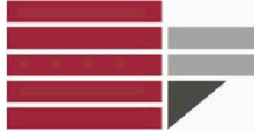

UNIVERSITA' DELLA CALABRIA

Dipartimento di Farmacia e Scienze della Salute e della Nutrizione

Dottorato di Ricerca in

Medicina Traslazionale

XXXIII CICLO

Evidence that ANG II/AGTR1 signaling stimulates aromatase activity, enhances local estrogen production involved in Glioblastoma cell growth and progression.

Settore Scientifico Disciplinare: MED/05

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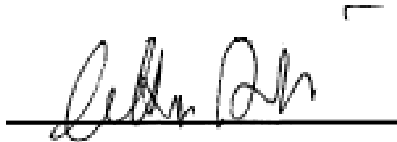

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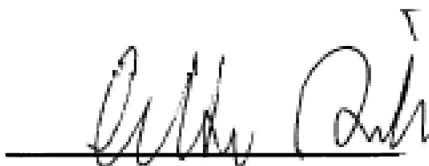

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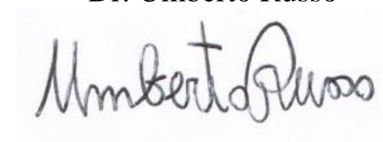

INDEX

	pag.
▪ **Abstract**	**4**
▪ **Introduction**	**6**
➢ **Glioblatoma risk factors**	**6**
➢ **Clinical manifestations of Glioblatoma**	**7**
➢ **Therapy of Glioblatoma**	**8**
➢ **The "classical" Renin-Angiotensin System**	**9**
➢ **Receptors of ANG II**	**12**
– ***ANG II Receptor 1 action and signaling pathways***	**12**
– ***ANG II Receptor 2 action and signaling pathways***	**15**
➢ **ANG II and angiogenesis**	**17**
➢ **Renin-Angiotensin System and cancer**	**19**
– ***Role of ACE***	**19**
– ***Role of AGTR1***	**19**
– ***Role of AGTR2***	**20**
➢ **Renin-Angiotensin System and Glioblastoma**	**20**
➢ **Estrogens and Aromatase in Glioblastoma**	**22**
– ***Aromatase gene and promoters***	**24**
▪ **Aim of the study**	**27**
▪ **Materials and Methods**	**28**
❖ *Materials*	**28**
❖ *Cell Culture*	**28**
IN VITRO EXPERIMENTS	**28**
❖ *RNA isolation, Reverse transcription and Real-time PCR*	**28**

❖ *Immunoblot Analysis* **29**

❖ *Immunofluorescence* **30**

❖ *Cell proliferation assays* **31**

– *Anchorage-independent soft agar growth assays* **31**

– *[^{3}H]-Thymidine incorporation assay* **31**

❖ *Aromatase activity assay* **32**

❖ *ELISA assay* **32**

❖ *Wound Healing Assays* **33**

❖ *Boyden chamber transmigration* **33**

❖ *Cell invasion assay* **34**

❖ *Transient transfections assays* **34**

❖ *Chromatin immunoprecipitation assay* **35**

❖ *DNA Affinity Precipitation Assay.* **35**

IN VIVO EXPERIMENTS **36**

❖ *Xenograft model analysis* **36**

❖ *Histopathological analysis* **37**

❖ *Immunohistochemical analysis* **37**

STATISTICAL ANALYSIS **38**

▪ **Results** **39**

1. *High AGTR1 expression is associated with glioblastoma progression* **39**
2. *Angiotensin II increases local estrogen production in glioblastoma cells* **42**
3. *Blocking ANG II signaling inhibits tumor growth in GBM xenografts* **46**

▪ **Discussion** **50**

▪ **References** **54**

ABSTRACT

Glioblastoma Multiforme (GBM) is the most malignant form of glioma causing 3-4% of all cancer-related deaths. Specific cancer treatment for GBM includes surgery, radiotherapy and chemotherapy with temozolomide. The angiogenesis induced by Vascular Endothelial Growth Factor (VEGF), determines alteration of the blood-brain barrier and chronic administration of glucocorticoid is usually required to minimize peritumoral edema inducing neurological deficits. However, the average survival of GBM patients is around 15 months. Angiotensin II (ANG II)/ANG II receptor type 1 (AGTR1) is part of the Renin-Angiotensin System (RAS). Local expression of the RAS has been reported in normal and tumoral tissues, including human GBM. On the basis of the evidence that ANG II up-regulates tumoral angiogenesis through the enhanced expression of VEGF, ANG II inhibitors have been utilized to reduce angiogenesis and tumor growth. It has been reported in different experimental models that ANG II is able to stimulate aromatase expression through the AGTR1 activation. Different studies have demonstrated that aromatase enzyme is expressed in GBM cells and local estrogen production may act as an autocrine or paracrine factor in enhancing glioblastoma growth and progression. Thus, our aim was to investigate if ANG II enhances aromatase activity and local estrogen production, sustaining glioblastoma survival and growth. Under treatment with ANG II the aromatase expression has been evaluated in terms of mRNA, protein content and enzymatic aromatase activity in U-87 MG and T98G cells. The biological correlate of the enhanced aromatase activity was represented by an increased estradiol production. Indeed, ANG II exposure enhances significantly E2 production in the presence of an aromatizable steroid such as androst-4-ene-3,17-dione, which was reversed by losartan. It has been demonstrated how ANG II may activate the two mostly identified aromatase gene promoter in glioblastoma such as pI.f and in a higher extent in pI.4. In the latter promoter a GAS consensous sequence has been indentified as putative STAT binding site effectors of ANG II/AGTR1 signaling.

The *in vivo* studies using human glioblastoma U-87 MG xenografts nude mice, have shown that the treatment for 24 days of ANG II increased tumor size by 30%, which was completely reversed by anastrazole. Treatment with losartan reduces drastically by 50% tumor size below the control size addressing how ANG II/AGTR1 signaling is working in basal condition.

The severe tumor growth regression upon losartan treatment can be explained by the marked antiangiogenic effects of this compound able to reduce vascular stroma sustained by the strong inhibition of VEGF expression as well documented in the last session of the present study.

Finally, immunochemistry analysis revealed how upon ANG II exposure the increased Ki-67 immunostaining confirms the enhanced tumor proliferation rate concomitantly with a clear enhanced immune reactivity of aromatase, while both events were repressed by losartan.

It has been widely documented how glioblastoma cell displays immune evasion weakening antitumor immunity. Thus, in the final session of the previous study we investigated how ANG II/AGTR1 interacting with estrogen signaling may influence the expression of immune check-point molecules (ICs) such as PD-L1/PD-1, as well as with the stromal enzyme hampering immune cell function such as arginase. Immunohistochemistry reveals how all the three immunosuppressive markers enhanced by angiotensin II treatment were drastically down regulated by losartan.

Conclusively, glioblastoma may be considered an estrogen responsive tumor wherein local estrogen production potentiated by angiotensin signaling may contribute to its growth and progression. Losartan appears to antagonize other than angiotensin signaling, also its functional interaction with local estrogen production resulting in a clear downregulation of glioblastoma growth rate.

Finally, losartan on the basis of its antiproliferative and antiangiogenic properties displayed in glioblastoma cell together with its capability to make tumor microenvironment less immunosuppressive may reasonably be repurposed in the novel therapeutical strategies for glioblastoma treatment.

INTRODUCTION

Glioblastoma Multiforme (GBM) is the most common primary malignant brain tumor, comprising 16% of all primary brain and central nervous system neoplasms (*Thakkar et al., 2014*). The average age-adjusted incidence rate is 3.2 per 100000 population (*Ostrom et al., 2014; Ostrom et al., 2015*). Although GBMs occur almost exclusively in the brain, they can also appear in the brain stem, cerebellum, and spinal cord. Sixty-one per cent of all primary gliomas occur in the four lobes of the brain: frontal (25%), temporal (20%), parietal (13%), and occipital (3%) (American Association of Neuroscience Nurses [AANN], 2014). Originally, GBMs were thought to be derived solely from glial cells; however, evidence suggests that they may arise from multiple cell types with neural stem cell-like properties. These cells are at multiple stages of differentiation from stem cell to neuron or to glia, with phenotypic variations determined, in large part, by molecular alterations in signaling pathways rather than by differences in cell type of origin (*Phillips et al., 2006*).

GBMs present at a median age of 64 years (*Thakkar et al., 2014*) but can occur at any age, including childhood. Incidence is slightly higher in men than women (1.6:1) and in Caucasians relative to other ethnicities (*Ellor et al., 2014*). GBMs can be classified as primary, or de novo, arising without a known precursor, or secondary, where a low-grade tumor transforms over time into GBM. A majority of GBMs are primary, and these patients tend to be older aged and have a poorer prognosis than patients with secondary GBMs (*Wilson et al., 2014*).

Glioblatoma risk factors

A potential link existing between glioblastoma and environmental occupational cues have been definitely inconclusive, while an increasead risk of glioblastoma development related to ionizing radiation exposure appears definitely demonstrated (*Johnson et al., 2015*). It was mentioned how an increase risk of glioma development is associated with some specific diseases such as neurofibromatosis 1 and 2, tuberous sclerosis, Li-Fraumeni syndrome, retinoblastoma, and Turcot

syndrome but it appears how a very low percentage (less than 1%) of patients with glioma have a known hereditary disease (*Figure 1*) (*Ellor et al., 2014*).

Monogenic Mendelian disorders associated with increased risk of glioma	
Gene	Disorder/syndrome
NF1	Neurofibromatosis 1
NF2	Neurofibromatosis 2
TSC1, TSC2	Tuberous sclerosis
MSH2, MLH1, MSH6, PMS2	Lynch syndrome
TP53	Li-Fraumeni syndrome
p16/CDKN2A	Melanoma-neural system tumour syndrome
IDH1/IDH2	Ollier disease/Maffucci syndrome

FIGURE 1. Monogenic Mendelian disorders associated with increased risk of glioma *(Ostrom et al., 2014).*

Clinical manifestations of Glioblatoma

Size, location, tumor and anatomic structures of the brain may greatly vary in a patient with primary GBM (*Lobera, 2015; Young et al., 2015*). Glioblastoma patients display frequently symptoms of increased intracranial pressure, such as headache and neurologic deficits present in almost 50% of patients, seizure may occur frequently in as many as of 50% patients (*Perry et al., 2006; Schiff et al., 2015*) and only in such circumstances is recommended the use of antiepileptic drugs (AEDs) (*Glantz et al., 2000; Perry et al., 2006*). In many patiens is indicated the use of corticosteroids at the diagnosis to help control vasogenic edema. Diagnostic imaging may be relaied on computed tomography or magnetic resurrance, necrosis is considerated to be a hallmark feature of GBM and this required for a brain tumor to be grade IV. Surrounding vasogenic edema, hemorrhage, ventricular distortion may be detected in diagnostic imaging (*Figure 2*) (*Ellor et al., 2014; Johnson et al., 2015*). In about 13% of cases GMB could be multifocal (*Chamberlain, 2011; Johnson et al., 2015*).

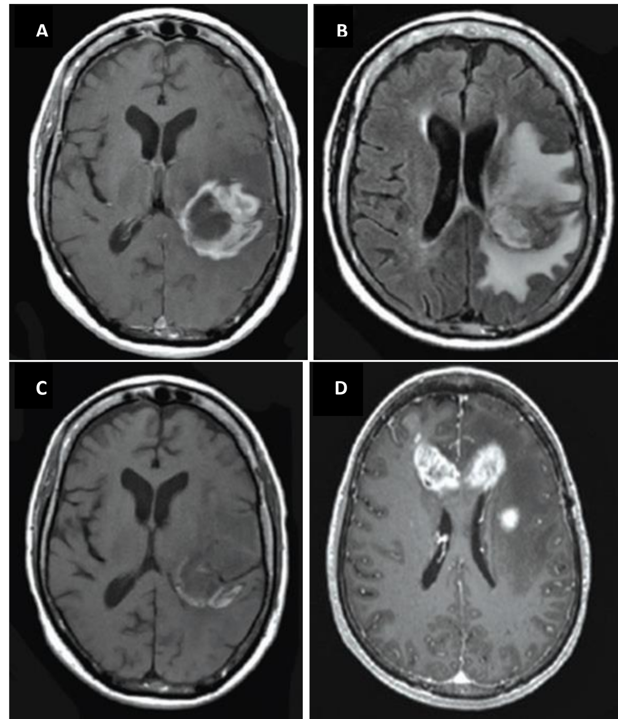

FIGURE 2. Radiographic Features of Glioblastoma on Magnetic Resonance Imaging Note. *(A) T1 post-gadolinium contrast with dense rim enhancement;* ***(B)*** *axial flair showing extensive vasogenic edema causing mass effect on the left lateral ventricle;* ***(C)*** *T1 pre-gadolinium showing hemorrhage (white areas) along posterior lateral margin of tumor;* ***(D)*** *multifocal bihemispheric disease. Images used with permission. (Davis, 2016).*

Therapy of Glioblatoma

At the present the instant therapy includes surgical resection, followed by radiation and oral adjuvant chemotherapy with TMZ (temozolomide), and then the adjuvant chemotherapy with the latter drug (*Wilson et al., 2014*). Due to the invasiveness of this tumor is really difficult to get a complete surgical resection of this tumors and tumor invasiveness compromise brain areas of control speech, motor function and the senses (*Kuhnt et al., 2011; Roder et al., 2014*). Statistically it has been reported a significant association between greatear extent of resection and longer progressive-free survival and overall survival that has been seen in several studies (*Keles et al., 1999; Lacroix et al., 2001; Mukherjee & Quiñones-Hinojosa, 2011; Stummer et al., 2006*). Notwithstanding recent advances obtained in surgical resection, the prognosis is still poor and the average survival of GBM patients is around 15 months (*Mukherjee & Quiñones-Hinojosa, 2011*). Overall survival (OS) appears to be related other than to the extent of surgical resection also to the age of patients since lower age and

higher performance status appear to confer longer survival (*Walid, 2008; Johnson and O'Neill, 2012*). Tumor greater than 5-6 cm and those that cross the mid-line have been associated with negative outcomes (*Ellor et al., 2014*).

The "classical" Renin-Angiotensin System

Renin-angiotensin-aldosterone system (RAAS) is the powerful vasoconstrictor system of the body relaied on the release of renin from the juxtaglomerular cells in the kidney wherein ischemia is one of the main activators of the system. Angiotensinogen, a globulin synthesized by the liver, is a plasmatic substrate of the renin (*Matsusaka et al., 1997; Gavras et al., 1993*).

The latter catalytic reaction generates angiotensin I (ANG I) after cleavage of the amino terminal residue of the decapeptide angiotensinogen. ANG I is an unstable peptide quicky cleaved by ANG II Converter Enzyme (ACE II) into ANG II (*Figure 3*) (*Johnston et al., 1992; Goodfriend et al., 1996; Dzau et al.,2001*). ANG II is a peptidic hormone and it is the main effector in the RAAS (*Figure 4*) (*Matsusaka et al., 1997*).

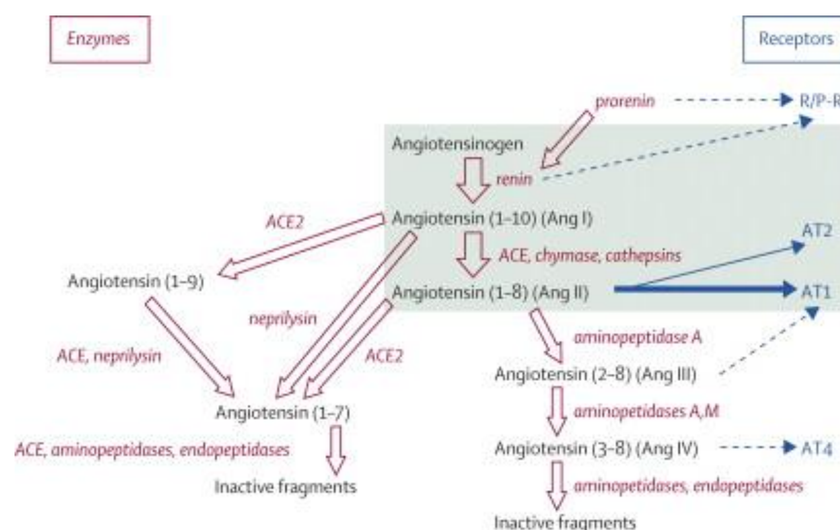

FIGURE 3. The catalytic reactions of clivage of angiotensin peptides *(Schmieder et al., 2007).*

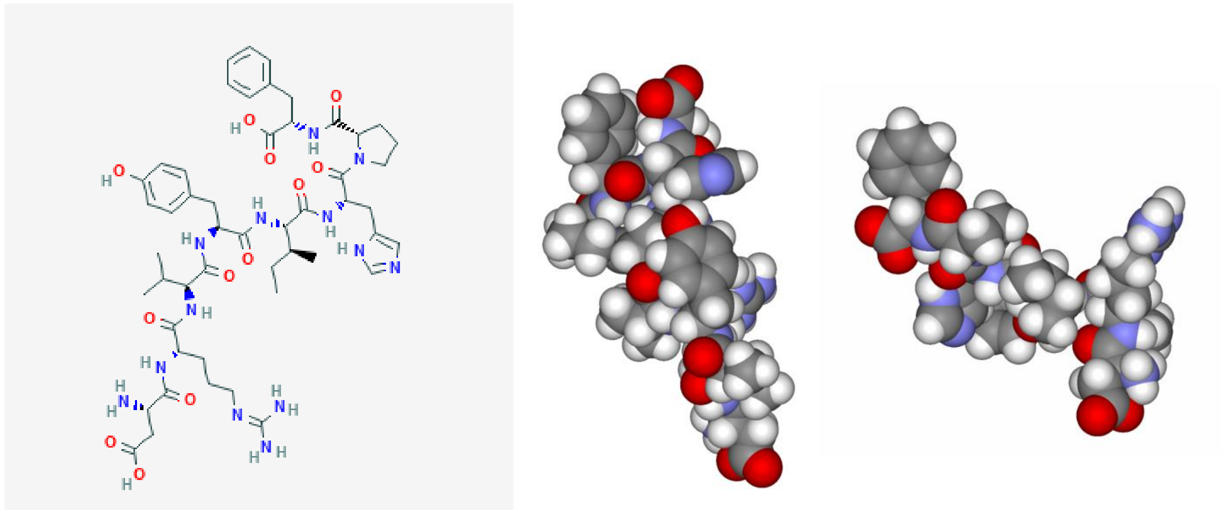

FIGURE 4. The 2D and 3D structure of Angiotensin II.
(http://www.hmdb.ca/metabolites/HMDB0001035 -https://en.wikipedia.org/wiki/Angiotensin)

RAAS plays the most important regulation of blood pressure as well as in the liquid and electrolyte balance. Its alterated regulation contributes on the pathophysiology of hypertension which lead progressively to cardiac hypertrophy remodeling, nephropathy, coronary artery disease and heart failure (*Matsusaka et al., 1997; Gavras et al., 1993*). The functional correlates of the activation of this system includes vasoconstriction in smooth vascular muscle, release of aldosterone by adrenal glands, gluconeogenesis in the liver, sodium absorption by the kidneys and intestine, increased fibroblast proliferation in the heart and increased β-adrenergic activity in the nervous system. (*Ferrario et al., 1990*) (*Figure 5*).

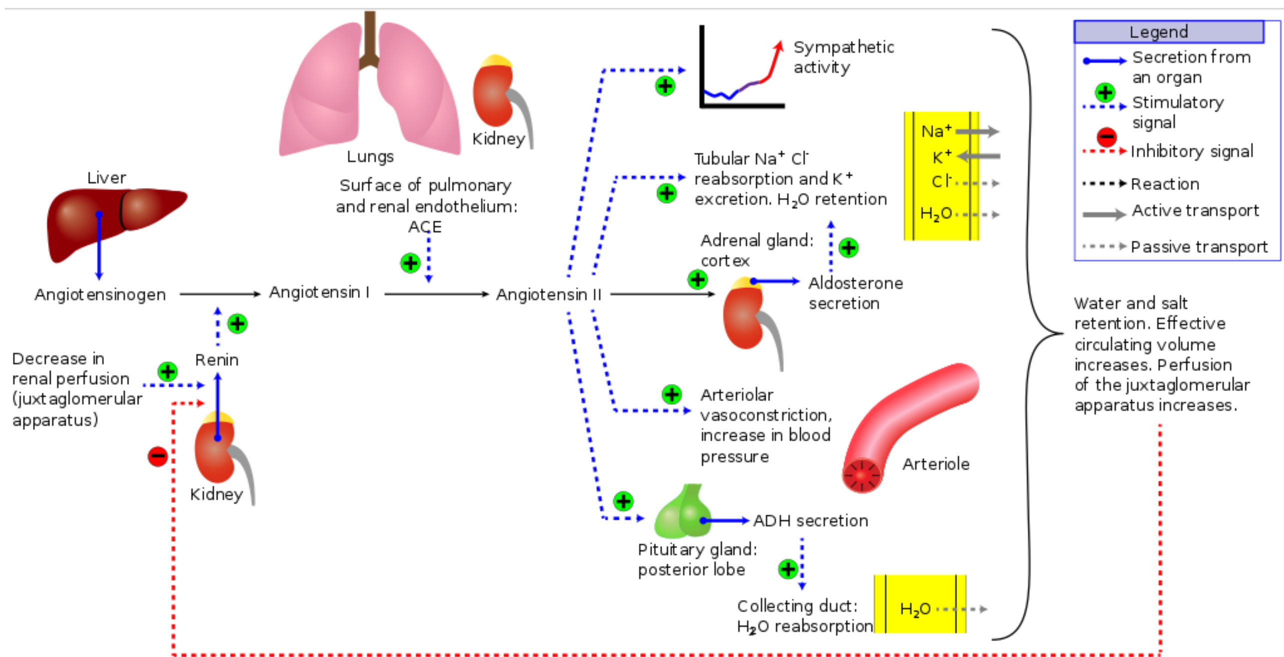

FIGURE 5. The renin-angiotensin system (RAS). *Start reading this schematic from the left, where it says "Decrease in renal perfusion (juxtaglomerular apparatus)". Alternatively, the RAAS can also be activated by a low NaCl concentration in the macula densa or by sympathetic activation. Legend info: Blue and red dashed arrows indicate stimulatory or inhibitory signals, which is also indicated by the +/-. In the tubule and collecting duct graphics, the grey dashed arrows indicate passive transport processes, contrary to the active transport processes which are indicated by the solid grey arrows. The other solid arrows either indicate a secretion from an organ (blue, with a starting Spot) or a reaction (black). These 2 processes can be stimulated or inhibited by other factors. (https://en.wikipedia.org/wiki/Renin%E2%80%93angiotensin_system)*

It has been recently reported how ANG II, generally described as a vasoconstrictor peptide, displays functional features of a growth factor. Indeed, an involvement of this peptide has been demonstrated to occur in the control of cell growth and cell differentiation as well as cell migration, extracellular matrix conformation and inflammation. In addition, it has been highlighted how ANG II stimulates the production of several growth factors such as PDGF, EGF, TGFβ and IGF (*Kim et al., 2000; Chua et al., 1998; Tamarat et al., 2002; Naftilan et al., 1989; Stachowiak et al; 1994; Peng et al., 2001; Scheidegger et al., 1999*).

It also stimulates the production of some vasoconstrictors as endothelin 1 (ET-1) and it helps to transactivation of growth factor receptors (EGF receptor and IGF receptor) (*Fujiyama et al., 2001*). Besides, it induces the expression of several proto-oncogenes in smooth vascular muscle in rats and humans, including c-fos, c-jun, c-myc, erg-1, VL-30, and the activator of the protein 1 complex

(*Touyz et al., 2002; Hsueh et al., 1995*). Besides its effect on blood pressure, ANG II displays several biological effects on the endothelium through its receptors.

Receptors of ANG II

Functional ANG II signaling is activated from its interaction with specific cell surface receptors on the target cells: AGTR1, AGTR2, AGTR3, AGTR4. AGTR3 has been identified in neuroblastoma cells and in rat mesangial cell cultures, AGTR4 have been reported in bovin adrenal cortex and in human placenta. (*De Gasparo et al., 1995; Timmermans et al., 1993*). AGTR1 and AGTR2 receptors display a homologous sequence of only 30% and their genes are located in chromosome 3 and in the X chromosome respectively (*Goodfriend et al., 1996*).

- ***ANG II Receptor 1 action and signaling pathways***

Angiotensin Receptors 1 (AGTR1) belonging to the superfamily of G protein coupled receptors (*Sayeski et al., 1998*), has 359 aminoacids and present 7 transmembrane domains exhibiting the C-terminus located within the cytoplasm and the glycosylated N-terminus at the surface of the cell (*Bergsma et al., 1992; Tamargo et al., 2002*). The affinity of ANG II binding to its own receptors dependes on serum concentrations since AGTR1 has a limited binding capacity (*Goodfriend et al., 1996*).

All tissues present AGTR1 receptors. One subtype has been described in the humans while in the rodent have been identified as $AGTR1_a$ and $AGTR1_b$ receptors (*Matsusaka et al., 1997*).

AGTR1 receptor are mainly expressed in smooth muscle cells, while in the heart they are mainly localized in myocardium and fibroblasts (*Touyz et al., 2002*).

The autocrine regulation of ANG II receptor by its own ligand appears to be different in the different tissue. For istance ANG II exposure decrease AGTR1 receptor in the smooth muscle cells, liver and mesangial cells while it’s upregulated by its own ligand in the kidney. (*Tamargo et al., 2002*).

AGTR1 stimulation produces several effects, such as:

a) ***Vascular effects**:* periferal vascular resistance and blood pressure mainly raise from a powerful arterial and venus vasoconstrictor induced by ANG II/AGTR1 signaling which directly affects the cells releasing aldosterone, endothelin-1 (ET-1) and vasopressin, an increase in sympathic tone, a stimulation of protein and DNA synthesis resulting in an increased volume and number of smooth muscle vessel cells, fibroblasts and middle sheath of the arteries (*Griffin et al., 1991; Van Bilsen et al., 1997; Laragh et al., 1960*);

b) ***Actions on myocardium**:* ANG II/AGTR1 enhances myocardium contractility, heart rate and oxygen requirements as well as an intense coronary artery vasoconstriction (*Moravec et al., 1990; Sudoshima et al., 1993*);

c) ***Renal effects**:* ANG II/AGTR1 decreases renal blood flow, enhances mesangial cells contraction and impairs glomerular filtration, increasing proximal tubular sodium reabsorption and potassium excretion and subsequent inhibition of renin secretion (*Tamargo et al., 2002; Mitchell et al., 1992; Schubert et al., 2001*).

d) ***Cell proliferation and apoptosis**:* an enhanced DNA synthesis is induced by ANG II/AGTR1 signaling in heart cell, vascular cell and fibroblasts cultures. MAPK mitogen activated protein kinases (MAPK) is also induced by ANG II exposure together with an increased expression of several metalloproteinase inhibitors. In addition, ANG II/AGTR1 signaling through an increased expression of the plasminogen activator inhibitor type 1 (PAI-1) that inhibits extracellular matrix degradation and concomitantly increases the synthesis of collagen type I stimulating transforming growth factor β1 (TFG-β1). In contrast to AGTR2 it's well documented the AGTR1 antiapoptotic effects (*Tamargo et al., 2002; Cook et al., 2001; Horiuchi et al., 1999*; *Silvestre et al., 2002; Bascands et al., 2001*);

e) ***Proinflammatory and proatherothrombotic actions**:* AGTR1, through the enhanced expression of the adhesion molecules (VCAM-1), stimulates the interaction between monocytes and endothelial cells, also sustained by proinflammatory cytokines release (TNFα, IL-6), monocyte

migration to subendothelial tissue and enhanced oxidation of low density lipoproteins (LDL*)* (*Tamarat et al., 2002; Tamargo et al., 2002*);

The binding of ANG II to AGTR1 determinates the dissociation of its subunits which activate a Gqα protein (*Goodfriend et al., 1996*), then the phospholipase C-β1 that hydrolyses 4,5-phosphatidylinositol biphosphate into 1,4,5-phosphatydilinositol triphosphate (IP3) and diacylglycerol (*Van Bilsen et al., 1997; Berk et al., 1997*). The effect of IP3 action is located in the sarcoplasmic reticulum membrane facilitating the release of stored calcium and its entrance through calcium channels (L and T channels of heart muscle cell membrane) which are generally activated. The latter process is associated to the exit of negative charges from the cells, depolarizing the membrane and opening L calcium channels particularly when intracellular calcium deposits are void (*Dzau et al., 2001; Touyz et al., 2002; Tamargo et al., 2002*).

Mitogenic effects of ANG II are mediated by different kinases such as PKC, which activates G Ras and mitogen activated protein kinases (MAPK) which in turn phosphorylate several proteins able to translocate into the nucleus wherein enhance the transcription of several genes involved in cell hypertrophy and tissue hyperplasia (*Touyz et al., 2002; Touyz et al., 1997*).

Upon ANG II exposure, phospholipase 1 and 2 are activated in some tissue leading to an increased synthesis of proinflammatory prostaglandine E2.

The enhanced calcium entrance above mentioned through L type channels leads to an increase to cardiac contractility and vascular tone concomitantly with block of several potassium channel. ANG II/AGTR1 signaling (*Figure 6*) activates a Gi protein and inhibits adenylate cyclase with a consequent decrease of intracellular cAMP contributing to the vasoconstrictor effect of ANG II (*Dzau et al., 2001; Tamargo et al., 2002; Sayeski et al., 1998; Van Bilsen et al., 1997; Berk et al., 1997*).

In response to ANG II stimulation several proteins such as c-Src, Jak-2 and FAK are phosphorylated at tyrosines residues favoring the generation of IP3 and the described entrance of calcium to the cell.

The latter event activates intracellular MAPK pathway, phosphorylation of signal transducers and activators of transcription (STATs) and stimulates the transcription of early growth response genes as c-fos, c-jun, c-myc *(Sayeski et al., 1998).*

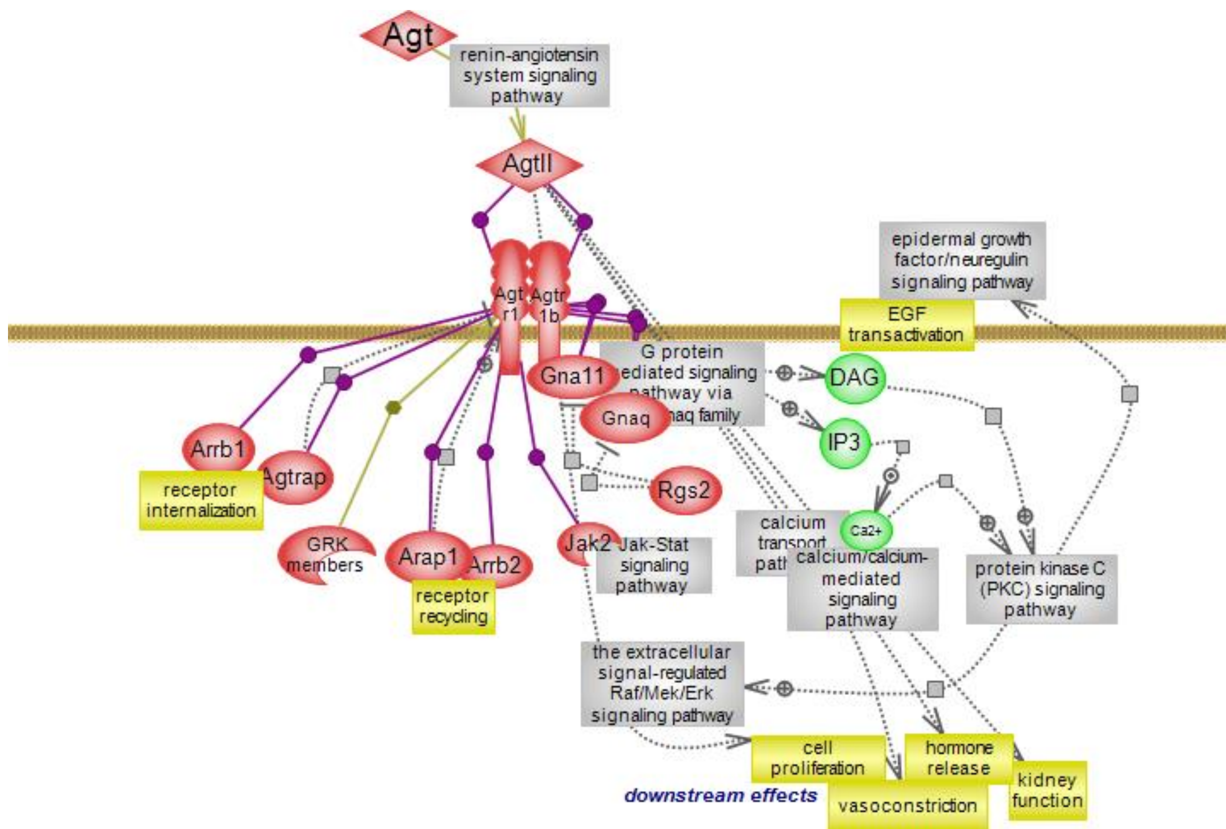

FIGURE 6. Angiotensin II (AgtII) signaling pathway via AGTR1 receptor.
(https://rgd.mcw.edu/rgdweb/pathway/pathwayRecord.html?processType=view&species=Mouse&acc_id=PW:0000244)

- ***ANG II Receptor 2 action and signaling pathways***

ANG II receptor 2 (AGTR2) is constituted by 363 aminoacids and 7 transmembrane domains (*Tsuzuki et al., 1994; Mukoyama et al., 1993*) and is highly expressed in fetal tissues descreasing rapidly after birth. During adult life is mainly located to adrenal cells, brain, myometrium, endothelial cells and to a lesser extent in pancreas, heart and kidney. Heart damage, myocardial hypertrophy, ovarian atrophy is generally associated with his overexpression which is also

maintained upon ACE inhibitors and AGTR1B treatment (*Dzau et al., 2001; Touyz et al., 2002; Nahmias et al., 1995*).

AGTR2 stimulation has different effects, such as:

a) ***Central actions,*** as mentioned before this receptor is an antagonist of AGTR1 receptor effects under physiologic conditions since is inhibits cell growth, induces apoptosis and vasodilation. As a consequence, it induces brain dilation, sustained by prostaglandin release (*Wolf et al., 2002*).
b) ***Proliferative, differentiation and apoptotic effects.*** The antiproliferative effect has been clearly documented in coronary endothelial cells, mesangial cells and pheochromocytoma cell lines wherein proapoptotic effect has been described (*Horiuchi et al., 1999*).
c) ***Vascular effects.*** Whereas AGTR1 induces angiogenesis and cell proliferation (*Pupilli et al., 1999; Leung et al., 2003*), AGTR2 appears functionally antagonist of these actions (*Tamarat et al., 2002*). It has been well demonstrated how the agonist of AGTR2 (CGP42112A) down-regulated the expression of VEGF in endothelial cell and how the combination of losartan with CGP42112A had significantly greater effect in down-regulating VEGF compared to each agent alone (*Park et al., 2014*). Thus, alterations of the signal AGTR1 and AGTR2 could influence apoptosis or survival of endothelial cells in response to the RAS dysregulation.
d) ***Effects on diuresis and natriuresis.*** It has been demonstrated that the AGTR2 plays a role in mediating the natriuretic/diuretic effects of AGTR1 blockers in obese Zucker rats. This may indicate that AGTR2, by promoting sodium excretion, may protect obese Zucker rats against blood pressure increase associated with sodium and water retention (*Hakam et al., 2005*).

The antagonizing effect of AGTR2 on AGTR1 leads to the growth inhibition effects mediated by tyrosine phosphatase (*Figure 7*). Some of these phosphatases are SHP-1, MKP-1 and PP2A, and they are able to inhibit STAT-1 phosphorylation, generating an antimitogenic and/or proapoptotic effect. Through an enhanced production of nitric oxide AGTR2 leads to cGMP stimulation in vascular cells

of coronary arteries and aorta. AGTR2, through Gi proteins, is able to stimulate phospholypase 2 releasing arachidonic acid and several serine/threonine phosphatase proteins able to dephosphorylate several regulatory proteins and decrease the cell concentrations of cGMP (*Horiuchi et al., 1999; Stroth et al., 2000*).

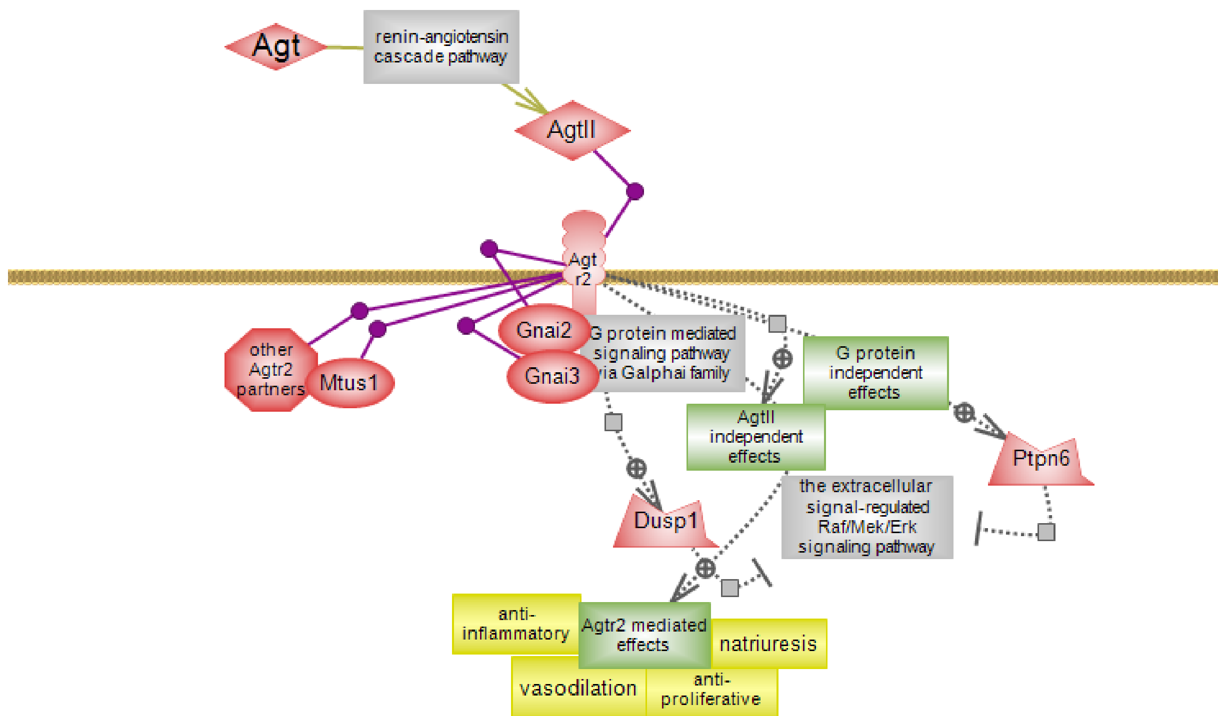

FIGURE 7. Angiotensin II (AgtII) signaling pathway via AGTR2 receptor.
(https://rgd.mcw.edu/rgdweb/pathway/pathwayRecord.html?acc_id=PW:0000528)

ANG II and angiogenesis

Angiogenesis comes from the balance of angiogenesis and stimulated factors. Among the endogenus factors generally consideratedasangiogenic are TGF β, granulocytic colony stimulating factor (GSF), FGFb, hepatocytic growth factor (HGF), tumor necrosis factor α (TNFα), PDGF, IL-8, angiogenin and VEGF (*Tamarat et al., 2002; Folkman et al., 1995*). The latter is the most important factor in the control of normal and pathologic angiogenesis since is able to induce a cascade of responses in endothelial cells, such as proliferation, migration, increase of vascular permeability and expression of pro-inflammatory genes (*Folkman et al., 1995*).

Neovascularization appears to characterize malignant cell invasiveness with ability to spread and generate metastases thus angiogenesis inhibition is an attractive therapeutic target for several malignant neoplasms; in fact, there are several studies that have explored this therapeutic option (*D'Amaro et al., 1994; Arrieta et al., 1998; Kisker et al., 2001; Arrieta et al., 2002; Folkman et al., 2002)*.

It is widely documented how in different experimental model the administration of exogenous ANG II increases the blood flow recovery in the muscles of lower extremities as observed in nephrectomized rats (*Fernandez et al., 1982; Fernandez et al., 1985*). When gerbils with progressive occlusion of the carotid artery were treated with ACE inhibitors, mortality increased, suggesting that ANG II has a protective effect against chronic ischemia (*Kaliszewski et al., 1998; Fernandez et al., 1986*). However, it was mentioned how antagonists of AGTR1, such as losartan, and AGTR2 agonists increased survival in this stroke model addressing speculatively AGTR2 stimulation involved in such circumstances (*Fernandez et al., 1994*).

Angiogenic effect of ANG II was also demonstrated in other models such as in the chicken embryo chorioalantoid membrane wherein this octapeptide caused precapillary and postcapillary vessel neoformation (*Le Noble et al., 1991*). It was mentioned how angiogenesis stimulation by ANG II was not blocked by losartan (AGTR1 receptor antagonist), but it was blocked by an AGTR2 ligand. (*Le Noble et al., 1993*). This finding suggests that the angiogenic effect of ANG II is mediated by different receptor subtypes.

It's worth to mention how ischemia is able to stimulate angiogenesis, a process that is experimentally inhibited in mice with limb ischemia with the pharmacological blockade of AGTR1 or ACE inhibitors (*Emmanueli et al., 2002*). The process above mentioned is affected in AGTR1 receptor knockout mice suggesting how AGTR1 receptor is involved in angiogenesis induced by ischemia *in vivo* (*Sasaki et al., 2002*). Anyway, it is mentioned as many components of RAAS may have different and opposing function. In addition, the regulation of angiogenesis by RAAS can be modified by many factors in different experimental or clinical situations, generating paradoxical results.

Renin-Angiotensin System and cancer

The interest to study RAS in a wider area of biological processes beyond the blood pressure and body fluids regulation is progressively increasing (*Munro et al., 2017*). Indeed, the signalling of individual RAS components and their crosstalk with other pathways including the IGF/IGFR1 and VEGF pathways, are implicated in numerous biological processes, such as angiogenesis, cell migration and proliferation, tumorigenesis (*Munro et al., 2017*). Thus, it emerges from this integrated and complex system a favourable microenvironment for promoting the proliferation and differentiation of CSCs (*Bradshaw A. et al., 2016; Jokubaitis et al., 2008; Sinka et al., 2012; Featherston et al., 2016; Bradshaw A.R. et al., 2016; Yu et al., 2016*). All these candidates RAS and AGTR1 receptors are potential targets to prevent self-renewal of CSCs blocking their capability to drive cancer progression and spread (*Munro et al., 2017*).

- ***Role of ACE***

The role of ACE in cancer has been investigated among different tumour and particularly in infantile haemangioma, the most frequent tumour in infancy (*Munro et al., 2017*). ACE is expressed on the endothelium of the microvessels within the peritumoral stroma surrounding tumour nests (*Featherston et al., 2016; Baillie et al., 2016; Ram et al., 2017; Bradshaw A.R. et al., 2016*). ACE inhibitors have been reported to have a cancer protective effect reducing tumour growth in necks cervical cancers (CCs) as well as metastasis and angiogenesis in orthotopic models of metastatic colorectal cancers to the lung (*George et al., 2010; Ager et al., 2008; Destrayes et al., 2005; Zambidis et al., 2008; Rosenthal et al., 2009*).

- ***Role of AGTR1***

It is well documented how AGTR1 expression is increased in breast, ovarian and gastric cancers (*George et al., 2010; Ager et al., 2008*). Particularly AGTR1 is overexpressed only in estrogen receptor positive breast tumors establishing a link between RAS and estrogen signaling (*George et*

al., 2010). Besides RAS dysregulation is also associated with an enhanced VEGF (*George et al., 2010; Ager et al., 2008*). AGTR1 has been associated with tumor differentiation status while it appears a marker of tumour invasiveness in cervice cancer (*Deshayes et al., 2005*). It has been documented how the connective tissue around the tumour displays an high expression of both AGTR1 and VEGF levels (*Deshayes et al., 2005; Ino et al., 2006*). The tissue-associated macrophages around the tumour also display increased AGTR1 levels and release more VEGF indicating how AGTR1 can condition tumour microenvironment by modulating immune cell infiltration (*Deshayes et al., 2005*). Particularly, AGTR1 plays a crucial role in the recruitment of tumour-associated macrophages which are the most abundant cells in the stroma, able to promote inflammation angiogenesis, migration, metastasis and suppression of anti-tumour immune responses (*Cortez-Retamozo et al., 2013*). Reduction of AGTR1 production leads to a lower number of HSPC (hematopoietic stem and progression cells), and TAMs in the spleen suggesting that ANG II/AGTR1 signalling when overexpressed, promotes endothelial-mesenchymal-transition (EMT), a process essential to cancer invasiveness and metastasis (*Cortez-Retamozo et al., 2013; Tawinwung et al., 2015*). Finally, administration of ANG II Receptor Blockers (ARBs) is concomitant with a reduction of tumour size, vascularization, lower occurrence of metastasis and lower VEGF levels (*Deshayes et al., 2005*).

- ***Role of AGTR2***

It is well known how AGTR2 counteracts the effects of AGTR1 in cancer (*Munro et al., 2017*). AGTR2-deficient mice demonstrate a strong increase in AGTR1-mediated VEGF upregulation upon ANG II exposure (*Ager et al., 2008*). In addition, AGTR2 blocks VEGF to reduce cell migration and metastasis (*Ager et al., 2008; Deshayes et al., 2005*).

Renin-Angiotensin System and Glioblastoma

The angiogenic effect of ANG II may influence carcinogenesis as well as the growth of several neoplasms. In the same line is worth to mention how in two epidemiologic studies involving

hypertensive patients the treatment with ACE inhibitors could decrease cancer risk although the results had no statistical significance (*Escobar et al., 2004*).

However, a retrospective study involved in more than 4900 hypertensive patients has shown that patients treated with ACE inhibitors display a significantly reduced cancer risk compared to patients without this treatment (*Escobar et al., 2004*).

We are aware that vessel perfusion conditions oxygen delivery in tumors (*Chauhan et al., 2013; Tsai et al., 2003; Jain et al., 2013*). Cancer and stromal cells proliferate in a limited microenvironment made up by cross-linked matrix molecules accumulating a high pressure by solid tissue components defined as "solid stress" which are transmitted trough the tumor collapsing blood vessel and limiting perfusion (*Helmlinger et al., 1997; Stylianopoulos et al., 2012; Stylianopoulos et al., 2013; Janmey et al., 2007; Griffon-Etienne et al., 1999; Padera et al., 2004*). The latter event determinates extensive hypoxia and limited drug delivery with a conseguent poorer chemioterapy response and short survival versus patients with high perfusion (*Park et al., 2009; Sorensen et al., 2012*). So, it is mandatory to identify drugs able to target solid stress and to improve blood perfusion and therapeutics delivery in tumors (*Chauhan et al., 2013*).

Previous studies have highlighted the role of CAF in producing tumor matrix components responsible of vessel compression, ANG II blockade reduces CAF density and inhibits the production of matrix components (*Chauhan et al., 2013*). Thus, ARBs such as losartan reduces the solid stress recompressing tumor vessels, increases drug delivery with a better oxygen supply improving chemotherapy efficacy (*Williams et al., 1995; Chua et al., 1998; Montezano et al., 2008; Carbajo-Lozoya et al., 2012*).

Local expression of RAAS has been demonstrated in normal tissue and in most cancers (*Deshayes et al., 2005; Fujita et al., 2002; Egami et al., 2003; Suganuma et al., 2005*) including human GBM and normal astrocytes (*Milsted et al., 1990; Fogarty et al., 2002; Juillerat-Jeanneret et al., 2004; Arrieta et al., 2005*). For istance, anaplastic astrocytoma and glioblastoma multiforme, are aggressive tumors

featured by enhanced endothelial cell proliferation and angiogenesis as well as a local production of growth factors such as PDGF, VEGF and HGF.

In contrast, some studies have shown that renin is synthesized by glioblastoma multiforme cells, unlike low grade reactive gliosis tumors, indicating a possible link existing between endogenous intratumoral renin and angiogenesis (*Ariza et al., 1988*; *Ganong et al., 1984*). Besides, it has been demonstrated how rat glioblastoma exhibits AGTR1/AGTR2 receptors subtypes and that incubation of these cells with different types of angiotensins stimulates cell proliferation regardless AGTR1/AGTR2 receptors expression (*Fogarty et al., 2002*). In the same way, in an *in vivo* study with C6 rat glioma has been evidenced as losartan inhibits in a dose related manner cell proliferation and vascular density (*Rivera et al., 2001*).

Estrogens and Aromatase in Glioblastoma

Previously it has been demonstrated how ANG II/AGTR1 signaling induces estradiol secretion from human placenta and the latter event was reversed by losartan in a dose related manner (*Kalenga et al., 1995*). Estrogens (*Figure 8*) stimulate cell proliferation in tumor such as in breast, lung, endometrium and prostate (*Dueñas Jiménez et al.,2014*).

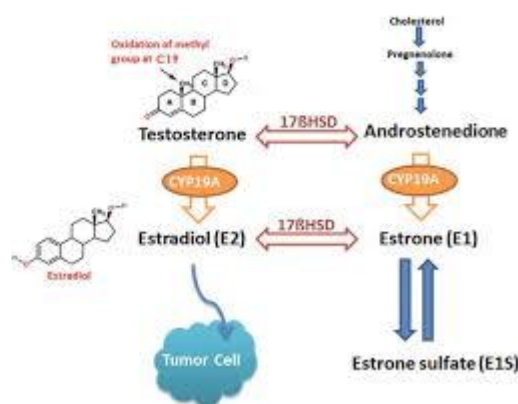

FIGURE 8. Estrogens are oncogenic hormones *(https://www.intechopen.com/books/genetic-polymorphisms/genetic-polymorphisms-in-aromatase-cyp19-gene-and-cancer)*

Estrogen biosynthesis is catalized by aromatase enzyme (*Figure 9*) which has two components: cytochrome P450 arom, a microsomial member of the cytochrome P450 superfamily, encoded by the

cyp19a1 gene and the ubiquitous NAPDH cytochrome P450 reductase (*Kamat et al., 2002; Simpson et al., 2001*).

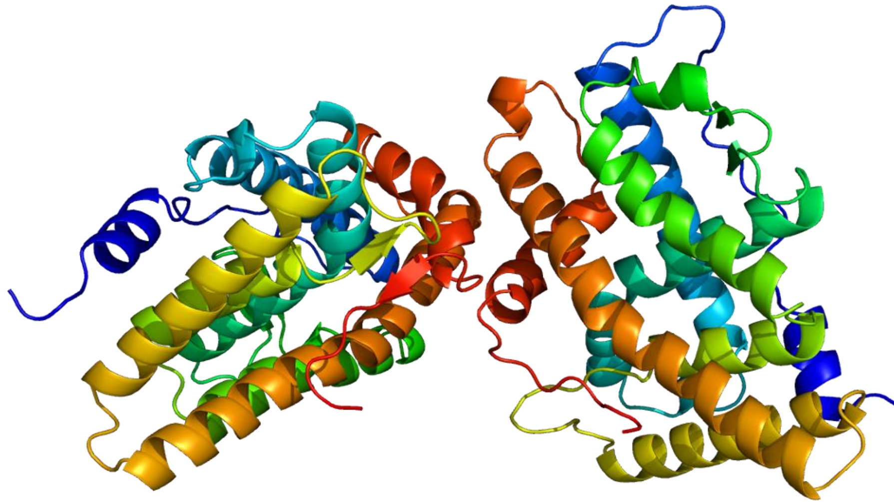

FIGURE 9. The 3D structure of Aromatase. *(https://en.wikipedia.org/wiki/Aromatase).*

Most vertebrates express aromatase in the gonads and in the brain (*Simpson et al., 2003*) and also in the hypophysis (*Carretero et al., 2002*) and in the placenta (*Ryan et al., 1959*) of mammals including humans. The enzyme is also expressed in other tissue such as adipose tissue (*Grodin et al., 1973*), as well as in mammarian brain (*Naftolin et al., 1971*) where the enzyme as being is mainly detected in the hypothalamus and limbic system where in the normal conditions the expression in restricted to neurons (*Balthazart et al., 1991; Lephart, 1996*). The enzyme has been also reported in glial cells of birds and mammals in primary cultures (*Azcoitia et al., 2003; Schlinger et al., 1994; Zwain et al., 1997*) and in radial glia of some teleosts (*Forlano et al., 2001; Menuet et al., 2003*). Finally, it's worth to mention how after brain injury reactive astrocytes of birds and mammals express aromatase (*Peterson et al., 2001; Garcia-Segura et al., 1999*). Local estrogen production from androgen precursor convertion is involved in neuronal differentiation, neuronal plasticity, neuroendocrine functions and sexual behaviors (*Hutchison, 1991; MacLusky et al., 1981; Lephart, 1996; Zwain et al., 1997; Garcia-Segura et al., 2003*). Following brain injury estradiol appears to be neuroprotective and prevents neural loss (*Dhandapani et al., 2002; Garcia-Segura et al., 2001; McCullough et al., 2003; Wise et al., 2001*). In contrast, aromatase expression by the tumors providing local estrogen

production has a negative impact on tumor growth (*Garcia-Segura et al., 2003; Azcoitia et al., 2001; de Jong et al., 2001; Kitawaki et al., 1993; Risbridger et al., 2003; Sasano & Harada, 1998; Tekmal et al., 1996*). The role of estradiol in affecting glioblastoma cells has some limited evidences (*Fujimoto et al., 1984; Leslie et al., 1994*). Estrogens modulate the growth of different tumors throught the two receptors ERα and ERβ (*Hua et al., 2018*). Estrogen receptor β appears to decrease in breast, ovarian, prostatic and colon carcinoma, vascular neoplasia (*Deyrup et al., 2004; Konstantinopoulos et al., 2003; Pujol et al., 1998; Speirs et al., 2002*) and in astrocytes tumors. Estrogen receptor β has a prognostic value for the survival time of patients with astrocytic tumors (*Batistatou et al., 2004*). Estrogen receptor α decreases in astrocytoma together with tumor grade progression (*Kefalopoulou et al., 2012*). Selective estrogen receptor modulators exhibit cell proliferation of glioma (*Patel et al., 2012*), while natural compounds, that act as ERβ agonist, inhibit the growth of glial tumors (*Sareddy et al., 2012*). There are few data related to the capacity of glioblastoma to produce estradiol while expression of aromatase has been displayed in different glioblastoma cell lines (*Yague et al., 2004*).

- ***Aromatase gene and promoters***

The aromatase protein in humans is encoded by the CYP19A1 gene, which extends roughly 123 kb on chromosome 15q21.2 and consists of a 93 kb 5′-untranslated region (UTR), 30kb of coding region, and the 3′-end (*Bulun et al., 2005; Boon et al., 2010*). The aromatase coding region consists of nine exons (II–X) with the ATG translational start site located in exon II. The 5′-UTR consists of a number of alternative untranslated first exons that are regulated by tissue-specific promoters (*Figure 10*). Until now, in humans have been found ten alternative tissue-specific promoters, including promoters I.1, I.2 and I.2a in placenta; I.4 in adipose tissue and skin; I.5 in fetal tissues; I.f in brain; I.7 in endothelial cells; I.6 in bone; I.3 in adipose tissue; and PII in gonads and adipose tissue (*Bulun et al., 2005; Bulun et al., 2012*). These promoters differentially regulate aromatase expression in gonads, adipose tissue, bone, brain, skin, fetal liver and placenta (*Bulun et al., 2005; Bulun et al., 2012*).

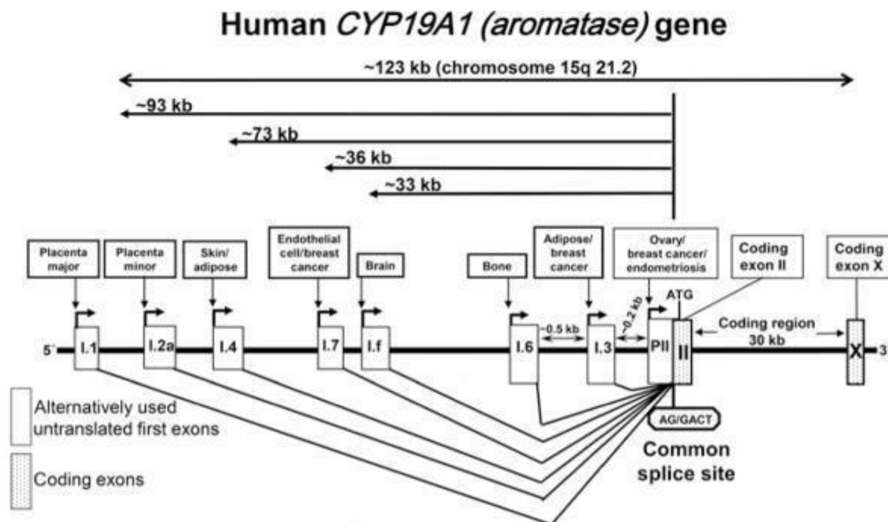

FIGURE 10. Human aromatase (*CYP19A1*) gene. *Expression of the aromatase gene is regulated by the tissue-specific activation of a number of promoters via alternative splicing. Aromatase mRNA species contain promoter-specific 5'-UTRs. The coding region and encoded protein, however, are identical regardless of the promoter used. (Zhao et al., 2016)*

Aromatase promoter I.4 (*Figure 11*) does not have a TATA or CAAT box upstream of the transcription start site for the untranslated exon I.4 (*Zhao et al., 1995; Chen et al., 2009*). The 5′-UTR of exon I.4 contains a putative silencer and several positive cis-acting elements, including an AP-1 site, an interferon γ activation site (GAS), a glucocorticoid response element (GRE), and an SP1-binding site, which can recruit and bind the transcriptional factors c-Fos/c-Jun, phosphorylated signal transducer and activator of transcription 3 (phospho-STAT3), glucocorticoid receptor (GR), and SP1, respectively (*Zhao et al., 1995*). Promoter I.4 is prevailing used and least activated in normal breast adipose tissue to keep basal levels of aromatase expression (*Bulun et al., 2005*).

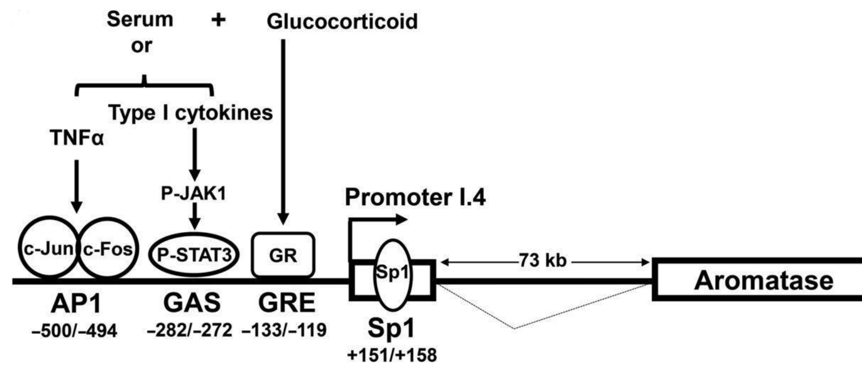

FIGURE 11. Activation of aromatase promoter I.4. *Glucocorticoid plus serum stimulates aromatase promoter I.4. Serum can be substituted with TNFα or one of the type I cytokines. Glucocorticoid is obligatory for promoter I.4 stimulation by binding to and activating the glucocorticoid receptor (GR), which interacts with the glucocorticoid response element (GRE) in promoter I.4. TNFα plus glucocorticoid induces expression of c-Jun and c-Fos, which heterodimerize and bind to the AP1 site in promoter I.4. Type I cytokines plus glucocorticoid, on the other hand, activate the JAK1/STAT3 pathway, resulting in binding of tyrosine phosphorylated STAT3 to the interferonγactivation site (GAS) in promoter I.4. Sp1 protein binding to its binding site is also essential for promoter I.4 stimulation (Zhao et al., 2016).*

The promoter I.f is the brain specific first promoter located ~33 kb upstream of exon 2 and it has precisely the same length in human and mouse (139 bp) with a high sequence homology of 94% (*Chow et al., 2009*). Are not well comprehended the signaling pathways or molecular mechanisms that regulate the brain-specific aromatase promoter I.f. Many groups of researchers have found that protein kinases A and C and cAMP regulate in the brain expression and activity of aromatase (*Balthazart et al., 2001; Lavaque et al., 2006*). Other groups demonstrated that testosterone also upregulates in the brain mRNA and enzyme activity of aromatase (*Zhao et al., 2007; Abdelgadir et al., 1994; Lephart et al., 1992; Negri-Cesi et al., 2001; Roselli et al., 1997*). Furthermore, was seen that estradiol upregulates or downregulates mRNA and enzyme activity of hypothalamic aromatase under both *in vivo* and *in vitro* circumstances (*Zhao et al., 2007; Negri-Cesi et al., 2001; Kretz et al., 2004; Iivonen et al., 2006*).

It is uncertain if aromatase promoter expressed and working in glioma are the same of those present in normal neurons and glia. Recent findings have report that glioblastoma cell line T98G displayed the same aromatase exon I transcript in human temporal cortex such as pII, pI.3, pI.f and pI.4 (*Tan et al., 2017; Yague et al., 2006*). It is observed how, according to this finding, the two latter promoters

exhibit the same exhibition pattern in glioblastoma cells and in temporal cortex (*Tan et al., 2017; Yague et al., 2006*) .

So, in the present study we focused our attention on the effect of ANG II treatment on the aromatase pI.f and pI.4 promoters, just to investigate its direct capability in modulating aromatase gene transcription.

AIM OF THE STUDY

Previous findings obtained in different experimental model have highlighted how ANG II treatment stimulates local estrogen productions in different tissues. The aim of the present study is to ascertain in two glioblastoma cell lines (U-87 MG and T98G) the molecular link existing between ANG II/AGTR1 signaling and estrogen local production, in sustaining tumor growth and progression. The whole experimental design pursued in the present study attempts to evaluate if the use of RAS inhibitor with/without antiestrogens may represent a potential pharmacological tool to be implemented in the novel therapeutical strategies of glioblastoma treatment.

MATERIALS AND METHODS

Materials

ANG II, androstenedione, anastrozole, aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), and sodium orthovanadate were purchased from Sigma-Aldrich (St. Louis, Missouri, U.S.A.). losartan was purchased from D.B.A. Italia s.r.l. (Segrate, Italy). Antibodies used in this study were: AGTR1 (Sigma-Aldrich); β-actin (Sigma-Aldrich); aromatase (Bio-Rad Laboratories; Berkeley, California).

Cell Culture

Human fetal glial cells SVG p12 and human glioblastoma cell lines U-87 MG and T98G were purchased from ATCC (Manassas, VA, USA). The SVG p12, U-87 MG, and T98G cells were cultured in Minimum Essential Medium (Life Technologies, Monza MB, Italy), including 10% heat-inactivated fetal bovine serum (FBS), 200 mM L-glutamine, 1% penicillin-streptomycin, 1% Eagle's nonessential amino acids, and 1% sodium pyruvate (Sigma Aldrich). Cells were cultured at 37 °C in a humidified atmosphere with 5% carbon dioxide. Cells were stored following the supplier's recommendations, and authenticated every six months after frozen aliquot resuscitations and regularly tested for mycoplasma negativity (MycoAlert Mycoplasma Detection Assay, Lonza, Basilea, CH, Switzerland).

- **<u>IN VITRO EXPERIMENTS</u>**

RNA Isolation, Reverse transcription and Real-time PCR

RNA was isolated using TRIzol (Life Technologies; Carlsbad, California, U.S.A.) following the manufacturer's protocol. U-87 MG, T98G and SVG p12 cells (6×10^6) were plated in 60-mm plates in MEM medium containing 10% FBS, 1% L-glutamine, 1% Eagle's nonessential amino acids, 1% Sodium Pyruvate and 1 mg/ml penicillin/streptomycin, serum-starved for 24 hours and then treated in 5% dextran charcoal stripped (CS) FBS.

cDNA was synthesized from 2 μg of RNA using High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific; Waltham, Massachusetts, U.S.A.), as indicated by the manufacturer. Analysis of gene expression was assessed by real-time PCR, using SYBR Green Universal PCR Master Mix (Bio-Rad, Segrate, Italy). mRNA expression levels of different genes were normalized on GAPDH mRNA content.

The primers were:

(*AGTR1* - forward) 5'-TCAGCCAGCGTCAGTTTCAA-3';

(*AGTR1* - reverse) 5'-CTACAAGCATTGTGCGTCGAAG-3';

(*CYP19A1*/Aromatase - forward) 5'-CCCTTTGATTTCCACAGGACT-3';

(*CYP19A1*/Aromatase - reverse) 5'-CGGGTTCAGCATTTCCAAAACCAT-3';

(*GADPH* - forward) 5'-CCCACTCCTCCACCTTTGAC-3';

(*GADPH* - reverse) 5'-TGTTGCTGTAGCCAAATTCGTT-3'.

Immunoblot Analysis

Protein expression or complex formation were assessed as described (*De Amicis et al., 2010*) by Western blotting (WB) using nuclear, total or cytoplasmic protein lysates, where appropriate. U-87 MG, T98G and SVG p12 cells (6×10^6) were plated in 100-mm plates in MEM medium containing 10% FBS, 1% L-glutamine, 1% Eagle's nonessential amino acids, 1% Sodium Pyruvate and 1 mg/ml penicillin/streptomycin, serum-starved for 24 hours and then treated in 5% dextran charcoal stripped (CS) FBS. The day after the cells were harvested to be analyzed using 300 μL of lysis buffer containing 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, 10 mmol/L EGTA (pH 7.5), 10% glycerol, and inhibitors (0.1 mmol/L Na_3VO_4, 1% PMSF, and 2.0 mg/mL aprotinin) to obtain cytoplasmic proteins. After the collection using a scraper, incubation of 30' on ice, we lysed the nuclei for 15' at 4˚C using 300 μL of nuclear buffer containing 20 mmol/L HEPES (pH 8), 0.1 mmol/L EDTA, 5 mmol/L $MgCl_2$, 0.5 mol/L NaCl, 20% glycerol, 1% NP-40, and inhibitors (1.7 mg/mL aprotinin, 1 mg/mL leupeptin 200 mmol/L PMSF, 200 mmol/L sodium

orthovanadate, and 100 mmol/L sodium fluoride). Then lysates were collected and centrifuged at 12000 × g for 10’ at 4˚C.

For total protein extracts, 300 µL RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na deoxycholate, plus inhibitors 1.7 mg/mL aprotinin, 1 mg/mL leupeptin 200 mmol/L PMSF, 200 mmol/L sodium orthovanadate, and 100 mmol/L sodium fluoride) was added to the 100 mm cell culture plate for 15’ at 4˚C. Then lysates were collected and centrifuged at 12000 × g for 10’ at 4˚C. The protein content was determined using Bradford dye reagent (Bio-Rad Laboratories). For WB, 50 µg of total or cytoplasmic lysates were separated on an 12% polyacrylamide denaturing gel (SDS-PAGE) and transferred to nitrocellulose membranes. Immunoblots show a single representative of three separate experiments. The images were acquired using Odissey FC (Licor, Lincoln, NE, USA). The Scion Image laser densitometry scanning program was used to quantify the band of interest. Standard deviations along with associated p values for the biological replicates were determined by using the GraphPad-Prism7 software program (GraphPad Inc., San Diego, CA, USA).

Immunofluorescence

U-87 MG, T98G and SVG p12 cells were cultured in 6-well plates in 6-well plates in MEM medium containing 10% FBS, 1% L-glutamine, 1% Eagle’s nonessential amino acids, 1% Sodium Pyruvate and 1 mg/ml penicillin/streptomycin and serum-starved for 24 hours. The day after the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 20’ at room temperature. Next, cells were permeabilized with 0.2% Triton X-100 in PBS for 5’, blocked with 5% BSA for 30’, and incubated with anti-AGTR1 and anti-Arom antibodies (overnight at 4˚C). The day after the cells were washed three times with PBS and incubated with the secondary antibody anti-rabbit IgG-FITC (1:500) for 1 hour at room temperature. To check the specificity of immunolabeling, the primary antibody was replaced by normal rabbit serum (negative control). 4’,6-Diamidino-2-phenylindole (DAPI; Sigma Aldrich) staining was used for nuclei detection. Immunofluorescence analysis was

carried out on an OLYMPUS BX51 (Tokyo, Japan) microscope using a ×100 objective. Images are representative of three different experiments.

Cell proliferation assays

Anchorage-independent soft agar growth assays

U-87 MG and T98G cells (5000/well) were plated in 2 ml of 0.35% agarose with 5% CS FBS in phenol red free media, on a 0.7% agarose base in 12-well plates. Two days after plating, media containing control vehicle or treatments were added to the top layer, and the appropriate media was replaced every 2 days. After 14 days, 150 µl of MTT was added to each well and allowed to incubate at 37˚C for 4 hours. Plates were then placed in 4˚C overnight and colonies ≥50 µm diameter from triplicate assays were counted. Data are the mean colony number of three plates and representative of two independent experiments analyzed for statistical significance ($p < 0.05$) using a two-tailed student's test, performed by Graph Pad Prism 7 (GraphPad Software, San Diego, CA, USA). SDs are shown.

[^{3}H]-Thymidine incorporation assay

U-87 MG and T98G cells were seeded in 24-well plates at 1.5×10^5 per well and grown for 24 hours in MEM medium containing 10% FBS, 1% L-glutamine, 1% Eagle's non-essential amino acids, 1% Sodium Pyruvate and 1 mg/ml penicillin/streptomycin and serum-starved for 24 hours. Next treatments were added in 5% dextran charcoal stripped (CS) FBS. After 24 hours incubation 0.5 µCi of [^{3}H]-thymidine was added to each well. Cells were incubated for 5 h at 37 ˚C. Next the medium was removed and cells were washed twice with cold (4 °C) 0.05 M Tris-HCl and 5 % trichloroacetic acid. At the end, cells were collected and placed into the scintillation cocktail. The level of [^{3}H]-thymidine incorporated in the newly synthesized DNA strand in relation to cells proliferating during the S phase of the cell cycle was assessed by a scintillation counter. The cells from passage 11 to 13 were used.

Aromatase activity assay

Aromatase activity in U-87 MG and T98G cells was measured by the tritiated water release assay, based on the formation of tritiated water during aromatisation of an androgenic substrate such as [1β-^{3}H(N)]-androst-4-ene-3,17-dione. Glioma cells were seeded onto 6-well plates (2 x 10^{5} cells per well) in 1,5 ml of MEM medium containing 10% fetal bovine serum (10% FBS), 1% L-glutamine, 1% Eagle's nonessential amino acids, 1% Sodium Pyruvate and 1 mg/ml penicillin/streptomycin. After 24 hours of starvation cells were treated. After 24 hours, when a homogeneous monolayer of preconfluent U-87 MG and T98G cells was reached, media were aspirated and replaced by fresh media (1,5 ml per well) containing 100 nM [1β- ^{3}H(N)]-androst-4-ene-3,17-dione] (NEN Life Science Products, Boston, MA, USA) (25–30 Ci mM^{-1}) in the presence of its diluent (ethanol at a final concentration lower than 0.0001%). At 5 h of incubation at 37 ˚C, the media were transferred to tubes containing 5 ml of chloroform, vortexed for 1 min and then centrifuged at 3000 g for 15 min. The resulting aqueous supernatants were adsorbed with 10% dextran-coated charcoal, vortexed for 1 minute, centrifuged at 3000 g for 15 min and the supernatant added to vials with scintillation cocktail and counted in a beta counter. The amount of radioactivity in [^{3}H]-water measured was corrected by subtracting the blank values from each sample, obtained by incubating dishes containing medium with the tritiated androgen but no cells. The values were also corrected by taking into account the fractional retention of tritium in medium water throughout the procedure of incubation and processing, utilising parallel dishes containing medium plus known amounts of [^{3}H]-water (NEN Life Science Products) through incubation and assay. The fractional retention of tritium in medium water throughout the incubation and processing of samples was always higher than 85%.

ELISA assay

Enzyme-Linked Immunosorbent Assays (ELISA) were performed using kit for 17β-estradiol (# ES180S-100, Enzo Life Sciences; Pero (MI), Italy) following the manufacturers protocol.

ELISA was used to test aromatase activity. U-87 MG and T98G cells were seeded onto 100-mm Petri plates MEM medium containing 10% fetal bovine serum (10% FBS), 1% L-glutamine, 1% Eagle's nonessential amino acids, 1% Sodium Pyruvate and 1 mg/ml penicillin/streptomycin. After 24 hours of starvation cells were treated and incubated for 24 hours. Next cell medium was recovered and the samples were lyophilized. The lyophilized samples were resuspended in 100 µL of private serum medium. Three micrograms of media extract were placed into each well of 96-well ELISA plates. 50 µl of each sample was added into the wells, followed by the addition of 50 µl of E_2-HRP (estradiol conjugate to horseradish peroxidase) and 50 µl of anti-estradiol. The plate was incubated for 2 hours at 37˚C. The liquid was removed and the wells were washed with wash buffer three times. Then, 200 µl of chromogen solution (tetramethylbenzidine) was added. The plate was incubated in the dark for 30 min. at room temperature, followed by the addition of 50 µl of stop solution (1.8 N H2SO4). The solution color changed from blue to yellow and absorbance was read on a spectrometer at 450 nm. A standard E_2 curve was used to compare the absorbance values of the samples.

Wound Healing Assays

U-87 MG and T98G cells were cultured until more than 70 % of confluent growth for each well of 6-well plates in MEM medium containing 10% fetal bovine serum (10% FBS), 1% L-glutamine, 1% Eagle's nonessential amino acids, 1% Sodium Pyruvate and 1 mg/ml penicillin/streptomycin, at 37 °C in a humidified atmosphere of 5 % CO_2. Seeded cells in well plates were scratched with a 2-10 µl micropipette tip to the same length and width. U-87 MG and T98G cells were treated in 5% dextran charcoal stripped (CS) FBS and then incubated at 37˚C for 12 hours. The images of each treatment group were captured at 10× magnification, using an OLYMPUS BX51 (Tokyo, Japan) microscope.

Boyden chamber transmigration

U-87 MG and T98G cells (5 x 10^3) were loaded into the upper Boyden chamber of a 24-well. The lower well contained cell culture MEM medium containing 10% fetal bovine serum (10% FBS), 1%

L-glutamine, 1% Eagle's nonessential amino acids, 1% Sodium Pyruvate and 1 mg/ml penicillin/streptomycin. Lower and upper chambers were separated by a 8-mm pore-size polycarbonate. Treatments were performed after 24 hours of serum starvation prior to the start of migration. Transmigrated cells were stained with blue fluorescent DAPI and counted by microscopy, using an OLYMPUS BX51 (Tokyo, Japan), ×10 objective. All assays were carried out at least in triplicate, and wells were counted by an investigator blinded to the experimental setup.

Cell invasion assay

24-well plates (6.5 mm, 8.0μm pore Polycarbonate membrane; Corning®) with matrigel-coated transwell inserts were used. U-87 MG and T98G cells (5×10^3) were seeded into the top of the matrigel. In the bottom of the well, 650 μL of MEM medium, containing 10% fetal bovine serum (10% FBS), 1% L-glutamine, 1% Eagle's nonessential amino acids, 1% Sodium Pyruvate and 1 mg/ml penicillin/streptomycin, was used as a chemoattractant during 12 hours under different conditions. Treatments were performed, after 24 hours of serum starvation prior to the start of migration. Transmigrated cells were stained with blue fluorescent DAPI and counted by microscopy, OLYMPUS BX51 (Tokyo, Japan), ×10 objective. All assays were carried out at least in triplicate, and wells were counted by an investigator blinded to the experimental setup.

Transient transfections assays

U-87 MG cells were transiently transfected using the Lipofectamine 2000 reagent (Invitrogen; Carlsbad, California, U.S.A.) with different aromatase promoters conjugated with luciferase reporter gene constructs (p1.f, p1.4, p1.4 GASmut, p1.4 GREmut). After transfection, U-87 MG cells were treated with ANG II 5 w/o LOS for 24 hours. Luciferase assays were performed with 20 μl of cell lysate employing a Dual-Luciferase Reporter Assay System kit (Promega; Madison, Wisconsin, U.S.A.). Luminescence was measured with a LUMAT LB9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). Results are presented as the average of luciferase activity from triplicate

experiments and expressed as the ratio to the internal standard Renilla luciferase. Purity of plasmids used in transfection experiments was verified by spectrophotometry and agarose gel electrophoresis. All transfection assays were performed using equimolar amounts of plasmids.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as described (*Giordano et al., 2012*). Chromatin extracts were precipitated with anti-STAT3, and anti-Polymerase II (all from Santa Cruz Biotechnology). Normal rabbit IgG (Santa Cruz Biotechnology) was used instead of primary Abs as negative control. Immuno-precipitated DNA was analyzed by qRT-PCR, and the human aromatase promoter I.4 sequence containing the GAS Element was amplified using the following pairs of primers: forward 5′-ATAGTTGCGCAATGAATGCA-3' and reverse 5′-CCAGCATGAACCACATATTTC-3'. Data were normalized with respect to unprocessed lysates (input DNA). Input DNA quantification was performed by using 5 µl of diluted (1/50) template DNA. The results were expressed as fold differences with respect to the relative inputs.

DNA Affinity Precipitation Assay.

The binding of nuclear STAT3 to on the aromatase promoter I.4 was assessed *in vitro* using a modified version of the DNA Affinity Precipitation Assay (DAPA) protocol of *Zhu et al., 2002*. Briefly, nuclear protein extracts were obtained from starved cells treated with ANG (5 µM) for 3 hours. One hundred g of nuclear proteins was mixed with 2 g of specific biotinylated DNA probes in 400 µl of buffer D (20 mM HEPES, pH 7.9, 10% glycerol, 50 mM KCl, 0.2 Mm EDTA, 1.5 mM MgCl2, 10 M ZnCl2, 1 mM dithiothreitol, and 0.25% Triton X-100) and then incubated on ice for 45 min. After that, 20 µl of streptavidinagarose beads (Promega) was added, and the samples were incubated under rotation for 2 hours at 4°C. Next, the agarose bead-protein complexes were collected by brief centrifugation and washed twice in buffer D. Proteins were uncoupled from DNA probes by the addition of 40 µl of 2x Laemmli's sample buffer and by heating them at 96°C for 10 min. The

beads were removed by centrifugation, and the supernatants were analyzed by immunoblotting for the presence of STAT3. The sequence of GAS oligonucleotide used as probe or the unlabelled competitor was 5′-GTGTTTCCTGTGAAAGTT-3′, mutated 5′- GTGTTTCCAATCTAAGTTCC-3′.

- **IN VIVO EXPERIMENTS**

Xenograft model analysis

All animal studies were carried out according to the Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of University of Calabria, Calabria, Italy. The *in vivo* experiments were performed in 35-day-old female nude mice (nu/nu Swiss; Charles River, Milan, Italy). At day 0, mice were inoculated with 3.0 x 10^6 U-87 MG cells/mouse into the intrascapular region. After the development of a palpable tumor (about 6 days, 200 mm^3), tumor size was measured with a caliper every 3 day by caliper measurements along two orthogonal axes: length (L) and width (W). The volume (V) of tumors was estimated by the following formula: V = L x (W2)/2. Mice were divided into five groups: vehicle, ANG II, ANG II+LOS, ANG II+ANA, LOS. On the first day of treatment, the ANG II group underwent aseptic surgery to subcutaneously implant omotic minipump (DURECT Corporation, Cupertino, CA). Each minipump administered ANG II at a subhypertensive dose of 10.8 ng/min.

The ANG II+LOS group receveid LOS, administered via drinking water, at a concentration of 66.7 mg/L. Based upon a presumed water intake of 4.05 ml/mouse/day (*Harkness JE et al. 1995*), this amounted to 0.27 mg of LOS per mouse per day.

The ANG II+ANA group receveid ANA, administered by subcutaneous injection, at concentration of 80 mg/kg/day. At day 24 the animals were sacrificed following the standard protocols and tumors were dissected from the neighboring connective tissue. Specimens of tumors were frozen in nitrogen and stored at -80 °C. The remaining tumor tissues of each sample, livers, lungs, spleens, and kidneys were fixed in 4% paraformaldehyde and embedded for the histological analysis.

Histopathological analysis

Tumors, livers, lungs, spleens and kidneys were fixed in 4% formalin, sectioned at 5 μm and stained with hematoxylin and eosin Y, as suggested by the manufacturer (Bio-Optica, Milan, Italy).

Immunohistochemical analysis

For immunohistochemistry, antigen retrieval was performed on 5 μm paraffin sections in 0.01 mol/L citrate buffer (pH 6) in a microwave at low setting. Incubations with primary antibodies were performed at room temperature overnight in a humidified chamber. Primary antibodies used were anti-Ki-67, anti-Aromatase, anti-AGTR1, anti-VEGF, anti-PD-L1, anti-PD-1 and anti-arginase 1.
Normal horse or goat serum was used as blocking agent. Biotinylated horse anti-mouse/rabbit (1:100) or biotinylated goat anti-rat (1:100) was used as the secondary antibody and revealed with a Vectastain ABC Kit Elite (Vector Laboratories, Burlingame, CA, USA, PK-6200) and a Peroxidase Substrate Kit DAB (Vector Laboratories, Burlingame, CA, USA, SK-4100). All stained slides were visualized using an Olympus BX41 microscope and the images were taken with CSV1.14 software, using a CAM XC-30 for image acquisition.
Paraffin embedded sections, 5 μm thick, were mounted on slides precoated with polylysine, and then they were deparaffinized and dehydrated (seven to eight serial sections). Immunohistochemical experiments were performed as described, 26 using rabbit polyclonal Ki67 primary antibody at 4°C overnight. Then, a biotinylated goat antirabbit IgG was applied for 1 hr at room temperature, followed by the avidin biotin horseradish peroxidase complex (Vector Laboratories, CA). Immunoreactivity was visualized by using the diaminobenzidine chromogen (SigmaAldrich). Counterstaining was carried out with methylene blue (Sigma-Aldrich). The primary antibody was replaced by normal rabbit serum in negative control sections.

- **STATISTICAL ANALYSIS**

- *In vitro*: Each datum point represents the mean ±SD of three different experiments. Data were analyzed by Student's t-test using the GraphPad Prism 7 software program. $p<0.05$ was considered as statistically significant.
- *In vivo*: The active tumor volumes in control (vehicle-treated), ANG II alone or in combination with losartan or anastrozole treated mouse were expressed as mean ± SD. The pre-treatment (day 0) and post-treatment (day 6) active tumor volumes were assessed for statistical differences employing single factor ANOVA followed by Student's t-test. A $p<0.05$ was interpreted as the level of statistical significance. Data were analyzed using GraphPad Prism version 7.0 (GraphPad software).

RESULTS.

1. High AGTR1 expression is associated with glioblastoma progression.

First, we evaluated in human GBM cells (U-87 MG and T98G) the expression of the receptor of ANG II, AGTR1. In both cell lines tested we observed an increased mRNA levels and protein content revelead by real-time PCR, western blotting and immunofluorescence assay, of AGTR1 compared to human glial cell line (SVG p12) assumed as control (*Figure 12 A, B and C*).

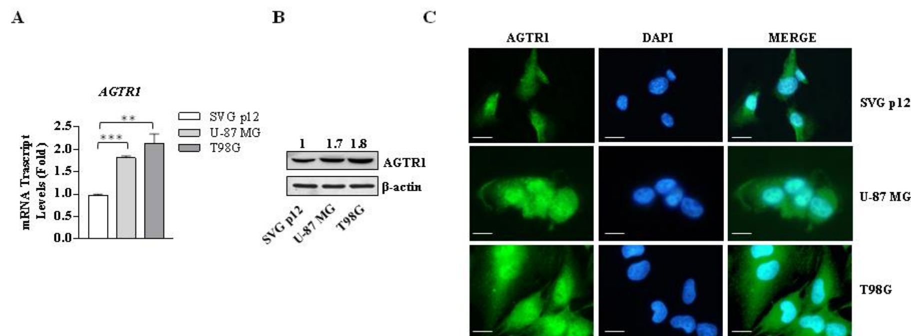

FIGURE 12. AGTR1 expression in normal glial cell and glioblastoma cell lines. *(A) Real-time RT-PCR for AGTR1 in U-87 MG and T98G cells; mRNA is shown relative to SVG p12 normal glial cells. (**B**) Immunoblotting showing AGTR1 protein expression. β-actin was used as a control for equal loading and transfer. Italicized numbers above blots represent the mean of the band optical density expressed as fold over SVG p12 for U-87 MG and T98G. (**C**) Immunofluorescence of AGTR1 in U-87 MG, T98G, and SVG p12 cells. DAPI staining for nuclear detection. Scale bars = 5 µm. Original magnification, X100. FITC, fluorescein isothiocyanate. Data are expressed as means ± SD of three different experiments, each performed in triplicate. **P < 0.01, and ***P < 0.001.*

This brought us to wonder if AGTR1 expression could represent a prognostic marker for the clinical outcoumes of GBM patients. According to TGCA-GBM dataset high AGTR1 expression shows low overall survival probability compared to the patients with low AGTR1 levels (*Figure 13 A*). In addition, high AGTR1 expression, shows lower progression-free survival (*Figure 13 B*) and displays upon chemotherapy treatment lower overall survival with respect to the patients with low AGTR1 expression (*Figure 13 C*).

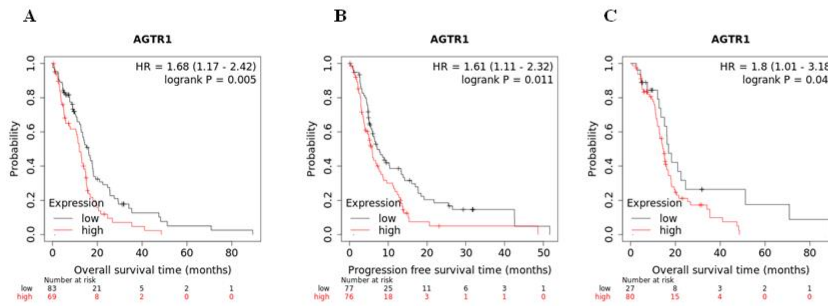

FIGURE 13. ***Kaplan-Meier survival analysis relating AGTR1 levels and overall survival (OS) in GBM patients.*** *(**A**) Overall survival (OS) in GBM patients (TCGA dataset). (**B**) Progression-free survival (PFS) in GBM patients (TCGA dataset). (**C**) Overall survival (OS) in chemotherapy treated GBM patients (TCGA dataset).*

Moreover, as shown in *Figure 14* we evidenced how ANG II upregulates in both GBM cell lines its own receptor, confirming positive feedback mechanism as previously described (*Chauhan et al., 2013*). The latter event was completely abrogated by losartan, a selective antagonist of AGTR1, evidencing how AGTR1 signaling is crucial in mediating ANG II-induced upregulation of its own receptor.

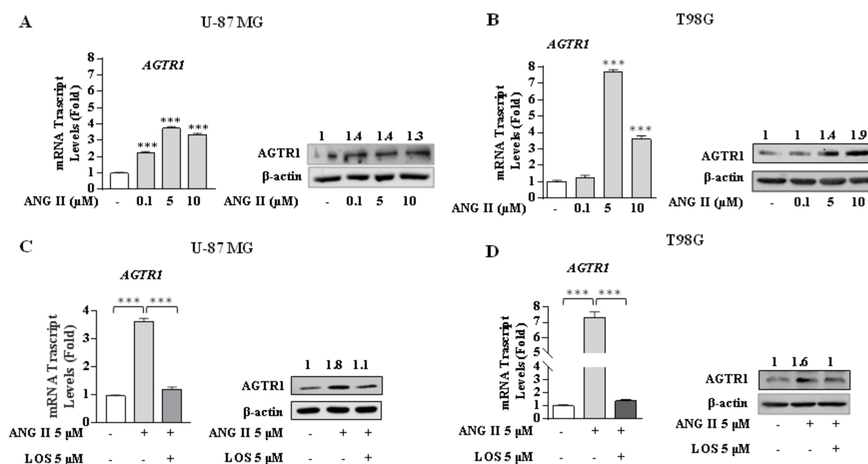

FIGURE 14. *(**A** and **B**) Real-time RT-PCR and immunoblotting assay for AGTR1 mRNA and protein expression, in GBM cells, treated with vehicle (-) or the angiotensin II (ANG II, 0.1, 5, 10 μM) for 24 h. β-actin was used as a control for equal loading and transfer. Italicized numbers above blots represent the mean of the band optical density expressed as fold over vehicle (-) for ANG II treatment. (**C** and **D**) AGTR1 mRNA and protein expression, in U-87 MG and T98G treated with vehicle (-) and angiotensin II (ANG II, 5 μM) alone or in combination with losartan (LOS, 5 μM) for 24 h. Italicized numbers above blots represent the mean of the band optical density expressed as fold over vehicle (-) for ANG II treatment, or fold over ANG II for ANG II in combination with LOS. β-actin was used as a control for equal loading and transfer. Data are expressed as means ± SD of three different experiments, each performed in triplicate. ***P < 0.001.*

ANG II exposure increases the proliferation rate in both GBM cell lines and enhances their motility and invasiveness. All these upregulatory events were reversed in the presence of losartan (*Figure 15 A-L*).

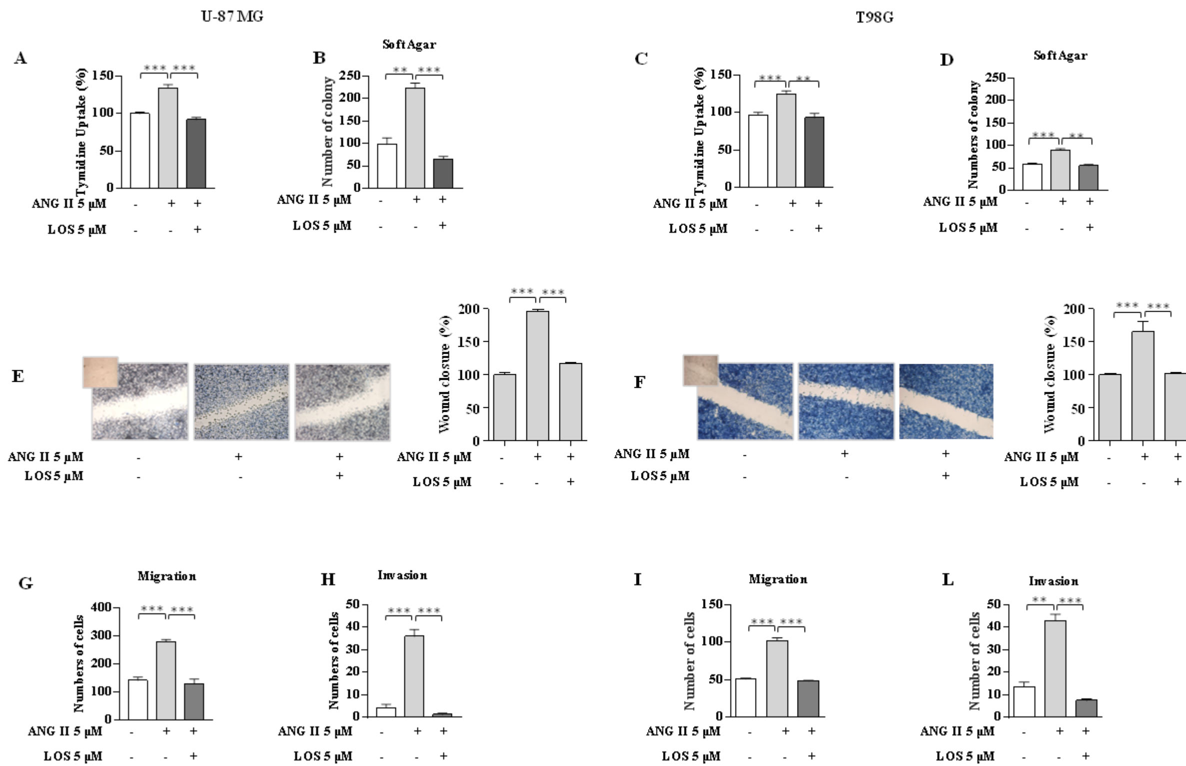

FIGURE 15. Effects of Angiotensin II receptor antagonist losartan on ANG II-induced U-87 MG and T98G cell proliferation, migration and invasiveness. *Cell proliferation was determined by the [^{3}H]thymidine (**A** and **C**) and soft agar growth (**B** and **D**) assays in cells treated with vehicle (-) and angiotensin II (ANG II, 5 µM) alone or in combination with the angiotensin II receptor antagonist losartan (LOS, 5 µM) for 24 h. (**E** and **F**) Wound healing assays in U-87 MG and T98G cells treated for 12 h with vehicle (-) and angiotensin II (ANG II, 5 µM) alone or in combination with the angiotensin II receptor antagonist losartan (LOS, 5 µM). Images are representative of three independent experiments. The histograms represent the relative percentage of wound closure calculated by ImageJ software version 1.51q. Small squares, time 0. Original magnification, ×10. Boyden chamber transmigration (**G** and **I**) and invasion (**H** and **L**) assays in U-87 MG and T98G cells treated with vehicle (-) and angiotensin II (ANG II, 5 µM) alone or in combination with the angiotensin II receptor antagonist losartan (LOS, 5 µM) for 12 h. Data are expressed as means ± SD of three different experiments, each performed in triplicate. **P < 0.01, and ***P < 0.001.*

2. Angiotensin II increases local estrogen production in glioblastoma cells.

Previously it has been reported in different experimental models that ANG II is able to influence local estrogen production modulating aromatase activity (*Kalenga et al., 1995*). Here we have shown how aromatase expression is significantly increased in term of mRNA and protein content revelead by real-time PCR, western blotting and immunofluorescence assay in GBM cell lines compared to normal glial cell line (*Figure 16 A, B and C*).

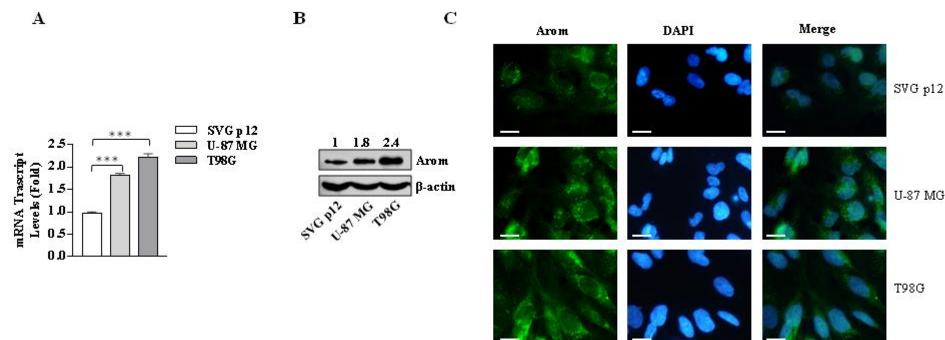

FIGURE 16. Aromatase expression in human normal glial cell and GBM cell lines. *(A) Real-time RT-PCR for CY19A1 in U-87 MG and T98G cells; mRNA is shown relative to SVG p12 normal glial cells.* ***(B)*** *Immunoblotting showing Arom protein expression. β-actin was used as a control for equal loading and transfer. Italicized numbers above blots represent the mean of the band optical density expressed as fold over SVG p12 for U-87 MG and T98G.* ***(C)*** *Immunofluorescence of Arom in U-87 MG, T98G, and SVG p12 cells. DAPI staining for nuclear detection. Scale bars = 5 µm. Original magnification, X100. FITC, fluorescein isothiocyanate. Data are expressed as means ± SD of three different experiments, each performed in triplicate. ***P < 0.001.*

In agreement with these data, Kaplan-Meier analysis of TCGA-GBM dataset revealed that patients with high aromatase expression show lower overall survival (*Figure 17*).

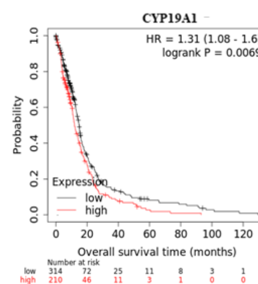

FIGURE 17. Correlation between CYP19A1 mRNA levels and overall survival (OS) in GBM patients. *Kaplan-Meier survival analysis in GBM patients with high and low CYP19A1 expression for OS. Kaplan Meier survival graph, Hazard Ratio (HR) with 95% confidence intervals and logrank P value.*

Furthermore, ANG II exposure enhances, in both GBM cell lines, the expression of aromatase in term of mRNA (*Figure 18 A and C*), protein content (*Figure 18 B and D*) and enzymatic activity (*Figure 18 I and M*). All these upregulatory events were reversed in the presence of losartan (*Figure 18 E-N*).

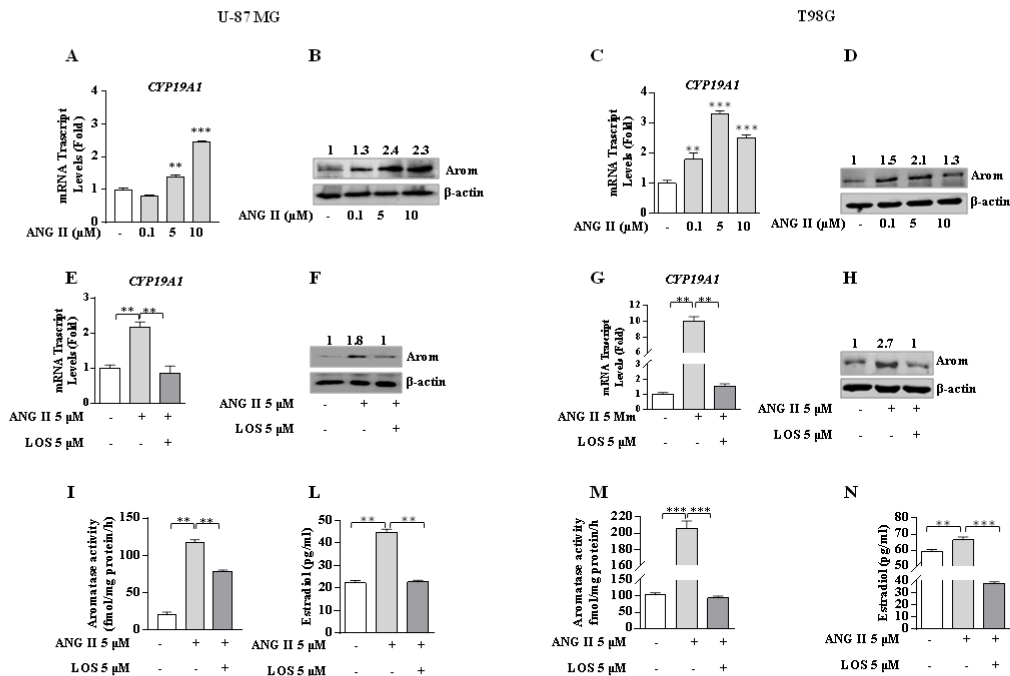

FIGURE 18. Effects of angiotensin II and Losartan, on aromatase expression and activity in U-87 MG and T98G cells. *(**A** and **C**) Real-time RT-PCR and (**B** and **D**) immunoblotting assay for CYP19A1 mRNA and protein expression, in GBM cells, treated with vehicle (-) or the angiotensin II (ANG II, 0.1, 5, 10 µM) for 24 h. β-actin was used as a control for equal loading and transfer. Italicized numbers above blots represent the mean of the band optical density expressed as fold over vehicle (-) for ANG II treatment. (**E** and **G**) CYP19A1 mRNA and (**F** and **H**) protein expression, in U-87 MG and T98G treated with vehicle (-) and angiotensin II (ANG II, 5 µM) alone or in combination with losartan (LOS, 5 µM) for 24 h. Italicized numbers above blots represent the mean of the band optical density expressed as fold over vehicle (-) for ANG II treatment, or fold over ANG II for ANG II in combination with LOS. β-actin was used as a control for equal loading and transfer. (**I** and **M**) Aromatase activity in U-87 MG and T98G treated with vehicle (-) and angiotensin II (ANG II, 5 µM) alone or in combination with losartan (LOS, 5 µM) for 24 h. (**L** and **N**) ELISA for Estradiol secretion in U-87 MG and T98G cells treated with vehicle (-) and angiotensin II (ANG II, 5 µM) alone or in combination with losartan (LOS, 5 µM) for 24 h. Data are expressed as means ± SD of three different experiments, each performed in triplicate. **P < 0.01, and ***P < 0.001.*

In order to ascertain whether ANG II/AGTR1 signaling was able to directly affect aromatase at transcriptional level, we investigated its effect on the activity of promoters pI.4 and pI.f, mainly expressed in brain tissue (*Yague et al., 2006*).

To this aim we transfected U-87 MG cell line with constructs containing these two promoters fused to luciferase reporter gene. Upon ANG II exposure, a significant increase of both promoters activities was observed even though (*Figure 19 A and B*), in a higher extent on promoter I.4.

These findings lead us to identify the functional sequence present in the promoter I.4 as possible effector of ANG II signalling.

We identified in the promoter I.4 a GAS element site (Interferon γ Activation Site), localized between nt -282/-272, and we performed site-directed mutagenesis assay. Disruption of the putative GAS consensus site resulted in a significant reduction of the upregulatory effects induced by ANG II on pI.4 promoter activity (*Figure 19 C*) addressing the GAS motif as a critical sequence in mediating promoter activation upon ANG II exposure.

To further investigate the functional importance of the identified GAS sequence, we carried out DAPA assay by using a double-stranded oligonucleotide containing the core GAS sequence. Endogenous STAT3 was found associated with the putative consensus oligonucleotide following ANG II administration. A mutant oligonucleotide abolished STAT3 binding, confirming the specificity of the site identified in vitro and that DNA-GAS binding is sequence-specific (*Figure 19 D*). Furthermore, to demonstrated the involvement of GAS element in ANG II-mediated aromatase upregulation at the pI.4 promoter level, ChIP assay was performed. Using specific antibodies against STAT3 and RNA-polymerase II, protein-chromatin complexes were immunoprecipitated from cells cultured with or without ANG II for 3h. The resulting precipitated DNA was then quantified using real-time PCR with primers spanning the STAT3 binding element in the aromatase pI.4 promoter region. STAT3 recruitment was significantly upregulated upon ANG II treatment in U-87 MG cell line (*Figure 19 E*). This result was well correlated with a high association of RNA-polymerase II to the aromatase regulatory region (*Figure 19 F*).

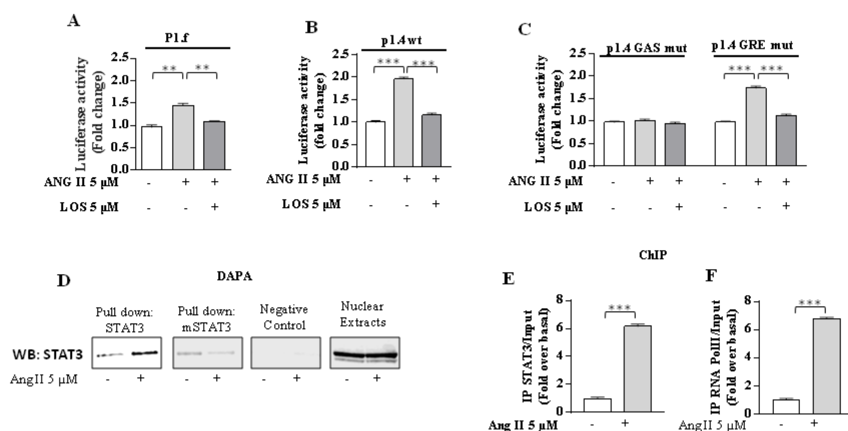

FIGURE 19. Aromatase promoters PI.f/1.4 in U-87 MG cells are stimulated upon ANG II exposure. *(**A** and **B**) Transcriptional activity of U-87 MG cells with human P450arom I.f and I.4 luciferase construct. The cells were treated with vehicle (-) and angiotensin II (ANG II, 5 µM) alone or in combination with losartan (LOS, 5 µM) for 24 h. (**C**) Transcriptional activity of U-87 MG cells with human P450arom I.4 luciferase construct mutant in positive cis-acting elements: interferon γ activation site (GAS, -282/-272), pI.4 mutGAS and glucocorticoid responsive element (GRE, -133/119) pI.4 mutGRE. (**D**) Nuclear extracts from U-87 MG cells treated with ANG II 5 µM or untreated, were incubated with a biotinylated oligonucleotide containing the CYP19A1-STAT3 or CYP19A1-mSTAT3 site and subjected to DNA affinity precipitation assay. Specifically bound proteins were subjected to western blotting analysis using an antibody specific by STAT3. The specificity of the binding was tested by loading the unbound fraction (Negative Control). U-87 MG cells nuclear extracts were used as positive control. (**E** and **F**) U-87 MG cells were treated in the presence of vehicle (-) or ANG II 5 µM for 3 hours, then cross-linked with formaldehyde, and analyzed. The precleared chromatin was immunoprecipitated with anti-STAT3 or anti-RNA Pol II. In control samples, normal mouse or rabbit IgG was used, respectively, instead of the primary Abs as control of Ab specificity. The 5' flanking sequence of the CP19A1 gene containing the STAT3 site was detected by Real-time PCR with specific primers. Input DNA were amplified as loading controls. Data are expressed as means ± SD of three different experiments, each performed in triplicate. **P < 0.01, and ***P < 0.001.*

The biological correlate of the upregulatory effects induced by ANG II on aromatase expression is represented by an enhanced production of estradiol secreted by U-87 MG cells upon ANG II exposure. This event was abrogated in the presence of losartan (*Figure 20 A*). The enhanced local estradiol production upon ANG II may contribute to the ANG II stimulatory effect on tumor cell proliferation. For instance, estradiol as well as an aromatizable steroid, such as androstenedione (AD), stimulate GBM cell proliferation rate. Besides, the stimulatory effect induced by AD was potentiated by ANG II. The latter event was no longer noticeable when AD was combined with losartan (*Figure 20 B*).

All this confirms again the intrinsic property of ANG II/AGTR1 signaling to stimulate aromatase enhancing local estrogen production.

FIGURE 20. Angiotensin II potentiates the proliferation effects of an aromatizable steroid androst-4-ene-3,17-dione in glioblastoma cells. *(A) ELISA for Estradiol secretion in U-87 MG cell treated with vehicle (-), androst-4-ene-3,17-dione (AD 10 nM) alone or in combination with angiotensin II (ANG II, 5 µM) alone or in combination with losartan (LOS, 5 µM) for 24 h.* ***(B)*** *Cell proliferation was determined by the [^{3}H]thymidine assay in cells treated with vehicle (-), estradiol (E2, 10 nM), androst-4-ene-3,17-dione (AD 10 nM) alone or in combination with angiotensin II (ANG II, 5 µM) alone or in combination with losartan (LOS, 5 µM) for 24 h. Data are expressed as means ± SD of three different experiments, each performed in triplicate. *P < 0.05, **P < 0.01, and ***P < 0.001.*

3. Blocking ANG II signaling inhibits tumor growth in GBM xenografts

To investigate *in vivo* the effects of ANG II/AGTR1 signaling on cell growth and progression we performed xenograft experiments. To this aim we injected U-87 MG cell line into the intrascapular region of female nude mice and monitored tumor growth after administration of ANG II, losartan (LOS), ANG II with LOS or anastrozole (ANA). Tumor was detected at day 6 and tumor size was monitored every 3 days up to day 24 (*Figure 21 A*). This administration was well tollerated because no changes in body weight or in food and water consumption were observed along with no evidence of reduced motor function. In addition, no significant differences in the mean weights or histologic features of the major organs (liver, spleen, and kidney) after sacrifice were observed between vehicle and treated mice, indicating a lack of toxic effects. Treatment with ANG II increases tumor growth significantly with respect to control group, which was reversed by both LOS and ANA. However, it was extremely impressive the evidence that treatment with LOS induces a drastically regression of tumor size which is the half of that one detected in the control group animals, addressing how the

elevated functional activity of ANG II/AGTR1 signaling system is basically present in GBM cell (*Figure 21 B and C*). The latter finding could be explained in all likelihood with the antiangiogenic effect of LOS as revealed by the induced regression of tumor vascular stroma (*Figure 21 D*) due to the drastic inhibition of VEGF expression as confirmed by immunostaining (*Figure 22*).

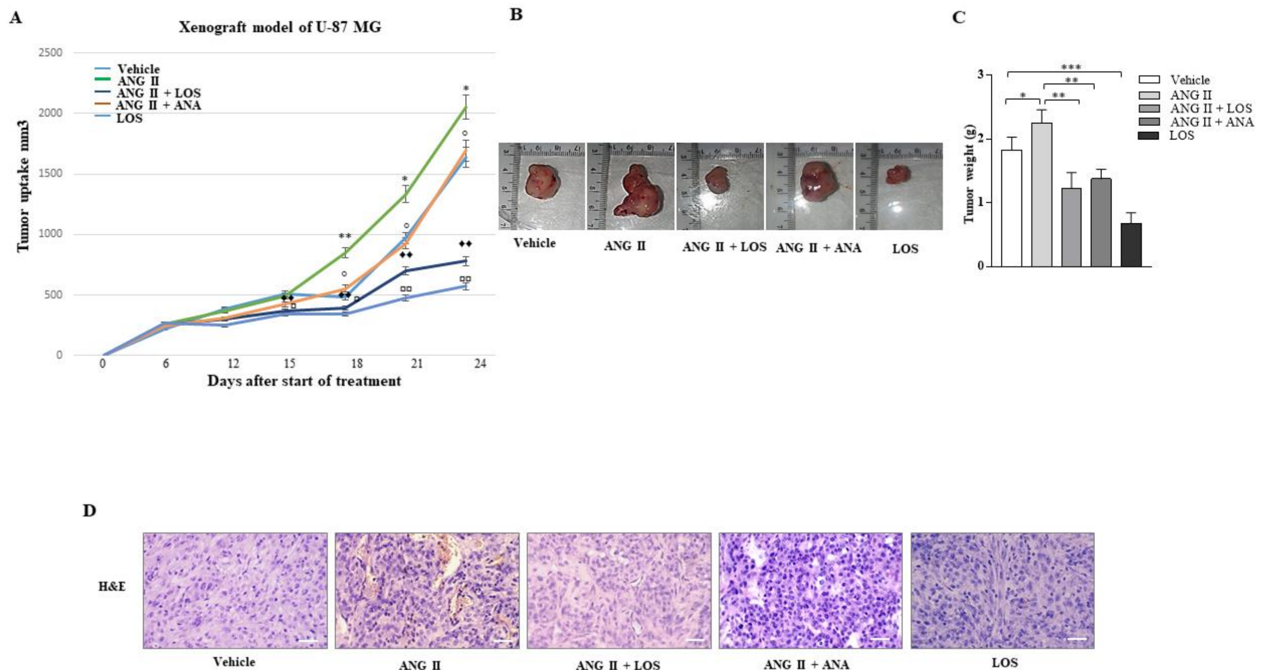

FIGURE 21. Losartan Inhibits Angiotensin II-Induced tumor growth of U-87 MG xenografts. *(**A**) U-87 MG cells were injected subcutaneously in female nude mice (five mice per group) and then treated with vehicle, ANG II, LOS, and ANG II in combination with LOS or ANA. Tumor growth was monitored over time. (**B**) Images of a representative individual tumor from each treatment group. (**C**) Average tumor weight from vehicle. (**D**) Hematoxylin and eosin (H&E) staining of tumor sections from vehicle (-), ANG II, LOS, and ANG II in combination with LOS or ANA. Scale bars = 12.5 µm. *P < 0.05, **P < 0.01; °P<0.05; ♦♦ P< 0.01; ¤ P< 0.05, ¤¤ P< 0.01.*

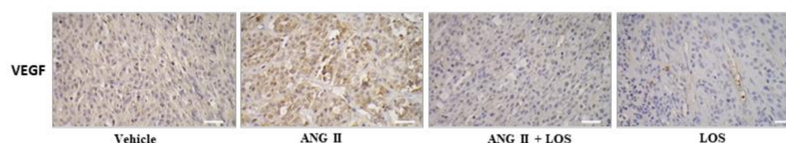

FIGURE 22. Losartan reverses the upregulatory effects of ANG II on VEGF expression. *Immunohistochemical analysis in U-87 MG xenograft tumors of VEGF upon ANG II treatment, ANG II in combination with LOS and LOS alone. Scale bars = 12.5 µ m.*

In agreement with our *in vitro* findings, we observed in GBM xenograft tumors from mice treated with ANG II an increased expression of Ki67, a well-known cell proliferation marker, along with an enhanced expression of AGTR1 and aromatase. In xenograft tumors from mice treated with ANG II in combination with LOS these effects were drastically abrogated (*Figure 23 A*).

On the basis of the large evidence that local Renin-Angiotensin System (RAS) may have immunosuppressor role especially in the stromal cells such as macrophage Myeloid-Derived Suppressor Cells (MDSC) and Cancer-Associated Fibroblast (CAF), we wondered how the functional interactions existing between RAS and estrogen signaling could also contribute to the local RAS immunosuppression tumor microenvironment.

To this aim we have focused our attention on the activation of the immune check point blockades mainly involving programmed cell death ligand 1 (PD-L1) and programmed cell death protein 1 (PD-1) and together with the expression of enzyme hampering immuno cells function (arginase, ARG 1). Immunohistochemically analysis revealed how PD-L1/PD-1 and ARG 1 were markedly increased upon ANG II exposure and reversed by treatment with either losartan and still scantly detectable with anastrozole (*Figure 23 B*).

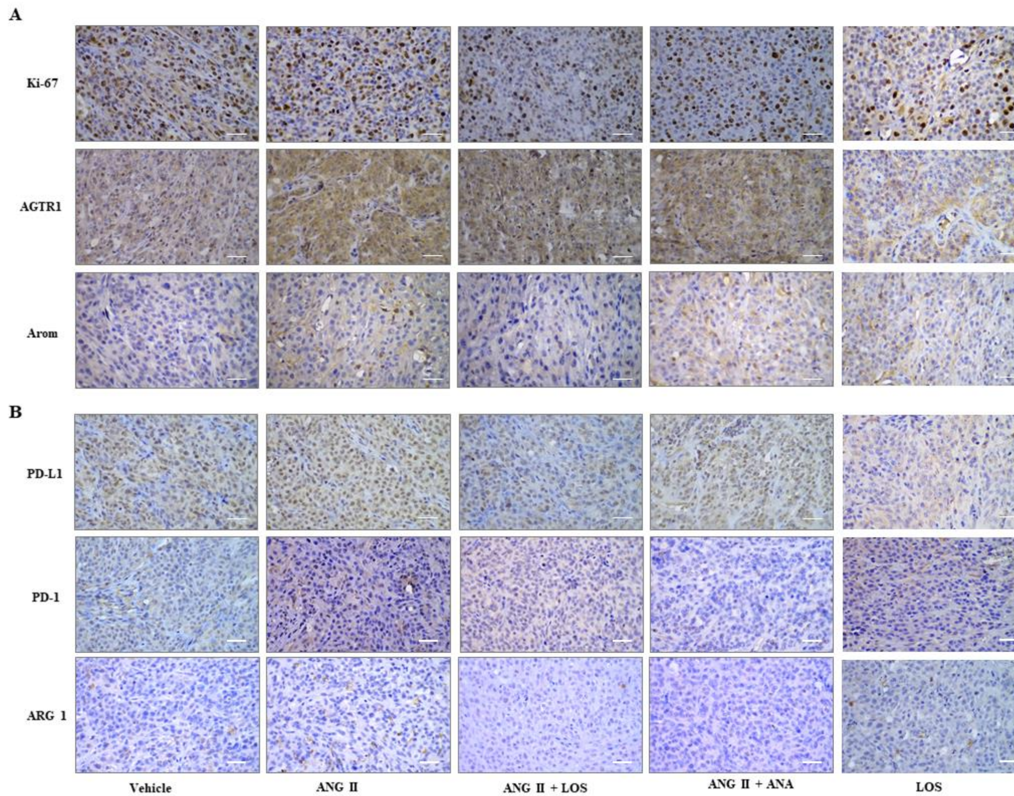

FIGURE 23. Losartan reverses the upregulatory effects of ANG II on Ki-67, AGTR1, Arom and in the protein involved in immune check point blockade PD-L1/PD-1 and ARG 1 expression. *(**A**) Immunohistochemical analysis in U-87 MG xenograft tumors of Ki-67, AGTR1, Arom, upon ANG II treatment in combination with LOS or ANA and LOS alone. (**B**) Immunohistochemical analysis in U-87 MG xenograft tumors of PD-L1, PD-1 and ARG 1 upon ANG II treatment in combination with LOS or ANA and LOS alone. Scale bars = 12.5 μm*

DISCUSSION

In 1980 it was demonstrated that ANG II, does not penetrate the blood-brain barrier (BB) and consequently ANG II content in the brain, does not come from systemic RAS (*Fuxe et al. 1980*).

Later it was shown that all essential substrates and enzymes needed for the synthesis, of bioactive angiotensin peptides can be produced locally in the brain a part from the peripheral system (*Xia et al., 2008*).

All RAS components were not found in a single cell, thus brain RAS requires complete network of intercellular interaction to produce its bioactive neuropeptides (*Cuadra et al., 2010*). Multiple experiments have demonstrated the effect of RAS on sustaining proliferative signaling, evading growth suppression, resisting apoptosis, inducing angiogenesis and deregulating cellular energetics as well as playing a role in inflammation, invasion and metastasis (*Arrieta et al., 2005*).

Accumulating evidences have suggested that ATGR1 is expressed in various tumors such as the most malignant brain tumor GBM and is associated with tumor growth and with more aggresive phenotype (*Banscands et al., 2001*).

Previously, it has been reported in different experimental models how ANG II enhances estradiol production through a stimulatory effect on the aromatization step of steroids precursors (*Kalenga et al., 1995*).

Previously, aromatase has been assessed by RT-PCR and immunohistochemistry in the rat and in human GBM cells T98G and U373, displaying a citoplasmatic pattern of immunoreactivity (*Yague et al., 2004*).

In the present study we demonstrated how aromatase in GBM cells is highly expressed and in analogy to its role in other estrogens sensitive cancers, it can reasonably be assumed that it supports local E2 production stimulating cell growth and progression. We have shown how E2 secretion by GBM cells is well correlated with aromatase enzymatic activity. A previous study did report that intratumoral estradiol concentrations in 36 biopsies patients with astrocytoma were: 11.51 ± 0.3892, 13.62 ± 0.6784, and 14.42 ± 0.8473 ng/μL for grades II, III, and IV, respectively (*Dueñas Jiménez et al.,*

2014). The E2 concentration in grade II tumors was significantly lower than that in grade III and IV tumors and was found that intratumoral E2 concentration increases with the severity of malignancy (*Dueñas Jiménez et al., 2014*).

The same authors found: 1) a positive correlation between estradiol concentration and aromatase expression wich is significantly increased in the patients with GBM; 2) a positive link existing between patient death risk increased by 1.256 %, in relation to a relative unit of aromatase mRNA expression; 3) a negative link existing between patient Overall Survival and aromatase mRNA units as reported in the present study.

In the above-mentioned study was reported how a decrease in ERα expression was inversely correlated with estradiol concentration (*Dueñas Jiménez et al., 2014*). We may reasonably explain the latter finding with the fact that intratumoral estradiol concentrations down-regulate the expression on ERα in terms of mRNA and protein content as classic biological feature of the functional transactivation of wild-type ERα.

Our findings show how local estrogen production in GBM is markedly enhanced by ANG II/ AGTR1 signalling through the induced upregulation of aromatase gene expression.

This will fits with the evidence that AGTR1 and aromatase are both involved in the clinical outcomes being both significantly linked with the overall survival of GBM patients.

In recent years, it has been widely demonstrated how central nervous system (CNS) interaction with the immune system (*Black et al., 1994*) may provide a new exciting theoretical basis and promising opportunity for brain tumor immunotherapy.

Tumor cells can display immune evasion to weaken antitumor immunity by activating the so-called Immune Checkpoint molecules (ICs) (*Topalian et al., 2015*). Programmed cell death ligand-1 (PD-L1), a “classic” IC molecule, is able to induce a T-cell-mediated immune tolerance in tumor microenvironment, leading to tumor immune escape and growth stimulation, by combination with programmed cell death-1 (PD-1) located on the surface of activated T cells (*Wei et al., 2013*). PD-L1 has been shown to be upregulated in various cancer cells and associated with unfavorable prognosis

(*Thompson et al., 2006; Nakanishi et al., 2007; Chen et al., 2009; Mu et al., 2011; Qing et al., 2015; Enkhbat et al., 2018*).

Over the past decade, immunotherapies targeting PD-1/PD-L1 axis have made a series of remarkable breakthroughs in prognosis improvement of hard-to-treat solid tumors (including head and neck squamous cell carcinoma, non-small cell lung cancer, gastric cancer, urothelial cancer, cervical cancer and melanoma) and have entered in the standard clinical practice (*Wu et al., 2006; Schalper et al., 2014; Faghfuri et al., 2015; Reck et al., 2016; Zhu et al., 2017; Rotte et al., 2018*).

Previous report has demonstrated that STAT3 signaling is a regulator of PD-L1 expression in mouse models and various cancer cell lines (*Lewis et al., 2006)*.

Since we highlighted in GBM how ANG II/AGTR1 signaling cascade involves STAT3 it was reasonable, in the same circumstances, to investigate how ANG II and its functional interaction with aromatase may affect the antitumor immunomicroenvironment.

In the present study we have demonstrated how ANG II exposure, concomitantly with a clear increase of aromatase expression, induces the upregulation of expression of PD-L1 detected in tumor cell, while PD-1 and an enzyme hampering immune cell function, such as Arginase, appears to be up-regulated in the tumor stroma. Losartan exposure abrogates the above mentioned up-regulatory effects which, however, were downregulated to a lesser extent by aromatase inhibitor, anastrazole. The latter finding suggests how the local estrogen production upon ANG II exposure may be somehow involved in its induced immunosuppressive effects on glioblastoma.

An important issue still not covered by the present study concerns the angiogenetic action of ANG II though the induced expression of VEGF. Angiogenesis is recognized as key event in the progression of glioma (*Würdinger et al., 2009*).

Among all solid tumors, GBM has been reported to be the most angiogenic because it displays the highest degree of endothelial cell hyperplasia and vascular proliferation (*Onishi et al., 2013*).

This could explain why losartan by itself is able to reduce by 60% glioblastoma growth in xenografted mice upon ANG II exposure and by 50% with respect to control growth suggesting how RAS system is overexpressed also in GBM cells in basal conditions.

More recently, has been highlighted how losartan may improve drug and oxygen delivery to tumors by reducing intratumoral stromal collagen and hyaluronan production, associated with decreased expression of profibrotic signals TGF-β1 (transforming growth factor β1), CCN2 (also known as CTGF, connective tissue growth factor) and ET-1 (endothelin 1) downstream of ANG II receptor inhibition. As consequence, losartan reduces solid stress in tumor resulting in increased vascular perfusion (*Chauhan et al., 2013*).

In conclusion from our finding it emerges how losartan blocks ANG II signaling in glioblastoma, down-regulates local estrogen production and weakens the immunosuppression of tumoral microenvironment, resulting in a strong inhibition of glioblastoma cell growth and progression. Thus, all this suggests how losartan may be reasonably repurposed as pharmacological tool to be implemented as the novel therapeutical strategies for glioblastoma treatment.

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