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The role of the placenta on the RANK/RANKL/OPG axis: a glance at multiple sclerosis

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ABSTRACT

The placenta is an organ of fundamental importance for a successful pregnancy; it's involved in cross-talk between mother and fetus, not only delivering essential oxygen and nutrients to the developing baby but also playing an active role in the synthesis and release of "information" factors in maternal and fetal circulation. Pregnancy is also known to suppress the inflammatory response in some autoimmune diseases, like multiple sclerosis. These beneficial effects of pregnancy have always been traced back to the changes that the maternal immune system makes to accept new life. Of particular interest is the potential involvement of the placenta in the production of the decoy receptor Osteoprotegerin (OPG), which controls the binding of the receptor activator of nuclear factor-kB (RANK) to its ligand (RANKL). However, the role of the placenta in the RANK/RANKL/OPG axis is currently elusive.

We hypothesized that OPG secreted by the placenta could modulate the activity of the RANK/RANKL system and thus contribute to a positive pregnancy outcome. Therefore, this study aimed to determine the expression levels of OPG and RANKL in the physiological human placenta during gestation. To this end, fresh placental tissue and cultures of placental explants were collected at different times of gestation, to monitor changes in the RANK/RANKL/OPG system during pregnancy. We also focused on the placenta-brain axis, being central in pathological conditions associated with significant placental insufficiency, which represents a risk factor for the development of diseases related to the nervous system. We hypothesized that the placental secretome regulates the physiology of a particular type of glial cell: the astrocytes. Using human placenta explants conditioned media, we investigate the astrocyte response following their activation towards an inflammatory phenotype.

In vitro studies have highlighted the pivotal role of the placenta in the production and release of OPG. Furthermore, we found that OPG levels were higher in maternal blood than in cord blood and they increase as pregnancy progresses, reaching maximum levels at the end of gestation. The results for soluble RANKL (sRANKL) showed an increase in serum during pregnancy but no change in its production by the placenta. Concerning the analysis conducted on the serum of patients with multiple sclerosis, we can highlight that the levels of sRANKL remain high, both in pregnancy and not. Interesting is the behaviour of the molecule OPG, whose levels increase in pregnancy going to balance the high levels of sRANKL.

Finally, by analysing the gene expression of *in vitro* activated astrocytes towards a pro-inflammatory phenotype, an up-regulation of pro-inflammatory chemokines was seen, which returned to physiological levels when treated with placental secretome containing 'protective' molecules such as OPG.

LIST OF ABBREVIATIONS

cDNA: Cyclic Deoxyribonucleic acid

CNS: Central nervous system

DAPI: 4',6-diamidino-2-phenylindole

DMEM: Dulbecco's modified Eagle Medium

E2: estradiol

E3: estriol

EAE: experimental autoimmune encephalomyelitis

ELISA: enzyme linked immuno-sorbent assay

FBS: fetal bovine serum

GFAP: Glial fibrillary acidic protein

IHC: Immunohistochemistry

IL: Interleukin

LPS: Lipopolysaccharide

MS: Multiple Sclerosis

Nf-kB: Nuclear factor kB

OPG: Osteoprotegerin

PBS: phosphate buffer saline

Pg: progesteron

RANK: Receptor activator of Nf-kB

RANKL: Receptor activator of Nf-kB ligand

RT-PCR: Real-Time Polymerase Chain Reaction

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis

sRANKL: soluble RANKL

SRB: Sulforhodamine B

TBS: tris buffered saline

TNF: tumor necrosis factor

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INTRODUCTION

1. The Human Placenta

The placenta is the life-supporting organ for the *in utero* development and growth of the fetus. Macroscopically, the human placenta has a discoid shape, with a diameter of about 22 cm, a central thickness of 2.5 cm and a weight of about 470 grams (Huppertz, 2008). The human placenta can be defined as a discoid, villous, haemochorial organ, respectively based on different characteristics such as macroscopic, structural and maternal-fetal interface (Lobo et al., 2016). It is a fetal annexe of dual origin, consisting of an embryonic part, the trophoblast, and a maternal tissue derived from the endometrium, the decidua (Benirschke et al., 2012). Between the maternal and fetal parts is the intervillous space containing the chorionic villi, the functional units of the placenta, widely branched and containing the fetal blood vessels (Dunk et al., 2020). As a haemochorial organ, the placental cells of the embryonic trophoblast are placed in direct contact with the maternal blood, allowing an exchange of nutrients and waste products with maximum efficiency. In addition, to ensure the transport of oxygen and nutrients to the fetus and the removal of carbon dioxide and metabolic waste products, the placenta acts as a barrier protecting the fetus from certain xenobiotic molecules, infections and maternal diseases (Huppertz, 2008). Moreover, the placenta releases a plethora of factors including hormones, cytokines, growth factors and microvesicles into the maternal and fetal circulation, that are involved in the regulation of maternal and fetal metabolism, pregnancy, fetal growth and delivery (Gude et al., 2004). The implantation of the blastocyst, the formation and functions of the placenta are finely regulated processes to ensure maximum efficiency in maternal-fetal exchanges. Changes in the development and function of the placenta can have dramatic effects on the health of both the fetus and its mother, with a lifelong impact on their future wellbeing (Jones and Lopez, 2014). Abnormal placentation is recognized as the major cause of pregnancy diseases, such as pre-eclampsia, fetal growth restriction, recurrent miscarriage and stillbirth (Brosens et al., 2011).

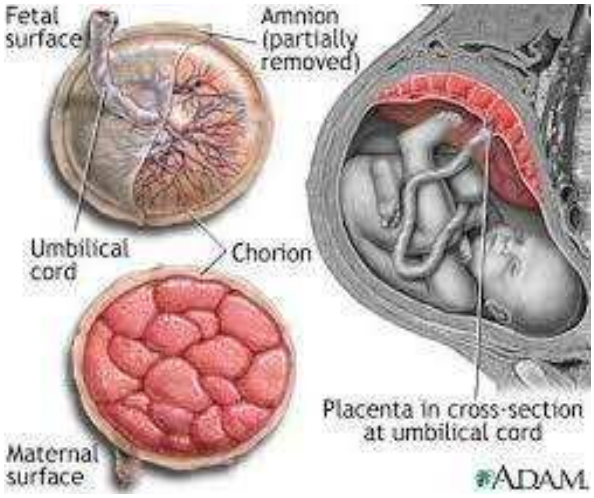

Figure 1. Diagram of the surfaces of the placenta and its location in the body. *(Source: ADAM (http://adameducation.com)*

2. Placenta development

Fertilization occurs in the fallopian tubes 24-48 hours after ovulation. The initial stages of development from zygote to morula occur as the embryo, enclosed in a protective envelope known as the zona pellucida, proceeds through the fallopian tubes. During the migration into the tube, in the morula is formed a cavity, the blastocoel, and two main cell layers: the inner cell mass, which constitutes the embryonic disc and a peripheral cell layer deputy to embryo nutrition, the so-called trophoblast. The layer of trophoblast tissue that will form the placenta progressively adheres to the endometrium, while the inner cell mass, formed by embryoblasts, will give rise to the embryo (Huppertz, 2008).

The implantation of the blastocyst in the maternal endometrium is a crucial event in the formation of the placenta and it is divided into three main phases: apposition, adhesion and invasion. The apposition begins 6-7 days after conception, the microvilli on the endometrial surface of the trophoblast are interdigitated and as the interactions become tighter, the adhesion of the blastocyst to the endometrium stabilizes (Norwitz et al., 2001). When the blastocyst is firmly attached, the trophoblast begins to differentiate, forming an innermost layer of mononuclear cells, the cytotrophoblast, and a coating layer formed by the fusion of mononuclear cells, the

syncytiotrophoblast (Gude et al., 2004). The syncytiotrophoblast extends into the endometrial epithelium and invades the connective tissue, thanks to the secretion of enzymes, such as metalloproteases, which enable the invasion of the endometrium and allow the blastocyst to nest. This invasion process is also promoted by cytokines, such as interleukin 1α and β (IL-1α and IL-1β) produced by the blastocyst, adhesion molecules, surface receptors and hormones (hCG from the embryo, estrogen and progesterone from the corpus luteum) that ensure proper endometrial receptivity. The blastocyst subsequently sinks below the endometrial surface, which is gradually repaired by day 10 (Norwitz et al., 2001).

About 12 days after conception, implantation is considered complete and placentation begins (Pijnenborg et al., 1981). The embryo is fully incorporated in the endometrium and the fetal side cytotrophoblast penetrates through the syncytiotrophoblast reaching the maternal side of the placenta and differentiating into two main cell lines: the villous and extravillous trophoblast (Gude et al., 2004). The villous trophoblast consists of all the chorionic villi of the placenta and is involved in the transport of oxygen and nutrients from mother to fetus. The villi are immersed in the maternal blood and therefore have exclusive contact with the maternal peripheral circulation. The extravillous trophoblast (EVT) is composed of all trophoblast cells that acquire invasive characteristics within the uterine mucosa (Dunk et al., 2020; Gude et al., 2004).

The decidua is the maternal tissue most intimately associated with the feto-placental unit and plays a critical endocrine and immunological role. The cellular components of the decidua change during the menstrual cycle and pregnancy due to the action of locally produced hormones (Ng et al., 2020). Endometrial decidualization involves extensive reprogramming of gene networks related to cytoskeletal organisation, extracellular matrix remodelling and cell adhesion, resistance to oxidative stress, secretion and responses to growth factors, cytokines and chemokines. Decidualization denotes the transformation of stromal endometrial fibroblasts into specialised secretory decidual cells that provide nutritional and immunological factors essential for embryo implantation and development (Gonzalez et al., 2011).

3. The chorionic villi

The chorionic villi represent the functional unit of the placenta (Wang and Zhao, 2010) and are branched protrusions of the chorion that protrude from the embryo towards the maternal tissue

(Gude et al., 2004). All villi have the same structure: outermost syncytiotrophoblast, underlying cytotrophoblast supporting syncytiotrophoblast growth, basement membrane and stroma. The stroma contains connective tissue fibers, macrophages (Hofbauer cells) and fetal vessels that channel into the umbilical cord (Benirschke et al., 2012). The chorionic villi are arboriform structures, which can be divided into two main types: the floating villi (90%) in the chorionic lacunae filled with maternal blood, and the anchoring villi (10%), attached to the wall of the uterus. The floating villus is formed from the anchoring villus thanks to proliferative processes of apical cytotrophoblast cells that invade and break the syncytium reaching the maternal decidua; here they come into direct contact with the spiral arteries always ensuring an appropriate blood flow towards the fetus (Jones and Lopez, 2014). The development of the villi begins about 12-18 days after conception when mononuclear cells merge to form the syncytiotrophoblast, which expands in the form of digitiform projections. Within these projections, cytotrophoblast mononuclear cells deepen: these structures are the primary villi. Two days later, cells from the extraembryonic mesoderm of the chorionic plate invade the primary villi forming the secondary villi. Then there is the development of the first fetal capillaries, which identify the formation of the tertiary villi. The first generation of tertiary villi is represented by the mesenchymal villi, the first structures capable of providing the mother-fetus exchange functions (Castellucci et al., 2000).

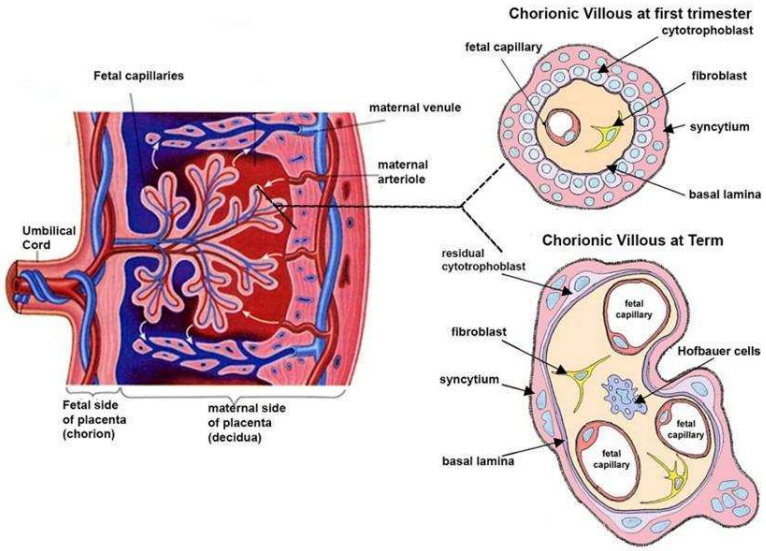

Figure 2. Schematic representation of human placenta and chorionic villi at first trimester and term. Chorionic villi at the first trimester of pregnancy show the presence of an external layer (syncytiotrophoblast) and an internal layer of cytotrophoblast. At term, the epithelial layer is thin for the strong reduction in the cytotrophoblast layer and the syncytium is in close contact with fetal capillary endothelium (Murthi et al., 2014).

4. Maternal-fetal circulation

The fetal microvascular capillary networks within the terminal villi are the culmination placental vascular tree originating from the arteries and vein of the umbilical cord. The umbilical arteries carry venous deoxygenated blood and waste products from the fetus to the placental villi and the umbilical vein carries arterial oxygenated blood from the placenta to the fetus. The umbilical cord, therefore, consists of two umbilical arteries and one vein, the only one providing vital support to the fetus (Hasegawa, 2018). The fetal blood is carried to the placenta via the two umbilical arteries and at the level of the placenta, through the numerous branches of these vessels within the villi, down to the terminal capillary branches of each villus, the blood is oxygenated and returns to the fetus via the umbilical vein (Finnemore and Groves, 2015).

Nutrients, oxygen, hormones and immunoglobulins pass from the maternal to the fetal blood. Under physiological conditions, there is never any mixing of maternal and foetal blood. The tissues separating the maternal and fetal bloodstreams form the so-called blood-placental barrier. The well-being of the embryo depends on an adequate supply of maternal blood to the embryonic villi. A

reduction in uteroplacental circulation leads to hypoxia of the embryo with a consequent delay in intrauterine growth; more significant reductions can lead to fetal death (Sibley and Dilworth, 2020).

5. How the placenta changes during gestation

The human placenta is a rapidly developing organ that undergoes structural and functional changes throughout pregnancy. Molecular, histological, and functional placental rearrangements are required during pregnancy to ensure adequate fetal development and maternal health (Sitras et al., 2012).

The differentiation and proliferation of trophoblast cells must be tightly controlled, and oxygen tension is one of the main regulators during placentation. Although hypoxia stimulates cytotrophoblast cell proliferation, high oxygen tension promotes cytotrophoblast differentiation. From implantation until the 10^{th} week of gestation, the placenta is in a low oxygen tension (around 18 mmHg) environment, which stimulates cytotrophoblast proliferation and inhibits differentiation and invasion of EVT cells. From week 12, the O_2 tension will increase until it reaches 60 mmHg during the last trimester of pregnancy (Gharesi-Fard et al., 2015).

With the advance of gestation, the needs of the fetus change and the placenta adapts to these changes, in addition, the weight of the placenta is related to the growth of the fetus, going to increase at term almost threefold (Salafia et al., 2006).

Moreover, differences in global gene expression profile between first and third-trimester human placenta reflect temporal changes in placental structure and function (Sitras et al., 2012). In a study of genome differences during pregnancy, it was seen that more than half of the genes that are expressed in the human placenta change their expression profile from the first to the third trimester of pregnancy (Sitras et al., 2012). For example, it has been seen that in early gestation placenta, genes involved in cell proliferation and related to cell cycle regulation, DNA, amino acids, and carbohydrate metabolism were significantly overrepresented compared to term placentae, which reflects rapid placental growth in early gestation (Sitras et al., 2012; Yong and Chan, 2020).

These findings support that the placenta undergoes profound molecular rearrangement to adapt to the changing needs of the fetus.

6. Placenta functions

The main functions of the placenta can be classified under the headings of transport function, metabolism function, protection function, endocrine function.

The placenta serves to supply oxygen, water, carbohydrates, amino acids, lipids, vitamins, minerals and other nutrients to the fetus while removing carbon dioxide and other waste products. It metabolises numerous substances and can release metabolic products into the maternal and/or fetal circulation. The placenta can help protect the fetus from certain xenobiotic molecules, infections and maternal diseases. It also releases hormones into the maternal and fetal circulation so that it influences pregnancy, fetal growth, birth and other functions (Gude et al., 2004).

Villi tissue is supplied with oxygen and nutrients directly from the mother's blood. From the villi, most substances that have passed the placental barrier reach the fetus directly via the fetoplacental circulation. The placental barrier controls the passage of substances by various mechanisms: simple diffusion, active transport, phagocytosis and pinocytosis (Griffiths and Campbell, 2015).

The placenta in addition to delivering essential oxygen and nutrients to the developing baby plays an active role in the synthesis and release of “informational” factors in maternal and fetal circulation. Indeed, the placenta is an endocrine organ, which produces several important peptides, steroid hormones, cytokines, pregnancy-associated glycoproteins and microvesicles. Such biologically active factors serve to regulate the maternal systems’ physiology for adaptation to pregnancy and fetal development and growth (Petraglia et al., 1996). It is noteworthy that the pool of molecules produced by the placenta change through pregnancy by producing specific signals at a particular period of gestation (Ietta et al., 2007; Phillips et al., 2001). This process leads to a completely different repertoire of factors expressed and produced by the placenta at term as compared to the first trimester.

Pregnancy is a unique physiologic condition that guarantees the survival of the semiallogenic embryo during the long period of gestation. The placenta plays a key role in the maintenance of local tolerance that is established at the maternal-fetal interface and allows the mother to accept the embryo until the completion of pregnancy. Gestation is the only physiological condition characterized by close physical contact between defense cells and foreign cells - the fetal ones - that because they express paternal alloantigens, should be recognised as non-self by the mother immune system (Bulla et al., 2003). Recent studies have shown that the implantation of blastocysts in the

uterus requires an environment with pro-inflammatory characteristics leading us to think of implantation as a Th1-dependent phenomenon (Mor, 2008). During placental development, however, there is a decrease in pro-inflammatory Th1 cytokines in favour of Th2 cytokines, suggesting that the progression of pregnancy is a Th2-dependent phenomenon (Wegmann et al., 1993). Th1 cytokines, IL-2, IFN-γ and Tumor Necrosis Factor (TNF)-α can promote the mother's immune response, leading to fetal loss; on the other hand, Th2-type cytokines such as IL-4 and IL-10 have immunosuppressive properties and promote the maintenance of pregnancy. In polyabortive women, an increase in NK cells has been demonstrated; these cells, stimulated by IL-2, release increased amounts of IFN-γ and TNF-α, cytokines that cause excessive apoptosis of EVT cells, thus compromising implantation (Thellin et al., 2000).

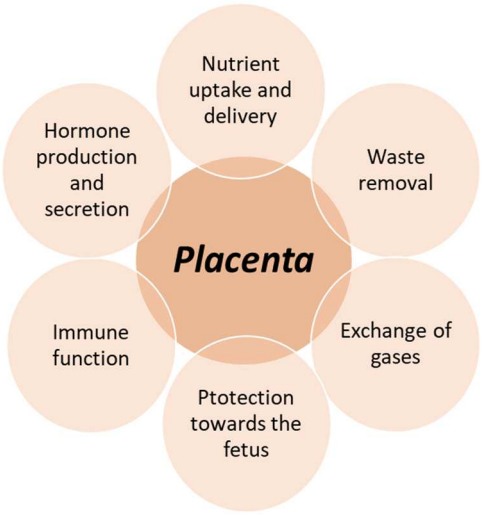

Figure 3. Schematic representation of some of the placenta functions

7. The RANK/RANKL/OPG axis

The receptor activator of NF-kB (RANK), receptor activator of NF-kB ligand (RANKL) and osteoprotegerin (OPG) triad was originally discovered through parallel efforts in the late 1990s as an important axis to immunity, primarily via actions on dendritic cells' survival and function (Anderson et al., 1997). Interestingly, more recent studies on this system have highlighted its profound involvement in macrophage polarization and T-cell differentiation, activation and

recruitment (Ahern et al., 2018). RANK/RANKL/OPG are members of the TNF and TNF receptor superfamilies and share signalling characteristics common to many members of each. RANK/RANKL/OPG are the main components of this signalling system.

Developmentally regulated and cell-type-specific expression patterns of each of these factors have revealed key regulatory functions for RANK/RANKL/OPG in bone homeostasis, organogenesis, immune tolerance, and cancer. The RANK/RANKL/OPG biological system is the ultimate mediator of many of the endocrine and paracrine factors that regulate the bone remodelling process (Walsh and Choi, 2014).

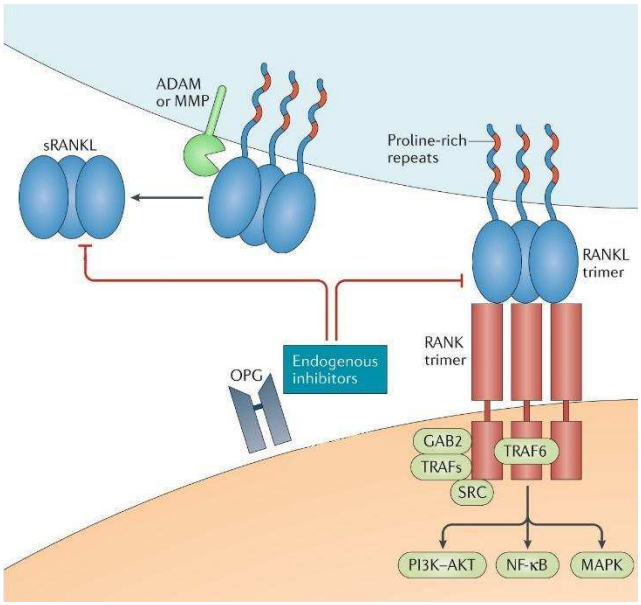

Figure 4. RANKL-RANK interaction and its inhibitor OPG. RANK is a type I transmembrane protein. Ligation of this receptor by RANKL leads to the activation of downstream signalling pathways, such as the canonical and non-canonical NF-κB, MAPK and PI3K-AKT pathways (Ahern et al., 2018).

RANK is a type I transmembrane protein member of the TNF receptor superfamily, expressed in different tissues and organs (bone, bone marrow, spleen, skeletal muscle, brain, heart, liver, lung, mammary gland, skin). Its primary expression is limited to oligodendrocytes precursor cells (OPCs), dendritic cells (DC) and mature osteoclasts. In particular, in bone tissue, the activation of RANK is essential in the osteoclast differentiation process. Activation of RANK is initiated by binding to its ligand, RANKL. The ligation leads to cellular downstream signalling that promotes translocation and

activation of transcription factors including NFATc1, CREB, NF-kB, AP-1 and MITF depending on the cell-type (Kearns et al., 2008; Walsh and Choi, 2014).

RANKL is a type II membrane protein and it is found in both membrane-bound and soluble forms (sRANKL). RANKL expression has been identified in many cells and tissues as well as T lymphocytes, osteoblasts (OBs), osteocytes and lungs (Ono et al., 2020). Binding studies show that RANKL can bind to both the functional receptor RANK and the decoy receptor OPG (Walsh and Choi, 2014). RANKL exists in at least three isoforms, two of which possess trans-membrane domains: the latter can remain anchored to the cytoplasmic membrane, or be cleaved and function as a soluble ligand (Vega et al., 2007). The sRANKL is a result of proteolytic division or alternative splicing of the membrane form. Matrix metalloproteases such as MMP3 or 7, and ADAM (a disintegrin and metalloprotease domain) are responsible for RANKL proteolytic cleavage (Lynch et al., 2005).

OPG is a soluble factor produced by osteoblasts structurally similar to RANK. It has been named OPG because of its protective effect on bone and in fact in Latin '*os*' means bone and '*protegere*' means to protect. OPG is also known as osteoclastogenesis inhibitory factor (OCIF) or a member of the TNF11b receptor superfamily. OPG is a secretory glycoprotein consisting of 401 amino acids with a monomeric weight of 60 kilo Daltons (kDa). It is composed of seven structural domains. It is assembled at the cys-400 residue of heparin to form a 120-kDa dimer for secretion. Before secretion of the monomeric and/or dimeric form, the 21aa signal peptide is cleaved at the N-terminus, making the mature 380aa OPG protein (Rochette et al., 2019). Initially, OPG is synthesized as a monomer within the cell, next converted to a disulfide-linked dimer, and is then secreted into the media. While smaller amounts of monomeric OPG also are secreted, the predominant extracellular form of OPG is a disulfide-linked dimer (Simonet et al., 1997). Therefore, circulating OPG exists as a free monomer and/or as a homodimer or as OPG bound to RANKL and sRANKL.

OPG is believed to function primarily as a decoy receptor, modulating interactions between ligands and signalling receptors. OPG takes part as a decoy receptor for RANKL and inhibits RANKL-RANK binding through it and effectively blocks its biological activities, and so it plays an anti-osteoclastogenesis role (Theoleyre et al., 2004). OPG is expressed primarily by bone marrow stromal cells but can be induced in B-lymphocytes and DCs.

7.1. In bone physiology

RANK activation by the binding of RANKL initiates an internal signalling cascade via the cytoplasmic adaptor proteins called TRAFs. This signalling cascade leads to the expression of various genes involved in osteoclastogenesis and thus facilitates the differentiation of monocytes into osteoclasts and the activation of mature osteoclasts. Increased production of RANKL by osteoblastic cells leads to osteoclast differentiation, activation and survival, which results in increased bone resorption (Wright et al., 2009). OPG acts as a decoy receptor by binding with high affinity to RANKL, therefore, preventing the interaction with RANK. Because of the binding to RANKL, OPG acts as an effective inhibitor of osteoclast differentiation, activation and survival and therefore inhibits bone resorption (Kong et al., 2000).

Bone remodelling allows adaptation to mechanical constraints and maintains phosphorus and calcium homeostasis through coordinated phases consisting of bone formation by osteoblasts and bone resorption by osteoclasts. It has been shown that the RANK signalling pathway plays a crucial role in the differentiation and activation of osteoclasts (Naylor et al., 2003). This system is regulated by many osteotropic hormones and cytokines which either reduce [glucocorticoids, inflammatory cytokines *e.g.*, IL-1, parathyroid hormone (PTH), prostaglandin E2 (PGE2), vitamin D3] or increase [transforming growth factor-β (TGF- β) and estrogens] the OPG/RANKL ratio (Wright et al., 2009). Various proinflammatory and proresorptive cytokines (IL-1, IL-6, IL-11, IL-17, M-CSF, PTH, and TNFα) have been detected in inflamed synovial tissues and have been implicated as mediators of bone and cartilage loss through stimulation of osteoclastic bone resorption (Hofbauer and Heufelder, 2001).

Intriguingly, all factors that inhibit or enhance bone resorption by osteoclasts act via regulation of RANKL-RANK and/or OPG, which leads to the surprising conclusion that the complex system of osteoclast regulated bone-remodelling is only controlled by these three molecules (Kong et al., 2000). It has been reported that RANKL and RANK deficient mice show severe osteopetrosis accompanying the lack of osteoclast differentiation while OPG deficient mice show severe osteoporosis. For this reason, RANK and OPG have been investigated as therapeutic targets and, in particular, a human anti-RANKL antibody, denosumab, has been discovered for the treatment of osteoporosis (Udagawa et al., 2021).

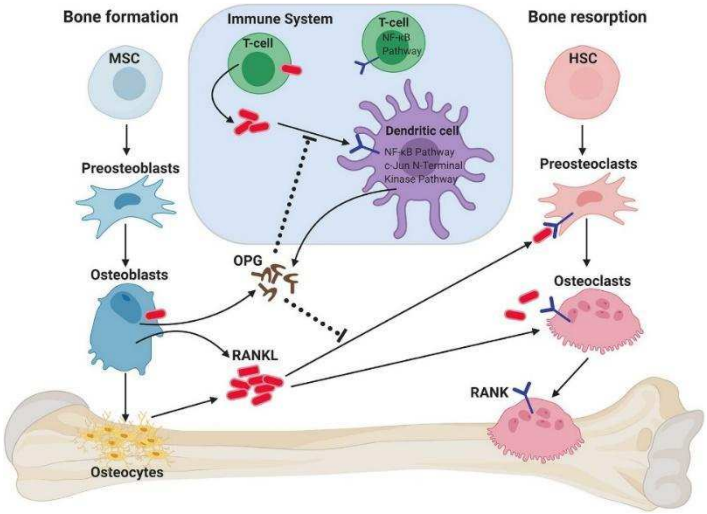

Figure 5. Role of RANKL/RANK/OPG axis on bone homeostasis and immune system. RANKL, secreted by osteoblasts and osteocytes, binds to RANK on the membrane of osteoclast progenitors (preosteoclasts), which results in bone resorption by mature osteoclasts. OPG binds to RANKL, thus inhibiting RANK signalling and bone resorption (Ming et al., 2020).

7.2. In Immune system

Remarkably, the same molecules that regulate osteoclastogenesis were also identified as regulators of early differentiation of thymocytes and B-cell precursors and as essential factors required for the formation of lymph nodes and Peyer's patches (Kong et al., 2000). RANKL is implicated in the development of medullary thymic epithelial cells, which are important for central tolerance and protecting the body from self-reactive T cells. Furthermore, RANKL, produced by activated T cells, supports the survival of dendritic cells, their interaction with T cells (Mueller and Hess, 2012), and their potential to stimulate T cell proliferation. In humans, mutations of the RANK gene *TNFRSF11A* lead to an impairment of immunoglobulin production by B cells (Guerrini et al., 2008).

Among immune cells, RANK and RANKL can be expressed on several immune cells involved in cell-mediated autoimmune diseases including rheumatoid arthritis (RA), uveitis, psoriasis, and multiple sclerosis (MS). Immature dendritic cells, immunosuppressive M2-type macrophages, myeloid-derived suppressor cells and natural killer cells express RANK, whereas CD8+ and CD4+ T cells (including regulatory T cells) express RANKL (Akiyama et al., 2012).

7.3. In tumorigenesis

The RANKL/RANK system has been studied in other human cancer subtypes, including bladder, colon, and cervical cancer, lymphoma, myeloma, and thyroid cancer (Göbel et al., 2020). RANKL promotes angiogenesis in cooperation with vascular endothelial growth factor, which increases RANK in endothelial cells. RANKL enhances their survival and the production of inflammatory cell adhesion molecules as well as *in vitro* and *in vivo* angiogenesis (Min et al., 2007).

Data from Jones et al. (2006) show that local differentiation factors such as RANKL have an important role in cell migration and the tissue-specific metastatic behaviour of cancer cells. RANKL triggers the migration of human epithelial cancer cells and melanoma cells that express the receptor RANK. RANK is also expressed on cancer cell lines and breast cancer cells in patients. In addition to its central role in tumor-induced osteolysis, bone destruction and skeletal tumor progression, there is emerging evidence for direct pro-metastatic effects of RANKL, independent of osteoclasts (Lleo and Gershwin, 2021). For example, RANKL also stimulates metastasis *via* activity on RANK-expressing cancer cells, resulting in increased invasion and migration. Pharmacological inhibition of RANKL may also reduce bone and lung metastasis through blockade of the direct action of RANKL on metastatic cells (Dougall et al., 2014). In a mouse model of melanoma metastasis, in vivo neutralization of RANKL by OPG results in complete protection from paralysis and a marked reduction in tumur burden in bones but not in other organs (Mori et al., 2009).

7.4. In the mammary gland

The development of a lactating mammary gland during pregnancy is essential for the nutrition, passive immunity, and survival of the new-born. The mammary ductal and alveolar epithelium undergoes expansion and proliferation to increase ductal side branching and to form lobule-alveolar structures. Mechanistically, progesterone, prolactin, and parathyroid hormone-related peptides are upregulated during pregnancy and are important for lactation, and they induce RANKL expression in mammary epithelial cells (Göbel et al., 2020).

This pathway has also been implicated in progestin-driven breast cancer development; indeed, the mammary gland epithelium is the predominant site for the development of breast cancer (Gonzalez-Suarez et al., 2010) was found to be expressed on primary breast cancer cells, and treatment with recombinant RANKL enhanced migration of both normal and malignant mammary epithelial cells

(Jones et al., 2006). Moreover, by inhibiting RANK/RANKL-mediated activation we can prevent carcinogenesis in the breast (Gonzalez-Suarez et al., 2010; Schramek et al., 2010).

7.5. In central fever regulation

The RANK/RANKL/OPG system is operational also in the Central Nervous System (CNS). This axis is an important thermoregulatory system; RANKL and RANK are both expressed in the brain (Kartsogiannis et al., 1999), where they play an important role in the regulation of central fever. RANKL specifically stimulates thermoregulatory brain regions and induces fever by activation of prostaglandins. An injection of RANKL into the cerebro-ventricular region in mice and rats has been shown to induce a strong febrile response, which can be attenuated with OPG (Göbel et al., 2020).

8. Pregnancy and Autoimmune diseases

Autoimmune diseases are characterized by tissue damage caused by the self-reactivity of several effector mechanisms of the immune system, namely antibodies and T cells. There is much evidence that pregnancy can influence the onset and progression of autoimmune and inflammatory diseases (Piccinni et al., 2016) and can influence it positively, by improving the disease status, or negatively by going on to worsen the autoimmune disease status.

During pregnancy, many autoimmune diseases go into remission, only to flare up again in the early postpartum period; multiple sclerosis (MS) and Graves' disease are examples. On the other hand, in Th-2 type autoimmune diseases such as Systemic lupus erythematosus (SLE), a worsening of the disease is found during pregnancy (Piccinni et al., 2016).

Table 1. *Autoimmune diseases progression in pregnancy*

Autoimmune disease	***Influence on pregnancy***
Rheumatoid arthritis (RA)	Improved
Systemic lupus erythematosus (SLE)	Worse
Multiple sclerosis (MS)	Improved

MS is a chronic autoimmune demyelinating disease affecting the white and grey matter of the CNS while the peripheral nervous system and other organs are not targeted by the disease process. The most typical progression pattern is an initial phase of relapsing and remitting symptoms (relapsing-remitting MS, RRMS) that can develop to progressive disease (secondary progressive MS, SPMS), leading to a deterioration of neurological functions (Aharoni et al., 2021).

One of the pathological hallmarks of MS is the T cell-mediated destruction of the myelin sheath, which results in axonal damage and subsequent neurological dysfunction. Autoreactive effector T-cells, activated in the periphery, such as T helper (Th)-1 and Th-17 that recognize specific myelin antigens, mediates the immunological attack.

Astrocytes in these lesions also show signs of cellular damage, in particular a retraction of foot processes from the perivascular glia limitans have been proven (Sharma et al., 2010).

The inflammatory infiltrates contain mainly T-lymphocytes, anyway B-cells and plasma cells are also present, although in much lower numbers (Lassmann, 2013). In MS, cytokines and chemokines produced by activated T lymphocytes play a crucial role in triggering the disease onset, as well as in the further development and progression of the disease. In this context a critical role is played by astrocytes, a type of glial cells, that control the recruitment of leukocytes into perivascular spaces and the CNS parenchyma through the secretion of multiple molecules. Interactions between activated astrocytes and endothelial cells increase Blood Brain Barrier (BBB) permeability and facilitate leukocyte infiltration, while bidirectional communication between astrocytes and peripheral immune cells potentiates CNS inflammation and contributes to disease progression (Linnerbauer et al., 2020). According to the most accredited thesis while the passage across the BBB of auto-aggressive T-cells, activated in the periphery by CNS extrinsic factors is considered the triggering event, the interactions between activated immune cells and CNS cells would strengthen and perpetuate the inflammatory response within the CNS (Hemmer et al., 2015). Pregnancy itself does not affect the integrity of the BBB (Johnson and Cipolla, 2015), on the contrary, a breakdown of the barrier has been reported in MS patients (Hawkins et al., 1990); so it is possible that during MS pregnancy, factors produced by a fully functional placenta may be active on tissues and cells of the pregnant mothers with effects either on immune cells of adaptive and innate immunity, cells within the CNS and/or on their reciprocal interaction.

Nevertheless, epidemiological and clinical evidence suggest a clear beneficial effect of pregnancy in women with MS, especially in the third trimester of pregnancy (Confavreux et al., 1998). In

particular, there is no evidence of pregnancy-related pathologies, such as an increased incidence of fetal complications, negative pregnancy outcome, and a placenta histologically comparable to a healthy woman's placenta (Bove et al., 2014; Jalkanen et al., 2010). Therefore, the maternal MS does not associate with an altered uterine-placental interface or abnormalities in the placenta (Spadaro et al., 2019), suggesting that the placenta in pregnancies with multiple sclerosis is healthy and functional.

Substantial experimental evidence suggests that the protective effect of pregnancy may be related to the immunological changes that occur during normal pregnancy allowing tolerance to fetal antigens and to autoimmune targets by preventing pathogen infections (Graham et al., 2021).

During pregnancy there are elevated levels of circulating hormones that have potent immunomodulatory properties, such as changes in cytokine production, downregulation of adhesion molecule and matrix metalloproteinase expression, decreased antigen presentation and induction of regulatory T cells (Voskuhl and Gold, 2012), all of which could positively influence MS pathology. The beneficial effects of pregnancy in MS may not be limited to modulation of the maternal immune system, but may in part also reflect effects on the maternal central nervous system, increasing resilience against immune-mediated attacks or promoting endogenous repair mechanisms (Patas et al., 2013).

Most studies have focused on the contribution of the maternal immune response, tracing these beneficial effects of pregnancy to the changes that the maternal immune system makes to "accept" the product of conception, but the role of the placenta in this context has never been studied.

RATIONALE

During physiological pregnancy the structure and function of the placenta change with gestation. This process leads to a completely different repertoire of factors expressed and produced by the placenta at term, compared to the first trimester.

These changes in placental physiology could contribute to the remission of MS in pregnant women, particularly in the last trimester of pregnancy acting on cells involved in the inflammatory process characterizing the MS disease.

It is also well recognized that pregnancy, labour, delivery and the incidence of fetal complications are not affected by MS with no difference with healthy women (Jalkanen et al., 2010). While most of the studies focused their attention on deciphering the contribution of the maternal immune response on MS remission during pregnancy, very few looked at the role of the placenta, the organ that coordinates maternal adaptation to pregnancy.

On this basis, we hypothesized that the RANK/RANKL/OPG system is useful in normal pregnancy for maternal tolerance versus the semi-allogenic fetus, which is active in MS pregnancy and contributes to the remission of disease in patients.

In synthesis, the rationale of our hypothesis is based on the following highlights:
the placenta orchestrates maternal adaptation to pregnancy through the synthesis and release of factors in maternal and fetal circulation that change throughout pregnancy in a gestational time-dependent manner; pregnancy exerts beneficial effects on MS women, particularly during the third trimester; the RANK/RANKL/OPG axis is involved in MS.

AIMS OF THE STUDY

1. *AIM 1*

This study aims to test the involvement of the placenta in a well-known receptor/ligand/decoy receptor system, such as the RANK/RANKL/OPG, which has been poorly investigated in the context of pregnancy.

The main goals of this part of the study were:

- to determine the pattern of expression of OPG and sRANKL in human placenta in normal gestation;
- to determine the OPG and sRANKL concentration in culture media of placental explants from normal pregnancy at first trimester and term of gestation;
- to determine the OPG and sRANKL concentration in the mother and cord blood;
- to determine the OPG and sRANKL concentration in the serum of healthy and MS-affected pregnant women collected during the third trimester of gestation and in the post-partum period;
- to determine the OPG and sRANKL concentration in the serum of non-pregnant MS women of childbearing age.

2. *AIM 2*

We also set ourselves the second main objective of the project to find out whether there is a relationship between the placenta and the brain in pregnancy. Astrocytes, a particular type of glial cells, play an important and central role in the recruitment of immune cells in the CNS during inflammation. Moreover, in conditions such as MS, astrocytes respond to the inflammatory stimuli with a process of morphological, transcriptional, biochemical, and functional remodelling (Aharoni et al., 2021).

Therefore, we evaluated whether:

- the placental secretome can regulate the physiology of astrocytes;
- the placental secretome could mitigate the neurotoxic action of activated astrocytes, and thus pregnancy can improve the inflammatory status of these cells even during MS.

MATERIALS AND METHODS

1. SAMPLE PREPARATION

- Placenta and serum collection

The collection of placenta samples for research purposes has already obtained the approval of the Ethics Committee CEAVS of the University of Siena and the Azienda Ospedaliera Senese hospital (Siena, Italy). After being properly informed by the physician, all patients undergoing placental tissue collection for research signed an informed consent form. The collection of the donor's tissues and personal data were processed following the Italian privacy law and the guidelines defined by the Declaration of Helsinki. At the time of collection, each sample is associated with a descriptive card indicating the gestational age, obtained by the doctor through a combination of anamnestic data and ultrasound examinations.

***Table 2**. Patients' characteristics*

	Non-pregnant	First trimester	Term
Maternal age (years)	35.8 ± 7.2	28.2 ± 6.8	37.4 ± 9.0 (33-45)
Caucasian ethnicity (%)	100	100	100
Pre-pregnancy weight	-	-	64 ± 10.7
Weight gain (kg)	-	-	10.9 ± 8.8
Gestational age (weeks)	-	9.3 ± 2.4	38.13 ± 0.6
Nulliparous (%)	-	-	23.2
Female (%)	-	-	77
Male (%)	-	-	23
Placental weight (g)	-	12.3±3.5	449.8 ± 83.6
Birth weight (g)	-	-	2679.1 ± 581

Tab. 2: The study group consists of ten (n=10) non-pregnant women, of ten (n=10) pregnant women who completed a physiological pregnancy and underwent an elective caesarean section, because of a previous caesarean section or a fetus in a podalic position, and three (n=3) first-trimester placenta from elective terminations of pregnancies.

After collection, the tissues were immediately transported in an insulated bag to the laboratory, where they were washed in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM $KH2PO_4$, pH 7.3) and dissected to collect only small portions of placental tissue. All tissue samples immediately after collection were in part snap-frozen and stored at −80 °C and in part, formalin-fixed for immunohistochemical analysis; the remnant tissue was processed for chorionic villous explant cultures. Small terminal fragments of chorionic villi were isolated using the dissection microscope, each consisting of a central shaft with 5-6 interdigitated lateral branches.
Peripheral blood from the mothers within the third trimester of pregnancy and blood from the umbilical cords after double clamping, reflecting fetal state, were collected in pyrogenic-free tubes. In addition, we analysed serum samples from women affected by multiple sclerosis of childbearing age, during the third trimester of pregnancy and the postpartum period.

The explants are placed in a 96-well plate (BD Falcon Becton Dickinson) and incubated overnight in 200 µl of Dulbecco's Modified Eagle Medium (DMEM) F-12 medium without phenol red (Gibco, Thermo Fisher, Waltham, USA), supplemented with 100U/mL of penicillin/streptomycin (Sigma Aldrich, Milan, Italy) in a humidified atmosphere at 3% O_2 and 8% O_2 for the first trimester and term explants respectively, and 5% CO_2 at 37°C. The morphology of the explants was constantly monitored under an optical microscope. The day after, the medium was replaced with fresh medium and cultures were incubated for 48h. At the end of the experiment, the explants and the conditioned media were collected and processed for biochemical analyses.

- Experimental setup

Placenta explants at the term of gestation (n=3) were treated for 24 hours with lipopolysaccharide (LPS) at two concentrations 100ng/mL and 1µg/mL to simulate an acute inflammatory situation.
In other experiments, term placenta explants (n=3) were treated for 48 hours with hormones to assess the effects on molecules production and secretion. The hormone concentration administered was 10^{-8}M Estradiol (E2), $7x10^{-8}$M Estriol (E3) and 10^{-6}M Progesterone (Pg) (Sigma Aldrich, Milan, Italy). The hormone concentrations used are in agreement with levels detected in the serum of women in the third trimester of pregnancy. Hormones were added to the medium individually.

Moreover, explants were exposed for 24h to a cytokines cocktail of 10 ng/mL of interleukin (IL)-1β (Abcam, Cambridge, UK) and IL-6 (R&D System Inc., Minneapolis, MN, USA).

- U373 cell culture

U373 MG (Uppsala), a human glioblastoma astrocytoma derived from a malignant tumor by explant technique, were cultured at 37°C, 97% air humidity and 5% CO_2. Astrocytic tumour cells were maintained in culture in DMEM supplemented with 4.5g/L glucose, 100U/mL penicillin/streptomycin, 10% fetal bovine serum (FBS) and 1% L-glutamine.

- Experimental setup

U373 cells were seeded in 6-well plates at a density of $2.5x10^5$cell/mL, and the day after they were activated by exposure to cytokine IL-1β at two different doses, 1ng/mL and 20 ng/mL, for 24h.
In another experiment, U373 cells were exposed to the conditioned media of term placenta explants for 24h, after activation with 1ng/mL of IL-1β.
The treated cells are monitored for viability, morphology and functionality.
In addition, U373 cells were activated by exposure to recombinant human RANKL (R&D Systems Inc., Minneapolis, MN, USA) (100ng/mL) and simultaneously to three different doses of recombinant human OPG (R&D Systems Inc., Minneapolis, MN, USA), namely 25-50-100 ng/mL for 24h.

- Sulforhodamine B proliferation assay

The Sulforhodamine B (SRB) assay has been used since its development in 1990 (Skehan et al., 1990) to inexpensively conduct various screening assays to investigate cytotoxicity in cell-based studies (Vichai and Kirtikara, 2006). This method relies on the property of SRB to bind stoichiometrically proteins under mildly acidic conditions and then can be extracted using basic conditions. The amount of bound dye can be used as a proxy for cell mass, which can then be extrapolated to measure cell proliferation.
U373 cells were seeded in 96-well plates at a density of $2.5x10^4$cell/mL and incubated until the following day to allow adhesion in the control medium. In plate T0, representative of the initially seeded number of cells, immediately after the adhesion, the medium was eliminated and the wells were washed three times with PBS, while the other plate was treated with two different doses of IL-1β (1ng/mL and 20 ng/mL) or with 100 ng/mL of rhRANKL for 24h. The fluorescent plates were

read at an excitation/emission wavelength of 488/585nm. To obtain the percentage of cell proliferation the following formula was applied:

$$\%\ cell\ proliferation\ =\ \frac{absorbance\ x}{absorbance\ T0}\ \text{X}\ 100$$

2. *HISTOLOGICAL ANALYSIS*

- Immunohistochemistry

Immunohistochemistry analysis allows the identification and visualisation of specific target molecules both on the surface and inside the cells. In this method, primary antibodies bind their respective antigen and a conjugated secondary antibody, in turn, targets them. Analysis with an appropriate microscope (light or fluorescence) shows the distribution of the antigen of interest throughout the sample.

Human placenta tissues were first fixed in 4% (v/v) buffered formalin (pH 7.2-7.4), to block cellular autolysis and preserve the morphology, afterwards they were dehydrated through passages in alcohol with increasing gradation. Later they were included in paraffin, a mixture of saturated hydrocarbons that becomes fluid when heated while solidifying at room temperature, giving the tissue the right consistency to obtain thin microtome sections. The 4µm slides obtained by microtome cutting were heated to 60°C for 10 minutes and rehydrated through passages in decreasing alcohol gradation and finally washed in Tris-buffered saline (TBS) (Tris-HCl 20mM and NaCl 150mM, pH 7.6). Subsequently, the slides are subjected to the unmasking of antigenicity in a 10mM citrate buffer at pH 6.0 in a microwave at 750W for 5', then left to cool at room temperature and washed in TBS for 15' (3 washes of 5'). The slides were incubated in 3% (v/v) H_2O_2 for 15’ to block endogenous peroxidase reaction, then, were incubated with Protein block serum-free (0.25% (w/v) casein in PBS solution, with stabilizing protein and sodium azide 0.015mol/L) (Dako) for 20 minutes to block non-specific binding of antibodies. After blocking, the slides were incubated overnight at 4°C with a primary antibody directed against OPG (Listarfish, Milan, Italy) diluted 1:2000 in TBS. The slides were then washed and incubated with the appropriate secondary antibody conjugated with peroxidase diluted 1:500 in TBS. The reaction was detected by diaminobenzidine (DAB) and the sections were contrasted with Meyer's Haematoxylin, mounted on an aqueous medium (Sigma Aldrich, Milan, Italy) and examined under an optical microscope. Negative controls were performed using the appropriate normal isotype antibody in TBS.

- Immunofluorescence

A total of 10^4 U373 cells were seeded on rounded coverslips in 6-well plates. After 24h of incubation, slides were washed and fixed with 4% formalin for 10 minutes and then some permeabilized in 0.1%

Triton X-100 in PBS for 5 minutes, and others not. The slides were blocked in 5% Bovine Serum Albumin (BSA) for 30 minutes and incubated overnight with mouse anti-human GFAP (R&D systems, Minneapolis, MN, USA) diluted 1:100 in PBS and with mouse anti-human RANK (R&D systems, Minneapolis, MN, USA) diluted 1:200 in PBS. Slides were then washed in PBS and incubated for 1h with goat anti-mouse Alexa Fluor diluted 1:200 in PBS. GFAP analysis and images acquisition were performed with LEICA CTR6500HS fluorescence microscope; instead RANK images were carried out with confocal microscope LEICA LAS-X cellular imaging (LEICA Microsystem).

3. *BIOCHEMICAL ANALYSES*

- Protein extraction

The tissue samples were solubilized in a RIPA^{++} lysis buffer, consisting of: Tris 50mM, NaCl 150mM, Triton X-100 1%, sodium deoxycholate 1%, SDS 0.1% added with PMSF (phenylmethylsulfonylfluoride) 1mM, sodium orthovanadate 100mM, 0.1% protease and phosphatase inhibitors (Sigma Aldrich, Milan, Italy). The samples were treated with 100µl of Lysis buffer and to solubilize the samples, they were pounded by hand with a potter on ice, made two hot-cold cycles, and sonicated in 2 cycles of 15 seconds each. Finally, they were centrifuged at 12000g for 15 minutes at 4°C and then frozen at -80°C. The placenta tissues were first pulverized with liquid nitrogen in a mortar, the material obtained was collected in a new Eppendorf and then 150µl of RIPA buffer and 0.1% protease and phosphatase inhibitors (Sigma Aldrich, Milan, Italy) were added. Finally, the samples were sonicated in 3 cycles of 15 seconds each, centrifuged at 12000g for 15 seconds at 4°C, then the supernatants were aliquoted and frozen at -80°C for subsequent analysis.

- Polyacrylamide gel electrophoresis (PAGE)

The concentration of the proteins contained in the protein lysates was determined by the method of Bradford (Bradford, 1976). The absorbance peak of the dye is at 595 nm. The standard curve of BSA (bovine serum albumin) at a known concentration (2 mg/ml) was set up in series (1:2 dilutions in PBS). The optical density values of the samples were read using a spectrophotometer and compared with the calibration curve; then by interpolation, the total protein concentration value of each sample was extracted.

A polyacrylamide gel was performed in the presence of Sodium Dodecyl Sulfate (SDS-PAGE). 40 µg of total protein were loaded on 10% Acrylamide/Bis-acrylamide electrophoresis gel as described by Laemmli (Laemmli, 1970) in condition of constant voltage 135 V for 80 minutes in a cell Mini-PROTEAN (BioRad Laboratories Inc., Cambridge, MA, USA) in TGS buffer (25mM Tris, 190mM glycine, 0.1% (w/v) SDS).

Protein electrophoresis was also performed in the absence of SDS, under "native" conditions, to assess the native size of OPG protein in dimeric form, both in placental tissues and the conditioned

media of explants. 20µg of tissue total protein were loaded on 8% Acrylamide/Bis-acrylamide electrophoresis gel without denaturing agents.

- Immunoblotting

After SDS-PAGE and "native" PAGE, proteins were transferred from the gels to the nitrocellulose membranes (Amersham Hybond) into the Mini TransBlot System (BioRad Laboratories Inc., Cambridge, MA, USA) transfer cell filled with Transfer Buffer (25 mM Tris, 190 mM glycine, 20% (v/v) methanol). An electric field at 100 Volt constant was applied for 2 hours. Therefore, the membranes were blocked in 5% (w/v) non-fat dry milk dissolved in Tris-buffered saline pH 7.3 (TBS) containing 0.1% (w/v) Tween20 (TBS-Tween20) for 1 hour at room temperature. Membranes were then incubated with mouse monoclonal antibodies anti-human OPG (Listarfish) diluted 1:1000 in 5% (w/v) non-fat dry milk dissolved in TBS-Tween20 overnight at 4°C.

The following day, membranes were washed 3 times for 5 minutes each in TBS-Tween20, to eliminate the primary antibody excess. Subsequently, the membranes were incubated for 1 hour at room temperature with goat anti-mouse horseradish peroxidase-conjugated IgG (BioRad Laboratories Inc., Cambridge, MA, USA) at dilution 1:6000 in 5% (w/v) non-fat dry milk in TBS-Tween20. The reaction was revealed using chemiluminescent reagents (BioRad Laboratories Inc., Cambridge, MA, USA) and then membranes were digitalized with CHEMIDOC Quantity One 1D Analysis Software (BioRad Laboratories Inc., Cambridge, MA, USA).

Subsequently, the membranes were stripped 3 times in strip buffer (0.05M glycine, 1% SDS (w/v), pH2.2) and after 1 hour of blocking in 5% (w/v) non-fat dry milk in TBS-Tween20, they were incubated overnight with mouse monoclonal antibody anti-human β-actin diluted 1:2000 in 5% (w/v) non-fat dry milk in TBS-Tween20.

- ELISA assay

The Enzyme-Linked Immunosorbent Assay (ELISA) is based on the concept of antigen-antibodies reaction, permitting the highly sensitive qualitative and/or quantitative analysis of antigens (Sakamoto et al., 2018).

The levels of OPG and sRANKL were measured: in circulating serum during healthy pregnancy and non-pregnant women; in maternal and cord serum; in placenta explants culture conditioned media;

in non-pregnant women with multiple sclerosis and in women with multiple sclerosis in the third trimester of pregnancy and in the postpartum period.

The levels of IL-6 and TNF-α were measured in the conditioned media of astrocytes activated with RANKL (100ng/mL) and with IL-1β (1ng/mL).

The kits used are those of the DuoSet enzyme-linked immunosorbent assay from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's recommendations.

Briefly, 96-well plates were coated overnight with the capture antibody at room temperature. The plates were then washed three times with the washing solution (10mM PBS, 0.05% Tween20) and non-specific binding was avoided by incubation with blocking solution (PBS-BSA 1%) for 2h at RT. The plates were washed and the standard and samples were added in duplicates and incubated for 2h at RT. Then the plates were washed three times and the detection antibody was added and incubated for 2h at RT. Plates were washed three times and streptavidin horseradish peroxidase was added and incubated for 20 minutes at RT. Then the plates were washed again and the Substrate solution (1:1 of H_2O_2 and Tetramethylbenzidine) was added for 20 minutes at RT in the dark. The reaction was stopped by adding H_2SO_4 2N. The optical density was determined at 450nm by a microplate reader (Multiskan).

***Table 3**. Standard range of the ELISA test performed*

Elisa Test	Standard range
OPG	4ng/mL - 0.06ng/mL
sRANKL	5ng/mL - 0.078ng/mL
TNF-α	1ng/mL - 0.015ng/mL
IL-6	600pg/mL - 9.38pg/mL

- Immunoprecipitation

Immunoprecipitation is a technique that leads to the enrichment or depletion of a protein of interest. The conditioned media of term explants from five (n=5) different placenta were incubated with the anti-OPG antibody (SantaCruz Biotechnology, Dallas, USA) overnight at 4°C in oscillation. The next day samples were centrifuged for 2 minutes at 12000g at 4°C and the supernatant depleted from the OPG was withdrawn and kept aside the pellet containing the matrix enriched of OPG. To verify immunodepletion and the relative percentage of depletion, an ELISA assay for OPG was performed with whole and OPG-deprived conditioned media.

4. GENE EXPRESSION ANALYSIS

Gene expression analysis allows the quantification of the mRNA content of a gene of interest. As this amount is proportional to the expression of the gene, its quantification provides information regarding the gene's activity. To perform gene expression analyses, RNA is isolated from tissues and cell lysate and then reverse transcribed into complementary DNA. The monitoring of DNA amplification in real-time is possible thanks to the monitoring of fluorescence. In Real-Time Polymerase Chain Reaction (RT-PCR), fluorescence is measured after each cycle and the intensity of the fluorescence signal reflects the momentary amount of DNA amplicons in the sample at that specific time (Kralik and Ricchi, 2017).

- RNA isolation

Total RNA extraction from placental tissues at first trimester and term and astrocytes was achieved using the zymo spin columns kit (ZymoResearch, Irvine, USA) according to the manufacturer's instructions. Genomic DNA was digested by DNase and several washing steps ensured further purification. The final elution step of RNA was conducted in 25µl of RNase-free water. The RNA concentration and purity were measured at 260 and 280 nm with the Nanodrop (Thermo Fisher, Waltham, USA). The absorbance at 280 nm provides information regarding RNA concentration while the purity of the RNA is determined by the absorbance ratio at 260 nm and 280 nm. Isolated RNA was stored at -80°C until further processing.

- cDNA transcription

For the reverse transcription of RNA into cDNA, the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, USA) was used. 1ug of RNA, for each sample, was reverse transcribed using a Bio-Rad thermal cycler, the samples were incubated 10 minutes at 25°C, 120 minutes at 37°C (optimum reverse transcriptase activity) and 5 minutes at 85°C (T enzyme inactivation). The cDNA obtained was diluted 1:2 and then stored at -20°C until the use.

Table 4. *List of the retrotranscriptase kit components*

Component	Volume
10X RT Buffer	2.0 µL
25X dNTP Mix (100 mM)	0.8 µL
10X RT Random Primers	2.0 µL
MultiScribe Reverse Transcriptase	1.0 µL
Nuclease-free H2O	4.2 µL
Total per reaction	10.0 µL

Table 5. *Thermal profile of the cDNA synthesis*

Settings	**Step 1**	**Step 2**	**Step 3**
Temperature	25°C	37°C	85°C
Time	10 minutes	120 minutes	5 minutes

- RT-PCR

The RT-PCR technique is a method that allows us to amplify and quantify DNA simultaneously using double-stranded DNA-binding dyes (SYBR Green) as reporters. The SYBR Green binds to all double-stranded DNA which was synthesized in the qPCR reaction, causing a fluorescent signal that can be detected. An increase in DNA product during the qPCR, therefore, leads to an increase in fluorescence intensity measured at each cycle. To check for specific RT-PCR products, the analysis of the melting curve (65°C-95°C) was included in the procedure.

RT-PCR experiments were conducted using the SsoAdvanced Universal SYBR Green Supermix kit (Biorad Laboratories Inc., Cambridge, MA, USA). The final 10µL qPCR mixture for each sample

consisted of 2µL of back-transcribed and 8µL of primers mix; first, the cDNA mix was pipetted to the bottom of the PCR microplate and then the primer mix was dispensed at the side of the well. The plate was centrifuged at 1000 rpm for 1 min before inserting it into the PCR machine. The PCR machine (Step-One RealTime-PCR System, Applied Biosystems, Waltham, USA) was programmed as shown in the table above. To control sampling errors and to normalize our data, RT-PCR for housekeeping genes 18S and HPRT1 were performed on each placenta sample and for 18S and GAPDH on the astrocyte's samples. After the analysis of the amplification plot, the Ct values of the samples were normalized against those of housekeepers according to the following formula: $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Table 6. *qRT-PCR reagent list*

Component	Volume
SsoAdvanced Universal sybr green Supermix (2x)	5 µL
Forward Primer	0.5 µL
Reverse Primer	0.5 µL
Nuclease-free H_2O	2.0 µL
Dna template	2.0 µL
Total per reaction	10.0 µL

Table 7. *List of genes of interest*

Gene symbol	Gene of interest	
OPG	Osteoprotegerin	Bio-Rad
RANKL	Receptor activator of nuclear factor kappa-B ligand	Bio-Rad
CCL2	Chemokine C-C motif ligand 2	Merck
CCL20	Chemokine C-C motif ligand 20	Merck
18S	18S ribosomal RNA	Bio-Rad
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	Merck

Table 8*. qRT-PCR thermal profile*

Cycling Step	Enzyme Activation	Denaturation	Annealing	Melt Curve
Temperature	95°C	95°C	60°C	65-95°C
Time	2 minutes	15 seconds	30 seconds	5 seconds/step

5. DATA ANALYSIS AND STATISTICS

All data sets were analysed for normality using Shapiro-Wilk or Kolmogorov-Smirnov followed by either parametric (Students' t-test or One-Way Anova) or non-parametric tests (Mann-Whitney test or Kruskal–Wallis) as appropriate. Densitometry was performed through Image J software. Graphs and statistical analysis present in this thesis were performed with GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, CA). P-values smaller than or equal to 0.05 were considered significant (****: $p < 0.0001$; ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$).

RESULTS

1. Serum OPG and sRANKL during pregnancy

To assess change in circulating OPG and sRANKL protein levels, sera from pregnant women and non-pregnant women were compared. In particular, we investigated how serum levels vary during pregnancy, at the first trimester as well as at term of pregnancy. For each condition, ten biological replicates (n=10) were analysed in a sandwich ELISA to determine the concentration of proteins of interest.

Concerning OPG, serum levels significantly increase at term compared to the first trimester of pregnancy; moreover, there is a statistically significant raise at the term of pregnancy compared to the non-pregnant condition; whereas no particular difference were observed between first trimester and non-pregnant condition.

As regards the sRANKL protein, the obtained results indicate that serum levels increase significantly during pregnancy compared to a non-pregnant situation. No differences are observed for this protein at different gestational ages.

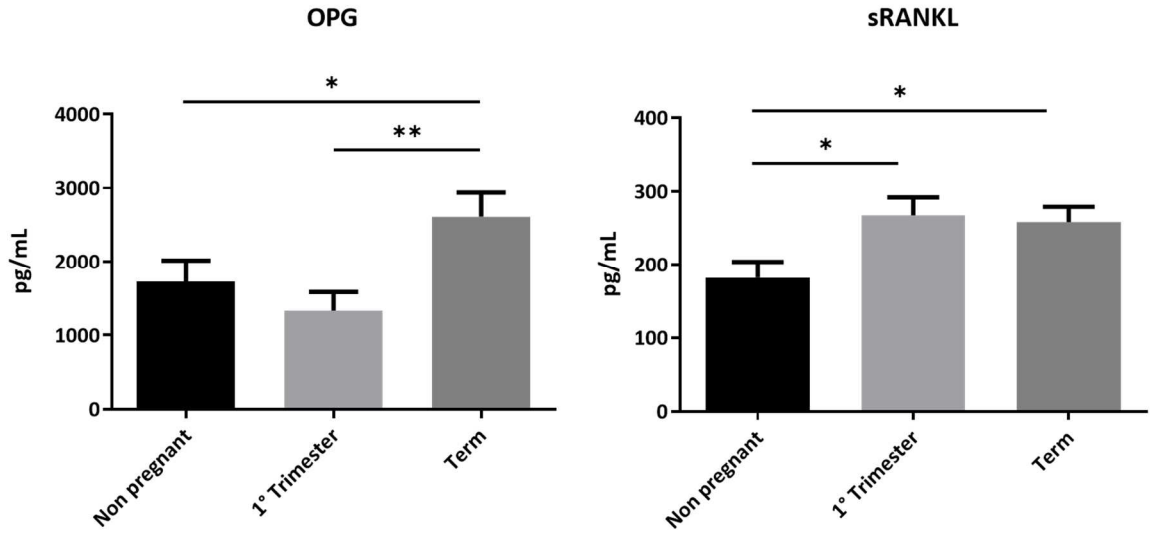

Figure 6. OPG and sRANKL serum levels during gestation. The ELISA test reveals an increase in OPG and sRANKL in the serum of pregnant women compared to non-pregnant women, with OPG significantly increasing in the third trimester of pregnancy. Data are means ± standard errors of the mean (SEM). (*p<0.05; **p<0.01)

2. *OPG and sRANKL in mother and cord serum*

We investigated possible differences in OPG and sRANKL levels between maternal serum and respective cord blood to establish the side of the major release of the two molecules by placenta. The ELISA quantification showed that OPG was significantly higher in maternal than in cord blood serum suggesting placental OPG is mainly released towards the maternal side. There was no difference in sRANKL protein levels between maternal and umbilical cord serum.

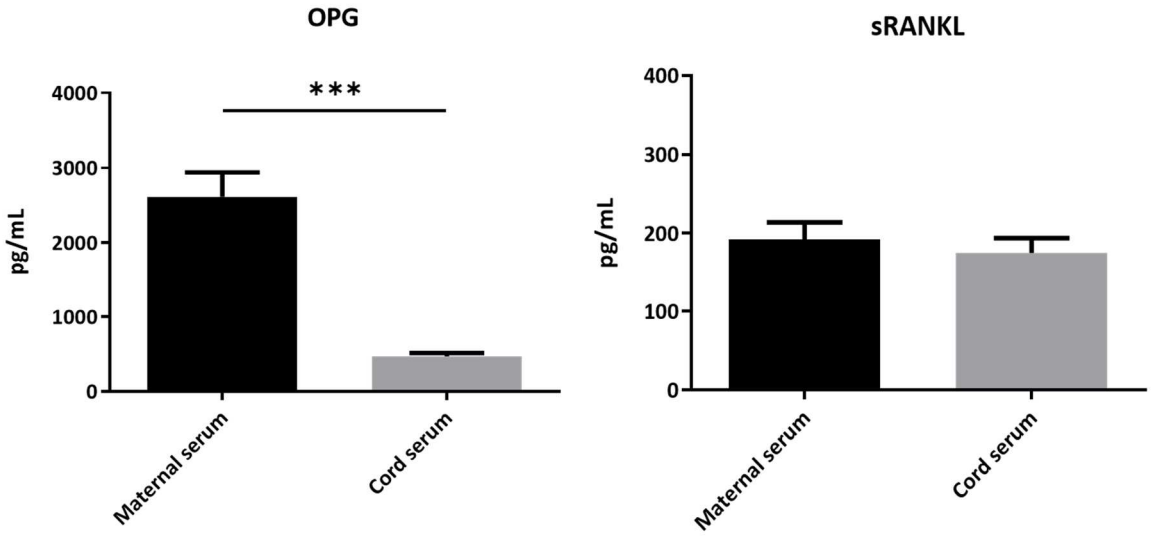

Figure 7. OPG and sRANKL levels in maternal and cord serum. OPG levels significantly decrease in cord blood compared to maternal blood. No significant difference in sRANKL. Data are means ± standard errors of the mean (SEM). (***p<0.001)

3. OPG and RANKL gene expression in placental tissues

Placenta tissues at the first trimester and at term of pregnancy were collected and the transcriptional levels of *OPG* and *RANKL* were analysed by RT-PCR.

OPG gene expression analysis revealed the presence of mRNA at both gestational times with a statistically significant difference in favour of term placenta tissues compared to the first trimester. Regarding the RANKL gene, we obtained extremely low expression levels at first trimester and non-detectable levels in term placenta samples (data not shown). Therefore, we may suggest that the placenta contribution to RANKL production is extremely low.

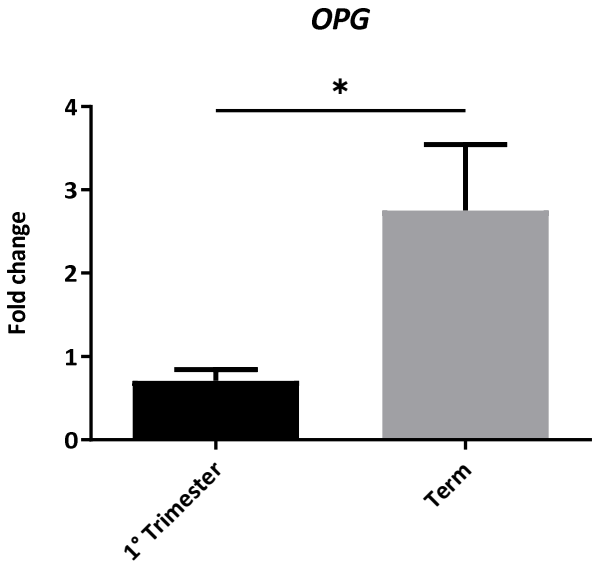

Figure 8. *OPG* gene expression in placenta tissues. *OPG* gene expression levels are elevated in placenta tissues at term of gestation. Data are means ± standard errors of the mean (SEM). (*p<0.05)

4. OPG and RANKL protein levels in placenta tissues

Immunoblot analysis for OPG protein, using SDS-PAGE, shows a clear band at 60kDa in both the first trimester and term placenta with no difference between the two groups. For both gestational times, ten samples were examined (n=10).

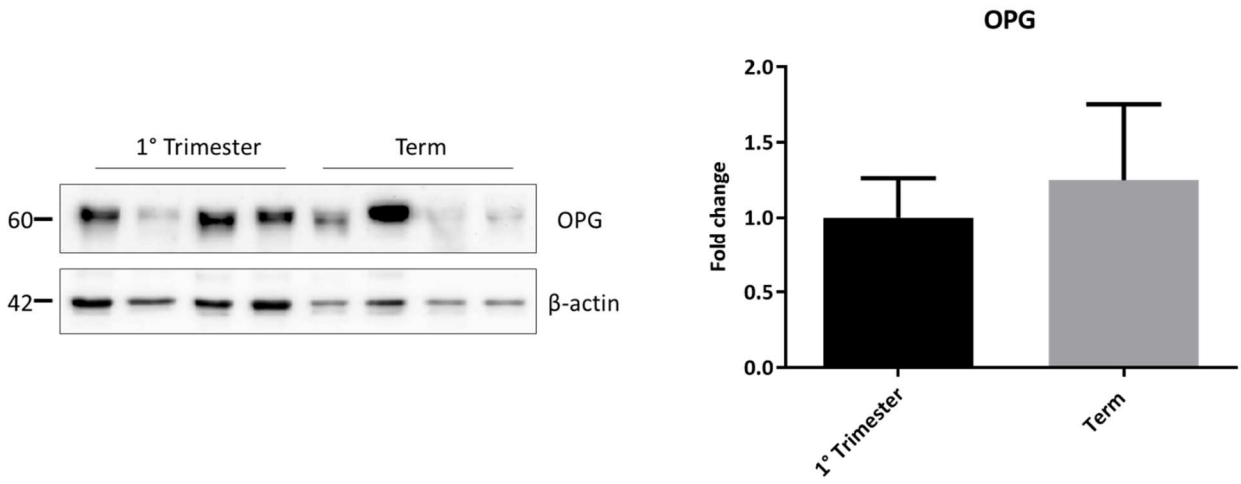

Figure 9. OPG protein expression levels in placenta tissues. The immunoblot analysis reveals a slight increase in OPG expression levels in term tissues compared to the first trimester. Data are means ± standard errors of the mean (SEM).

To better characterise the presence of the OPG dimer, electrophoresis was carried out under 'native' conditions, without reducing and denaturing agents. The immunoblot revealed the presence of OPG dimer mainly in placenta samples at term of pregnancy showing a statistically significant difference compared to the first trimester tissues. We also evaluated the expression of the dimeric form in cell lines, such as Caco2 and HT-29, distant and different from the gravid tissues. The results show the presence of the dimeric form mainly in the term placenta, while only a weak signal occurs in colon adenocarcinoma cell lines (data not shown).

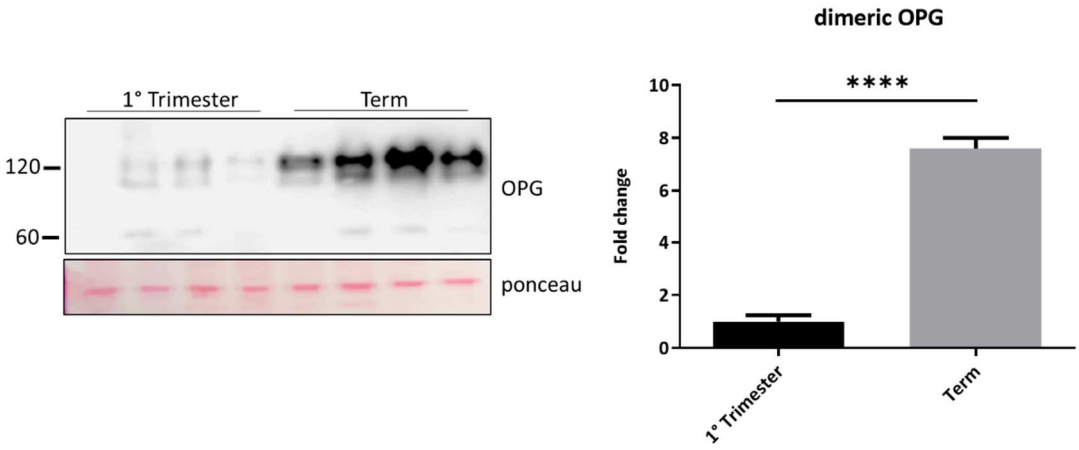

Figure 10. OPG native protein expression levels in placenta tissues. The immunoblot analysis in native conditions reveals a significant increase in dimeric OPG expression in term tissues compared to the first trimester. Data are means ± standard errors of the mean (SEM). (****p<0.0001)

Immunoblot analysis of RANKL revealed variability of expression, at the first trimester of pregnancy, when the protein is produced at a low level. Moreover, the secreted amount of sRANKL is close to the minimum detention level (data not shown).

5. OPG localization in human placenta

To evaluate OPG localization in the placental tissue, immunohistochemistry was performed in three biological samples for each gestational age.

In the first trimester placenta, a strong OPG immunoreactivity was found in the villous stroma and the perivascular cells of fetal capillaries within the villi core. OPG immunoreactivity was also observed in the cytotrophoblasts, the innermost trophoblast cellular layer of the placenta. The syncytiotrophoblast, the outermost trophoblast facing the maternal circulation, was negative for OPG staining.

In term placenta, OPG is still localized in the cytotrophoblast cells which are, at this gestational age, in closer proximity to the maternal blood as respect to first trimester ones.

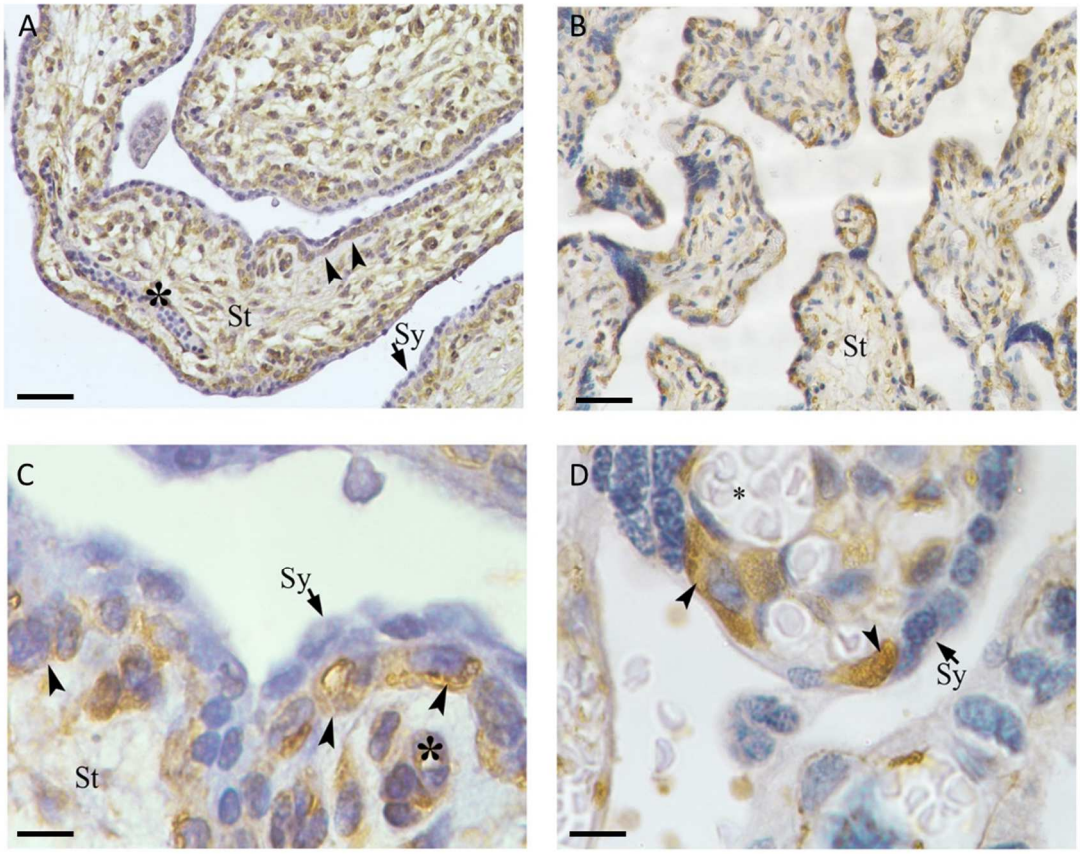

Figure 11. Immunolocalization of OPG in the first trimester and term placenta. The immunohistochemical analysis for OPG in the first trimester (A and C) and term (B and D) placenta. Cytotrophoblast (arrowhead); chorionic stroma (St); Perivascular cells of fetal capillaries (asterisk); syncytiotrophpblast (arrow). The brownish staining represents positivity. Bar in A and B, 50µm; Bar in C and D, 10µm.

6. *In vitro secretion of OPG by placental explants*

In vitro studies were conducted on chorionic villus explants from the first trimester and term placenta; we analysed three and ten biological samples for the first trimester and for the term placenta respectively. After 48h of culture, conditioned media were collected and processed by ELISA to determine the concentration of OPG secreted in the medium.

Term explant cultures released higher amounts of OPG into the conditioned medium compared to first-trimester explants. These results show a high statistically significant difference between the two tested groups.

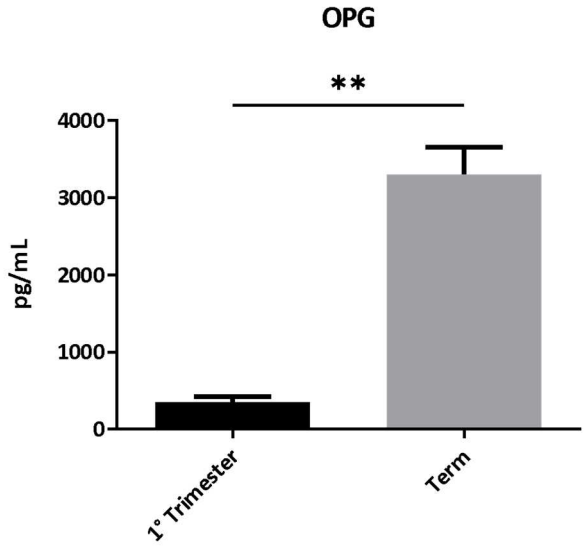

Figure 12. OPG levels in placenta explants conditioned media. The ELISA test reveals a significant increase in the levels of OPG secreted by the explants at term compared to those in the first trimester. Data are means ± standard errors of the mean (SEM). (**p<0.01)

Since the ELISA test we used does not allow us to discriminate between the monomeric or dimeric form of the OPG protein, we carried out electrophoresis under “native” conditions of the conditioned media from placenta explants both at the first trimester and at the term of pregnancy. The results confirmed the expression profile obtained from the native immunoblot on tissue lysates. There is an increased expression of the dimeric form of OPG in placental secretion at term of gestation, which is statistically significant compared to the first trimester of pregnancy.

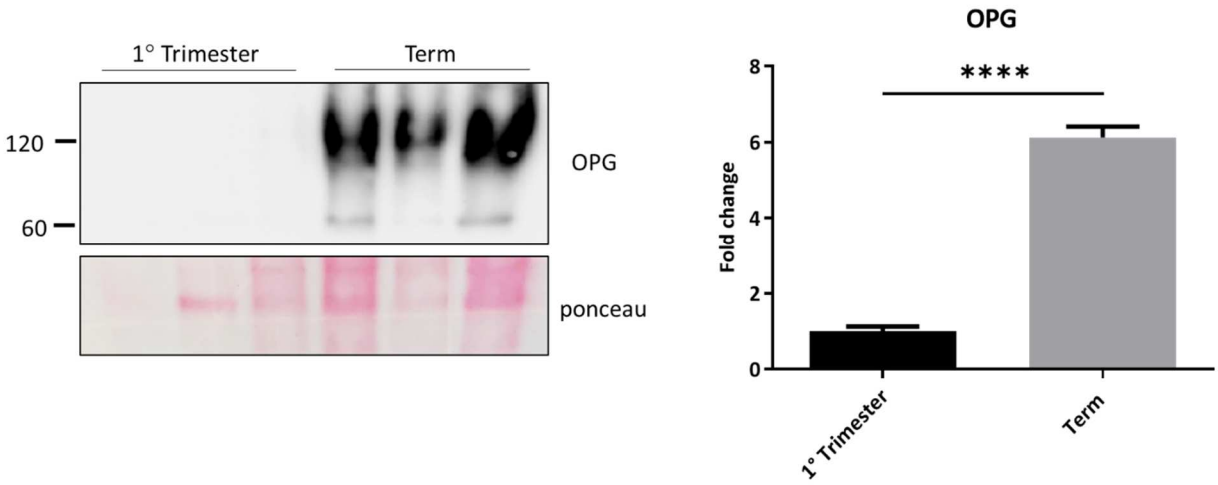

Figure 13. OPG native secreted protein expression levels in placenta explants conditioned media. The immunoblot analysis in native conditions reveals a significant increase in dimeric OPG secretion by term placenta explants compared to the first trimester. Data are means ± standard errors of the mean (SEM). (****p<0.0001)

7. LPS impact on OPG placenta explants secretion

In order to establish whether direct inflammatory insults to the placenta might have effect on OPG release, we exposed placental explant cultures to LPS a well-known proinflammatory stimulus. The effect of LPS on the OPG placental release was assessed by ELISA. Two different doses of LPS (100 ng/mL and 1 µg/mL) were added to the explant culture medium for 24 hours. As can be seen, the treatment with both doses of LPS significantly increases the release of OPG from placental explants.

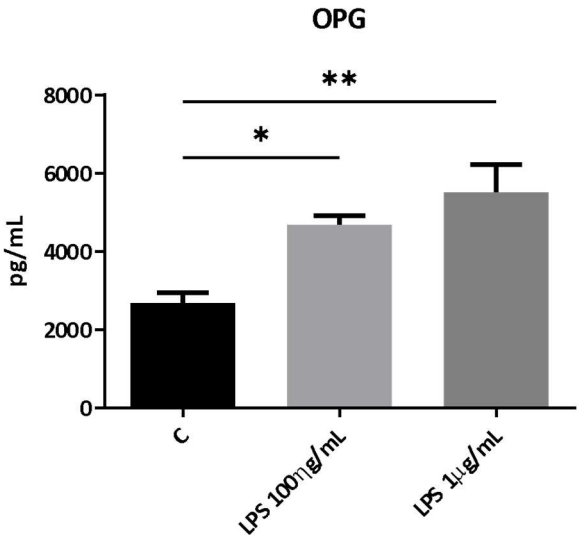

Figure 14. LPS impact on OPG placenta explants secretion. OPG release by term explants increases significantly in the presence of LPS at both doses after 24 h of treatment. Data are means ± standard errors of the mean (SEM). (*p<0.05, **p<0.01)

8. Hormones effect on OPG placenta explants secretion

The effect of estrogen (E2 and E3) and progesterone (Pg) on the expression of OPG has been assessed by ELISA. Placenta explants were treated with estradiol, estriol and progesterone for 48 hours at a dose (E2 10^{-8} M, E3 $7x10^{-8}$ M, Pg 10^{-6} M) corresponding to the physiological levels in the circulation at the end of pregnancy (Schock et al., 2016).

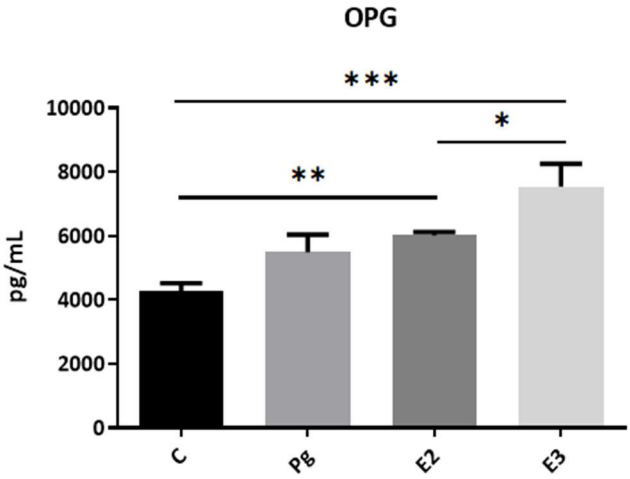

Figure 15. Hormone's impact OPG placenta explants secretion. Analysis shows a trend towards increased placental secretion of OPG with 48h hormone treatment and that both estradiol E2 and estriol E3 significantly increase OPG secretion. Data are means ± standard errors of the mean (SEM). (*p<0.5; **p<0.01; ***p<0.001)

9. Cytokines impact on OPG placenta explants secretion

The effect of a cytokines' cocktail on the expression of OPG has been assessed by ELISA. The cytokines IL-1β and IL-6, are known to be at high levels and to play an important role in inflammation during MS (Holmøy et al., 2013), were added to the explant culture medium for 24 hours at the dose of 10 ng/mL. As can be seen from the plot, treatment with cytokines did not affect the release of OPG from placental explants.

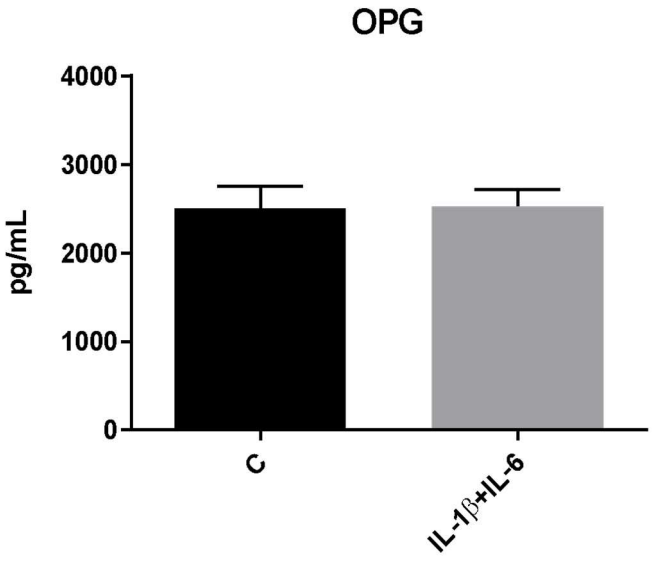

Figure 16. The impact of proinflammatory cytokines on OPG placenta explants secretion. No changes are observed in OPG release from term explants with 24 h cytokines treatment. Data are means ± standard errors of the mean (SEM).

10.Immunofluorescence for GFAP and RANK in astrocytes

Immunofluorescence was performed on the U373 astrocyte cell line to localise the RANK receptor and the GFAP, an intermediate filament protein expressed by the astrocytes. However, double labelling was not possible due to the lack of two different fluorescent secondary antibodies.

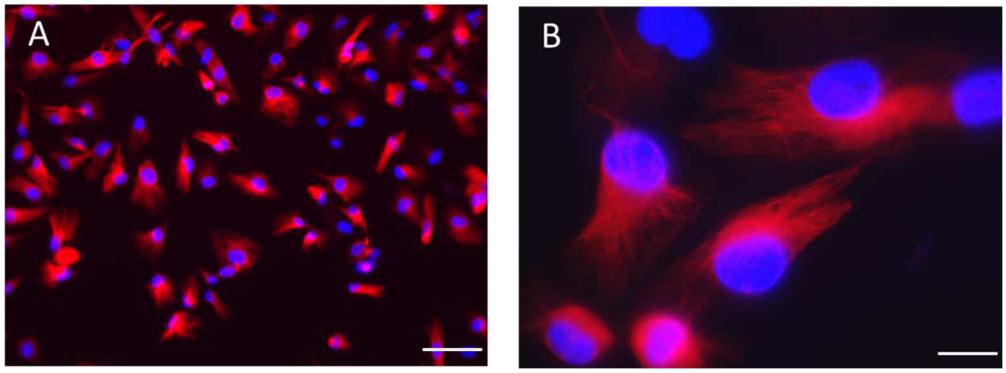

Figure 17.1. Immunofluorescence for GFAP. Immunolocalization of GFAP in astrocytes at low and higher magnification in A and B respectively. Scale bar= 100µm; scale bar= 30µm

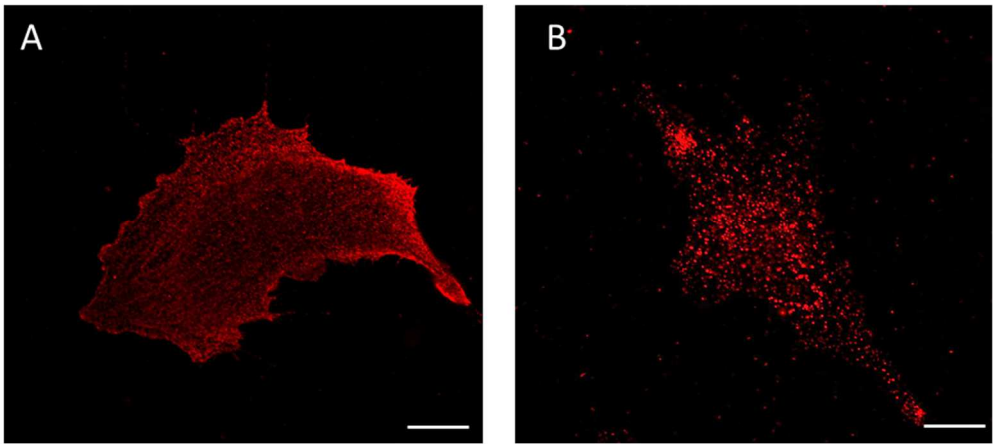

Figure 17.2. Immunofluorescence for RANK. Immunofluorescence performed in permeabilised or non-permeabilised astrocytoma cells shows the presence of the RANK protein in the cytoplasm (A) and at the membrane level (B), respectively. The point-shaped marking of the RANK receptor, in figure B, shows its presence on the astrocyte cytoplasmic membrane. Scale bar= 20µm.

11.Activation of Astrocytes

To assess whether treatment with two different doses of IL-1β (1ng/mL and 20ng/mL) or with rhRANKL (100 ng/mL) affected cell proliferation and viability, we carried out a Sulforhodamine B assay. As can be seen from the graph, there were no differences in cell proliferation and both treatments after 24 hours maintained viable cell cultures.

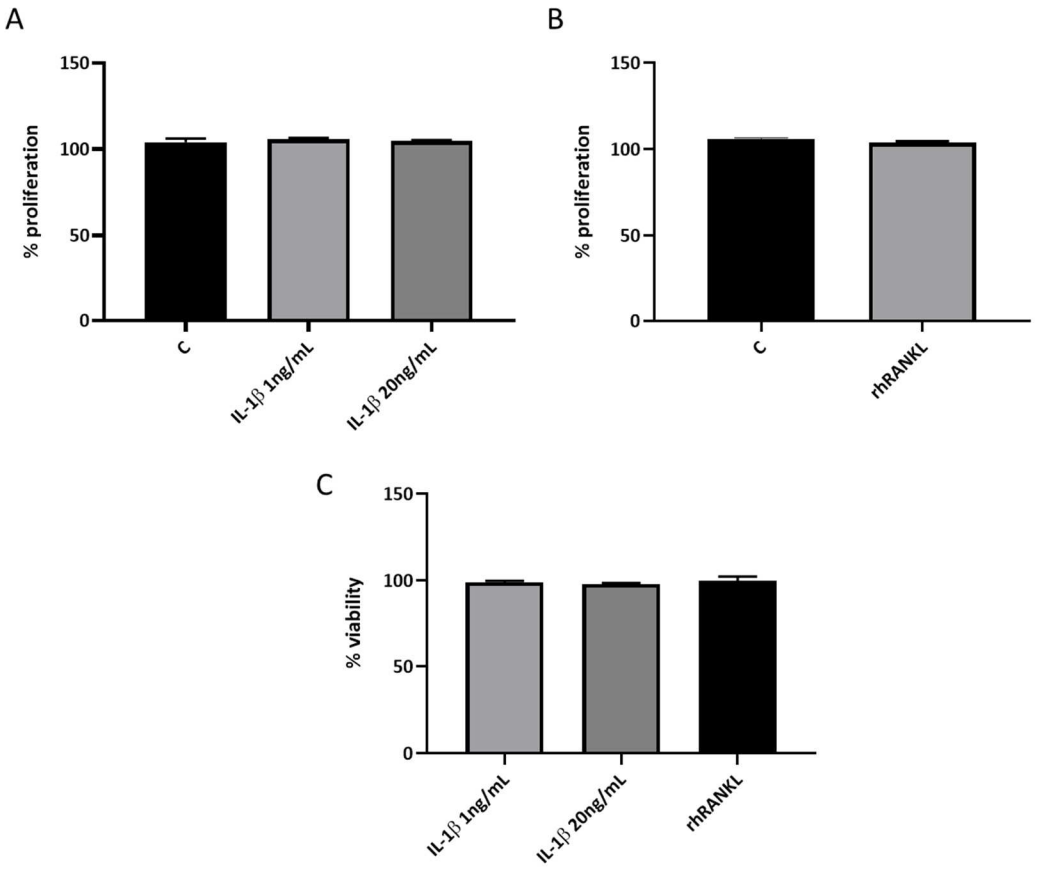

Figure 18. IL-1β and RANKL impact on astrocytes proliferation and viability. Cell proliferation in astrocytes exposed for 24h to 1ng/mL and 20ng/mL of IL-1β (A) and to 100 ng/mL of rhRANKL (B). Cell viability to IL-1β and rhRANKL in C. Data are means ± standard errors of the mean (SEM).

The administration of interleukin-1β (IL-1β) to astrocytes results in their activation, as we know from literature (Hyvärinen et al., 2019). Astrocytes respond to IL-1β treatment with a typical polygonal morphology transition. Therefore, as shown in figure 19, increasing doses of IL-1β (1ng/mL and 20ng/mL) administered to astrocytes induce a hypertrophic morphology, characterized by massive enlargement of the cell soma and reduced process density.

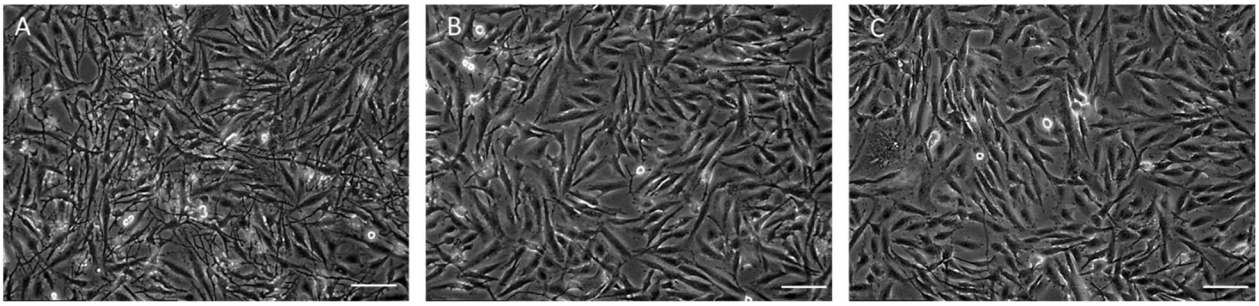

Figure 19. IL-1β impact on astrocytes morphology. Astrocytes change morphology after 24h of IL-1β treatment with 1ng/mL in B and 20ng/mL in C. In A control astrocytes. Scale bar= 50µm

Using the mathematical formula $4\pi * Area/Perimeter^2$, we calculated the roundness index of the cells, which was compared to the control astrocytes. We can observe that roundness statistically increased in a dose dependent manner.

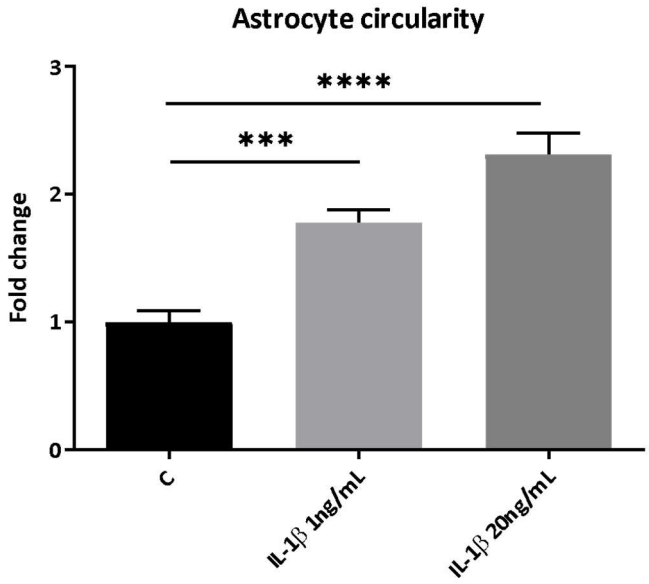

Figure 20. IL-1β impact on astrocytes morphology. Astrocytes change morphology after 24h of IL-1β treatment with 1ng/mL and 20ng/mL and assume a rounded shape. Data are means ± standard errors of the mean (SEM). (***p<0.001; ****p<0.0001)

In addition, an immunoblot for RANK was performed on IL-1β treated cells to verify if the treatment interfered with the expression of the receptor. As we can see from the relative plot, there was no change compared to the control.

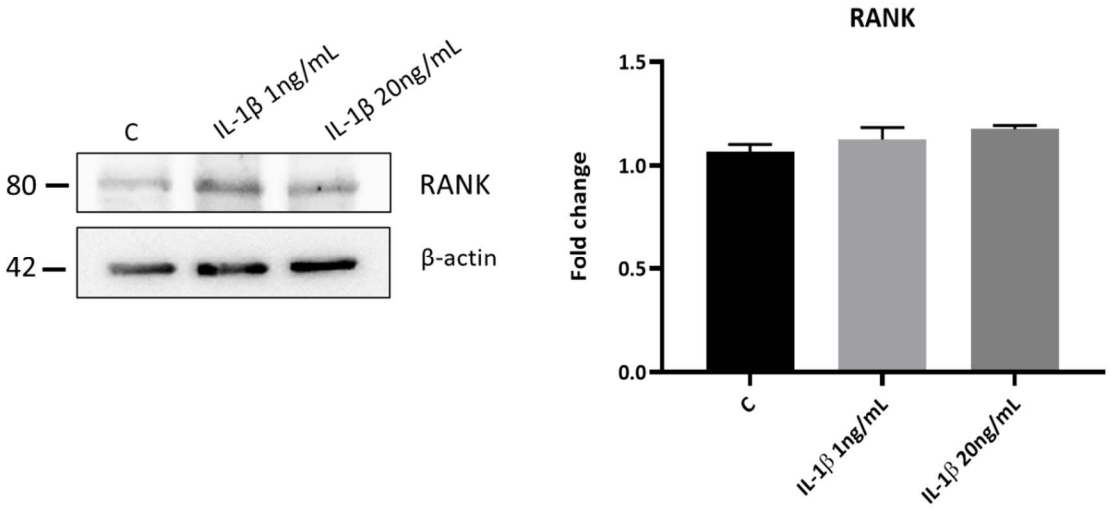

Figure 21. IL-1β impact on RANK. IL-1β treatment with 1ng/mL and 20ng/mL for 24 hours does not interfere with RANK receptor expression. Data are means ± standard errors of the mean (SEM).

12.Chemokine's gene expression in activated astrocytes

Astrocytes respond to IL-1β treatment not only by changing morphology but also by switching to an inflammatory phenotype characterised by altered gene and protein expression profiles. Therefore, we assessed the gene expression of two chemokines, *CCL2* and *CCL20*. The cells exposed to two doses of IL-1β for 24 hours, 1 ng/mL and 20 ng/mL respectively, showed a significant increase in *CCL2* mRNA after both IL-1β treatments.

Gene expression analysis of *CCL20* showed a statistically significant increase only after the administration of 1ng/mL IL-1β.

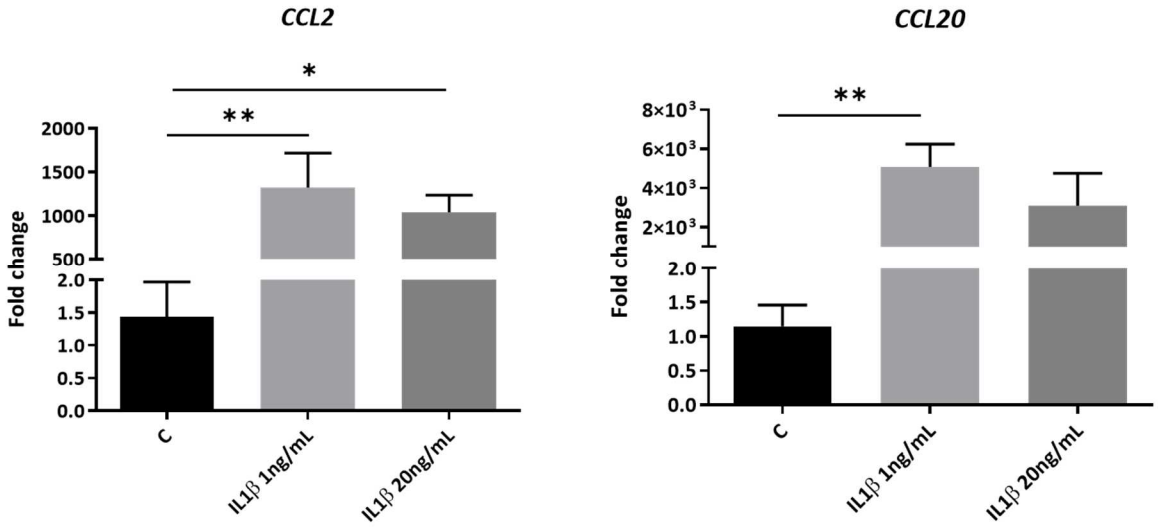

Figure 22. IL-1β impact on chemokines gene expression. IL-1β treatment with 1ng/mL and 20ng/mL for 24 hours up-regulates *CCL2* and *CCL20* mRNA. Data are means ± standard errors of the mean (SEM). (*p<0.05, **p<0.01)

13.Cytokines secretion in activated astrocytes

After activation of astrocytes with IL-1β, we evaluated, with ELISA assay, the secretion of cytokines as IL-6, and TNF-α. Cells were exposed to two doses of IL-1β for 24 hours: 1 ng/mL and 20 ng/mL.

We observe a significant increase, compared to the control group, with both doses of IL-1β. On the other hand, the results obtained for TNF-α show no difference between control and treatment, furthermore, the levels were close to the minimum detention level (data not shown).

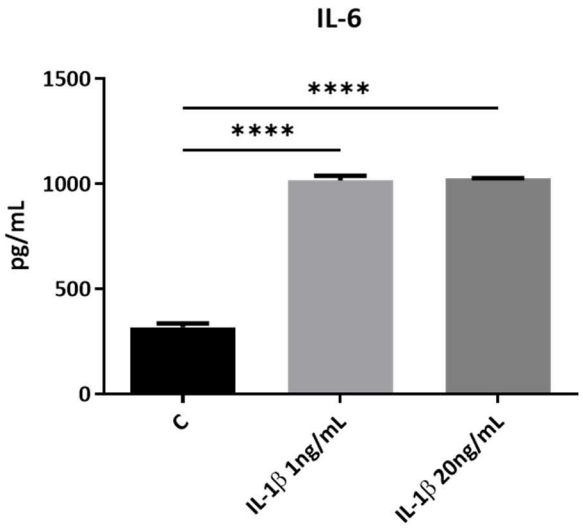

Figure 23. IL-1β impact on cytokines secretion. IL-1β treatment with 1ng/mL and 20ng/mL for 24 hours does not interest the TNF-α release, instead the secretion of IL-6 is incremented with both doses of IL-1β. Data are means ± standard errors of the mean (SEM).

14. How chemokines and cytokines change in presence of RANKL and OPG

Astrocytes were exposed for 24 hours with rhRANKL (R&D Systems Inc., Minneapolis, MN, USA) at the dose of 100ng/mL alone and in the presence of three increasing doses of rhOPG (R&D Systems Inc., Minneapolis, MN, USA) 25, 50, 100ng/mL to investigate possible changes in gene expression of the chemokines CCL2 and CCL20 and in the secretion of the cytokines IL-6, TNF-α and IL-1β.

As shown in figure 24, *CCL2* mRNA increases slightly with rhRANKL administration and it appears that rhOPG doses have little effect on the 'reparative' mechanism triggered by OPG.
The gene expression of the chemokine *CCL20* showed a statistically significant increase when rhRANKL was administered, whereas the transcription level decreased to very low levels, almost similar to the physiological levels, when 25ng/mL of rhOPG was administered.

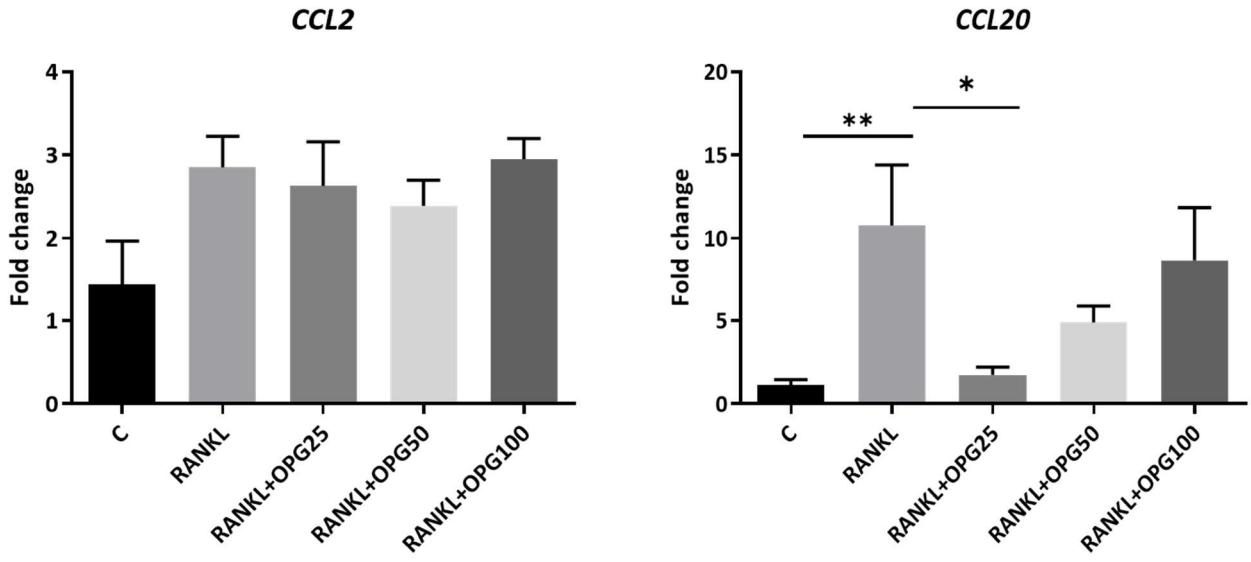

Figure 24. RANK/RANKL/OPG impact on chemokines gene expression. Chemokine CCL20 is the most sensitive and most responsive to the RANK/RANKL/OPG system. Data are means ± standard errors of the mean (SEM). (*p<0.05, **p<0.01)

By using specific ELISA tests for the cytokines IL-6, IL-1β and TNF-α, we evaluated changes in the secretion of these molecules in the conditioned medium of astrocytes, following exposure for 24 hours with the same treatments as above. The obtained results suggested no change in the secretion of these cytokines. Moreover, as regards the levels of IL-1 β and TNF-α they were close to the detection threshold (data not shown).

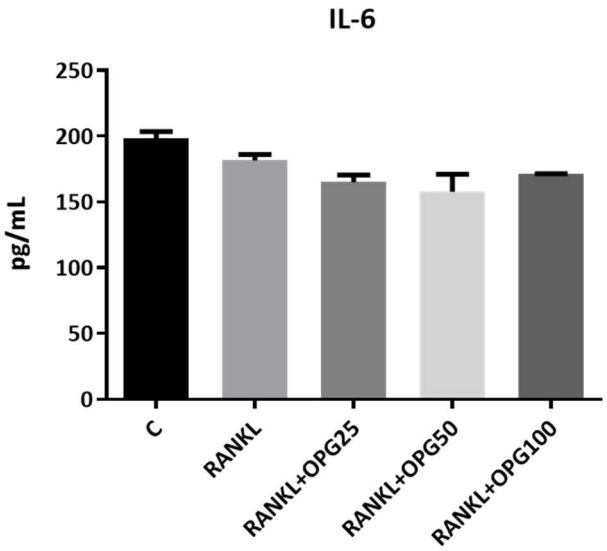

Figure 25. RANK/RANKL/OPG axis impact on cytokines secretion. Cytokines IL-6 and TNF-α do not appear to be involved in the RANK/RANKL/OPG system. Data are means ± standard errors of the mean (SEM).

15. Changes in gene expression of astrocytes after treatment with term placenta explants conditioned media

We administered conditioned media from term placental explants to activated astrocytes (with 1ng/mL IL-1β) and we observed a significant reduction in gene expression of *CCL2* compared to IL-1β-activated astrocytes. About *CCL20* chemokine gene expression, we also observed a statistically significant decrease after treatment with the conditioned media of the explants.

Conditioned media of term placenta added to non-activated astrocytes had no effect, as shown in Figure 26.

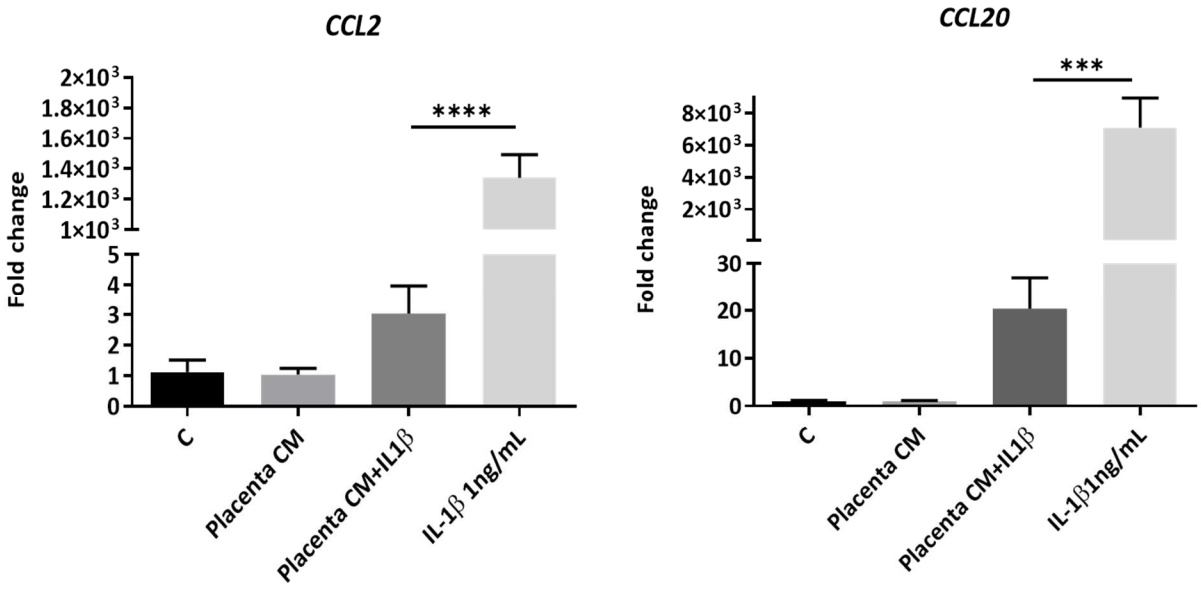

Figure 26. Term placenta conditioned media impact on chemokines gene expression. In activated astrocytes, chemokines *CCL2* and *CCL20* are downregulated thanks to term placenta conditioned media. Data are means ± standard errors of the mean (SEM). (***p<0.001, ****p<0.0001)

Furthermore, we performed an immunodepletion for OPG from the conditioned media of term placental explants. In particular, the conditioned media from term placental explants maintained for 48 hours in culture was in part OPG depleted. The percentage of immunodepletion after ELISA analysis was around 60%.

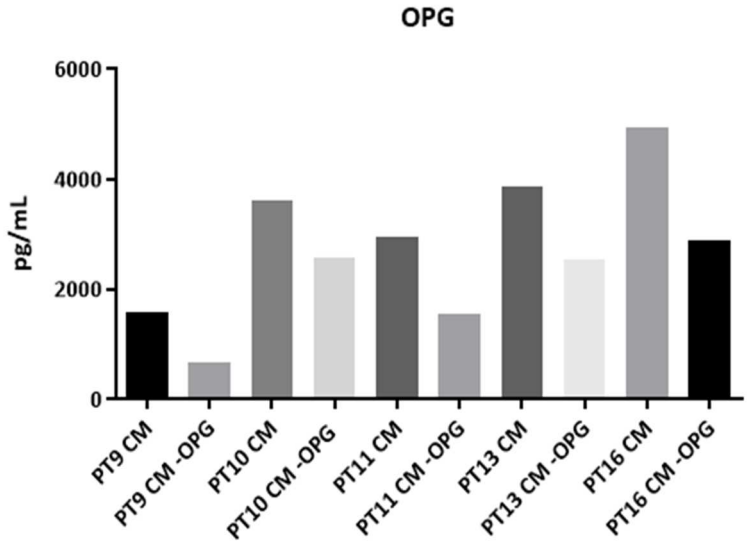

Figure 27. OPG immunodepletion in placenta conditioned media. ELISA analysis reveals a decrease in OPG levels in the conditioned media of the five placenta in question.

We, therefore, decided to administer the whole conditioned medium and its respective OPG-depleted medium to activated astrocytes (with 100ng/mL rhRANKL) to assess changes in gene expression of pro-inflammatory chemokines *CCL2* and *CCL20*.

In Figure 28, *CCL2* shows a non-significant increase in gene expression in astrocytes treated with OPG depleted medium compared to those treated with completely conditioned medium.

In Figure 28 is demonstrated a significant increase in gene expression of the chemokine *CCL20* in astrocytes treated with OPG-depleted medium.

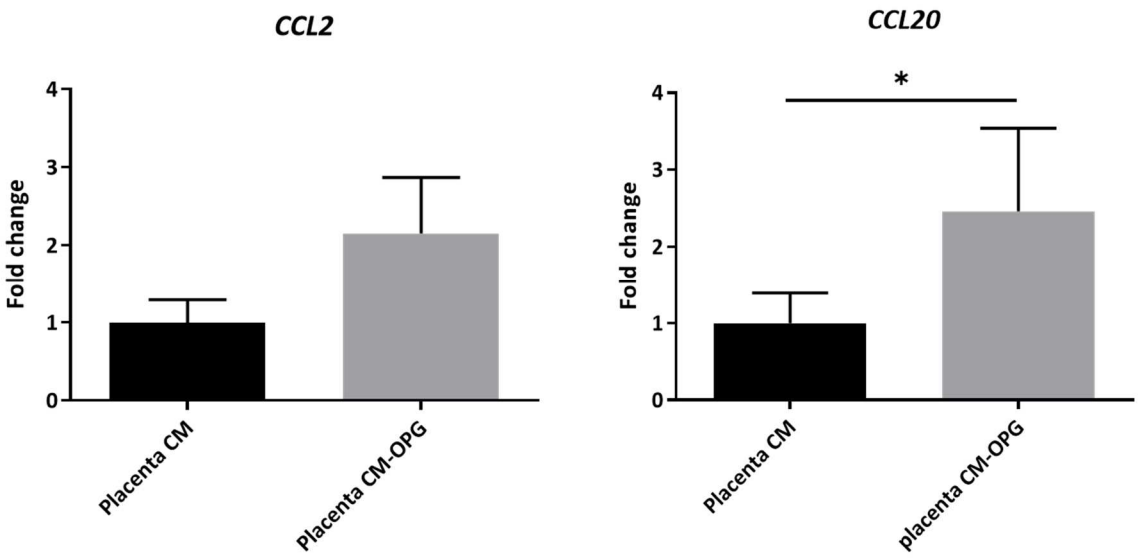

Figure 28. Impact of OPG-depleted conditioned medium on chemokine gene expression. Gene expression of *CCL2* and *CCL20* is upregulated in activated astrocytes treated with OPG-depleted conditioned medium. Data are means ± standard errors of the mean (SEM). (*p<0.05)

16.OPG and sRANKL in the sera of pregnant women with Multiple Sclerosis

Using the ELISA test, we assessed how the OPG protein varies in the serum of MS women during the third trimester of pregnancy and the post-delivery period. We detected a significant decrease in circulating OPG levels in the post-delivery period compared to the third trimester.

On the other hand, circulating levels of sRANKL in MS women did not change between pregnancy and the post-delivery period.

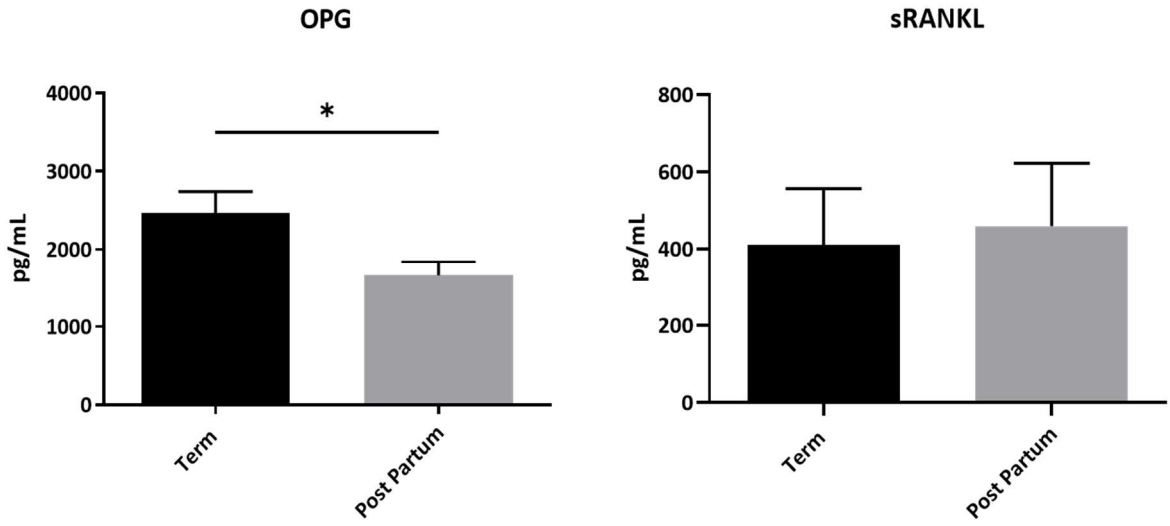

Figure 29. OPG and sRANKL serum levels in MS-pregnant women. OPG levels significantly decrease in the post-partum period compared to the third trimester of pregnancy. No significant difference in sRANKL. Data are means ± standard errors of the mean (SEM). (*p<0.05)

17.OPG and sRANKL in the sera of non-pregnant Multiple Sclerosis women

Using the ELISA test, we assessed how the OPG protein varies in the serum of non-pregnant women with MS compared to healthy non-pregnant women. The plot shows no differences between the two groups as regards OPG levels. In contrast, sRANKL levels increased significantly in MS non-pregnant women compared to non-pregnant healthy women.

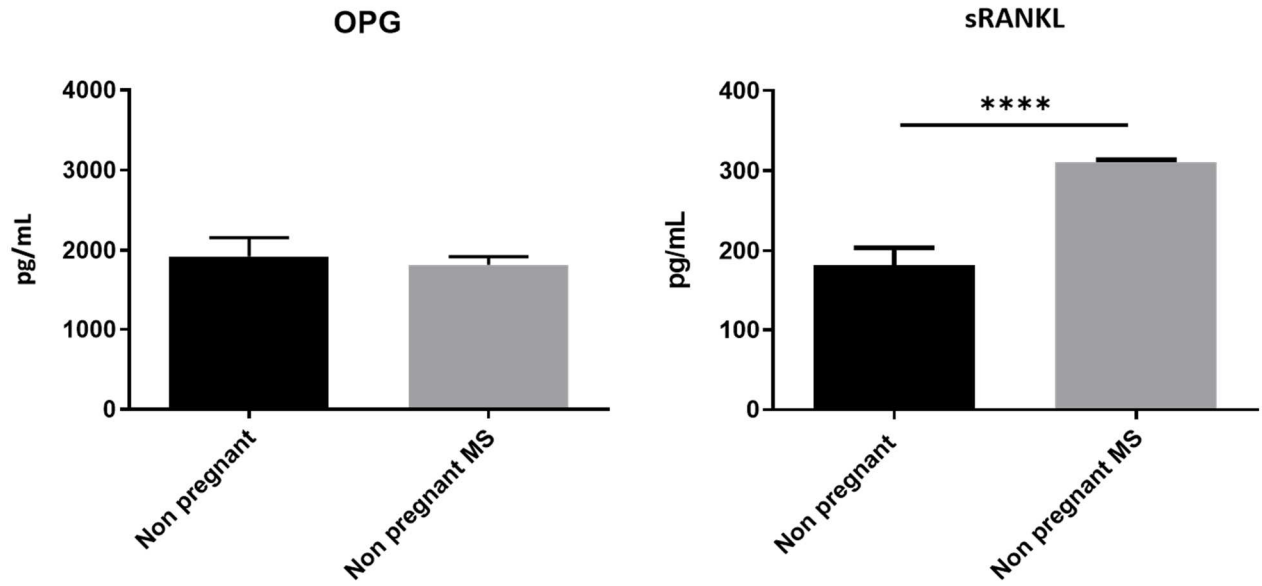

Figure 30. OPG and sRANKL serum levels in non-pregnant women. No significant difference is observed in OPG levels. sRANKL levels significantly increase in non-pregnant women with MS compared to healthy women. Data are means ± standard errors of the mean (SEM). (****p<0.0001)

Finally, since under physiological conditions, OPG and its soluble ligand sRANKL are in balance, we considered the ratio between sRANKL and OPG in MS and healthy women (Fig 31).

The results highlighted a significant decrease of the ratio in women with MS at term of gestation compared to non-pregnant women with sclerosis, reflecting the robust increase of circulating OPG at term of gestation (Fig 31 A).

On the other hand, the ratio remained unchanged in the group of healthy women (Fig 31 B), suggesting that in MS affected women the pregnancy at term has a pivotal role in reducing sRANKL bioavailability.

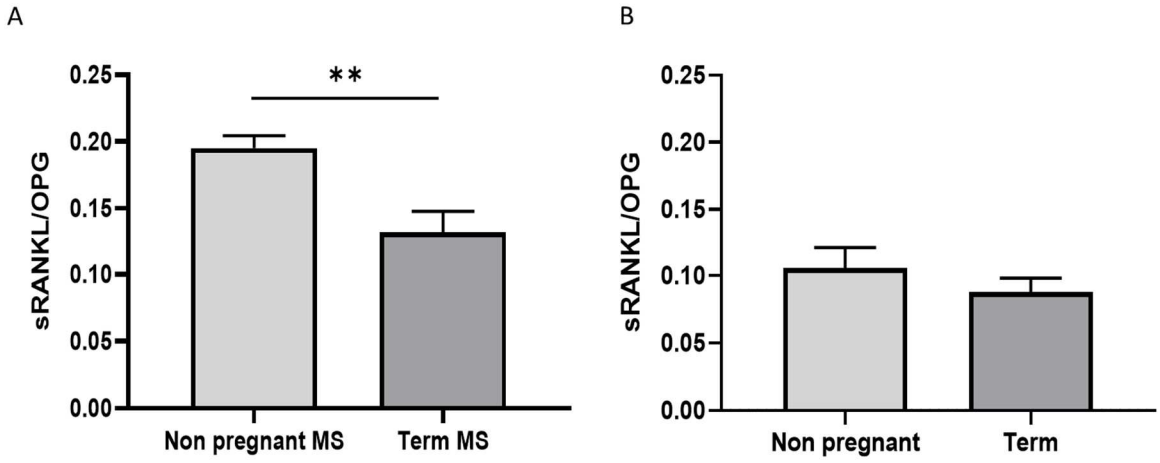

Figure 31. Serum sRANKL/OPG ratio in MS and healthy women. sRANKL/OPG ratio for MS patients (A), (non-pregnant: NP, n=19; at term of gestation: Term, n=10) and healthy women (B) (non-pregnant: NP, n=10; at term of gestation: Term, n=20). Data are means ± standard errors of the mean (SEM). (**p<0.01)

DISCUSSION

1. The RANK/RANKL/OPG axis in placenta

Pregnancy is a period in a woman's life when important changes occur in the mother. During pregnancy, the pregnant mother undergoes significant anatomical and physiological changes to bring up and accommodate the developing foetus (Soma-Pillay et al., 2016). For example, calcium metabolism and bone mineral status are central to satisfying the needs of the fetus for proper mineralisation and skeletal growth. Evidence shows that, at the end of pregnancy, the fetus needs about 20-30 g of calcium, and this is ensured by the passage of calcium from the maternal to the fetal compartment via the placenta (Prentice, 2000). One of the systems that have generated the greatest scientific interest in recent times is the role of the RANK/RANKL/OPG triad. These proteins belong to the superfamily of tumor necrosis factor (TNF) and exert a regulatory effect on bone metabolism (Sanz-Salvador et al., 2015). This axis is thought to play a central role in regulating bone metabolism, balancing osteoclastogenesis and osteogenesis (Wright et al., 2009). However, we know that it is present and plays a role in a whole series of mechanisms and systems in the body.

In the literature, it is reported that Osteoprotegerin is significantly increased in the sera of mothers at term of gestation compared to non-pregnant women, thus suggesting a protective role in the maternal skeleton (Hong et al., 2005), avoiding excessive bone resorption to compensate for the growing demands of the fetal skeletal system. Studies in the literature (Styczynska et al., 2009) show that the difference in OPG expression is not only found between non-pregnant and third-trimester women but also between the first and third trimesters of pregnancy. Our ELISA results on women sera confirm this trend and show that there is an increasing relationship in OPG expression between non-pregnant, first-trimester and term pregnant women. For the first time, we have also shown in our study that there is a higher concentration of OPG in maternal serum, at term of gestation, when compared with their cord blood sera. This result further supports the 'homeostatic' role of this protein concerning a stressed maternal environment and the fetus' demand for calcium.

Another purpose of this study was to evaluate the contribution of the placenta to circulating levels of OPG and sRANKL. In the literature, the concentration of OPG in women with preeclampsia and women with physiological pregnancies was reported (Shen et al., 2012). They claim that the

expression of OPG is greatly increased in women with severe preeclampsia compared to those with moderate preeclampsia and those with physiological pregnancies, suggesting a role for OPG not only as an important factor in maternal bone physiology but also as a protective factor for vascular endothelial cells (Rochette et al., 2019). This work of thesis shows that the placenta produces and releases OPG and the production of this protein is significantly increased at the term of pregnancy. The fact that OPG is produced by the placenta mainly at term reflects what we found in the serum of mothers at term of gestation and confirms what is already present in the literature (Shen et al., 2012).

The immunohistochemical analysis of placenta tissues shows the presence of OPG in the cytotrophoblast both in the first trimester and term placenta; however, the results suggest an important functional difference in OPG secretion between the two gestational periods. In the first trimester placenta, OPG is mainly localized in the cytotrophoblast layer, the innermost trophoblast cellular layer of the placenta, not in direct contact with the maternal blood. This localization suggests a role for the molecule in the fetal compartment at the beginning of pregnancy. At term of pregnancy, OPG is still localized in the cytotrophoblast cells, which are, at this gestational age, in closer proximity to the maternal blood as respect to first trimester ones, suggesting a role of the molecule on the maternal compartment at term of pregnancy.

Indeed, at the end of gestation, the presence of the protein in close contact with maternal blood supports the fact that the amount of OPG in maternal blood is higher than in cord blood. We can therefore state that the OPG produced by the placenta at this gestational age is released for the most part on the maternal side.

Regarding the assessment of soluble RANKL expression, there are few studies in the literature evaluating its expression in the serum during pregnancy and in the placenta. One study pointed out that circulating sRANKL levels seem to increase in parallel with the expression of OPG. However, it highlighted the difficulty in detecting serum sRANKL using commercial kits (Sanz-Salvador et al., 2015). Instead, we looked at the expression of sRANKL in both the sera of third-trimester pregnant women and their placenta explants and cord blood sera, showing that there is no change in sRANKL levels among the different compartments. These results likely suggest that the placenta does not play a major role in the synthesis and release of RANKL and that, therefore, serum levels of sRANKL may be attributable to other organs and/or tissues. This renders the role of the placenta in OPG

production and secretion even more interesting and complex since it is exactly during pregnancy that the synthesis of this protein may regulate several biological systems.

Furthermore, the data obtained from the analysis of the gene expression levels of OPG and RANKL in the first trimester and term placental tissues made the study of OPG even more interesting, focusing our attention specifically on the receptor. While there is a significant increase in *OPG* mRNA in term placenta tissues compared to the first trimester, *RANKL* gene expression is almost undetectable in both gestational ages.

Following gene expression analysis, we assessed OPG protein expression. At the tissue level, no particular differences were observed between the first trimester and the term placenta. However, studies carried out on the conditioned media of the explants revealed an important difference in the secretion of OPG between the two gestational ages. Structural studies demonstrated that an intact dimerization interface is essential for the biological activity of OPG and that the dimeric form of the protein is the most biologically active one (Xiao et al., 2018). Therefore, we wondered whether the OPG we find in placenta tissues and the explant conditioned media is in dimeric or monomeric form. To answer this question, we performed a native protein analysis on tissue and explants conditioned media by immunoblot. It is interesting to note that exactly at the end of pregnancy, when production and secretion of OPG by the placenta increase, a high amount of OPG in dimeric form can be detected. According to our results, we have shown that the placenta at the term of gestation expresses and secretes the biologically active form of OPG.

Intriguingly, we have found that the fetal organ produces predominantly the dimeric form of the protein known to increase of three orders of magnitude its affinity for RANKL, therefore, inhibiting the RANKL-RANK receptor interaction more powerfully (Schneeweis et al., 2005). In addition, the OPG dimer has greater ability than the monomeric one to distribute itself from the circulation to the peripheral compartments such as body tissues (Yano et al., 2001), suggesting that the OPG produced by the placenta could exert its biological activity far from the maternal-fetal interface.

We then examined whether the production and secretion of OPG by the placenta can be regulated by various stimuli, such as bacterial infections, inflammatory environment or even sex hormones

secreted during pregnancy. We have performed *in vitro* experiments exposing placenta cultures to LPS, cytokines and hormones characterizing the latest stages of pregnancy.

Infection is increasingly considered to contribute to pathological conditions in pregnancy. The placenta acts as a protective immunological feto maternal barrier, which recognizes microbes by pattern recognition receptors on the trophoblast (Hoo et al., 2020). Lipopolysaccharide (LPS) is a cell wall constituent of Gram-negative bacteria that stimulates a strong immune response (Wada et al., 2004). LPS is widely recognised as a potent activator of monocytes/macrophages, and its effects include altered production of key mediators, such as inflammatory cytokines and chemokines; the resulting exaggerated inflammatory responses are usually detrimental (Tucureanu et al., 2017). In our study, LPS from *E. coli* was used to treat the term placenta explants culture and examine its influence on OPG secretion. Our results showed that the release of OPG from term explants was significantly increased when they are subjected to a strong LPS insult. This result further supports the protective role played by OPG, since when the placenta is subjected to an intense inflammatory stimulus it reacts by producing and secreting OPG, which acts to mitigate this strong inflammation. This result confirms what was also observed by Wada et al in a different compartment: they detected an up-regulation of OPG gene expression following *E. coli* infection in periodontal ligament fibroblasts, protecting against excessive osteoclastogenesis (Wada et al., 2004).

It has also been recognised that inflammatory processes such as rheumatoid arthritis and periodontal infection predispose to osteoclastogenesis, leading to speculation that inflammatory cytokines such as IL-1, TNF-α and IL-6 may play critical roles in osteoclast formation (Wada et al., 2004; Weitzmann, 2013). Moreover, an inadequate remodelling of the uterine spiral arteries in preeclampsia leads to focal ischemia and production by the placenta of inflammatory cytokines such as TNF-α and IL-1β, responsible for endothelial dysfunction (Benyo et al., 2001). Therefore, we wondered whether an inflamed environment during pregnancy, loaded by a wide range of pro-inflammatory cytokines and chemokines, due to *e.g.*, immune-driven processes might play a role in the regulation of placental OPG. Data obtained from our experiments with pro-inflammatory cytokines show that an inflamed environment does not affect OPG secretion by the placenta at the term of gestation. Therefore, we can suppose that diseases of an autoimmune nature, such as multiple sclerosis (MS), which involve persistent inflammation, do not negatively influence the production and release of OPG.

To understand factors able to regulate placental OPG production throughout gestation, we have performed *in vitro* experiments exposing placenta cultures to hormones characterizing the latest stages of pregnancy.

Estrogens are essential for bone growth, development and maintenance of bone health in adulthood. For postmenopausal women, estrogen deficiency is one of the most common causes of osteoporosis. The RANK/RANKL/OPG triad is central to bone regulation and metabolism. OPG is implicated in the pathogenesis of postmenopausal osteoporosis and other metabolic bone diseases caused by estrogen deficiency (Jia et al., 2017; Rogers et al., 2002). Various studies have shown that estrogens can stimulate OPG expression in osteoblast cells, thus blocking the RANK/RANKL bond, which causes excessive bone resorption (Bord et al., 2003).

Moreover, increasing evidence supports the anti-inflammatory role of elevated levels of circulating hormones, such as estrogens, glucocorticoids, or progesterone in MS-pregnant patients, originating from the observation of a reduction in relapse rates during pregnancy. These hormones have potent immunomodulatory properties including shifts in cytokine production, downregulation of adhesion molecule expression and matrix metalloproteinase, decreased antigen presentation and induction of regulatory T cells (Voskuhl and Gold, 2012), all of which could beneficially affect MS pathology. It has also been demonstrated the protective role of estrogen in experimental autoimmune encephalomyelitis (EAE) and possibly MS (Palaszynski et al., 2004). During the last trimester of pregnancy, relapses are reduced by 70%, when estrogen and progesterone concentrations are higher. Indeed, pregnancy is a state of temporary immune modulation that allows the survival of the semi-allogeneic fetus (Voskuhl et al., 2016).

Our studies on term placenta explants treated with hormones, especially estriol, show that there is an increase in OPG secretion. This result suggests a positive effect of estrogen and particularly of estriol on the release of this protective protein. Moreover, estriol is an estrogen unique to pregnancy, released by the fetal-placental unit that reaches the highest concentration in the last trimester (Sicotte et al., 2002). Furthermore, Voskuhl and Gold in their studies reported that both estriol and estradiol have a beneficial effect in EAE, while progesterone treatment alone has a mild positive effect on the disease (Voskuhl and Gold, 2012). It is probably due to estriol, which already has beneficial effects on MS, that there is an increase in the production of OPG by the placenta. OPG synergizes with estriol to further improve MS symptoms during the third trimester of pregnancy.

MS is a chronic inflammatory disease of the central nervous system, mainly affecting the white matter of the brain and spinal cord. The major pathological features of MS are autoimmune destruction of myelin and subsequent axonal degeneration (Bove et al., 2014). Interestingly, pregnancy in patients with MS is associated with a substantial decrease in the relapse rate. Actually, the relapse rate in pregnant patients with MS is substantially reduced during the third trimester; indeed, the levels of two estrogens (estradiol and estriol) and progesterone increase progressively during pregnancy and peak in the third trimester (Patas et al., 2013). However, after childbirth, the relapse rate increases significantly compared to the rate before pregnancy (Vukusic et al., 2004). Large gaps remain in our understanding of the biological mechanisms underlying the pregnancy-related effect in MS patients. In addition, in non-pregnant MS patients, sRANKL serum concentrations were found to be higher. Notably, a significant decrease in cerebrospinal fluid OPG levels or an increase in plasma sRANKL leads to a higher sRANKL/OPG ratio observed in patients with MS at clinical onset and advanced remitting relapse, respectively (Glasnović et al., 2018). The results obtained from our study may explain the data reported in the literature. Indeed, during the third trimester of pregnancy, when there is a strong remission of the disease, high levels of OPG are observed in the serum of pregnant women with multiple sclerosis. The molecule at this stage protects against the inflammatory processes related to MS. After that, the OPG levels tend to drop significantly in the postnatal period. The results obtained from the serum of pregnant women with MS further support the hypothesis that the placenta produces much of the circulating OPG during pregnancy and that OPG is one of the molecules that contribute to the improvement of MS in pregnancy, probably also thanks to the synergic activity of steroid hormones which reach high levels during pregnancy.

As regards the serum levels of sRANKL, however, these remain stable during pregnancy and in the postpartum period; we can therefore assume that the placenta does not participate in the production and secretion of sRANKL, even in the case of MS.

As reported above, serum levels of sRANKL are significantly higher in patients with advanced MS, while OPG levels are comparable with control groups (Glasnović et al., 2018). Therefore, we decided to compare two groups of non-pregnant women of childbearing age with and without MS, to assess serum levels of OPG and sRANKL. The results obtained confirm and support the data of Glasnović et al (2018): while no differences are observed in OPG levels, sRANKL levels appeared significantly

increased in MS patients. From these results, it is possible to suggest an imbalance towards the increase of the RANKL/OPG ratio in women with MS, and the pregnancy condition plays in favour of the OPG secretion increase, which restores this relationship. These data again provide support for the hypothesis that the placenta itself produces OPG and that the placenta of women with MS is physiological, producing and releasing OPG, which is important in counteracting elevated sRANKL levels during MS.

2. The RANK/RANKL/OPG axis in the brain

The RANK/RANKL/OPG axis has been found to potentially play a role in murine EAE, an inflammatory demyelinating model of human MS (Pilli et al., 2017). In EAE, the production of RANKL by T cells induces the secretion of CCL20 by astrocytes, a type of glial cells. Otherwise, the lack of RANKL in T cells or the astrocytes-specific deletion of RANK reduces the infiltration of the T cells in the spinal cord and markedly protects mice from EAE. Interestingly, the pharmacological inhibition of RANKL exerted a reduction of T cell infiltration and a significant protective effect in murine EAE, without affecting the peripheral immune response (Guerrini et al., 2015). Current MS therapies are focused on immunosuppression as they are aimed at limiting the entry of immune cells into CNS thereby preventing neuroinflammation. Although these therapies are strong, disease-modifying agents they fail to prevent or reverse disease progression (Ponath et al., 2018). Moreover, Ponath et al. suggest that astrocytes are active participants in the development of MS lesions. Astrocytes make an important contribution to maintaining an optimal microenvironment for neuronal function and survival. They are versatile dynamic cells, expressing numerous receptors, which enables them to respond to neuroactive compounds, such as neurotransmitters, neuropeptides, growth factors, cytokines, and toxins (Volterra and Meldolesi, 2005). In MS, astrocytes respond to the inflammatory stimulation with an early robust process of morphological, transcriptional, biochemical, and functional remodelling. Astrocytes recruit lymphocytes contributing to tissue damage and suffer significant damage during the inflammatory process (Farina et al., 2007). Key signalling pathways regulate astrocyte reactivity and NF-κB is a key regulator of innate and adaptive immunity that controls cell survival, differentiation and proliferation (Hayden and Ghosh, 2012). The NF-κB signalling pathway is directly activated by stimulation with pro-inflammatory cytokines such as TNF-α and IL-1β (Shih et al., 2015). Thus, while MS is driven by dysfunction of the peripheral immune

system, CNS cells such as astrocytes may contribute to MS pathology by targeting dysregulated immune responses to the CNS. The role traditionally assigned to astrocytes in the pathogenesis of MS is the formation of the glial scar once the inflammation has subsided. Astrocytes are now recognised as early and highly active players during lesion formation and are crucial in providing access to the central nervous system for peripheral immune cells (Ponath et al., 2018).

Based on these literature data, we explored the possibility of a link between the part played by these glial cells in conditions of inflammation and the role of the placenta, which, through the secretion of molecules and active factors, such as OPG, could mitigate this inflammatory environment.

In neuroinflammatory conditions such as MS, numerous cytokines and chemokines are elevated including IL-6, IL-17, CCL2 and CCL20. CCL20, a CC motif chemokine, released by astrocytes, functions as a chemoattractant to facilitate the recruitment of CCR6-expressing cells, including Th17 cells (Meares et al., 2012); moreover elevated CCL20 serum levels were found in MS patients (Jafarzadeh et al., 2014). In mice with astrocyte-specific gene deletion of CCL2, the induction of EAE results in a less severe disease course with fewer macrophage and T cell infiltrates, and less activation of astrocytes and microglia (Kim et al., 2014). In addition astrocytes in active lesions secrete proinflammatory chemokine and assume a hypertrophic morphology, characterised by massive enlargement of the cell soma and reduced process density (Sofroniew and Vinters, 2010); we were able to reproduce *in vitro* this situation by activating astrocytes in culture media with IL-1β or recombinant human RANKL.

Our studies on IL-1β-activated astrocytes confirm data from the literature showing an increase in pro-inflammatory chemokines such as CCL2 and CCL20, thus suggesting that these chemokines may contribute to the amplification of the autoimmune response in diseases such as MS. A very interesting and innovative finding is extrapolated from our results combining IL-1β-activated astrocytes and conditioned media from term placental explants. Significant mitigation of gene expression of these two chemokines is observed due to the presence of the placental secretome, which contains many factors with a mitigating effect.

Another very interesting result comes from our studies on the gene expression of the chemokines *CCL2* and *CCL20* by RANKL-activated astrocytes. Chemokines are important molecules regulating leucocytes' recruitment and infiltration into the tissues. The result that immediately stands out is

that *CCL20* mRNA significantly decreases and returns to near-physiological levels when the OPG is administered. It should also be noted that the other pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) are not affected by this treatment. Therefore, we may suppose that the RANK/RANKL/OPG triad can be finely tuned to regulate the expression of main pro-inflammatory chemokines in autoimmune diseases, such as MS. Many cytokines and chemokines have been linked to the pathogenesis of MS, depending on its clinical form, severity, and applied therapy. Disturbed cytokine network is marked by upregulated proinflammatory cytokines such as IL-6, IL-17, IL-1, IL-22 and TNF-α, and downregulated immunosuppressive cytokines including IL-3 and IL-10 (Glasnović et al., 2018). Continuing with our studies, we investigated whether OPG produced and secreted by the placenta also has an active role in mitigating the inflammatory response following astrocytes activation by RANKL. The interesting datum that immediately becomes apparent is that the *CCL20* expression strongly decreased when the activated astrocytes were treated with conditioned media from term explants. In contrast, gene expression levels remain high when OPG was immunodepleted from the placental explant conditioned media.

Although the data obtained from our experiments on conditioned media and OPG depletion are encouraging, we cannot exclude that immunoprecipitation may have depleted the medium of other unknown factors. Despite this, the results from treatment with rh OPG support the role of placental OPG in reducing *CCL20* expression. Interestingly, our results from recombinant OPG, supplied as a disulfide-linked homodimer, report a greater effect at lower doses and are in line with Schneeweis *et al*. who clearly demonstrated that the high-affinity binding of OPG to RANKL is based on avidity, which is only achieved if OPG dimerizes (Schneeweis et al., 2005). Therefore, the production of a dimerized form of OPG by the placenta at the end of gestation is an event of great biological importance, through which the RANK-RANKL system could be physiologically and effectively inhibited in MS pregnancy.

Even though the placenta is a transient organ, its function is essential for communication between the mother and the fetus, as well as for the supply of nutrients and oxygen to the fetus. During pregnancy, the maternal brain undergoes temporal changes at different levels that are maintained for at least two years after birth. For example, changes in hormone levels during late pregnancy and early postpartum affect specific cognitive abilities in women (Hoekzema et al., 2017).

Moreover several ligand-receptor systems are regulated in a coordinated manner in the placenta compared to the brain, e.g. brain-derived neurotrophic factor (Bdnf) and its corresponding receptor (Trkb) (Behura et al., 2019) or the ephrin/receptor system (Efna/Eph) involved in neurite outgrowth. The *Efna1* gene is expressed in the placenta and its *Eph* receptor is expressed in the maternal brain. Similarly, the ligand genes Efna3 and Efna5 are expressed in the brain and their corresponding receptor (Eph1) is expressed in the placenta. Interestingly, the ephrin/receptor system is involved in the physiological development of the placenta but its alterations have been described in placental diseases, such as preeclampsia (Chatzizacharias et al., 2014). In general, these genes expressed in a coordinated manner by the placenta and the brain are associated with the regulation of the immune system, the development and differentiation of neurons, and the regulation of metabolism.

The placenta is exposed to various intrinsic and extrinsic factors that may increase the risk of fetal neurodevelopmental disorders and fetal death (Behura et al., 2019). Similarly, placental dysfunction has been shown to alter gene expression in the maternal brain, and these alterations potentially influence maternal behaviour (Rosenfeld, 2021). Thus, there is a strong interaction between the placenta and the maternal and fetal brain.

Overall, based on our study, we propose a model in which pregnancy through the placenta promotes the production of a dimeric form of OPG that creates a favourable environment capable of mitigating, by acting on the sRANKL/OPG ratio, the excessive presence of sRANKL in the peripheral blood of MS mothers with effects that may extend to the CNS on maternal astrocytes (Fig 9) and their interaction with immune cells.

The study of the RANK/RANKL/OPG axis in placenta-brain interaction may play a central role in the development of targeted drugs and therapies to fight diseases and adversities in both the mother and the developing fetus.

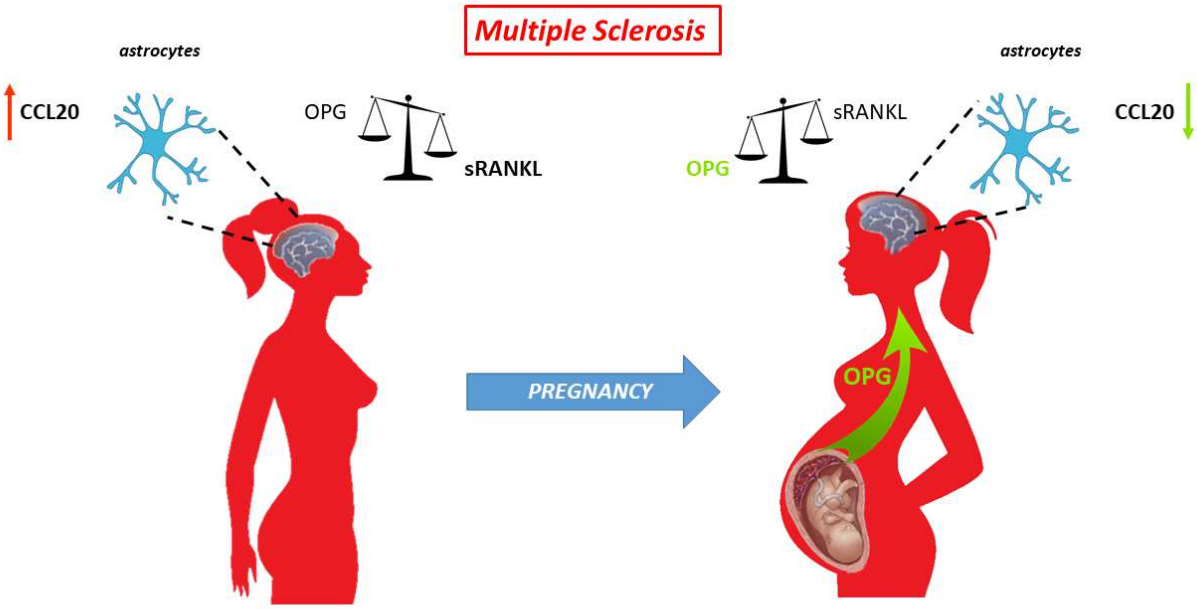

Figure 32. Schematic representation of the effect of pregnancy on sRANKL to OPG ratio in multiple sclerosis patients. MS patients have a dysregulated sRANKL to OPG ratio and increased expression of CCL20 within the CNS. During the third trimester of pregnancy, the placenta releases OPG in the maternal circulation. Placental OPG reverts the sRANKL/OPG balance, exerts inhibition on RANK-RANKL interaction within the maternal CNS, and leads to a reduction of CCL20 by astrocytes.

SUMMARY and CONCLUSIONS

This study aimed to examine the involvement of the placenta in the RANK/RANKL/OPG axis. Our results show that the placenta not only contributes importantly to the determination of circulating OPG levels during pregnancy, but also that this organ regulates its production based on maternal conditions. Moreover, we tried to find out if there is a relationship between the placenta and the brain, focusing our attention and interest on astrocytes and on MS as a model of study.

There are several indications, supported by the literature, that an unbalance of the RANK/RANKL/OPG system might represent a pathogenic mechanism in MS modulation. First, the importance of this axis in the modulation of adaptive and innate immunity and the control of inflammation has been clearly shown (Ahern et al., 2018). Secondly, changes in the RANK/RANKL/OPG axis have been reported in plasma and cerebrospinal fluid of MS patients (Glasnović et al., 2018). Thirdly, a consistent increase of plasma OPG levels during normal pregnancy exclusively at the third trimester of pregnancy, the period associated with disease remission in MS pregnant women has been shown (Hong et al., 2005).

The main goals achieved by our results are as follows:

1) OPG levels change during pregnancy in a gestational time-dependent manner and OPG levels are higher in maternal serum than cord serum.
2) The placenta at term of gestation produces and releases OPG in the dimeric form, the most biologically active. Therefore, we can strongly assert that the placenta is an important contributor to the circulating levels of OPG during pregnancy.
3) The expression of proinflammatory chemokines, such as CCL2 and CCL20, in activated astrocytes is mitigated by term placenta explants conditioned media, particularly through the presence of OPG.
4) In pregnant MS patients, serum OPG levels are elevated and comparable to those of a woman not affected by MS. OPG levels decrease after childbirth in MS women, while sRANKL levels remain the same, both during pregnancy and in the postpartum period. Concerning non-pregnant women with MS, we note that circulating levels of sRANKL are extremely high compared with healthy women, while OPG levels remain similar. Therefore, it is important

to focus on the RANKL/OPG ratio: if this shift in favour of OPG, as in women with MS during pregnancy, this counteracts the sRANKL levels that are instead high in the disease.

Determining the cellular and molecular mechanisms that mediate protection especially in the third trimester of pregnancy would reveal important targets for new drug development. Although evidence exists for changes in the expression of some immune markers in MS patients during pregnancy, no marker has so far been convincingly associated with the decreased relapse rates observed during the third trimester.

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