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# *The Heme Oxygenase-1 (HO-1) system in Glioblastoma Multiforme*

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*PhD Thesis*

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## ABSTRACT

Gliomas are the most common primary tumours of the central nervous system (CNS) in the adult. Glioblastoma represents half of the newly diagnosed gliomas, and it is the most aggressive brain tumour with a median patient survival of 14-17 months. Heme oxygenase (HO) is the rate-limiting enzyme in the catabolism of heme: this two-step enzymatic reaction results in the formation of equimolar amounts of biliverdin, iron and carbon monoxide (CO). HO exists in two forms, HO-1, the inducible form, and HO-2, the constitutive form which degrades heme in an identical stereospecific manner. In human glioma tumours, HO-1 is overexpressed both when compared with normal brain tissues and also during oligodendroglioma progression. So far, the molecular mechanisms triggered by HO-1 to promote glioblastoma remains unknown. In the first part of the project HO-1 was induced by hemin treatment (10 μM) whereas VP13/47 (100 μM) was used as a specific non-competitive inhibitor of its activity. Carbon Monoxide Releasing Molecules (CORMs) (i.e. CORM-3 and CORM-A1) were also used in a separate set of experiments to confirm the effect of HO-1 by-product. Our results were further validated using GSE4412 microarray dataset analysis and comparing biopsies from patients overexpressing HMOX-1 with the rest of the cases. Hemin, but not CORMs treatment, resulted in a significant increase of cell proliferation following 24h of treatment as measured by increased cell index and colony formation capacity and such effect was abolished by VP13/47. Both hemin and CORMs showed a significant effect on the wound healing assay also exhibiting a cell specificity. Moreover, our dataset analysis showed a positive correlation of HMOX-1 gene expression with ITGBI and ITGBII which are membrane receptors involved in cell adhesion, embryogenesis, tissue repair, immune response and metastatic diffusion of tumour cells.

In the second part of the project we analyzed the effects of estradiol (E2, 5nM) in human glioblastoma multiforme U87-MG cells and how it may impact on cell proliferation and mitochondrial fitness. In particular, mitochondrial fitness was evaluated by assessing the expression of gene involved in mitochondrial biogenesis (PGC1α, SIRT1, TFAM), oxidative phosphorylation (ND4, Cytb, COX II, COX IV, Ndufa6, ATP synthase) and dynamics (OPA1, MNF2, MNF1, FIS1). Moreover, Nrf2 nuclear translocation was evaluated by immunocytochemical analysis. Taken all together, our data suggest that HO-1 and its byproduct CO exhibit a cell-specific effect on various aspects of disease progression and is associated with a complex series of molecular mechanisms driving cell proliferation, survival and metastasis; furthermore, E2 promotes proliferation in glioblastoma cells and regulate the expression of genes involved in mitochondrial fitness and chemoresistance pathway.

# GLIOMAS

## *Classification*

Gliomas are the most common primary tumours of the central nervous system (CNS) in the adult. Glioblastoma represents half of the newly diagnosed gliomas and it is also the most aggressive brain tumour with a median patient survival of 14-17 months (Lefranc, Le Rhun, Kiss, & Weller, 2018; Ventura, Weller, & Burghardt, 2017). The cell of origin of gliomas is still controversial. Several studies in animal models suggest that different cell types can generate gliomas, including neural stem cells and oligodendroglial progenitor cells (Alcantara Llaguno & Parada, 2016). According to the World Health Organization (WHO) Classification of Tumours of the CNS published in 2016, gliomas are diagnosed following an integrated approach which combines the histological classification with molecular information (Louis et al., 2016; Reifenberger, Wirsching, Knobbe-Thomsen, & Weller, 2017). This method allows a more precise classification of gliomas with improved prediction of clinical outcome and treatment response.

The first type of classification is based on the histological tumour type considering cytological features and expression of lineage-associated proteins of astrocytic or oligodendroglial cells (Alcantara Llaguno & Parada, 2016).

The second type of classification is based on tumour grading, according to the WHO definition, taking into account the degree of cell anaplasia: defined and low-proliferative lesions are assigned grade I; grade II lesions are low proliferative too, but they show cytological atypia and are infiltrative; grade III tumours show nuclear atypia and high mitotic activity. Grade IV tumours show characteristics of grade III tumours, with microvascular proliferation and necrosis (Louis et al., 2007; Wen & Kesari, 2008) (Table 1).

| WHO grades of select CNS tumours | |
|---|---|
| **Diffuse astrocytic and oligodendroglial tumours** | |
| Diffuse astrocytoma, IDH-mutant | II |
| Anaplastic astrocytoma, IDH-mutant | III |
| Glioblastoma, IDH-wildtype | IV |
| Glioblastoma, IDH-mutant | IV |
| Diffuse midline glioma, H3 K27M–mutant | IV |
| Oligodendroglioma, IDH-mutant and 1p/19q-codeleted | II |
| Anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted | III |
| **Other astrocytic tumours** | |
| Pilocytic astrocytoma | I |
| Subependymal giant cell astrocytoma | I |
| Pleomorphic xanthoastrocytoma | II |
| Anaplastic pleomorphic xanthoastrocytoma | III |
| **Ependymal tumours** | |
| Subependymoma | I |
| Myxopapillary ependymoma | I |
| Ependymoma | II |
| Ependymoma, *RELA* fusion–positive | II or III |
| Anaplastic ependymoma | III |
| **Other gliomas** | |
| Angiocentric glioma | I |
| Chordoid glioma of third ventricle | II |
| **Choroid plexus tumours** | |
| Choroid plexus papilloma | I |
| Atypical choroid plexus papilloma | II |
| Choroid plexus carcinoma | III |
| **Neuronal and mixed neuronal-glial tumours** | |
| Dysembryoplastic neuroepithelial tumour | I |
| Gangliocytoma | I |
| Ganglioglioma | I |
| Anaplastic ganglioglioma | III |
| Dysplastic gangliocytoma of cerebellum (Lhermitte–Duclos) | I |
| Desmoplastic infantile astrocytoma and ganglioglioma | I |
| Papillary glioneuronal tumour | I |
| Rosette-forming glioneuronal tumour | I |
| Central neurocytoma | II |
| Extraventricular neurocytoma | II |
| Cerebellar liponeurocytoma | II |
| **Tumours of the pineal region** | |
| Pineocytoma | I |
| Pineal parenchymal tumour of intermediate differentiation | II or III |
| Pineoblastoma | IV |
| Papillary tumour of the pineal region | II or III |
| **Embryonal tumours** | |
| Medulloblastoma (all subtypes) | IV |
| Embryonal tumour with multilayered rosettes, C19MC-altered | IV |
| Medulloepithelioma | IV |
| CNS embryonal tumour, NOS | IV |
| Atypical teratoid/rhabdoid tumour | IV |
| CNS embryonal tumour with rhabdoid features | IV |
| **Tumours of the cranial and paraspinal nerves** | |
| Schwannoma | I |
| Neurofibroma | I |
| Perineurioma | I |
| Malignant peripheral nerve sheath tumour (MPNST) | II, III or IV |
| **Meningiomas** | |
| Meningioma | I |
| Atypical meningioma | II |
| Anaplastic (malignant) meningioma | III |
| **Mesenchymal, non-meningothelial tumours** | |
| Solitary fibrous tumour / haemangiopericytoma | I, II or III |
| Haemangioblastoma | I |
| **Tumours of the sellar region** | |
| Craniopharyngioma | I |
| Granular cell tumour | I |
| Pituicytoma | I |
| Spindle cell oncocytoma | I |

***Table 1: WHO grades of select CNS tumours –*** *Adapted from Louis, D.N., et al. "The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary." Acta Neuropathol, 2016. 131(6): p. 803-20.*

The third type of classification is based on the molecular characteristics of the tumour. Numerous genetic and epigenetic alterations have been identified in gliomas (C. W. Brennan et al., 2013; Parsons et al., 2008; Verhaak et al., 2010). These molecular alterations are used for the diagnosis or as predictive biomarkers of patient response to therapy, as described in the following. The determination of isocitrate dehydroidrogenase 1 or 2 (IDH 1/2) status represents the first layer of the molecular diagnosis. IDH status is first assessed by immunohistochemistry assay with a specific antibody for the most common IDH mutation in gliomas (IDH1-R132H). IDH mutation is one of the earliest events occurring in gliomas and identifies biologically different tumours with different clinical behaviour and improved prognosis when compared to wild-type gliomas (Parsons et al., 2008; Sanson et al., 2009; Suzuki et al., 2015; Yan et al., 2009). Mutated IDH proteins acquire the ability to convert α-ketoglutarate (α-KG) into D-2-hydroxyglutarate (D-2-HG) (Dang et al., 2009). D-2-HG acts as a competitive inhibitor of α-KG-dependent dioxygenase, including some DNA and histone demethylases (Xu et al., 2011). This inhibition results in an increase in DNA and histone methylation leading to the hypermethylation of CpG

islands and glioma phenotype known as “glioma CpG-island methylator phenotype” (G-CIMP) (X. Y. Liu et al., 2012; Noushmehr et al., 2010).

After the definition of the IDH 1/2 status, the molecular diagnosis may proceed with the immunohistochemical determination of the nuclear retention or loss of a-thalassemia/mental retardation syndrome X-linked (ATRX) transcription factor. ATRX is involved in chromatin remodelling (X. Y. Liu et al., 2012; Watson, Goldberg, & Berube, 2015). Another determination required for patients with histological diagnosis of diffuse astrocytic or oligodendroglial gliomas with IDH-mutant and nuclear ATRX retention is the combined loss of genetic material from the p arm of chromosome 1 and the q arm of chromosome 19, known as 1p/19q co-deletion. This co-deletion defines oligodendrogliomas and is used as a biomarker to predict benefit from radiotherapy and chemotherapy with procarbazine and vincristine compared to radiotherapy alone in patients with anaplastic gliomas (Cairncross et al., 2013; van den Bent et al., 2013).

The K27M mutation in the histone H3.3 gene characterizes gliomas developing in midline structures such as the thalamus, spinal cord and brainstem. O6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein which

repairs DNA alkylation induces by alkylating agents like temozolomide (TMZ). Glioblastoma with hypermethylated MGMT promoter shows reduced levels of MGMT protein, translating into a reduced ability of DNA repair. MGMT promoter hypermethylation is a predictor of benefit from temozolomide therapy and increased survival, in patients with IDH-wild-type status (Wick et al., 2012). Based on the new classification, glioblastomas are divided into two categories according to their IDH status: IDH-wild-type and IDH-mutant glioblastomas. IDH-wild-type glioblastomas enclose almost 90% of cases, and clinically they correspond to de novo glioblastomas with a predominance in patients older than 50 years. IDH-mutant glioblastomas cover the remaining 10% and correlate to glioblastomas originating in younger patients and derived from lower-grade diffuse gliomas (Reifenberger et al., 2017) (Table 2).

| | IDH-wildtype glioblastoma | IDH-mutant glioblastoma |
|---|---|---|
| Synonym | Primary glioblastoma, IDH-wildtype | Secondary glioblastoma, IDH-mutant |
| Precursor lesion | Not identifiable; develops de novo | Diffuse astrocytoma<br>Anaplastic astrocytoma |
| Proportion of glioblastomas | ~90% | ~10% |
| Median age at diagnosis | ~62 years | ~44 years |
| Male-to-female ratio | 1.42:1 | 1.05:1 |
| Mean length of clinical history | 4 months | 15 months |
| Median overall survival<br>Surgery + radiotherapy<br>Surgery + radiotherapy + chemotherapy | <br>9.9 months<br>15 months | <br>24 months<br>31 months |
| Location | Supratentorial | Preferentially frontal |
| Necrosis | Extensive | Limited |
| *TERT* promoter mutations | 72% | 26% |
| *TP53* mutations | 27% | 81% |
| *ATRX* mutations | Exceptional | 71% |
| *EGFR* amplification | 35% | Exceptional |
| *PTEN* mutations | 24% | Exceptional |

***Table 2: Key characteristics of IDH-wildtype and IDH-mutant glioblastomas***
*Adapted from Louis, D.N., et al., The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. Acta Neuropathol, 2016. 131(6): p. 803-20.*

*Alteration of molecular pathways in glioblastoma*

Many other molecular lesions have been identified in gliomas (C. Brennan et al., 2009; International Cancer Genome et al., 2010; Parsons et al., 2008; Verhaak et al., 2010).

In IDH-wild-type glioblastoma, it is possible to identify macroscopic genomic alterations with the loss of chromosome 10 and the gain of chromosome 7 (Aldape, Zadeh, Mansouri, Reifenberger, & von Deimling, 2015). Some frequent genomic alterations regard genes encoding for tumour suppressor proteins such as p53, retinoblastoma (Rb) and cyclin-dependent kinase inhibitor 2A (Henson et al., 1994; Watanabe et al., 2001; Weber et al., 2007). In particular, TP53 is mutated in 81% of IDH-mutant glioblastomas. Besides, the reduced activity of p53 and Rb pathways can also be the consequence of gene amplification events leading to increased levels of p53 and Rb repressors, like cycling-dependent kinase CDK-4 and mouse double minute MDM-2. Another group of frequent alterations in glioblastoma regard receptor tyrosine kinases/RAS/phosphatidylinositol 3-kinase (RTK/RAS/PI3K) pathways (McBride et al., 2010; Snuderl et al., 2011; Verhaak et al., 2010).

The epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor alpha are the most frequently altered RTK in glioblastoma. Around 40% of IDH-wild-type glioblastoma show EGFR amplification. Moreover, 50% of tumours showing EGFR amplification express a mutated and constitutively active form of EGFR known as EGFRvIII (Frederick, Wang, Eley, & James, 2000; Hegi, Rajakannu, & Weller, 2012; Lee et al., 2006): the mutation leading to EGFRvIII generates a novel epitope that may be potentially used as tumour-associated antigen for therapeutic vaccination approaches (Reifenberger et al., 2017). The increase in PDGFR signalling is usually associated with PDGFRA amplification (Verhaak et al., 2010) and promotes the proliferation of tumour cells. Subsequently to RTK, it is possible to identify commonly several mutations in RAS and PI3K pathways: 1) mutations in the gene encoding for PI3K (Suzuki et al., 2015); 2) deletions, epigenetic alterations or mutations affecting the gene encoding phosphatase and tensin homolog (PTEN) (Huse et al., 2009; Wiencke et al., 2007), inhibitor of PI3K pathway which are present in 24% of IDH-wild-type glioblastomas; 3) mutation in the gene of neurofibromin 1 (NF1) which is a negative regulator of RAS and mammalian target of rapamycin (mTOR) pathways.

Moreover, the V600E mutation in B-Raf proto-oncogene serine/threonine kinase (BRAF) is identified (Suzuki et al., 2015). Lastly, another frequent genetic alteration affects the telomerase reverse transcriptase (TERT) gene (Arita et al., 2013; Killela et al., 2013; Koelsche et al., 2013): in fact, mutations in the TERT promoter lead to aberrant expression of TERT, and these are detected in 72% IDH-wild-type and 26% IDH-mutant glioblastomas; these mutations are present in more than 95% of oligodendroglial gliomas (Reifenberger et al., 2017).

*Diagnosis and therapy*

The diagnosis of glioblastoma requires the histological and molecular analysis from tumour tissue obtained during an open resection or a biopsy, as shown in figure 1. Glioblastoma, as a glial brain tumour, has features of malignancy that include angiogenesis and focal necrosis. The new WHO classification recognises giant cell glioblastoma, gliosarcoma and epithelioid glioblastoma, in addition to the classical glioblastoma as histological defined variants (Louis et al., 2016).

Moreover, classical glioblastoma does not exhibit isocitrate dehydrogenase (IDH) 1 or 2 mutations: these mutations show a less aggressive clinical outcome (Brat et al., 2018).

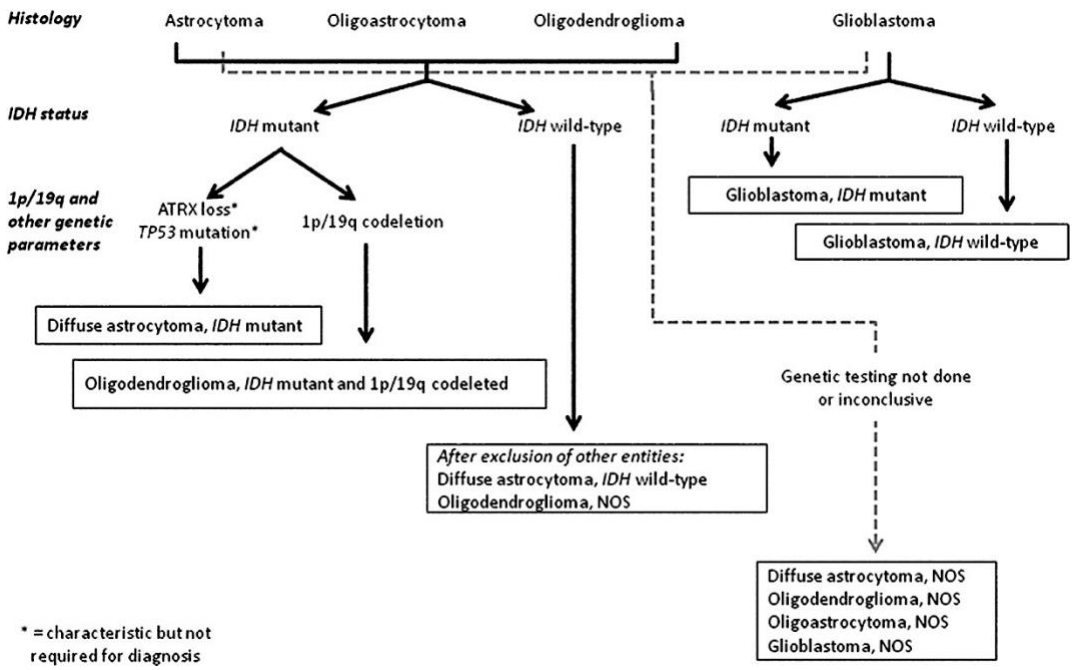


***Fig. 1: Simplified algorithm for classification of the diffuse gliomas based on histological and genetic features***
*Adapted from Louis, D.N., et al., The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. Acta Neuropathol, 2016. 131(6): p. 803-20.*

The standard of care is maximum surgical resection using microsurgical techniques as safely feasible; nevertheless, the role of surgery is difficult to be defined in controlled clinical trials (Stummer et al., 2006). The extent of surgery should be verified by MRI within 24-72 hours of surgery: it is impossible to clarify if the extent

of resection improves the outcome or if the tumours responsive to gross total resection have a less malignant course of the disease. When microsurgical resection is not available, due to medical reasons or refusal of the patient, it is possible to provide sufficient material for histological and molecular analysis using an image-guided stereotactic serial biopsy. It is firmly discouraged to administrate any treatment without obtaining tissue for a histological and molecular diagnosis (Eigenbrod et al., 2014). Radiotherapy (RT) is an additional standard of care for glioblastoma, established more than 40 years ago. It is demonstrated that RT improves local control and probably approximately doubles survival. Standard RT is delivered in 1.8-2 Gy fraction to a total dose of 54-60 Gy. A valid option for older patients or patients with limited life expectancy is a hypo-fractionated RT with a biologically equivalent dose of 40 Gy distributed in 15 fractions of 2.67 Gy. If tumour exhibits MGMT promoter methylation or in case of elderly patients, the RT can be rejected, and temozolomide is administrated alone (Wick et al., 2012). In addition, patients with glioblastoma can be also treated with temozolomide, a cytotoxic alkylating agent, which is administrated during

radiotherapy (75 mg/m2 daily) followed by six cycles of maintenance therapy (150-200 mg/m2, 5/28 days) (Perry et al., 2017; Stupp et al., 2005).

Limitations in this therapy include significant comorbidities such as haematological of hepatic disease. The most common side effect is myelosuppression with thrombocytopenia (Weller et al., 2017). Only patients with tumours with MGMT promoter methylation have benefit from temozolomide treatment (Hegi et al., 2005).

A new strategy of treatment involves the focal delivery of low-intensity, intermediate-frequency (200 kHz) alternating electrical fields to the tumour-bearing brain: this technique is defined as tumour-treating fields which are theorised to inhibit cell cycle progression during metaphase. Actually, an open-label randomised trial reported superior progression-free survival and overall survival compared with temozolomide alone (Stupp et al., 2017). Despite the safety and the well tolerance of the treatment, its acceptance by patients and healthcare professionals is low. In case of recurrence, the standard of care is far less defined than in the newly diagnosed glioblastomas (Gramatzki et al., 2018). It is possible to perform surgery if a gross total resection is feasible and if the time from first surgical intervention is longer than 6 months (Suchorska et al., 2016). Most patients with recurrent glioblastoma are

treated with the second administration of the alkylating agent, such as lomustine if they are eligible for salvage therapy. Up to 50% of patients may not qualify for salvage treatment (Gramatzki et al., 2018).

Similarly, Bevacizumab, a recombinant humanised monoclonal antibody directed against the vascular endothelial growth factor (VEGF), was approved in the USA and other countries as single agent based on response rate and progression-free survival rates; however, bevacizumab treatment as a single agent is not approved in the European Union. Up until now, the combination of bevacizumab and lomustine showed prolonged progression-free survival compared with lomustine alone: nonetheless, this combination does not show an improvement of the overall survival (Wick et al., 2017). A population-based analysis has not been able to find evidence that the approval of bevacizumab prolonged survival on a population level (Gramatzki et al., 2018). The assessment of the benefit from the therapy is performed with a neurological evaluation and MRI at least every three months. Pseudoprogression defined radiologically as a new or enlarging area(s) of contrast agent enhancement in the absence of actual tumour growth (Thust, van den Bent, & Smits, 2018), remains a concern within the first 3-6 months

after the conclusion of radiotherapy. In the case of pseudoprogression, essential attention is not to discontinue a potentially active treatment too early. Moreover, important tasks which need to be checked are the symptomatic seizures and the detection, treatment and prevention of venous thromboembolic events. Several clinical trials are currently carried out, but in particular, four-phase III trials could change the clinical practice in the upcoming years. For example, depatuxizumab, an antibody-drug conjugate, was tested in the same setting in the same setting in estimated glomerular filtration rate-amplied glioblastoma or nivolumab, a fully human immunoglobulin (Ig) G4 monoclonal antibody, is explored in the treatment of patients diagnosed with glioblastoma without and with MGMT promoter methylation. Moreover, the proteasome inhibitor marizomib is examined in newly diagnosed glioblastoma by the European Organisation for Research and Treatment of Cancer (Weller, Le Rhun, Preusser, Tonn, & Roth, 2019).

# HEME OXYGENASE

*Definition and characteristics*

Heme oxygenase (HO) is the rate-limiting enzyme in the catabolism of heme: this two-step enzymatic reaction results in the formation of equimolar amounts of biliverdin, iron and carbon monoxide (CO) (Ahmad, Salim, & Maines, 2002; Tenhunen et al., 1972) (Figure 2).

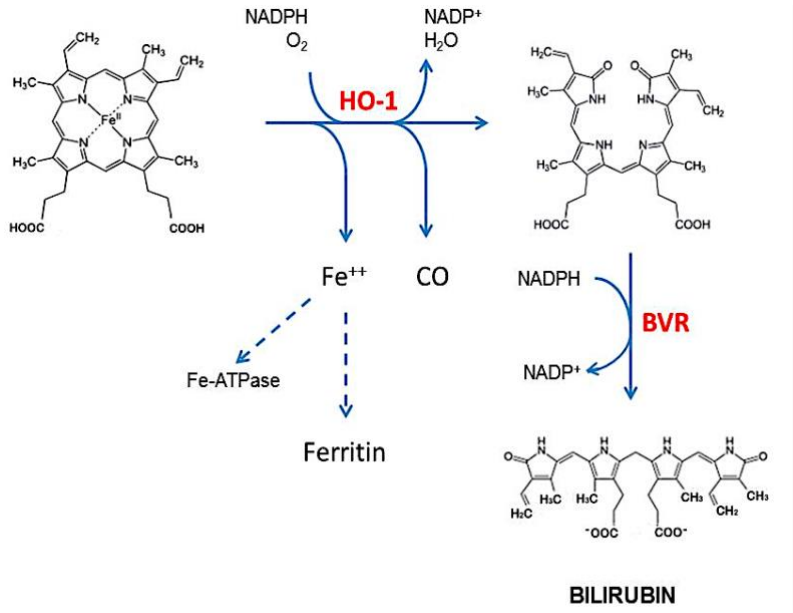


***Fig. 2: Heme degradation pathway***
*Adapted from Nitti, M., et al., HO-1 Induction in Cancer Progression: A Matter of Cell Adaptation. Antioxidants (Basel), 2017. 6(2).*

Tenhunen and collaborators described the catabolic reaction, the characterization of the enzyme and the induction with hemin (Tenhunen, Marver, & Schmid, 1968, 1969, 1970). HO exists in two isoforms, HO-1, the inducible form, and HO-2, the constitutive form which degrades heme in an identical stereospecific manner to biliverdin with the simultaneous release of CO and iron (Abraham, Feldman, Falck, Lutton, & Schwartzman, 1991). In mammals, biliverdin is rapidly converted into bilirubin by biliverdin reductase (Ahmad et al., 2002; Kapitulnik & Maines, 2009).

HO-1 is a 32-kDa inducible protein identified in 1990 as a stress protein involved in antioxidant and anti-inflammatory responses (Keyse & Tyrrell, 1989). HO-1 is present at low levels in most mammalian tissues, and it is highly upregulated by several oxidative stimuli: its substrate heme, heavy metals, UV irradiation, modified lipids, reactive oxygen species (ROS), growth factor and inflammatory cytokines (Foresti, Clark, Green, & Motterlini, 1997). The localization of HO-1 is mainly in cellular microsomes, although there are several demonstrations of its presence in the caveolae, mitochondria and nucleus (Converso et al., 2006; Grochot-Przeczek, Dulak, & Jozkowicz, 2012; H. P. Kim, Wang, Galbiati, Ryter, & Choi, 2004; Q. Lin et al., 2007).

HO-1 exhibits a particular cytoprotective activity which is exerted by its metabolites (Gozzelino, Jeney, & Soares, 2010; Loboda, Jozkowicz, & Dulak, 2015). Certainly, the release of free iron favours the synthesis of the heavy chain of ferritin and the activation of the membrane transporter Fe-ATPase, which allows cytosolic iron efflux: this transport decreases the intracellular Fe2+ concentration, preventing the generation of ROS through the Fenton reaction (Baker, Anderson, & Baker, 2003; Balla et al., 1992). Moreover, the production of bilirubin (BR) from biliverdin (BV) exerts potent antioxidant, anti-inflammatory and anti-apoptotic activity (Loboda, Damulewicz, Pyza, Jozkowicz, & Dulak, 2016; Loboda et al., 2015; Stocker, Yamamoto, McDonagh, Glazer, & Ames, 1987). In particular, bilirubin is able to scavenge hydroxyl radical, superoxide anions and singlet oxygen preventing protein and lipid peroxidation (Baranano, Rao, Ferris, & Snyder, 2002; Dudnik & Khrapova, 1998; He et al., 2015). Besides, carbon monoxide (CO) has an antiapoptotic and anti-inflammatory effect through the induction of soluble guanylyl cyclase (sGC), the modulation of mitogen-activated protein kinase (MAPK) pathway and the increase of cyclic guanosine monophosphate (cGMP) (Dennery, 2014; Gozzelino et al., 2010; Loboda et al., 2015).

These modulations exert different results, such as the stimulation of blood vessel formation, the induction of vascular endothelial growth factor (VEGF) synthesis and the production of endothelial cells (Dulak et al., 2002; Jozkowicz et al., 2003; Loboda et al., 2008).

Besides the well-characterized enzymatic activity defined as canonical, HO-1 can also act independently through non-canonical functions (Vanella et al., 2016). Certainly, HO-1 can translocate into the nucleus and regulate gene transcription, favouring in particular cancer tumour growth (Biswas et al., 2014; Q. Lin et al., 2007).

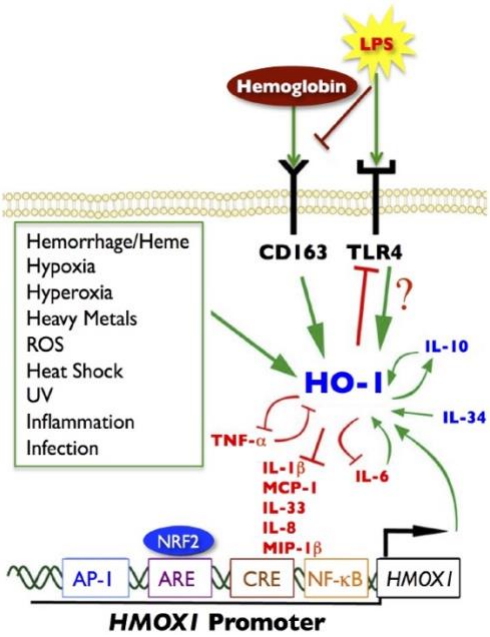


***Fig. 3: Signaling pathways of HO-1. Green arrows represent increased HO-1 expression or activity. Red blocked lines represent inhibition or decreased expression***
*Adapted from Ozen, M., et al., Heme oxygenase and the immune system in normal and pathological pregnancies. Front Pharmacol, 2015. 6: p. 84.*

HO-1 synthesis is regulated at a transcriptional level, and there are several binding sites for different transcription factor in its promoter region: in particular, this can be activated in oxidative stress conditions like AP-1, HIF-1, NF-$\kappa$B and Nrf2; Nrf2 is considered a potent regulator of HO-1 transcription (Alam & Cook, 2007; Anderson et al., 2001; Dennery, 2014).

The nuclear factor erythroid 2-related factor 2 (Nrf2) belongs to the Cap 'n' Collar (CnC)-bZIP (basic leucine zipper) transcription factor family, with Nrf1 and Nrf3 as well as the transcriptional repressors Bach1 and Bach 2: notably, Nrf2 plays a critical role in maintaining cellular redox homeostasis (Canning, Sorrell, & Bullock, 2015; Sykiotis & Bohmann, 2010). Nrf2 translocates into the nucleus when oxidative stressors modify Keap1 (Kelch ECH-associating protein 1): the transcription factor is dimerized inside the nucleus with Maf proteins and binds antioxidant/electrophile-response elements (ARE/EpRE sequences) exerting the transcription of target genes (Hirotsu et al., 2012; Tebay et al., 2015).

The binding of Nrf2 to ARE sequences is exceptionally complex, and Bach1 plays a crucial role in the regulation of the transcription of Nrf2 target gene, with particular attention to HO-1 (Davudian, Mansoori, Shajari, Mohammadi, & Baradaran, 2016).

Bach1 is a heme-binding protein with dimerizes with Maf proteins and binds to the ARE/EpRE sequences, acting as a transcriptional repressor of ARE-dependent genes. When the concentration of heme groups increases or under oxidative conditions, a conformational change off Bach1 supports its displacement from ARE sequences and its degradation to the proteasome, allowing Nrf2 to bind (Davudian et al., 2016; Ogawa et al., 2001).

HO-1 activity is also dependent from the specific localization of the enzyme: it is well described as being active in the mitochondria in lung epithelial cells exposed to LPS (lipopolysaccharide), hemin or cigarette smoke, while the plasma membrane localization prevents its catalytic activity (Slebos et al., 2007). Thus, in physiological conditions, the activation of Nrf2/HO-1pathway is involved in the maintenance of cellular homeostasis and plays a vital role in the adaptive response to stress: this kind of response represents a critical point in cytoprotection, prevention of carcinogenesis and cell survival (Guo, Yu, Zhang, & Kong, 2015; Marengo et al., 2016; Traverso et al., 2013). Otherwise, prolonged activation of Nrf2 and HO-1 in cancer cells is enigmatic and deleterious (Chau, 2015; Furfaro, Traverso, et al., 2016; Tebay et al., 2015).

*Heme oxygenase-1 role in cancer*

The expression of HO-1 correlates with cancer resistance to therapy and growth rate: this is demonstrated in different types of tumours such as human renal cell carcinoma, pancreatic and prostatic cancer, lymphosarcoma, hepatoma and melanoma (Goodman, Choudhury, da Silva, Schwartzman, & Abraham, 1997; Loboda et al., 2015; Maines & Abrahamsson, 1996; Schacter & Kurz, 1986; Was, Dulak, & Jozkowicz, 2010). Furthermore, the HO-1 expression is increased during chemotherapy, radiotherapy and photodynamic therapy, and it has been demonstrated that pharmacological inhibitors are able to sensitize cancers to therapies (Fang et al., 2012; Was et al., 2010). In particular, there are several studies which highlight the correlation between cancer and high levels of HO-1 with a loss of sensitivity to the treatment with etoposide, doxorubicin or cisplatin (Furfaro et al., 2012; Jeon et al., 2012; Lv, Song, Niu, & Wang, 2016; Tan et al., 2015). Whereas, HO-1 inhibition or silencing exacerbates cytotoxicity devouring apoptosis with downregulation of matrix metallopeptidase 9 (MMP-9) and VEGF levels (Jeon et al., 2012; Y. S. Liu et al., 2014; Lv et al., 2016; Yin et al., 2012).

HO-1 is also overexpressed in glioma cells, where the resistance to arsenic trioxide is reverted by HO-1 inhibition using Zinc II Protoporphyrin IX (ZnPPIX), which exerts enhanced apoptosis (Y. Liu et al., 2011). Furthermore, it has been demonstrated that Nrf2-dependent HO-1 induction prevents neuroblastoma cell death after glutathione (GSH) depletion or bortezomib treatment: HO-1 inhibition or silencing restore cell sensitivity (Furfaro et al., 2012; Furfaro, Piras, et al., 2016; Furfaro et al., 2014). However, the role of HO-1 in some tumour is controversial, and this different action of HO-1 overexpression is also related to invasiveness and metastatic potential. It has been proved that HO-1 is involved in the gain of metastatic phenotype of cancer, which shows more aggressive features such as the increase of cell ability to move, the capacity to surround tissue and favour the growth of new blood vessels to increase tumour mass and reach the bloodstream.

HO-1 overexpression increases viability, angiogenetic potential and proliferation of melanoma cells, decreasing tumour-bearing mouse survival (Was et al., 2006). Besides, the high expression of HO-1 is related to tumour invasiveness and poor clinical outcome in non-small cell lung cancer (NSCLC) patients (Tsai, Yeh, Huang, Tan, & Lu, 2012). Moreover, this overexpression contributes to higher microvessel

density, advanced tumour stage, metastasis and poor prognosis (Bronckaers, Gago, Balzarini, & Liekens, 2009). HO-1 upregulation is also involved in osteopontin-induced glioma cell invasion and migration and breast cancer proliferation (Deng et al., 2013; Lu et al., 2012). It is noteworthy that HO-1 upregulation is associated with tumour cell protection against anoikis, a particular type of apoptosis which follows matrix detachments, leading to metastatic properties of cancer cells (S. Dey et al., 2015). The protease inhibitor bortezomib (BTZ), approved for multiple myeloma, has been demonstrated to induce HO-1, even in brief treatment (Barrera, Rushworth, Bowles, & MacEwan, 2012). Nevertheless, the antiapoptotic and cytoprotective action of HO-1 seems to reduce the effect of BTZ (Yerlikaya, 2012): it is suggested that the nuclear localization, rather than the enzymatic activity, is involved in BTZ mediated chemoresistance (Tibullo et al., 2016). Moreover, the treatment with 17β-oestradiol (E2) has been demonstrated to increase HO-1 expression (Son et al., 2018): nowadays, the anti-carcinogenic effect of E2 is investigated in several studies, due to its capability to disrupt the intracellular iron metabolism with oxidative stress, membrane damage, and cell cycle arrest (Bajbouj, Shafarin, Abdalla, Ahmad, & Hamad, 2017).

The invasive and metastatic capabilities of tumours are related to the stimulation of angiogenesis. The role of HO-1 in angiogenesis is well reported both *in vitro* and *in vivo* in several studies (Bussolati et al., 2004; Grochot-Przeczek et al., 2012; Jozkowicz, Was, & Dulak, 2007; H. H. Lin, Chiang, Chang, & Chau, 2015; Loboda et al., 2008; Miyake et al., 2011). Carbon monoxide, derived from heme catabolism, stimulates blood vessel formation and induces VEGF and stomal-cell-derived factor 1 (SDF-1), leading to angiogenesis (Deshane et al., 2007; Jozkowicz et al., 2002; Loboda et al., 2008). In colorectal cancer cells, HO-1 inhibition suppresses the expression of HIF-1α and VEGF, with a remarkable decrease of angiogenesis in a mouse xenograft model (Cheng et al., 2016). Several studies have shown that the degradation pathway of heme is involved in the regulation of the immune system and that HO-1 plays a key role in the modulation of immune reactions within the tumour (Chauveau et al., 2005; Pae, Oh, Choi, Chae, & Chung, 2003; Wegiel et al., 2014). The tumour microenvironment is made of infiltrating immune cells, endothelial cells, extracellular matrix and signalling molecules.

The interaction mediates invasiveness, growth and metastatic abilities of tumours between cells and this environment (Friedl & Wolf, 2003; Schreiber, Old, & Smyth, 2011).

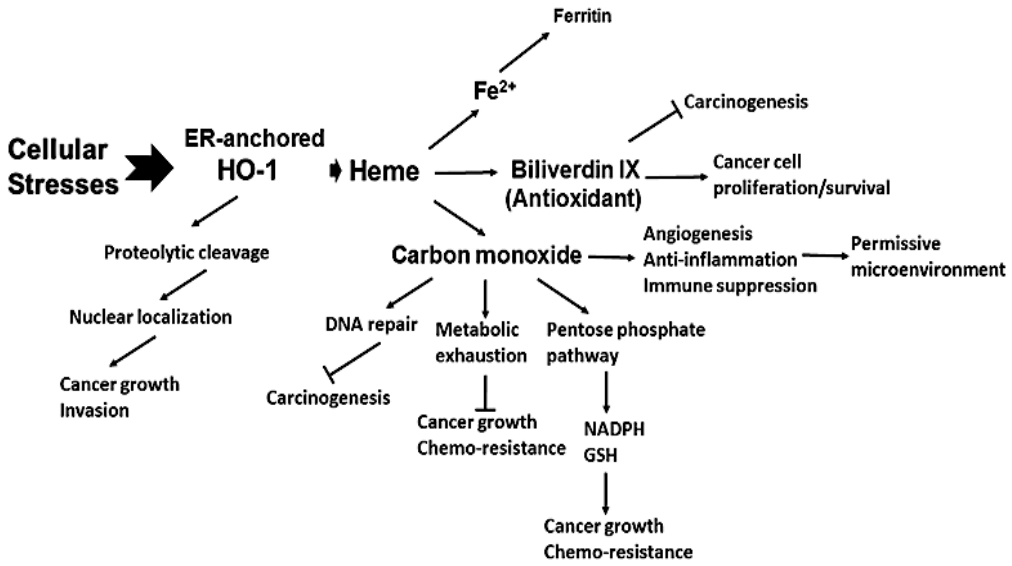


***Fig. 4: Multifaceted roles of HO-1 in cancer.***
*Adapted from Chau, L.Y., Heme oxygenase-1: emerging target of cancer therapy. J Biomed Sci, 2015. 22: p. 22.*

The main effector of HO-1 in the regulation of the immune system is CO which leads to pleiotropic effects in most immune cell types (Wegiel, Hanto, & Otterbein, 2013). For example, CO blocks dendritic cells (DC) maturation and modulates the cytokine release, inducing a tolerogenic phenotype.

Furthermore, it has been recently shown that bilirubin can act as an endogenous regulator of inflammation, exerting an impairment of adhesion molecules expression; although the role of HO-1-dependent bilirubin generation in cancer environment has not been demonstrated yet, it seems probable that it could play a role in immune-escape. Macrophages, which influences cancer progression, play another critical role in the tumour microenvironment. Indeed, macrophages with specific polarization phenotype can modulate the growth and the invasiveness of cancer cells, leading to a regulation of the immune response of T cells as well as angiogenesis and metastatic growth. Depending on their polarization state, macrophages (M1) can kill tumour cells and present tumour-associated antigens or exert tumour supportive activities (M2) by promoting invasion and metastasis (Siveen & Kuttan, 2009). The expression of HO-1 in stromal macrophages in different cancer tissue (e.g. gliomas) was described in the early 2000s (Nishie et al., 1999; Torisu-Itakura, Furue, Kuwano, & Ono, 2000). HO-1 is involved in macrophage polarization favouring a pro-angiogenic, interleukin 10 (IL-10) producing, M2 phenotype (Hao et al., 2012; Martinez, Sica, Mantovani, & Locati, 2008; Weis, Weigert, von Knethen, & Brune, 2009).

HO-1 positive macrophages induce the suppression of the immune response, leading to a poor outcome of cancer patients (Hao et al., 2012; Lewis & Pollard, 2006; Martinez et al., 2008; Murdoch, Giannoudis, & Lewis, 2004; Nishie et al., 1999; Siveen & Kuttan, 2009; Weis et al., 2009): for example, in a transplanted model of pancreatic ductal adenocarcinoma, a subset of M2 macrophages mediate immune suppression by the induction of HO-1 (Arnold, Magiera, Kraman, & Fearon, 2014). Moreover, an essential role in tumour recognition is played by natural killer (NK) cells which are actively involved in the early immune response to tumour cells (Orange, 2008). Indeed, activated NK cells express important receptors (NKp30, NKp44 and NKp46) that recognize specific ligands in the tumour inducing apoptosis or the recruitment of other immune cells (Bryceson et al., 2011; Gomez-Lomeli et al., 2014; Gross, Sunwoo, & Bui, 2013). Nevertheless, the connection between HO-1 and NK-mediated tumour recognition is still unclear. It is demonstrated that the inhibition of HO-1 in different cervical cancer cell lines (CCC) increases the expression of INF-α and TNF-α with restored expression of NK receptors and markers of NK activation (Gomez-Lomeli et al., 2014).

*Non canonical functions of HO-1*

HO-1 protein can localize in multiple subcellular compartments, including caveolae and mitochondria: it is well demonstrated that HO-1 can be localized in the nucleus after its c-terminal cleavage (Vanella et al., 2016).

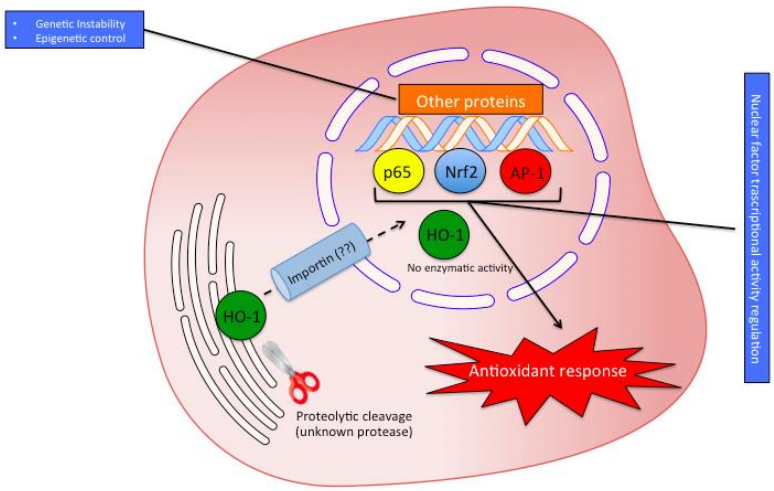


***Fig. 5: Possible significance of HO-1 nuclear translocation.***
*Adapted from Vanella, L., et al., The non-canonical functions of the heme oxygenases. Oncotarget, 2016. 7(42): p. 69075-69086.*

Several studied demonstrate that HO-1 is preferentially localized into the nucleus in a different type of cancer such as prostate, lung, oral cancer and chronic myeloid leukaemia with a strong correlation with cancer progression (Degese et al., 2012; Dennery, 2014; Gandini et al., 2012; Hsu et al., 2015; Sacca et al., 2007; Tibullo et al., 2013).

Moreover, HO-1 nuclear localization was also demonstrated in metastatic cells of prostate cancer and head and neck cancer (Gandini et al., 2012; Gueron et al., 2009). In lung cancer expression high levels of HO-1, the signal peptide peptidase (SPP) catalyse the cleavage of HO-1, with an evident positive nuclear staining. Also, it has been demonstrated that the overexpression of a truncated form of HO-1 in cervical carcinoma and lung cancer promotes proliferation and invasiveness (Hsu et al., 2015). Interestingly, it has been shown that the HO-1 nuclear translocation is responsible for imatinib resistance in chronic myeloid leukaemia: in particular, the treatment with the protease inhibitor Ed64, which prevents the nuclear translocation, enhances the cytotoxicity of imatinib (Tibullo et al., 2013). In support of the hypothesis that nuclear localization of HO-1 could be involved in cancer progression, it has been demonstrated that nuclear HO-1 interacts with Nrf2, increasing its stabilization (Beckner, Stracke, Liotta, & Schiffmann, 1990). Nonetheless, it is essential to underline that there are some opposite observations and demonstrations. Indeed, in prostate cancer, the nuclear localization of HO-1 exerts anti-tumorigenic effects inhibiting cell proliferation, invasion and migration both *in vitro* and *in vivo* or acting on the NF-κB pathway preventing the angiogenetic switch (Ferrando et al.,

2011). The mechanism which connects HO-1 nuclear translocation and its pathological significance in tumours need to be investigated further.

*HO-1 as a possible biomarker of disease*

Lately, some studies explored the prognostic significance of HO-1 overexpression in human cancer, likewise the possible correlation with clinical features and patients outcome (Miyake et al., 2010; Wang et al., 2010; Yokoyama, Mita, Okabe, Abe, & Ogawa, 2001).

For example, NSCLS patients with metastasis and advanced-stage disease (III-IV) show a higher and significant expression of HO-1 compared to early-stage or non-metastatic patients: in particular, HO-1 expression inversely correlates with patient survival (J. R. Tsai et al., 2012). HO-1 and Nrf2 expression are both elevates in bladder cancer: in tissue specimens from patients with primary non-muscle-invasive bladder cancer (NMIBC) the high expression was detected in 33% of all primary MNIBC cases; in particular, the expression was strongly associated with adverse pathological characteristics, tumour progression, progression-free survival and lower recurrence-free survival (J. H. Kim & Park, 2014; Kozakowska et al., 2016).

Moreover, a univariate Kaplan-Meier analysis on neuroblastoma samples revealed that high HO-1 mRNA expression levels are associated with an unfavourable prognosis (Fest et al., 2016). HO-1 expression was also higher in histological samples of human glioma compared to non-malignant brain tissue: no differences were demonstrated among the various tumour grades. Still, HO-1 protein expression was associated with a worse prognosis of grade II and grade II astrocytoma patients (Gandini et al., 2014).

However, the role of HO-1 in regulation is highly tissue- and cell-specific and differently involved in tumour biology (Loboda et al., 2015). The specific role of HO-1 in tumour progression is far from being completely cleared, but HO-1 and heme catalysis pathway can be modulated in a strategic way for cancer therapy.

A new strategy focuses the attention on the inhibition of HO-1, and different inhibitors have been identified (Pittala, Salerno, Romeo, Modica, & Siracusa, 2013). The metalloporphyrins, such as Sn-, Zn- or Cr-protoporphyrin, are structurally similar to heme, and they have been used as prototypic inhibitors even if their application is limited (Loboda et al., 2015). Indeed, ZnPPIX acts also on other pathways such as cyclin D1, Wnt/β-catenin and indoleamine-2,3-dioxygenase;

moreover, due to the poor solubility, its application *in vivo* is minimal (Lu et al., 2012). However, new modifications of ZnPPIX have been realized, such as the pegylated form PEG-ZnPPIX, which is characterized by an increased circulation time, permeability and retention (Sahoo et al., 2002).

Currently, new water-soluble imidazole-based molecules have been realized to be an HO-1 inhibitor with a better specificity against HO-1, increasing the possibilities to develop new therapeutic strategies (Pittala et al., 2013).

# CHAPTER I

Molecular Neurobiology (under review)

# HEME OXYGENASE-1 AND CARBON MONOXIDE REGULATE GROWTH AND PROGRESSION IN GLIOBLASTOMA CELLS

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## ABSTRACT

In human glioma tumours, heme oxygenase-1 (HMOX-1) is upregulated both when compared with normal brain tissues and also during oligodendroglioma progression. Besides, the molecular mechanisms triggered by HMOX-1 to promotes glioblastoma remains unknown. We therefore aimed at investigating the effect of HMOX-1 expression and enzymatic inhibition in two different cell lines (i.e. A172 and U87-MG). HMOX-1 was induced by hemin treatment (10 μM), and VP13/47 (100 μM) was used as a specific non-competitive inhibitor of HMOX-1 activity. Cell proliferation was measured by cell index measurement (xCelligence technology) and clonogenic assay, whereas cell migration was assessed by wound healing assay. Carbon Monoxide Releasing Molecules (CORMs) (i.e. CORM-3 and CORM-A1) were also used in a separate set of experiments to confirm the effect of HMOX-1 by-product in glioblastoma progression further. Our results were further validated using GSE4412 microarray dataset analysis and comparing biopsies overexpressing HMOX-1 with the rest of the cases. Our results showed that hemin was able to induce both HMOX-1 gene and protein expression in a cell-dependent manner being A172 more responsive to pharmacological upregulation of HMOX-1. Hemin, but not CORMs treatment, resulted in a significant increase of cell proliferation following 24h of treatment as measured by increased cell index and colony formation capacity and such effect was abolished by VP13/47. Interestingly, both hemin and CORMs showed a significant effect on the wound healing assay also exhibiting a cell specificity. Finally, our dataset analysis showed a positive correlation of HMOX-1 gene expression with ITGBI and ITGBII which are membrane receptors involved in cell adhesion, embryogenesis, tissue repair, immune response and metastatic diffusion of tumour cells. In conclusion, our data suggest that HMOX-1 and its byproduct CO exhibit a cell-specific effect on various aspects of disease progression and is associated with a complex series of molecular mechanisms driving cell proliferation, survival and metastasis.

## INTRODUCTION

Gliomas are the most common primary tumours of the central nervous system (CNS) in the adult. Glioblastoma represents half of the newly diagnosed gliomas, and it is the most aggressive brain tumour and has a median patient survival of 14-17 months [1,2]. Current treatment for gliomas remains suboptimal, and the promise for improved therapies rests mainly on a better understanding of the underlying biology and genetics of these tumours. The determination of isocitrate dehydroidrogenase 1 or 2 (IDH 1/2) mutation is one of the earliest events occurring in gliomas and identifies biologically different tumours with different clinical behaviour and improved prognosis when compared to wild-type gliomas [3-6]. The majority of patients with glioblastoma can be treated with temozolomide, a cytotoxic alkylating agent, which is administrated during radiotherapy (75 mg/m$_2$ daily) followed by six cycles of maintenance therapy (150-200 mg/m$_2$, 5/28 days) [7,8]. Limitations in this therapy include significant comorbidities such as haematological of hepatic disease. The most common side effect is myelosuppression with thrombocytopenia [9]. Targeted therapies directed to some of these ubiquitous cancer-associated targets (i.e., erlotinib and gefitinib) have unfortunately met with

limited success [10], further reinforcing the need for the identification of glioma-specific novel molecular targets. Heme oxygenase (HO) is the rate-limiting enzyme in the catabolism of heme: this two-step enzymatic reaction results in the formation of equimolar amounts of biliverdin, iron and carbon monoxide (CO) [11,12]. HO exists in two forms, HMOX-1, the inducible form, and HO-2, the constitutive form which degrades heme in an identical stereospecific manner to biliverdin with the simultaneous release of CO and iron [13]. In mammals, biliverdin is rapidly converted into bilirubin by biliverdin reductase [14,11]. Heme oxygenase-1 (HMOX-1) is a 32-kDa inducible protein identified as a stress protein involved in antioxidant and anti-inflammatory responses [15]. HMOX-1 is present at low levels in most mammalian tissues, and it is highly upregulated by several oxidative stimuli: its substrate heme, heavy metals, UV irradiation, modified lipids, reactive oxygen species (ROS), growth factor and inflammatory cytokines [16]. The localization of HMOX-1 is mainly in cellular microsomes, although there are several demonstrations of its presence in the caveolae, mitochondria and nucleus [17-20]. Besides the well-characterized enzymatic activity defined as canonical, HMOX-1 can also act independently through non-canonical functions [21].

Certainly, HMOX-1 can translocate into the nucleus and regulate gene transcription, favouring in particular cancer tumour growth [20,22]. The expression of HMOX-1 correlates with cancer resistance to therapy and growth rate: this is demonstrated in different types of tumours such as human renal cell carcinoma, pancreatic and prostatic cancer, lymphosarcoma, hepatoma, melanoma and multiple myeloma [23-28]. Furthermore, the HMOX-1 expression is increased during chemotherapy, radiotherapy and photodynamic therapy, and it has been demonstrated that pharmacological inhibitors can sensitize cancers to therapies [27,29]. In particular, there are several studies which highlight the correlation between cancer and high levels of HMOX-1 with a loss of sensitivity to the treatment with etoposide, doxorubicin or cisplatin [30-33]. Whereas, HMOX-1 inhibition or silencing exacerbates cytotoxicity devouring apoptosis with downregulation of matrix metallopeptidase 9 (MMP-9) and VEGF levels [34,35,32,33]. HMOX-1 is also overexpressed in glioma cells, where the resistance to arsenic trioxide is reverted by HMOX-1 inhibition using Zinc II Protoporphyrin IX (ZnPPIX), which exerts enhanced apoptosis [36]. Consistently, a univariate Kaplan-Meier analysis on neuroblastoma samples revealed that high HMOX-1 mRNA expression levels are

associated with an unfavourable prognosis [37]. HMOX-1 expression was also higher in histological samples of human glioma compared to non-malignant brain tissue: no differences were demonstrated among the various tumour grades. Still, HMOX-1 protein expression was associated with a worse prognosis of grade II and grade II astrocytoma patients [38]. The present study aimed to assess the effect of HMOX-1 on cell proliferation and migration in two different glioblastoma cell lines and the possible molecular mechanisms underlying such an effect in a clinical setting.

## MATERIAL AND METHODS

*Cell culture and pharmacological treatments*

Human glioblastoma cells (A172 and U87-MG) were purchased from ATCC Company (Milan, Italy). Cells were suspended in DMEM (Gibco, Cat. # 11965092) culture medium containing 10% fetal bovine serum (FBS, Gibco, Cat. #10082147), 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco, Cat. # 15070063). At 80% confluency, cells were passaged using trypsin-EDTA solution (0.05% trypsin and 0.02% EDTA, Gibco, Cat. # 25300054) (Sacerdoti et al., 2005). Pharmacological treatments were performed using Hemin 10μM (Sigma Aldrich, Cat. #51280), CORM-A1 25μM (Sigma Aldrich, Cat. #SML0315) and CORM-3 25μM (Sigma Aldrich, Cat. #SML0496) and VP13/47 100μM.

*Real-Time Monitoring of cell proliferation*

xCELLigence experiments were performed using the RTCA (Real-Time Cell Analyzer) DP (Dual Plate) instrument according to manufacturers' instructions (Roche Applied Science, Mannheim, Germany, and ACEA Biosciences, San Diego,

CA). The RTCA DP Instrument includes three main components: (i) RTCA DP Analyzer, which stays inside a humidified incubator maintained at 37°C and 5% CO2, (ii) RTCA Control Unit with RTCA Software preinstalled, and (iii) E-Plate 16 for proliferation assay. First, we defined the optimal seeding number by cell titration and growth experiments to obtain a significant cell index value and a constant cell growth (data not shown). We added 100µl of cell culture media in the E-plate 16, and we left it in the tissue culture hood for 30 minutes at room temperature: this procedure ensures the equilibrium between the culture media and E-Plate surface. We inserted into the E-plate 16 into a cradle pocket of the RTCA DP Analyzer, and we performed a blank reading to measures the background impedance of cell culture media. We added 100µl of a cell solution with a final concentration of 2500 cells/well in the E-plate 16 and, as recommended, we waited 30 minutes before starting the automatic monitoring every 15 min for 24h.

*Cell migration*

Cell proliferation was studied by employing the "wound healing" assay. Cells were seeded separately in 6 wells dishes and cultured until confluence. Cells were scraped

with a 200 µl micropipette tip and monitored at 0 h, 4 h, 8 h, 24 h and 48 h. The uncovered wound area was measured and quantified at different intervals with ImageJ 1.37v (NIH).

*Real Time PCR for gene expression analysis*

RNA was extracted by Trizol® reagent (Cat. # 15596026 Invitrogen, Carlsbad, CA, USA). First-strand cDNA was then synthesized with High-Capacity cDNA Reverse Transcription Kit (Cat. #4368814 Applied Biosystems, Foster City, CA, USA). High cDNA quality was checked, taking into consideration the housekeeping gene Ct values. Quantitative real-time PCR was performed in Step One Fast Real-Time PCR System Applied Biosystems, using the SYBR Green PCR MasterMix (Cat. #4309155 Life Technologies, Monza, Italy). The specific PCR products were detected by the fluorescence of SYBR Green, the double-stranded DNA binding dye. Primers were designed using BLAST® (Basic Local Alignment Search Tool, NBCI, NIH), considering the shortest amplicon proposed: primers': β-actin was used as the housekeeping gene. Primers were purchased by Metabion International AG (Planneg, Germany). The relative mRNA expression level was calculated by the

threshold cycle (Ct) value of each PCR product and normalized with β-actin by using a comparative $2^{-\Delta\Delta Ct}$ method.

*Clonogenic assay*

Colony assays performed by seeding cells in 6 wells plates at low density (5000 cells/well) and allowing growth for 10 days. Colonies were fixed, stained with crystal violet and manually counted.

*Microarray dataset selection and analysis*

The NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) was used to select GSE4412 (GPL96) transcriptome dataset of 85 brain biopsies of grades III and IV gliomas of various histologic types. In particular, we focused our attention only to the 59 glioma grade IV samples data. Complete experimental details can be retrieved in the following publication [39]. Also, we analyzed another microarray dataset, GSE50161 [40], to compare the expression levels of HMOX-1 to healthy control brain subjects and various brain tumour types.

To process and identify Significantly Different Expressed Genes (SDEG) in the datasets, we used the MultiExperiment Viewer (MeV) software. In cases where multiple genes probes have insisted on the same GeneID NCBI, we have used those with the highest variance[41]. The significance threshold level for all data sets was $p < 0.05$. The genes with $p < 0.05$ were identified as significantly differentially expressed genes (SDEG) and selected for further analysis. The analysis of microarray data by Z-score transformation was used to allow the comparison of microarray data independent of the original hybridization intensities. To identify the genes common modulated with HMOX-1 in glioma samples and the hypothetical network activated, we selected and stratified the data from glioma samples sorted from GSE4412, using the HMOX-1 z-score as a parameter. The samples with HMOX-1 high z-score ($2^{\wedge\text{-z-score}}$) (3.09± 1.06) formed the Q1 upper quartile ($75^{th}$ percentile), which included 16 subjects. The group with low z-scores ($2^{\wedge\text{-z-score}}$) of HMOX-1 (1.05±0.07) corresponded to the subjects in the 25th percentile (Q4, lower quartile) and included 15 subjects. We compared the two group and selected the SDEG. We obtained 2869 significantly upregulated and 2967 downregulated genes. The genes Ontology analysis was performed using the web utility GeneMANIA

(http://genemania.org/) [42] and STRING (https://string-db.org/) [43]. The STRING was also used for building the weighted gene networks commonly modulated. The database assembles all available interaction data in the dataset by creating massive networks, which captures the current knowledge on the functional modularity and interconnectivity of genes in a cell.

*Statistical Analysis*

Statistical significance ($p < 0.05$) of differences between experimental groups was determined by the Fisher method for analysis of multiple comparisons. For comparison between treatment groups, the null hypothesis was tested by either single-factor analysis of variance (ANOVA) for multiple groups or the unpaired *t*-test for two groups, and the data are presented as mean ± SD. For the microarray dataset, we performed statistical analysis with GEO2R, applying a Benjamini & Hochberg (False discovery rate) to adjust P values for multiple comparisons. For statistical analysis, Prism 7 software (GraphPad Software, USA) was used. Based on the Shapiro-Wilk test, almost all data were skewed, so nonparametric tests were used. Significant differences between groups were assessed

using the Mann–Whitney U test, and the Kruskal-Wallis test was performed to compare data between all groups followed by Dunn's post hoc test. Correlations were determined using Spearman's ρ correlation. All tests were two-sided, and significance was determined at $P < 0.05$.

## RESULTS

### *Effect of pharmacological modulation of HMOX-1 and its byproducts on cellular proliferation and migration*

We firstly aimed at studying the effect of pharmacological manipulation of HMOX-1 on cell proliferation and migration. For this purpose, we induced HMOX-1 in two glioma cell lines A172 and U87-MG by hemin treatment. Our results showed that hemin treatment resulted in a significant increase of HMOX-1 gene and protein expression (Figure 1A-D) in both cell lines. Interestingly, we observed a different pattern of induction for both gene and protein expression in the two cell lines. In particular, treatment with Hemin (10 μM) for 8h induced a significant increase of HMOX-1 gene expression of nine folds in A172 cells in comparison to untreated cells (Figure 1A) whereas the same treatment induced an increase of about

two folds in U87-MG cells in comparison to untreated cells (Figure 1B). Moreover, protein expression increased about seven folds in A172 cells (HO-1/Actin ratio, Figure 1C) whereas an increase of four folds in protein expression was found when U87-MG cells were treated with Hemin (HO-1/Actin ratio, Figure 1D). Furthermore, treatment with CORM-A1 and CORM-3 (both 25 and 50 μM) in A172 decreased HO-1 protein expression when compared to untreated cells. By contrast, only CORM-3 was able to induce HO-1 protein expression when compared to untreated cells, whereas CORM-A1 (50 μM) reduced protein expression when compared to control cells. We, therefore, tested the effect of such induction on cell proliferation and migration. As shown in Figure 2A, hemin treatment resulted in a significant increase in cell proliferation in A172 cells, as showed by cell index performed by xCelligence technology. By contrast, we observed that hemin was not able to modify cell proliferation in U87-MG cell line (Figure 2B). To further confirm the involvement of HMOX-1 in the effects induced on cell proliferation by hemin, cell cultures were treated concomitantly with hemin and VP13/47, a potent non-competitive and specific inhibitor of HMOX-1 activity. These set of experiments showed that HMOX-1 activity inhibition abolished the

proliferative effect of hemin in the A172 cell line (Figure 2A). Interestingly, selective HMOX-1 activity inhibition resulted in a significant decrease in cell proliferation when compared to untreated cells. By contrast, the selective HMOX-1 activity inhibition in U87-MG cell line resulted in a significant increase in cell proliferation when compared to untreated cells (Figure 2B). Also, CORM treatments resulted in a significant increase in cell proliferation in the A172 cell line (Figure 2C) whereas had no significant effects in U87-MG cell line (Figure 2D). These results were further confirmed by clonogenic assay showing that hemin treatment induced a significant increase of colony formation in the A172 cell line (Figure 3A). Again, hemin treatment had no significant effect on colony formation in U87-MG cell line (panel B). Interestingly, HMOX-1 activity inhibition following VP13/47 treatment resulted in a significant decrease of colony formation in the A172 cell line, but no significant effect was observed in U87-MG cell line. Infiltration throughout the brain is a prominent feature of low- and high-grade malignant gliomas usually involving the activation of several processes, including the migratory properties of the cells. Therefore, we analysed the involvement of HMOX-1 on human glioma cell migration through a wound closure assay. As shown in Figure 4A, hemin decreased

the migratory rate significantly in A172 cells. Treatment of A172 cells with Hemin *plus* VP 13/47 for 24 and 48h induced a significant inhibition of cell migration in comparison to untreated cells, maintaining the percent of wideness very similar to those of untreated cells at 0h, indicating the cooperative effect of the two molecules. No significant increase of migratory cells was observed following treatment of U87-MG cell line with Hemin alone and in association with VP 13/47 (Figure 4B) in comparison to untreated cells at the same time. Interestingly, CORM-A1 and CORM-3 were able to induce migration in A172 (Figure 4C). Finally, CORMs treatment had no significant migratory effect in U87-MG cell line (Figure 4D).

*HMOX-1 expression level is related to chemotaxis genes in glioma patients*

During our GSE4412 microarray dataset analysis, we showed that the expression levels of HMOX-1 in Gliomas grade IV brain biopsies were related to the chemotaxis genes (Figure 5) (Supplementary Figure 1). The analysis of microarray dataset highlighted that HMOX1 expression levels were significantly increased in Glioma Grade IV brain biopsies compared to Glioma grade III (Figure 5A). Further, to identify the genes common modulated with HMOX-1 in Glioma grade IV samples, we selected and stratified the data using the HMOX-1 z-score as a

parameter (Figure 5B and C). Among the Top 50 genes positively correlated to HMOX1 expression levels (Figure 5D), we highlighted two genes belonging to the family of cell surface receptor, the ITGBI (p=3.4E-9) and ITGBII (p=1.7E-8) (Figure 5E/F). These two genes are membrane receptors involved in cell adhesion, embryogenesis, tissue repair, immune response and metastatic diffusion of tumour cells. As regards the top 50 genes negatively correlated to HMOX1 expression levels in Glioma Grade IV brain biopsies (Figure 5G), we selected PUM2 (*pumilio RNA binding family member 2*) (p=1.05E-6) and FBXW11 (p=1.6E-6), the first most significantly modulated (Figure 5H/I). PUM2 play a role in repressor during cell differentiation. As regard FBXW11 is a member of the *F-box protein family* which function in phosphorylation-dependent ubiquitination.

## DISCUSSION

The present study aimed to investigate the effect of HMOX-1 expression and enzymatic inhibition in two different cell lines (i.e. A172 and U87-MG) and to unravel possible molecular mechanisms by which HMOX-1 promotes glioblastoma progression. In the results presented in our manuscript, there were also some similarities and differences between A172 and U87-MG cell lines.

In particular, hemin treatment was able to induce both gene and protein expression in both cell lines even though A172 cells showed to be more responsive in terms of folds of increase and timing of expression. Furthermore, we also observed a similar biological effect of HMOX-1 upregulation which results in an increased proliferation and colony formation capacity. These results are consistent with our previous studies showing that HMOX-1 and its by product play a significant role in controlling cell proliferation via regulating essential protein involved in cell cycle progression (i.e. p21) [44,45]. Additionally, some reports demonstrated that HMOX-1 has a cytoprotective role in glioma and provided evidence that HMOX-1 could be a potential therapeutic target in this cancer type [36]. Also, several groups showed that a decrease [36] or an increase [46] in HMOX-1 is necessary for the anticancer effects

of many compounds on human glioma cells. All these reports point to a role of HMOX-1 in glial cell pathophysiology and suggest that HMOX-1 expression in these cell types might have a role in glioma progression and/or treatment, thus supporting our results of HMOX-1 expression by the glial cells within the tumour.

An increasing body of evidence indicates that HMOX-1 also plays an essential role in cancer. It has been suggested that eight hallmark capabilities and two enabling characteristics are necessary for a complete tumour formation and progression. The first eight capabilities are: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, reprogramming of energy metabolism, evading immune destruction, and activating invasion and metastasis. The enabling characteristics are genome instability and inflammation [47]. There is evidence of HMOX-1 being related to most of these capabilities and the two enabling characteristics [48]. There are conflicting reports as to the role HMOX-1 plays in tumour initiation and progression since it has been demonstrated that HMOX-1 can play a role as a tumour-promoter molecule or have inhibitory effects on tumour progression [48]. For example, in prostate cancer, HMOX-1 overexpression has been shown to reduce cellular

proliferation and migration [49] and to negatively modulate the angiogenic switch [50], thereby exerting antitumoral effects. Contrariwise, HMOX-1 has been shown to stimulate the proliferation of melanoma, hepatoma, sarcoma, and pancreatic cancer cells, thus suggesting a permissive role of HMOX-1 in tumour growth [27]. In this sense, the study of HMOX-1 prognostic significance in human tumours is relevant, and again, the reports show contradictory results. For example, in bladder [51,4], and lung cancer [34] HMOX-1 expression was significantly associated with recurrence and progression, whereas in tongue squamous cell carcinoma, low expression correlated with lymph node metastasis [52]; in colorectal cancer, the expression of HMOX-1 was associated with a longer survival time [53]. Concerning the role of HMOX-1 in glioma progression, we investigated the correlation of HMOX-1 expression with some clinicopathological parameters important in the prognosis of gliomas. Interestingly, we observed that the HMOX-1 expression levels were higher in all histological subtypes and all grades analysed when compared to the non-malignant brain (an exception was medulloblastoma) (Supplementary Figure 1) and in Glioma Grade IV brain biopsies compared to Glioma grade III. Importantly, HMOX-1 protein expression was associated with worse prognosis of grade II and III

astrocytoma patients, and although this subgroup of astrocytoma was small, the results were corroborated at the mRNA level in univariate and multivariate analyses using the REM- BRANDT data. Furthermore, the results demonstrating an increase in HMOX-1 protein in all glioma subtypes are also reflected at the mRNA level, as shown in the REMBRANDT database. Altogether, these results show that HMOX-1 is an independent prognostic factor and that its expression could be relevant in determining the malignant behaviour of grade II and III astrocytomas (Supplementary Figure 1). In this work, we highlighted the correlation of HMOX1 gene expression with two other genes. PUM2 and FBXW11. PUM2 is an RNA binding protein and functions as a translational repressor. It promotes stem cell proliferation via repressing cell cycle regulators expression. One of the well-known cell cycle regulators, BTG1, functions as a tumour suppressor by keeping cell cycle at the G0 phase and its expression is downregulated in multiple malignant tumours. Studies on bladder cancer development showed that up-regulation of PUM2 is associated with inhibited cancer growth [54], while one recent study on leukaemia showed that PUM2 sustains myeloid leukaemia cell growth [55]. The analysis of microarray dataset highlighted that HMOX1 expression levels were inversely

correlated with PUM2 expression suggesting that PUM2 may be a repressor of cell proliferation in glioblastoma. Interestingly, we observed also a negative correlation with FBXW11 (betaTrCP2) an enzyme able to ubiquitinylate p-IκBα, thus promoting NF-κB heterodimers to translocate to the nucleus and activate the transcription of metastasis-related genes. Besides, betaTrCP can target Nrf2 for proteasomal degradation leading to an inactivation of HMOX1 gene expression. These data suggesting also that cell proliferation induced by upregulation of HMOX-1 was not connected with NF-κB activation.

In conclusion, our studies provide strong evidence of HMOX-1 overexpression in human gliomas compared with non- malignant samples. Furthermore, the expression of HMOX-1 was observed in tumour cells and was associated with a worse prognosis in patients with grade II and III astrocytoma. Preliminary analyses of the significance of HMOX-1 and its correlation with outcome in gliomas suggest that the enzyme might be involved in tumour cell proliferation. Altogether, these results point to a pro-tumoral role of HMOX-1 in glioma progression.

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**Author Contributions**

CCC and GLV: Made a substantial contribution to the concept and design, acquisition of data or analysis and interpretation of data; GLV: Drafted the article or revised it critically for important intellectual content; LL, AD, GL, DC, DT, VP and GLV: performed in vitro experiments; MDR: analyzed GEO datasets and performed statistical analysis; all the authors: Approved the version to be published.

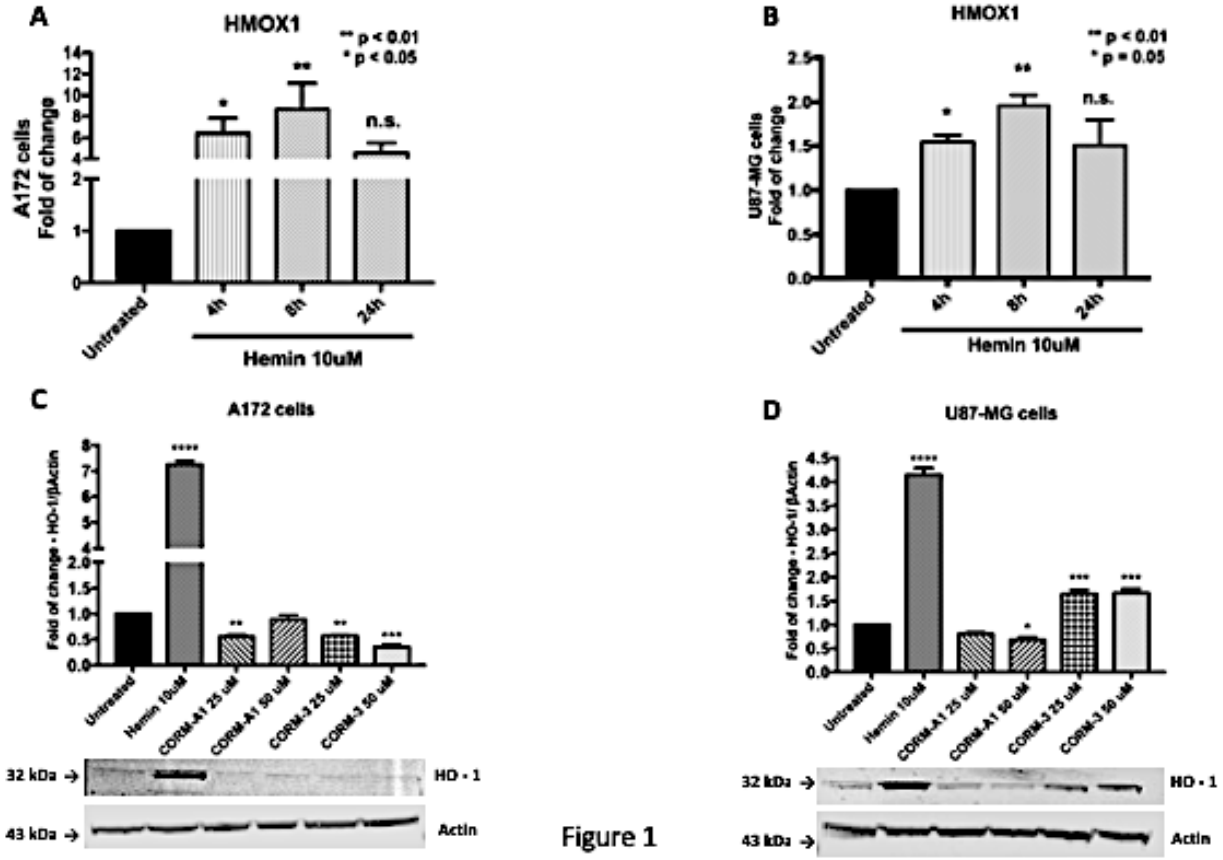



Figure 1

**Figure 1.** Heme Oxygenase-1 gene expression in A172 cells (A) and in U87-MG cells (B) and protein expression in A172 cells (C) and in U87-MG cells (D). Values represent the means ± SD of 4 experiments performed in triplicate. P values <0.05 were considered to be statistically significant (*p<0.05; **p<0.1 vs Untreated)

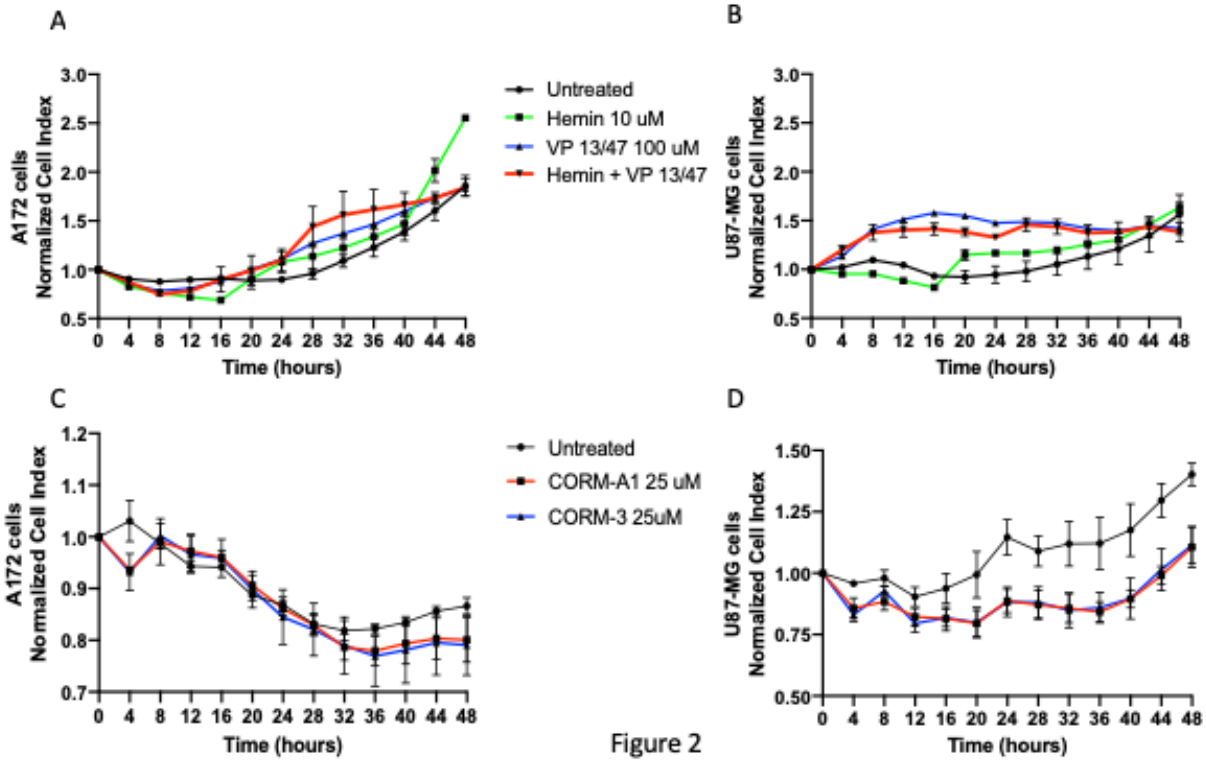



Figure 2

**Figure 2.** Real-time cell proliferation monitoring by xCELLigence system following treatments with Hemin, VP 13/47 and Hemin *plus* VP 13/47 of A172 cells (A) and U87-MG cells (B) and following treatments with CORM-A1 and CORM-3 of A172 cells (C) and U87-MG cells (D). Cell index values were normalized at the time of pharmacological treatments in order to obtain a normalized cell index. Each line is expressing the average of four different experiments. P values <0.05 were considered to be statistically significant.

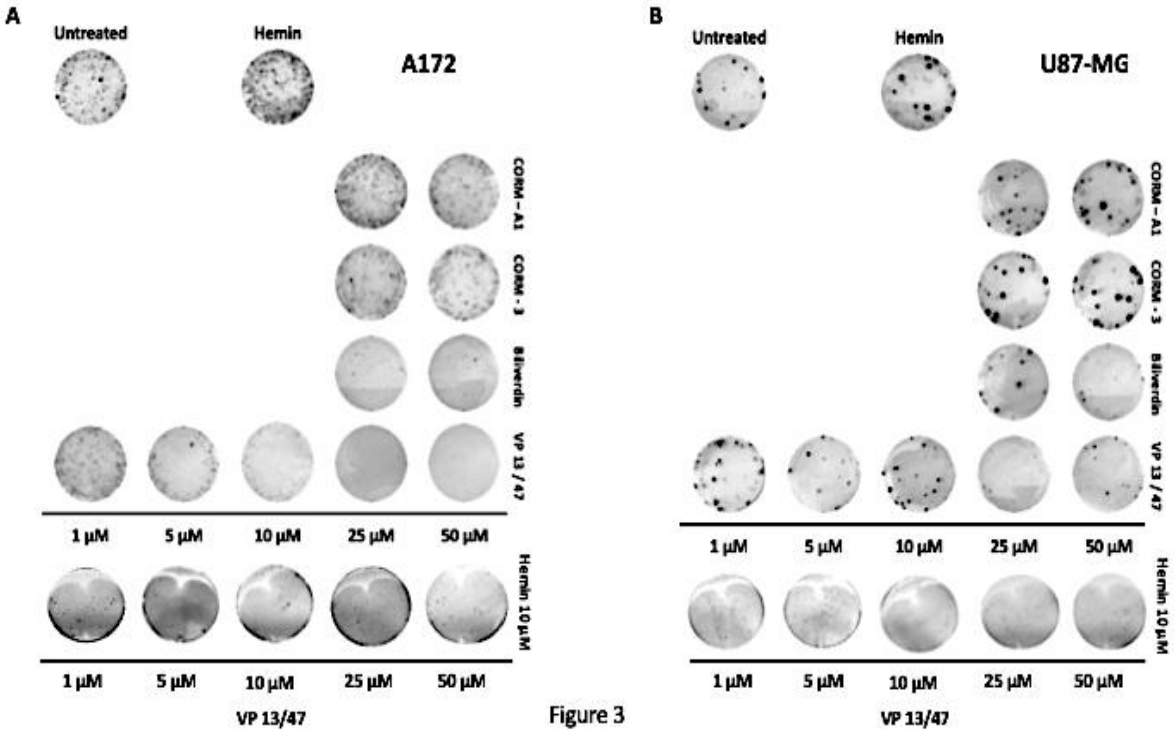



Figure 3

**Figure 3.** Colony formation capacity following treatments of A172 cells (A) and U87-MG cells (B) with Hemin, CORM-A1, CORM-3, Biliverdin and VP 13/47. Images are representative of four separate experiments.

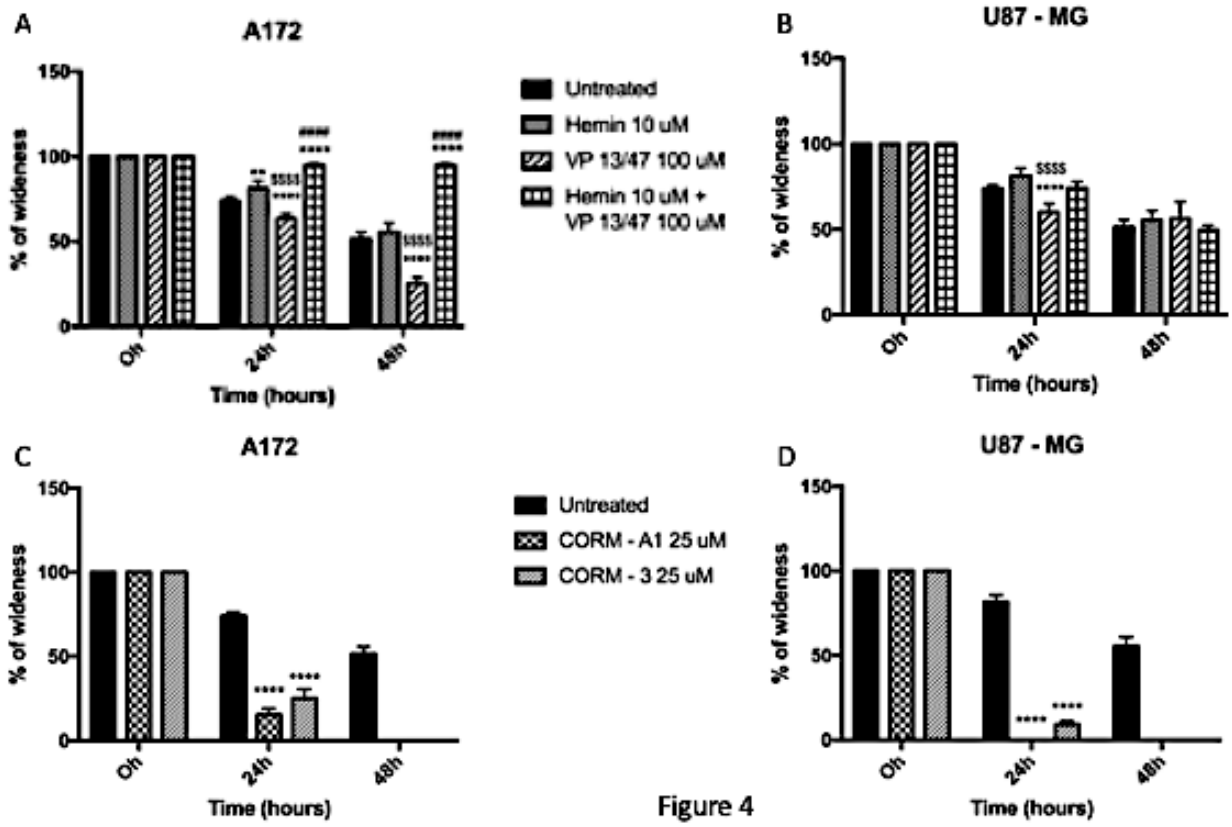



Figure 4

**Figure 4.** Analysis of human glioma cell migration through a wound-healing assay following treatments of A172 (A) and U87-MG (B) cells with Hemin, VP 13/47 and Hemin *plus* VP 13/47 and after treatments of A172 (C) and U87-MG (D) cells with CORM-A1and CORM-3.

Each column is expressing the average of four different experiments. P values <0.05 were considered to be statistically significant (**p<0.01 vs Untreated; ****p<0.0001 vs Untreated; ####p<0.0001 Hemin 10μM vs Hemin 10μM + VP 13/47 100μM; $$$$ p<0.0001 VP 13/47 vs Hemin 10μM + VP 13/47)

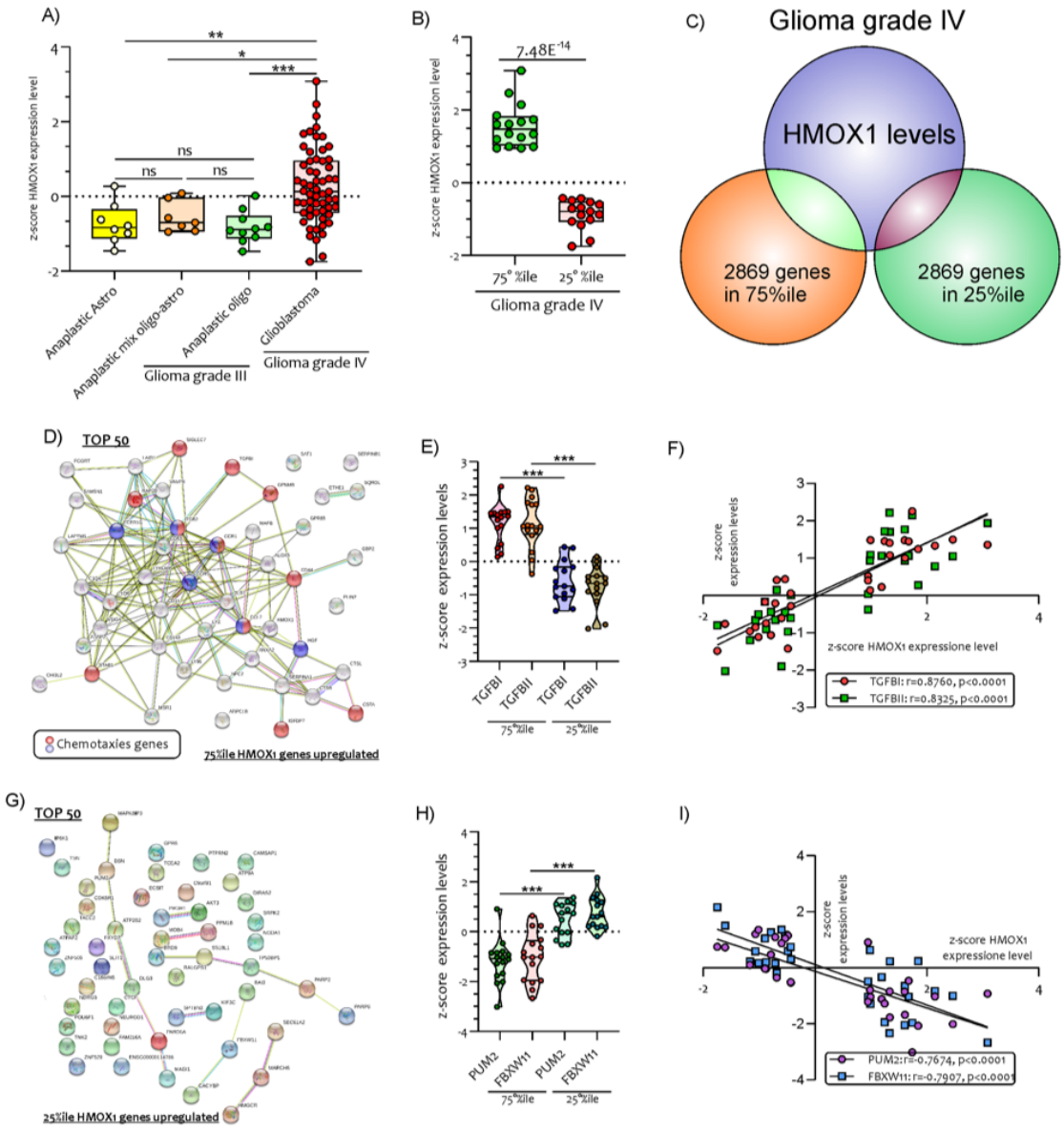
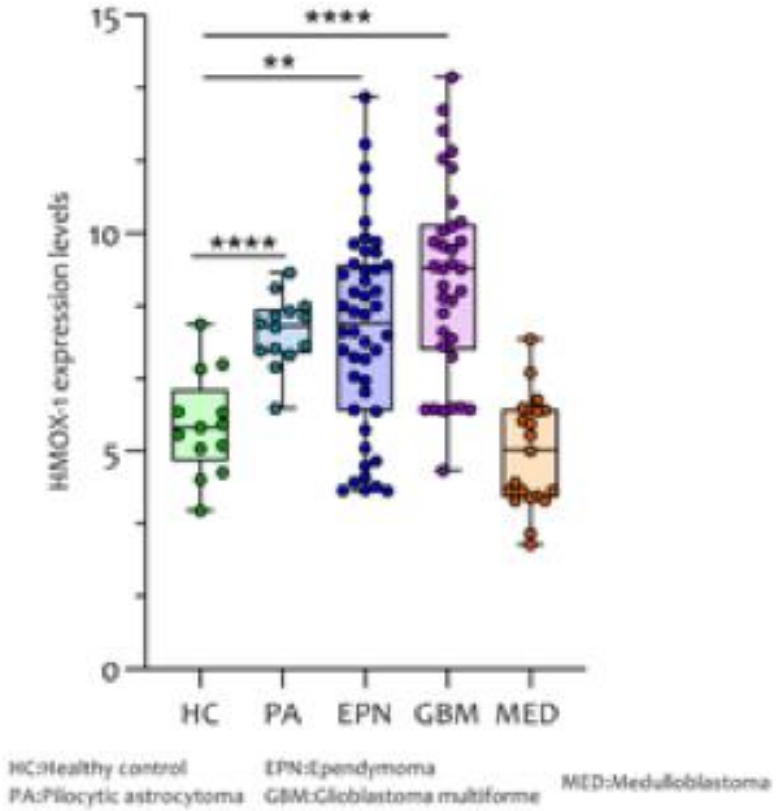



**Figure 5.** HMOX1 expression levels in glioma grade IV brain biopsies. GO analysis was performed with STRING online tool. The String networks have been downloaded and subsequently modified. Data are expressed as z-score intensity expression levels and presented as vertical scatter dot plots and violin plots. P values <0.05 were considered to be statistically significant (*p<0.05; **p<0.005;***p<0.0005; ****p<0.00005).




Supplementary Figure 1

**Supplementary Figure 1**. Expression levels of HMOX-1 in several brain tumours. Gene expression profiles were used to identify HMOX-1 gene that is differentially expressed between various brain tumour types. Data are expressed as z-score intensity expression levels and presented as vertical scatter dot plots and violin plots. P values <0.05 were considered to be statistically significant (*p<0.05; **p<0.005; ***p<0.0005; ****p<0.00005).

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# CHAPTER II

Journal of Oncology (under review)

# ROLE OF 17β-ESTRADIOL ON CELL PROLIFERATION AND MITOCHONDRIAL FITNESS IN GLIOBLASTOMA CELLS

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## ABSTRACT

Gliomas are the most common primary tumors of the central nervous system (CNS) in the adult. Previous data showed that estrogen affects cancer cells, but its effect is cell type-dependent and controversial. The present study aimed to analyze the effects of estradiol (E2, 5nM) in human glioblastoma multiforme U87-MG cells and how it may impact on cell proliferation and mitochondrial fitness. We monitored Cell proliferation by xCelligence technology and mitochondrial fitness by assessing the expression of genes involved in mitochondrial biogenesis (PGC1α, SIRT1, TFAM), oxidative phosphorylation (ND4, Cytb, COX II, COX IV, Ndufa6, ATP synthase) and dynamics (OPA1, MNF2, MNF1, FIS1). Finally, we evaluated Nrf2 nuclear translocation by immunocytochemical analysis. Our results showed that E2 resulted in a significant increase in cell proliferation, with a significant increase in the expression of genes involved in various mechanisms of mitochondrial fitness. Finally, E2 treatment resulted in a significant increase of Nrf2 nuclear translocation with a significant increase in the expression of one of its target genes (i.e., heme oxygenase-1). Our results suggest that E2 promotes proliferation in glioblastoma cells and regulate the expression of genes involved in mitochondrial fitness and chemoresistance pathway.

## INTRODUCTION

Gliomas are the most common primary tumors of the central nervous system (CNS) in the adult. Glioblastoma is the most frequent and aggressive brain tumor in humans with a median survival from 14 to 17 months after the diagnosis (Lefranc et al., 2018; Ventura et al., 2017). Targeted therapies directed to ubiquitous cancer-associated targets (i.e., erlotinib and gefitinib), had limited success (Haas-Kogan et al., 2005; L. Lin, Cai, & Jiang, 2017; Maugeri et al., 2015), further reinforcing the need for the identification of glioma-specific novel molecular targets. With the advent of new technologies, several recent studies have reiterated the importance of metabolic reprogramming in various cancers. The importance of glycolysis in the survival and progression of certain cancers is undeniable, and it is increasingly evident that cancer cells may use many alternative metabolic pathways to drive their phenotype (DeBerardinis & Chandel, 2016). Previous data showed that estrogen affects glioblastoma cells, since certain glioblastomas express estrogen receptors (ERs) (Sribnick, Ray, & Banik, 2006; Yague, Lavaque, Carretero, Azcoitia, & Garcia-Segura, 2004). Consistently with this evidence, the ER-modulator tamoxifen inhibits the growth of certain glioblastomas (Hui et al., 2004; Moodbidri & Shirsat,

2005; Tian et al., 2009). Furthermore, a previous study showed that high concentrations of 17-β-estradiol induce apoptosis in the estrogen-dependent human breast cancer cell line MCF-7, but not in the ER-negative human breast cancer cell line MDA-MB 231, under low growth-stimulated conditions (Altiok, Koyuturk, & Altiok, 2007). In addition to its nuclear functions, estradiol also plays an essential role in the mitochondria. The mitochondrial electron transport chain comprises of several complexes formed by proteins that are encoded by the nuclear or mitochondrial genome. Moreover, E2 plays a role in mitochondrial bioenergetic function, modulating the microviscosity of the inner membrane (Torres et al., 2018) and inducing mitochondrial biogenesis genes in hepatic cells (Galmes-Pascual et al., 2017). Others reported that long-term E2 treatment increased nuclear respiratory factor-1 (NRF-1) protein in cerebral blood vessels of ovariectomized rats (Mattingly et al., 2008). Interestingly, high estradiol concentrations (about 10-8 M) decrease the mitochondrial DNA contents, and ATP formation, and these effects were not showed at minor concentrations(Chou et al., 2019; Hirano, Furutama, & Hanafusa, 2007). For this reason, in this work, we study the effect of estradiol at low concentrations.

Given that, estradiol may induce cell growth or death under different conditions, depending on the concentration of estradiol and the expression of ERs in the brain and other tissues (Lewis-Wambi & Jordan, 2009; McCarthy, 2009; Waters & Simerly, 2009): we analyzed the effects of estradiol in human glioblastoma multiforme U87-MG cells and how it may impact on cell proliferation and mitochondrial fitness.

## MATERIALS AND METHODS

### *Cell culture and pharmacological treatments*

Human glioblastoma cells (U87-MG) were purchased from ATCC Company (Milan, Italy). Cells were suspended in DMEM (Gibco, Cat. # 11965092) culture medium containing 10% fetal bovine serum (FBS, Gibco, Cat. #10082147), 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco, Cat. # 15070063). At 80% confluency, cells were passaged using trypsin-EDTA solution (0.05% trypsin and 0.02% EDTA, Gibco, Cat. # 25300054) (Sacerdoti et al., 2005). A 20μg/mL 17β-estradiol (E2) (Cat. #E2758 Sigma–Aldrich, Milan, Italy) solution was prepared in 1

mL absolute ethanol (Cat. # 51976 Sigma–Aldrich, Milan, Italy) and it was added separately to the cell culture of all experiments at final concentrations of 5.0 nM.

*Real-Time Monitoring of cell proliferation*

xCELLigence experiments were performed using the RTCA (Real-Time Cell Analyzer) DP (Dual Plate) instrument according to manufacturers' instructions (Roche Applied Science, Mannheim, Germany, and ACEA Biosciences, San Diego, CA). The RTCA DP Instrument includes three main components: (i) RTCA DP Analyzer, which stays inside a humidified incubator maintained at 37°C and 5% CO2, (ii) RTCA Control Unit with RTCA Software preinstalled, and (iii) E-Plate 16 for proliferation assay. First, we defined the optimal seeding number by cell titration and growth experiments to obtain a significant cell index value and a constant cell growth (data not shown). We added 100µl of cell culture media in the E-plate 16, and we left it in the tissue culture hood for 30 minutes at room temperature: this procedure ensures the equilibrium between the culture media and E-Plate surface. We inserted into the E-plate 16 into a cradle pocket of the RTCA DP Analyzer, and we performed a blank reading to measures the background impedance of cell culture

media. We added 100µl of a cell solution with a final concentration of 2500 cells/well in the E-plate 16 and, as recommended, we waited 30 minutes before starting the automatic monitoring every 15 min for 24h.

*Real Time PCR for gene expression analysis*

RNA was extracted by Trizol® reagent (Cat. # 15596026 Invitrogen, Carlsbad, CA, USA). First-strand cDNA was then synthesized with High-Capacity cDNA Reverse Transcription Kit (Cat. #4368814 Applied Biosystems, Foster City, CA, USA). High cDNA quality was checked, taking into consideration the housekeeping gene Ct values. Quantitative real-time PCR was performed in Step One Fast Real-Time PCR System Applied Biosystems, using the SYBR Green PCR MasterMix (Cat. #4309155 Life Technologies, Monza, Italy). The specific PCR products were detected by the fluorescence of SYBR Green, the double-stranded DNA binding dye. Primers were designed using BLAST® (Basic Local Alignment Search Tool, NBCI, NIH), considering the shortest amplicon proposed: primers' sequences are shown in table 1, and β-actin was used as the housekeeping gene. Primers were purchased by Metabion International AG (Planneg, Germany). The relative mRNA expression

level was calculated by the threshold cycle (Ct) value of each PCR product and normalized with β-actin by using a comparative $2^{-\Delta\Delta Ct}$ method.

*Immunocytochemistry*

Cells were grown directly on coverslips before immunofluorescence and treated with 17β-estradiol (E2) at the final concentration of 5nM. After washing with PBS, cells were fixed in 4% paraformaldehyde (Cat. #1004968350 Sigma-Aldrich, Milan, Italy) for 20 min at room temperature. Subsequently, cells were incubated with primary antibody against TFAM at dilution 1:200, overnight at 4 °C. The next day, cells were washed three times in PBS for 5 min and incubated with secondary antibodies: TRITC (anti-goat, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at dilution 1:200 for 1 h at room temperature. The slides were mounted with medium containing DAPI (4′,6- diamidino-2phenylindole, Cat. #sc-3598 Santa Cruz Biotechnology, Santa Cruz, CA, USA) to visualize nuclei. The fluorescent images were obtained using a Zeiss Axio Imager Z1 Microscope with Apotome 2 system (Zeiss, Milan, Italy). As a control, the specificity of immunostaining was verified by omitting incubation with the primary or secondary antibody. Immunoreactivity was evaluated considering the signal-to-noise ratio of immunofluorescence.

*Statistical Analysis*

Statistical analysis was performed using SPSS11.0 software. Statistical significance ($p < 0.05$) of differences between experimental groups was determined by the Fisher method for analysis of multiple comparisons. For comparison between treatment groups, the null hypothesis was tested by either single-factor analysis of variance (ANOVA) for multiple groups or the unpaired *t*-test for two groups, and the data are presented as mean ± SD.

## RESULTS

### *E2 induces glioblastoma cell proliferation and mitochondrial metabolism gene expression*

We firstly aimed at studying the effect of E2 on cell proliferation. As shown in Figure 1, E2 treatment resulted in a significant increase in cell proliferation in U87-MG cells, as showed by cell index performed by xCELLigence technology. Increased cell index was already significant following 3h ($p<0.001$) treatment with E2, and such effect was still evident following 9h of treatment ($p<0.001$). We, therefore, investigated the effect of E2 on mitochondrial metabolism with regard to mitochondrial biogenesis, oxidative phosphorylation, and dynamics. As shown in Figure 2A, E2 resulted in a significant increase of PGC1α gene expression following 1h treatment ($p<0.001$), and such expression decrease in a time-dependent manner reaching the control levels following 24h. Consistently, we observed a significant increase in two additional biomarkers of mitochondrial biogenesis (i.e., SIRT1 and TFAM) (Figure 2B and 2C). This set of experiments showed that E2 resulted in a significant ($p<0.001$) increase in SIRT1 and TFAM gene expression following 1h of E2 treatment, and such increased expression was sustained during all other time of

observation. As shown in Figure 3, E2 treatment also resulted in a significant change in the expression of genes involved in oxidative phosphorylation. E2 treatment significantly increased ND4, Cyb4, COXII, COXIV, COX, and Ndufa6 gene expression following 1h treatment of E2 (Figure 3A-E). Similarly, ATP synthase gene expression was significantly increased treatment and peaking 3h following E2 pharmacological treatment (Figure 3F). Besides, E2 exhibited a significant effect on the expression controlling mitochondrial dynamics. E2 treatment resulted in a significant ($p<0.001$) increase of OPA1, MNF2, and MNF1 gene expression following 1h of treatment (Figure 4 A-C). Consistently, E2 treatment resulted in a significant ($p<0.010$) increase in FIS1 gene expression following 3h of E3 treatment (Figure 4D). Finally, these results were further confirmed by immunocytochemistry analysis, demonstrating increase TFAM protein expression and increased mitochondrial network as measured by mitotracker staining (Figure 5A-D).

*E2 induces Nrf2 nuclear translocation and increase heme oxygenase-1 expression*

To assess the effect of E2 on the activation of pathways involved in chemoresistance mechanisms, we evaluated the nuclear translocation of Nrf2. Our data showed that

E2 treatment resulted in a significant increase in nuclear translocation following 24h treatment when compared to untreated cells (Figure 6A and 6B). Consistently with this observation, we also showed that HO-1, one of Nrf2 targeted genes, was upregulated following E2 treatment ($p<0.001$) (Figure 6C).

## DISCUSSION

Previous studies showed that high concentrations of estradiol, under low growth-stimulated conditions, inhibit cell proliferation and increase apoptosis in ER-positive breast cancer cells through the sustained activation of the JNK pathway (Altiok et al., 2007), underlying the basis for the antitumor effects of high-dose estrogen therapy in postmenopausal women approximately 40 years ago (Lewis-Wambi & Jordan, 2009). Recently, high concentrations of estradiol were shown to trigger apoptosis in adrenal carcinoma cells (Prieto, Brown, Perez-Stable, & Fishman, 2008), indicating that the mechanisms of these cytotoxic effects of estradiol remain to be further elucidated. Glioblastomas are the most aggressive type of brain tumors, with a poor prognosis and a limited response to chemotherapy and other therapeutic strategies (Minniti, Muni, Lanzetta, Marchetti, & Enrici, 2009;

Quick, Patel, Hadziahmetovic, Chakravarti, & Mehta, 2010). Failure of therapy arises from the resistance of tumor cells to therapy-induced apoptosis (Lino & Merlo, 2009); therefore, new drugs targeting alternative pathways are required. In the present study, E2 induces cell proliferation and the expression of genes involved in mitochondrial metabolism in glioblastoma cells. Estradiol, the predominant form of estrogen, mediates its effects via the activation of intracellular signaling pathways on neurons and glial cells (Pozzi, Benedusi, Maggi, & Vegeto, 2006). Previous studies concerning the effects of estrogens in cancer cells exhibited controversial results (Russo et al., 2016). As far as concern glioblastoma, epidemiologic evidence suggests a tumor-suppressive role of E2 (Pares, 2010). The incidence of developing glioblastoma is greater in men than in women, and women of reproductive age have a survival advantage over men and postmenopausal women (Anic et al., 2014; Kabat, Park, Hollenbeck, Schatzkin, & Rohan, 2011; Pares, 2010). These correlative findings suggest that estrogen plays a significant role in the suppression of glioblastoma, but how they might do so is poorly understood. By contrast, our data suggest that E2 induces cell proliferation in the U87-MG glioblastoma cell line. However, a different expression of ERβ may explain, at least in part, the discrepancy

with previously published reports. In this regard, multiple isoforms of ERβ exist and may have distinct roles in various cancers (Leung, Mak, Hassan, & Ho, 2006; Thomas & Gustafsson, 2011). The ERβ2 isoform is overexpressed in chronic lymphocytic leukemia, prostate cancer, non-small cell lung cancer, breast cancer, and ovarian cancer (P. Dey, Barros, Warner, Strom, & Gustafsson, 2013). ERβ2 expression associated with worse disease-free survival and overall survival of patients and disease-free survival of tamoxifen-treated patients (Baek, Chae, Song, & Jung, 2015). Further, ERβ2 is implicated in prostate cancer metastasis (P. Dey et al., 2012). ERβ3 has limited tissue distribution restricted to testis (Moore et al., 1998). ERβ5 is overexpressed in ovarian cancer, prostate cancer and associated with poor prognosis (Leung et al., 2010) while ERβ5 expression associated with good prognosis in non-small cell lung cancer and confers sensitivity to chemotherapeutic agent-induced apoptosis in breast cancer cells (Leung et al., 2010). Different authors suggested that ERβ5 was highly expressed in the majority of primary and established GBM cells compared to ERβ1 and ERβ2, with ERβ4 is the least expressed (Li et al., 2013; J. Liu et al., 2018). The data regarding the effect of E2 on glioblastoma progression are further supported by our results showing that E2 induces Nrf2

nuclear translocation and HO-1 expression. Estradiol also exerts non-genomic rapid actions via direct interaction of estradiol with plasma-associated ERs and the activation of second messenger pathways (Levin, 2009). The late and sustained effects of estradiol described in this study suggest that non-genomic rapid actions of estradiol are not involved. In this regard, it has become evident that malignant cells benefit from having increased Nrf2 pathway activity: this was first observed in lung cancer (Padmanabhan et al., 2006), as well as subsequently in many other cancer types, such as pancreatic, ovarian, liver and gallbladder cancers (Lister et al., 2011). Aberrant Keap1-Nrf2 signaling leads to radio- and chemoresistance and provides growth advantage to cancer cells due to the constitutive expression of cytoprotective genes (Lau et al., 2010). Multiple mechanisms for Nrf2 overactivation have been found, such as somatic mutations in either KEAP1 or NFE2L2, deletion of exon 2 of NFE2L2, aberrant expression of inhibitory proteins and transcriptional induction by oncogenes and hormones (Goldstein et al., 2016). Previous results demonstrated that in GBM cells, inhibition of Nrf2 and p62 decreased tumorigenic properties, such as cell invasion and anchorage-independent growth (Polonen et al., 2019). Furthermore, Nrf2 could also function as a key balancing factor in metabolic reprogramming, as

Nrf2 can regulate both energy metabolism and antioxidant response to ROS to favor glioma growth and development. Our results are consistent with these observations and showed that E2 resulted in a significant increase in the expression of genes involved in mitochondrial metabolism, biogenesis, and dynamics. Furthermore, our results showed that E2 resulted in a significant increase of HO-1, which is associated with increased chemoresistance and proliferative phenotype (Gandini et al., 2014), thus further confirming our observations. Our data showed that E2 plays an important role in GBM progression, improving the mitochondrial fitness, highlighting its role in resistant mechanisms to the therapies: this can lead to a new therapeutic strategy for future studies.

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## Author Contributions

CCC, MA, GLV: Made a substantial contribution to the concept and design, acquisition of data or analysis and interpretation of data; GLV, CCC, DT: Drafted the article or revised it critically for relevant intellectual content; LL, AD, DA, SK, MC, DT, RA and GLV: performed in vitro experiments; all the authors: Approved the version to be published.

### Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon request.

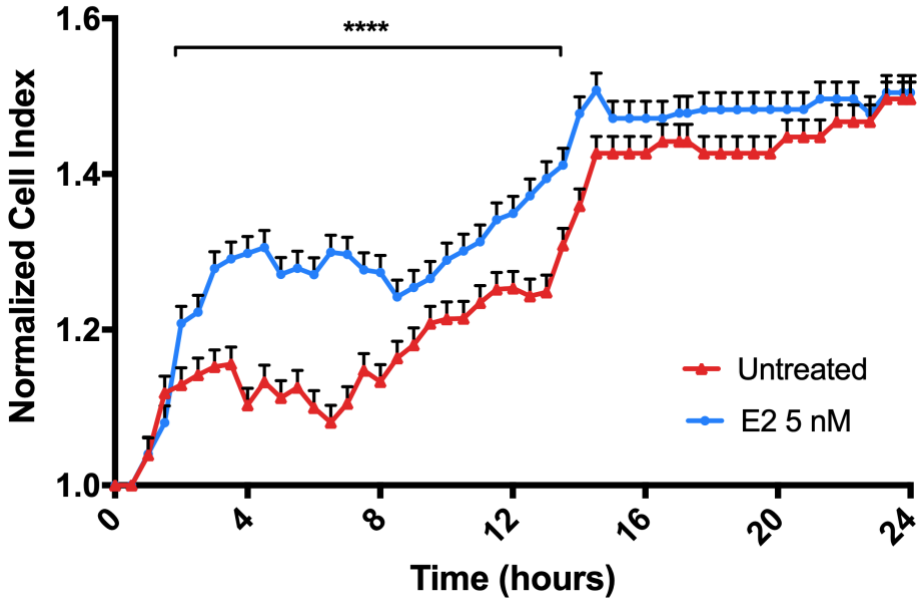


**Figure 1. Effect of E2 in glioblastoma cell proliferation.** E2 treatment resulted in a significant increase in cell proliferation in U87-MG cells following E2 5nM treatment. A normalized cell index was performed for 24 hours by xCELLigence RTCA technology.

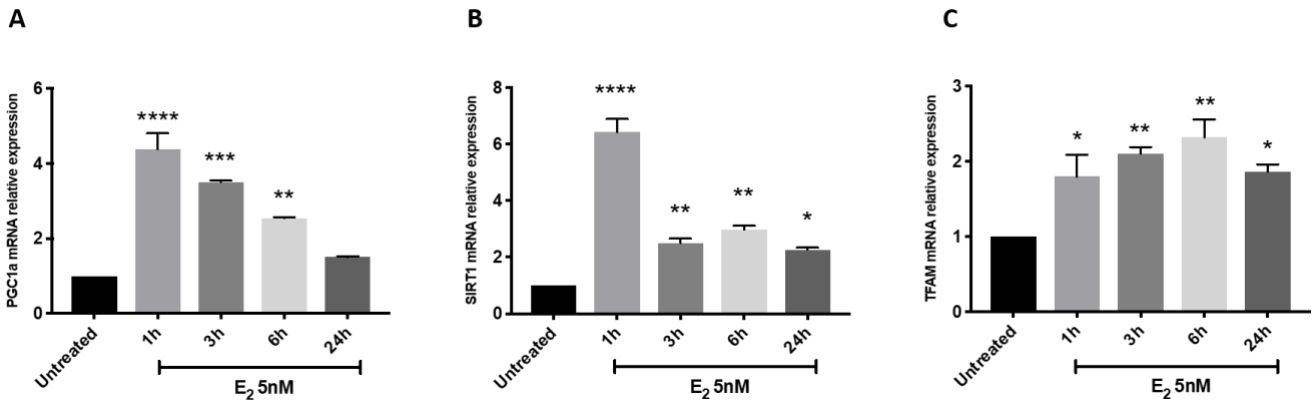


**Figure 2. Effect of E2 on mitochondria biogenesis**. E2 resulted in a significant increase of PGC1α gene expression following 1h treatment, and such expression decrease in a time-dependent manner reaching the control levels following 24h (Figure 2A). Consistently, SIRT1 and TFAM show a significant increase following 1h of E2 treatment (Figure 2B and 2C). The calculated value of $2^{-\Delta\Delta Ct}$ in untreated controls is 1. Data are expressed as mean ± SD of at least four independent experiments. *p<0.05; **p<0.001; ***p<0.0001

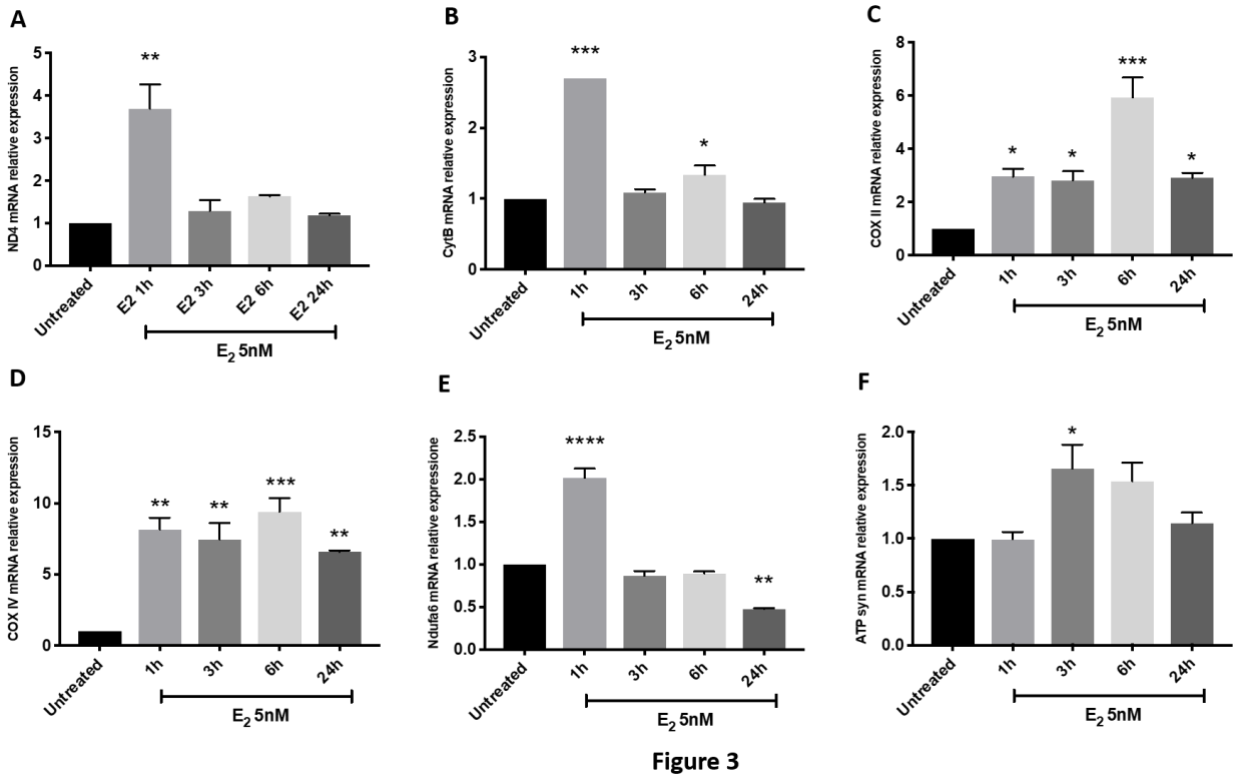


Figure 3

**Figure 3. Effect of E2 on mitochondria OXPHOS gene**. E2 treatment significantly increased ND4, Cyb4, COXII, COXIV, COX, and Ndufa6 gene expression following 1h treatment of E2 (Figure 3A-E). Similarly, the treatment increases ATP synthase gene expression and peaks at 3h (Figure 3F). The calculated value of $2^{-\Delta\Delta Ct}$ in untreated controls is 1. Data are expressed as mean ± SD of at least four independent experiments. *p<0.05; **p<0.001; ***p<0.0001.

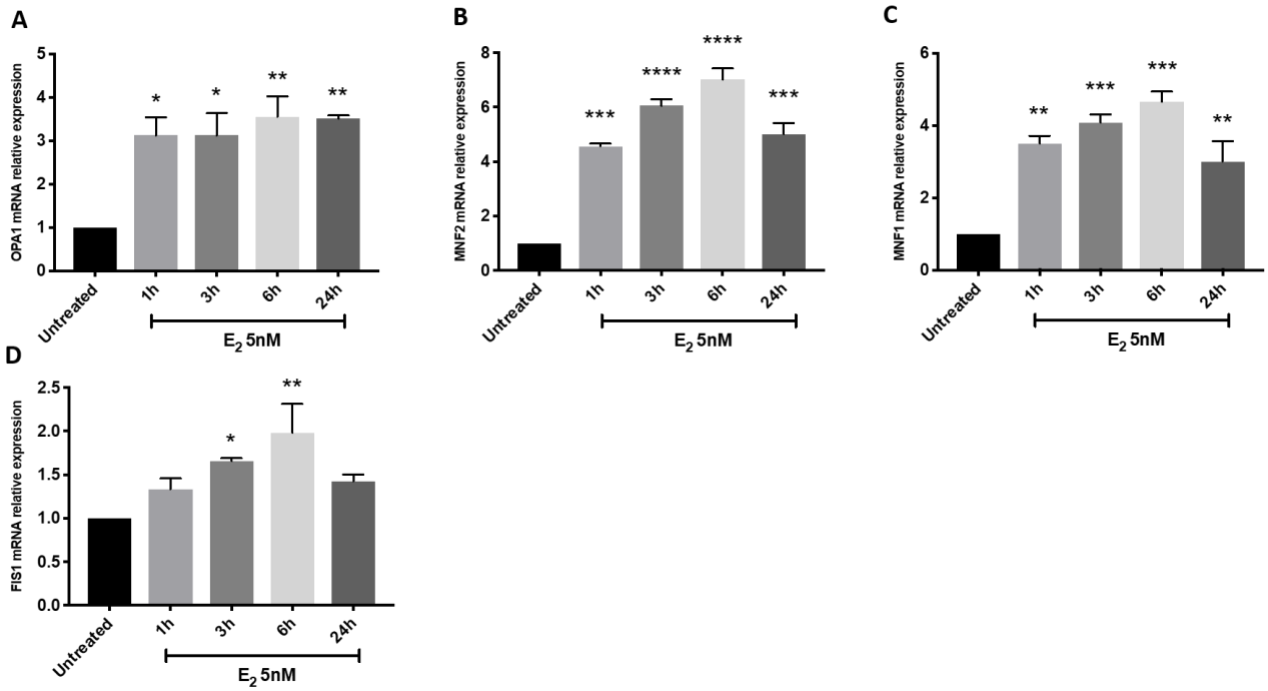


**Figure 4. Effect of E2 on mitochondria dynamics**. E2 treatment increases OPA1, MNF2, and MNF1 gene expression following 1h of treatment (Figure 4 A-C). Consistently, E2 treatment increases FIS1 gene expression following 3h of treatment (Figure 4D). The calculated value of $2^{-\Delta\Delta Ct}$ in untreated controls is 1. Data are expressed as mean ± SD of at least four independent experiments. *p<0.05; **p<0.001; ***p<0.0001.

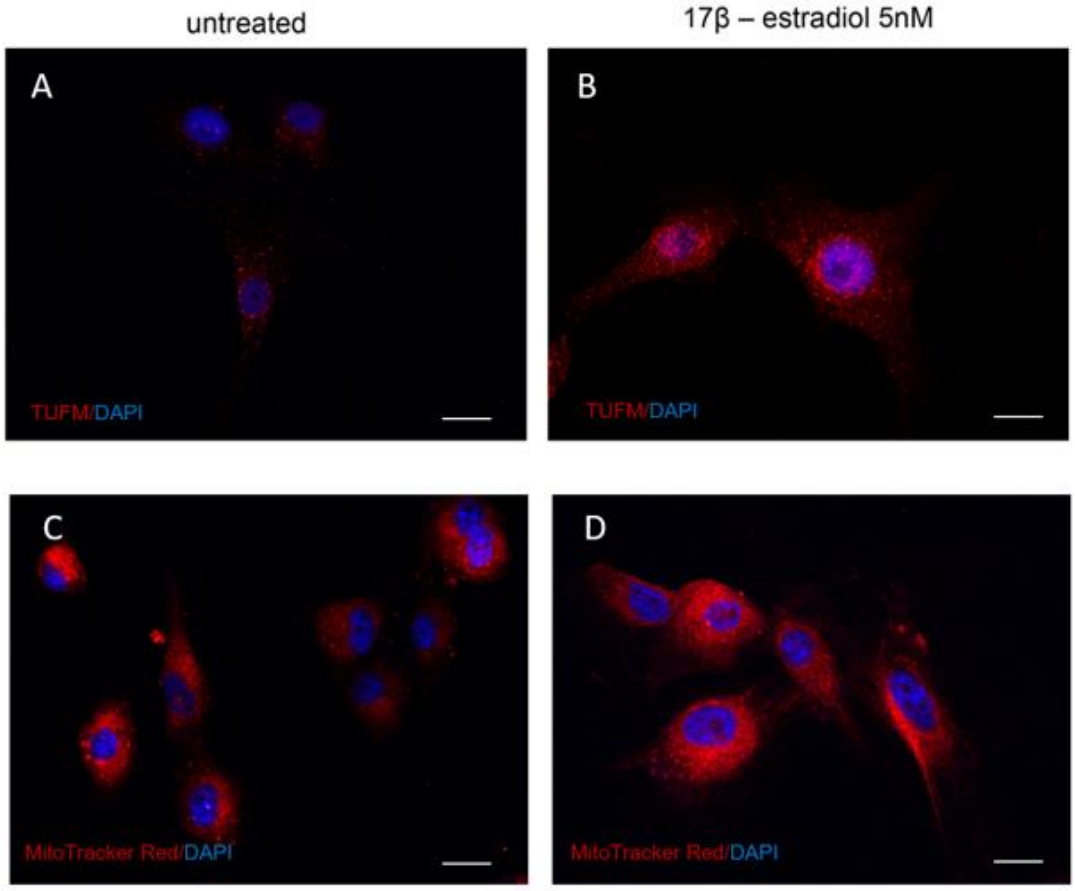


**Figure 5. E2 increases mitochondrial mass in glioblastoma cells**. The immunocytochemistry analysis demonstrates an increase of TFAM protein expression and mitochondrial network. Immunofluorescence staining of TFUM (red) was performed in U87-MG human glioblastoma cells in basal condition (Figure 5A) and after 24h treatment with E2 (Figure 5B). The Mitotracker Red staining was performed in U87-MG human glioblastoma cells after at basal condition (Figure 5C) and after 24h treatment with E2 (Figure 5D). DAPI was used to stain cell nucleus and the scale bar is set as 10 μm.

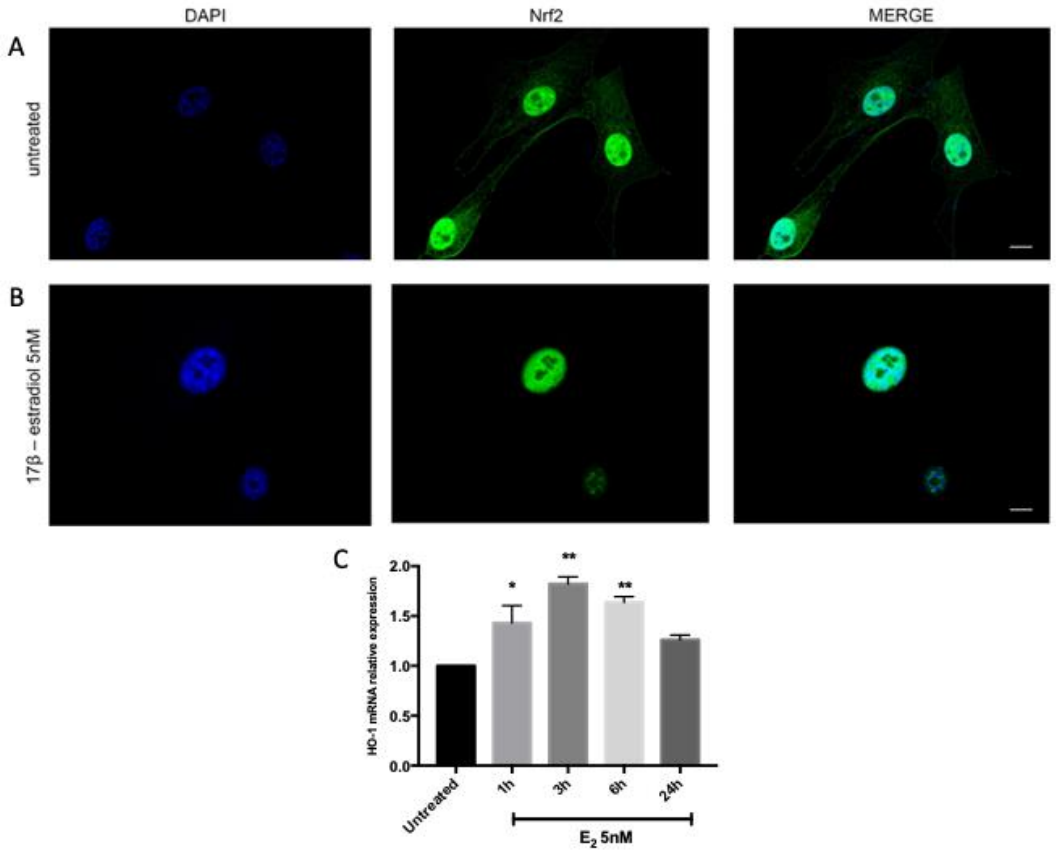


**Figure 6. E2 increases HO-1 expression and induces Nrf2 nuclear translocation.** E2 treatment increases Nrf2 nuclear translocation following 24h treatment when compared to untreated cells (Figure 6A and 6B). Consistently, HO-1, one of Nrf2 targeted genes, is upregulated following E2 treatment ($p<0.001$) (Figure 6C). Immunofluorescence staining of Nrf2 (green) was performed in U87-MG human glioblastoma cells at basal condition (Figure 6A) and after 24h treatment with E2 (Figure 6B). DAPI was used to stain the cell nucleus, and the scale bar is set as 10 μm. Gene expression analysis of HO-1 was performed after 24h of treatment with E2 (5nM) in glioblastoma cells. The calculated value of $2^{-\Delta\Delta Ct}$ in untreated controls is 1. Data are expressed as mean ± SD of at least four independent experiments. (Figure 6C) *$p<0.05$; **$p<0.001$;

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# GENERAL DISCUSSION AND CONCLUSIONS

The first part of the present project aimed to investigate the effect of HMOX-1 expression and enzymatic inhibition in two different glioblastoma cell lines, A172 and U87-MG, and to discover possible molecular mechanisms by which HMOX-1 promotes cancer progression. In the results presented, there are also some similarities and differences between A172 and U87-MG cell lines. In particular, hemin treatment was able to induce both gene and protein expression in both cell lines even though A172 cells showed to be more responsive in terms of folds of increase and timing of expression. Furthermore, we also observed a similar biological effect of HMOX-1 upregulation which results in an increased proliferation and colony formation capacity.

These results are consistent with previous studies showing that HMOX-1 and its by product play a significant role in controlling cell proliferation via regulating essential protein involved in cell cycle progression (Li Volti et al., 2005; Li Volti, Wang, Traganos, Kappas, & Abraham, 2002). Besides, literature shows a role of HMOX-1 in glial cell pathophysiology and suggest that HMOX-1 expression in these cell types might have a role in glioma progression and/or treatment, thus supporting our results of HMOX-1 expression by the glial cells within the tumour (Y. Liu et al., 2011; Long et al., 2007). Interestingly, we observed that the HMOX-1 expression levels were higher in all histological subtypes and all grades analysed when compared to the non-malignant brain (Supplementary Figure 1) and in Glioma Grade IV brain biopsies compared to Glioma grade III. Largely, HMOX-1 protein expression was associated

with worse prognosis of grade II and III astrocytoma patients, and although this subgroup of astrocytoma was small, the results were corroborated at the mRNA level in univariate and multivariate analyses using the REMBRANDT data. Moreover, the results demonstrating an increase in HMOX-1 protein in all glioma subtypes are also reflected at the mRNA level.

Altogether, these results show that HMOX-1 is an independent prognostic factor and that its expression could be relevant in determining the malignant behaviour of grade II and III astrocytomas (Supplementary Figure 1). In this work, we highlighted also the correlation of HMOX1 gene expression with two other genes: PUM2 and FBXW11. PUM2 is an RNA binding protein and functions as a translational repressor. It promotes stem cell proliferation via repressing cell cycle regulators expression. The analysis of microarray dataset highlighted that HMOX1 expression levels were inversely correlated with PUM2 expression suggesting that PUM2 may be a repressor of cell proliferation in glioblastoma.

The second part of the project we investigated the effect of high concentration of estradiol (E2): previous studies showed that under low growth-stimulated conditions, it inhibits cell proliferation and increases apoptosis in ER-positive breast cancer cells through the sustained activation of the JNK pathway (Altiok et al., 2007). Recently, high concentrations of estradiol were shown to trigger apoptosis in adrenal carcinoma cells (Prieto et al., 2008), indicating that the mechanisms of these cytotoxic effects of estradiol remain to be further elucidated. In the present study, E2

was found to induce cell proliferation and the expression of genes involved in mitochondrial metabolism in glioblastoma cells. Estradiol, the predominant form of estrogen, mediates its effects via the activation of intracellular signaling pathways on neurons and glial cells (Pozzi et al., 2006). As far as concern glioblastoma, epidemiologic evidence suggests a tumor-suppressive role of E2 (Pares, 2010). The incidence of developing glioblastoma is greater in men than in women, and women of reproductive age have a survival advantage over men and postmenopausal women (Anic et al., 2014; Kabat et al., 2011; Pares, 2010). These correlative findings suggest that estrogen play a significant role in suppression of glioblastoma. By contrast, our data suggest that E2 induces cell proliferation in U87-MG glioblastoma cell line. Furthermore, our results showed that E2 resulted in a significant increase of HO-1 which is associated with increased chemoresistance and proliferative phenotype (Gandini et al., 2014) thus further confirming our observations.

In conclusion, our studies provide strong evidence of HMOX-1 overexpression in human gliomas compared with non- malignant samples. Furthermore, the expression of HMOX-1 was observed in tumour cells and was associated with a worse prognosis in patients with grade II and III astrocytoma. Preliminary analyses of the significance of HMOX-1 and its correlation with outcome in gliomas suggest that the enzyme might be involved in tumour cell proliferation. Altogether, these results point to a pro-tumoral role of HMOX-1 in glioma progression.

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