commercialize the new medical device, 3) evaluating the advantage(s) and limitations of the new nanotechnological discovery 4) proposing whenever possible a benchmark comparison with existing gold-standard(s), 5) analyzing with objective measurements the perceived impact of nanodevices or nanodrugs on the patients and their relatives, 6) monitoring the long-term effect(s) of the nanoproduct (*Ganau M, et al. 2016*).

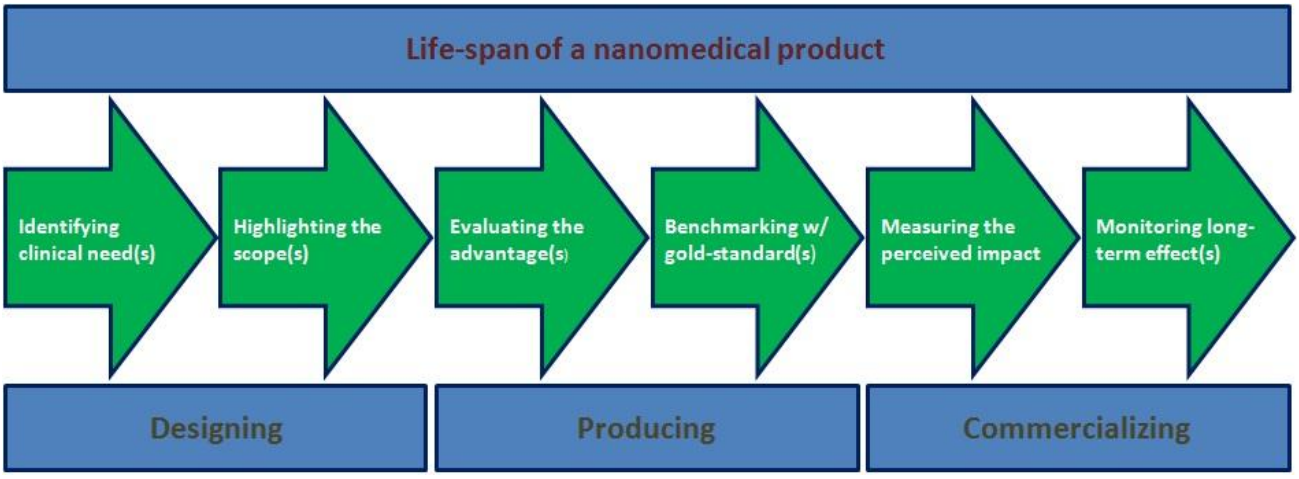

Fig. 5.1: *Nanoethical framework for ensuring adequate communication with the general public during each phase of the life-span of any given nanomedical product.*

5.3 Toward Bioengineering-Enhanced Neurosurgery

Delivering the surgical precision at the unprecedented cellular and subcellular length scale is a future goal along this continuum of minimally invasive neurosurgery, and the development of nanodevices capable of performing surgery at a molecular or atomic level is the first and foremost mandate of nanoneurosurgery. Some technologies already allow for operational accuracy at the nanoscale level, such as nanoknives, nanotweezers, and femtosecond-laser systems.

At present, nanoknives have been deployed and used effectively for microscale cellular surgery, allowing for precise targeted cutting. Among their greatest advantage is the possibility to constantly observe their tip in real time, allowing for used feedback, image capture, and even physiological recording (i.e., by electromyography techniques). Chang et al. examined the in vivo use of 10 to 100 μm long nanoknives with cutting edges of 20 nm in radius of curvature during experimental axonal reconstruction. Using those instruments they were able to make very small incisions (range of 50 to 100 μm long incisions) in nerve tissue in vivo and to repeat those incisions to progressively pare down the nerve as documented visually and by the accompanying incremental diminution of evoked motor responses recorded from target muscle (*Chang WC, et al. 2007*). Furthermore, these nanoknives showed to be safe in terms of induced neurotoxicity, as evidenced by the following robust growth of axons and neurons on this experimental material in vitro.

Subcellular surgery is much more complex: proteins and small-molecule metabolites constantly traffic among intracellular compartments, and it has become increasingly evident that biological specificity relies heavily on their spatial and temporal segregation and compartmentalization. The ability to isolate selectively single subcellular compartments for chemical analysis or transplantation opens new venues for intervening in the cellular pathologies. Silicon nanotweezers (SNTs), for instance, are a well-known microsystem for molecular manipulation. They can be used to trap molecules while sensing their biomechanical and bioelectrical response in minute operations. SNTs can be mass-produced by highly parallel microsystem technology and their exploitation in single-cell nanosurgery has widespread applications in biology but so far has been limited by difficulty in maintaining the functionality of the transported subcellular organelles.

However, beside few exceptions, such as those described above, much of the nanosurgical instrumentarium is still in the developing phase. Although those instruments serve the needs of proof-of-concept experiments, more refined equipment will need to be designed specifically to enable their efficient translation out of the laboratories in the surgical theater.

A fundamental question concerning the surgical use of microdevices that have the characteristic size of only several tens or hundreds of microns is whether such small instruments can be repeatedly used in an operative field: to reach this stage nanoinstruments must be mechanically strong and ensure adequate performances. Commonly, the properties of nanomaterials provide significant advantages when compared to their conventional benchmarks: the ultimate strength of silicon nitride nanoknives, which range from 2 to 8 GPa, is actually stronger than bulk steel (on average 0.5 GPa). Moreover, the nanomaterials chosen for the fabrication of instruments designed for neurosurgery must not be subjected to plastic deformation, which could alter their performance after repeated use. As a result, although the manufacture of miniature-scaled surgical devices is based on well-developed and reasonably mature fabrication technology, the actual profile of such instrumentation in vivo and its potential use in cellular- and subcellular-scale surgical procedures has yet to be optimized.

Regarding the surgical manipulation of cells, one point concerns the development of miniaturized micromanipulators to economize on space utilization around the operative field; another area of critical need is the development of surgical microscopes with sufficient magnification to visualize down to unprecedented small scales (i.e., isolation of specific parts of neurons such as axons in peripheral nerve surgery for reconstruction of their functionality) and to provide optimal working distances between the optical elements and the tissue. For instance, Chang et al. suggest that the design of future surgical microscopes for cellular-scale neurosurgery should include on-board lighting and likely also incorporated fluorescence imaging, which provides more contrast and far better signal-to-noise ratios over bright-field imaging (*Chang WC, et al. 2007*).

On one hand, the greatest promise of tissue engineering is to deliver artificial cells and organs for a myriad of purposes while ensuring their maximal biocompatibility; on the other, biosensors will be combined with improved electrodes and pacing devices to control impaired neurological functions. In the last decade, the interest toward strategies for tissue repair in neurodegenerative and inflammatory diseases (i.e. Amyotrophic Lateral Sclerosis, Alzheimer's and Parkinson's disease) and traumatic injuries (i.e. axonotemesis and neurotemesis following peripheral nerve and spinal cord injuries) increasingly rose. In the future, should such approaches prove to be safe and effective, they will likely replace treatments like DBS or nerve grafts from autologous donor roots.

Due to the complexity of the CNS and PNS: conventional repair approaches used in other body compartments are usually pointless; in this framework, technologies based on covalent and non-convalent modification of carbon nanotubes (CNT) and graphene, both allotropes of carbon and part of the fullerene family, emerged as innovative tools due to their outstanding physical

properties and the documented ability to interface synapses. Novel functionalized single-walled and multi-walled CNT nanoscaffolds have overcame the toxicity limitations, due to oxidative stress and reduced cell viability, typical of non-functionalized CNTs, and proved to deliver safely neural stem cells to injured sites of the CNS in animal models (*Vidu R, et al. 2014*).

Finally, achievements in miniaturizing chip technology, along with progresses in optics and micro mechanics led to the development of micro- and nanosized robots designed to navigate human biological systems. For instance, some prototypes using embedded nanoelectronics chemical sensors have been programmed for the proteomic detection of intravascular levels of nitric oxid synthase, their interpretation as pattern signals of the early stages of intracranial aneurysm development, and the communication of those information to the treating physician through radiofrequency wireless communication allowed by the nanorobots' antenna (*Cavalvanti A, et al. 2009*). The rationale of this proposed screening would be to monitor patients with medical or family history of previous aneurismal subarachnoid hemorrhage replacing the need for serial follow up with angioCT.

Similarly, others are working on swarm of nanorobots propelled into the bloodstream and able to recognize glioma cells, destroy them, and then forward information about the presence of cancer formation to other nanorobots, through acoustic signals in a distributed and decentralized fashion (*Loscrí V, et al. 2015*). As far as nanorobots have been conceived aiming at creating nonbiological entities that do not generate any harmful activities, that can be useful in any kind of surgery both under general as well as regional anesthesia, that are highly specific and target oriented, reducing drug-related mortality and morbidity (*Sengupt S, et al. 2012*).

Given the striking pace of advancement of those technologies, the translation of such prototypes from laboratory settings to clinical trials is hopefully not far away.

In conclusion, all the progresses highlighted in this thesis which find application in several aspects of neurosurgery have proved not only to be potentially game-changing, but also to be meant to evolve ubiquitously. In this PhD thesis the reasons why this bioengineering-enhanced revolution has what it takes to push forward the boundaries of our clinical practice has been constantly pinpointed. As such, we will probably witness in the forthcoming of our clinical and surgical careers that what is deemed untreatable today, has realistic chances to become treatable if not curable in the coming decades.

Abbreviation List

ATP:	Adenosine Triphosphate
BBB:	Blood Brain Barrier
CA:	Cinnamaldehyde
CBF:	Cerebral Blood Flow
CI:	Confidence Interval
CNS:	Central Nervous System
CNT:	Carbon Nanotubes
CPP:	Cerebral Perfusion Pressure
CSF:	Cerebro-Spinal Fluid
CT:	Computed Tomography
CTA:	Computed Tomography Angiography
CTC:	Circulating Tumour Cell
DBS:	Deep Brain Stimulation
DC:	Decompressive Craniectomy
DECRA:	Decompressive Craniectomy in Patients with Severe Traumatic Brain Injury
DSA:	Digital Subtraction Angiography
DTI:	Diffuse Tensor Imaging
ECM:	Extra Cellular Matrix
EPR:	Enhanced Permeability and Retention
EVD:	External Ventricular Drainage
FGF:	Fibroblast Growth Factor
Glut-1:	Glucose Transport-1
GK:	Gamma Knife
HA:	Hyaluronc Acid
HGG:	High Grade Gliomas
HIF-1:	Hypoxia Inducible Factor 1
HYAL:	Hyaluronidasis
ICH:	Intracerebral Haemorrhage
ICP:	Intracranial Pressure
I-ICPM:	Invasive ICP Monitoring

ICU:	Intensive Care Unit
IIH:	Idiopathic Intracranial Hypertension
IOM:	Intraoperative Neurophysiological Monitoring
IRB:	Institutional Review Board
LGG:	Low Grade Gliomas
LINAC:	Linear Particle Accellerator
MEMS:	Microelectromechanical System
MRI:	Magnetic Resonance Imaging
MWI:	Molecular Weight
NEMS:	Nanoelectromechanical System
NI-ICPM:	Non-Invasive ICP Monitoring
ONSD:	Optical Nerve Sheath Diameter
P.gP:	P-Glycoprotein
PET:	Positron Emission Tomography
PLN:	Platelet-like Nanoparticles
PNS:	Peripheral Nervous System
PRx:	Cerebrovascular Reactivity
RHAMM:	Receptor for HA-Mediated Motility
ROC:	Receiving Operating Characteristics Analysis
RT:	Radiotherapy
SAH:	Subarachnoid Haemorrhage
SNTs:	Silicon Nanotweezers
SPECT:	Single Photon Emission Computed Tomography
TGF:	Transforming Growth Factor
TNS:	Tumour Neurosphere
TNF:	Tumour Necrosis Factor
5-ALA:	5-Aminolevulinic Acid
vWF:	von Willebrand Factor
VEGF:	Vascular Endothelial Growth Factor

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- **Chapter IV**

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