Characterization of the Expression of Angiogenic Factors in the Feline Placenta During Development and in Feline Cutaneous Squamous Cell Carcinoma

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ABSTRACT

Angiogenesis in the placenta is critical during embryonic development, guided by the interaction of endothelial growth factors (VEGF-A, PlGF) with their receptors Flt-1 and KDR. Dysregulation of angiogenic factors during pregnancy is associated with pregnancy pathologies. Despite the importance of placental morphologic development and VEGF family gene expression, they have not been thoroughly correlated across gestation. We postulated that changes in placental vessel density and architecture can be appreciated as consequences of dynamic expression of VEGF family and receptors in placenta.

We characterized changes in placental morphology and vasculature, particularly in the lamellar zone (LZ) where feto-maternal exchange occurs, alongside expression analysis of angiogenic factors, their receptors, and their respective splice variants throughout pregnancy in the domestic cat. Our morphological observations suggest that increased vascular perfusion in the LZ occurs as expansion of lamellar thickness during mid-pregnancy and continuously increasing lamellar density and microvascularity as pregnancy progresses. Using RT-PCR, we detected two PLGF isoforms (PLGF-I, PLGF-II) and three VEGF-A isoforms (VEGF-A119, VEGF-A163, and VEGF-A187). Immunohistochemical analysis localized KDR to endothelial cells of the maternal and fetal microvasculature. Flt-1 and PLGF were located in the trophoblasts and fetal vasculature. VEGF-A was immunolocalized in trophoblast cells and associated with endothelial cells. Relative gene expression studies showed upregulation of panVEGF-A and all specific VEGF-A isoforms at gestational day 30-35. VEGF-A119 showed a marked relative increase in expression during mid-pregnancy, consistent with the pro-angiogenic changes seen in the LZ at day 30-35.

We postulate that the smaller, more diffusible VEGF-A isoforms may reach KDR receptors endothelial cells increasing capillary length, diameter and permeability at mid-pregnancy. Flt-1 was upregulated during late pregnancy. PlGF variants showed stable expression during the first 2/3 of pregnancy, followed by a marked increase toward term, especially PLGF-

II. Increased ratios of PLGF:panVEGF-A expression during late pregnancy may affect VEGF-A bioavailability, since PlGF can displace VEGF from Flt-1, freeing it to signal through KDR. VEGF-B and KDR were expressed stably throughout pregnancy. Taken together, these findings revealed specific spatiotemporal localization and expression patterns of VEGF-A family members consistent with pivotal roles in the development of normal placental vascular architecture and fetal nourishment.

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GENERAL AUDIENCE ABSTRACT

Throughout gestation, the blood vessel network of the placenta is formed sequentially by processes known as vasculogenesis and angiogenesis, which together meet the needs of the growing fetus. Normal placental angiogenesis is critical to support adequate fetal growth and assure the health of the offspring. Proper angiogenesis requires precise regulation of expression of agents that modulate this process; otherwise, pathologies of pregnancy such as preeclampsia may occur. The placenta is composed of different layers of tissue, including the lamellar (LZ), junctional, and glandular zones, each with a vascular morphology attuned to its function. We hypothesized that higher expression of pro-angiogenic factors is associated with increased morphological metrics in the LZ, the major vascularized zone. Thus, we aimed to characterize the major changes in morphology and vascular development in the placenta throughout pregnancy in cats, alongside a compressive analysis of the expression of major angiogenic factors and their receptors in the placenta, with an emphasis on the identification and interaction of different isoforms of the VEGF family.

Microscopic analysis of tissue specimens from different stages of pregnancy revealed increased thickness of the LZ, especially during early to mid-gestation, at which time the tissue is composed of abundant materno-fetal interdigitations that appears rich in capillaries. VEGF proteins were detected in placental tissue in both fetal and maternal cells of the placenta, suggesting stimulatory interactions between different cell types to promote growth and angiogenesis. Gene expression analysis of placenta revealed upregulation of the pro-angiogenic factor VEGF-A in mid-pregnancy, followed by a steady decline toward term, consistent with morphologic changes in the LZ. In contrast, another pro-angiogenic factor, PlGF, showed a marked increase toward term; Flt-1, which acts as a receptor or reservoir for PLGF and VEGF A, was also upregulated at late pregnancy. Increased ratios of PLGF:VEGF-A may contribute to LZ proliferation in the last trimester. These findings are consistent with the creation of a pro-angiogenic placental state during gestation. Overall, we expect that this research will help

elucidate mechanisms of placental vascularization, which can be applied to the design of improved strategies to treat vascular complications of pregnancy.

Lastly, we applied the tools developed for placental studies to investigate pathologic angiogenesis in cutaneous squamous cell carcinoma (CSCC), a common skin cancer with major economic and medical impacts in humans and veterinary species. The creation of a new blood supply is essential for growth and metastasis of many tumor types. The goal of this study was to measure expression of variants of proteins that stimulate angiogenesis or transmit an angiogenic stimulus in feline CSCC. The results were mixed, with differences detected in expression of some regulatory agents and, for others, unexpectedly lower expression in CSSC compared to controls. Interestingly, the expression of VEGF-A relative to the protein that transmits its signal (KDR) was elevated in CSCC, suggestive of an altered signaling relationship. This finding supports our hypothesis and is consistent with human SCC studies. Our results encourage further studies on angiogenic factor variants in feline CSCC.

Dedication

This work is dedicated to my family, my parents, Erwin Gudenschwager and Veronica Basso, for their unconditional support, guidance and encouragement even from the distance; I could not ask for better parents, and I know I will always be in debt to both of you. Thank you very much for all you have done for me. Also, want to thanks my brother J. Tomas and Sister, Katalina. For their encouragement during these years. My sincere thanks to all of you

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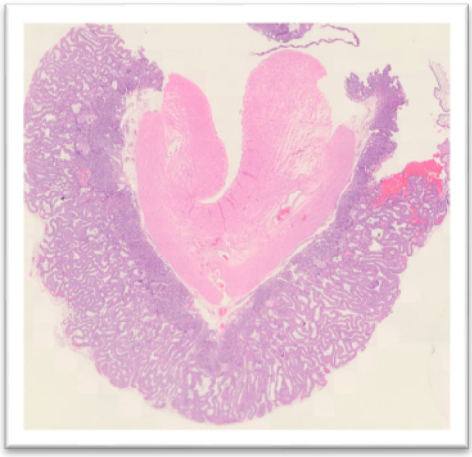

Domestic cat placenta of ~d25

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Chapter 1
Introduction and Literature Review

1.1 General concepts about the placenta.

All viviparous vertebrates during pregnancy form a system of extraembryonic membranes that englobe the fetus and deliver the adequate environment for the embryonic development. The apposition or fusion of embryonic membranes with the maternal mucosa in the endometrium for nutrient exchange from the feto-maternal interface, also known as the placenta (Benirschke, Burton et al. 2012). This very broad definition of the placenta accounts for the great variation in placental anatomy and histology, even among eutherian mammals, and reflects the relatively recent evolutionary origin of the placenta compared to other organs (Lewitus and Soligo 2011). An adequate establishment and growth of this temporary feto-maternal organ can be the key to a successful pregnancy and even determine the future health of the offspring. One of the key components of the placenta is the formation of two separate circulatory systems, a maternal and a fetal, that oppose each other in variable degrees of intimacy, but under normal conditions do not communicate directly, forming the placental barrier (Pang, Bates et al. 2017). In the placental barrier, there is an intricate growing and dynamic vascular network where the materno-fetal physiological exchange occurs.

Throughout gestation, the vasculature of the placenta is continually growing and expanding in order to accommodate the increasing fetal needs. Normal placental angiogenesis is critical to guarantee an adequate blood flow to the placenta and therefore to provide the nutrients that maintain normal fetal growth (Reynolds, Borowicz et al. 2010). Supporting this is the fact that the reduction in blood flows from the uterine or umbilical veins, results in a reduction of transplacental exchange rates (Wang and Zhao 2010). In humans, an increase in vascular resistance and reduced uterine blood flow can be used to identify high-risk pregnancies even before fetal changes are noted. Continued gestation under these conditions can result in fetal hypoxia, reduced fetal nutrient uptakes, hypoglycemia, or even abortion. If these conditions are maintained, they can lead to intrauterine growth restriction or even fetal growth retardation (Roescher, Timmer et al. 2014). Therefore, placental blood flow is highly dependent on placental vascularization, and placental angiogenesis is consequently critical for the successful

development of viable and healthy offspring (Reynolds and Redmer 2001, Guimaraes Filho, da Costa et al. 2008).

The placenta is one of the most versatile organs of the body. Despite its rapid and transitorily development, the placenta has to participate in all the basic functions of a fully-grown mammal before fetal organs are ready to function properly. Accordingly, the placenta can mimic multiple organs: it acts like a lung for gas exchange, absorbs the nutrients from maternal blood as a digestive system, removes and metabolizes waste products from the fetal blood, provides water balance and helps with pH balance similar to the kidney and liver. It is the first place to initiate erythropoiesis, preceding the bone marrow. In addition, the placenta provides immunological protection to the fetus acting as an immune system, synthesizes and metabolizes many hormones and growth factors that regulate, maintain the pregnancy, and can initiate parturition, much like an endocrine gland. Finally, the placenta helps with fetal thermoregulation and environment isolation/protection, functions are latter assumed by the skin. An early formation of vasculature is critical to establish and maintain all placental functions correctly and assure an adequate embryo and fetal development (Guimaraes Filho, da Costa et al. 2008, Wang and Zhao 2010, Carter 2012).

1.2 Placental types with emphasis in the placenta of the domestic cat

In mammals, the fertilized oocyte uses the energy stored in its cytoplasm to start mitosis. Then, during the first days of development, the blastocyst survives by simple diffusion of oxygen and nutrients from the surrounding uterine environment. In cats and all other mammals, as embryogenesis continues, simple diffusion is not capable to provide enough oxygen to the entire embryo. In support of embryonic development, the circulatory system is one of the first specialized tissues to develop, concurrent with emergence of a primitive choriovitelline placenta around day 12 post-fertilization in the cat (Leiser 1981). This event provides an early system for nutrient and oxygen exchange, offering nutrient extracted from the vitelline sac to the embryo (Tiedemann 1977). Also during this period, embryonic hematopoiesis begins inside “blood islands” at the wall of the vitelline sac **(Figure 1.1D),** and, in the same islands, new blood vessels are formed from mesodermal embryonic precursors. (Zygmunt, Herr et al. 2003, Baron, Isern et al. 2012). Around day 20 of pregnancy, a definitive chorioallantoic placenta is established and will replace the choriovitelline placenta **(Figure 1.1B)**. The chorioallantoic

placenta will continue to grow around the equatorial region of the chorion, forming a girdle where fetal structures will penetrate into the maternal endometrium and blood vessels will spread and grow by angiogenesis.

In order to study the placenta and understand its differences between species, many classification systems have been established. Main criteria of classification include: Overall shape and size of the placenta, type of the materno–fetal interdigitations, number of tissue layers in the materno-fetal interface, and distribution or direction of blood within fetal and maternal vessels (Benirschke, Burton et al. 2012). Based on shape and distribution of the placenta in the chorion, the main types of placenta are described as *diffuse*, *cotyledonary*, *zonary*, and *discoidal*. In the diffuse placenta, materno-fetal interdigitations cover the entire area of the chorion; pigs and whales are typical examples of this distribution. The cotyledonary placenta consists of a localized, relatively small circular or elliptical patches where maternal-fetal interdigitations grow into the chorion; this type of placenta is typical of ruminants (Sammin, Markey et al. 2009, Haeger, Hambruch et al. 2016). In the zonary placenta, the type typical of carnivores including the cat, the feto-maternal interaction occurs in an equatorial region of the chorion**.** In the queen, this placenta is classified as a zonary with an incomplete girdle **(Figure 1.1A)** (Miglino, Ambrosio et al. 2006). Finally, the discoidal distribution is characterized by oval or plate shape in one or two poles of the chorion. This is the most common type of higher primates and in humans is single discoidal. (King 1993, Leiser and Kaufmann 1994, Carter and Enders 2004, Enders and Carter 2004).

Based on the criteria of materno-fetal interdigitation, there are four types: *folded*, *lamellar*, *trabecular* and *villous*. The cat is described as lamellar, reflecting that these structures develop as multiple layers called lamellas as the essential structures of the feline placenta. Lamellas are located in the lamellar zone of the feline placenta **(Figure 1.2)** where nutrient and oxygen exchange occurs. Further, this zone of the chorioallantoic placenta shows the highest rates of proliferation and vascular expansion of all the layers during pregnancy (Wooding and Burton 2008). This type of placentation differs from that in humans, where a villous type of placenta occurs, and in mice, which develop a labyrinthine type.

Based on the tissue layers composing the maternal-fetal interface, the feline maternal–fetal interface is endotheliochorial, meaning that the epithelium of the fetal chorion penetrates the superficial layers of the endometrium and reaches up to the endothelium of the maternal

blood vessels (Leiser and Koob 1993, Miglino, Ambrosio et al. 2006, Satoshi, Yusuke et al. 2014) **(Figure 1.3).** In contrast, in humans and rodents the chorion develops a deeper invasion of the endometrium and even erodes the maternal vessels producing extravasation of maternal blood. Thus the trophoblastic surface is bathed directly in blood, termed a hemochorial placentation. Finally, with respect to the materno-fetal blood flow relationship, the felines have a simple crosscurrent type, which is very effective in passive exchange of oxygen and nutrients. (Miglino, Ambrosio et al. 2006)

1.3 The development of blood vessels in the placenta

The formation of blood vessels is a key early developmental process that is accomplished by two main processes: it starts by vasculogenesis and then is expanded by angiogenesis. The first primary vascular plexus in the embryo is formed by vasculogenesis. This consists of the *de novo* formation of blood vessels, beginning from mesodermal cells, which under the appropriate stimuli differentiate into endothelial cells precursors, forming aggregates scattered throughout the embryonic and extraembryonic mesoderm. These aggregates, called blood islands, start to hollow and form elongated spheres; primitive endothelial cells differentiate in the peripheral lining of the island and mesenchyme. Mesenchymal cells in the periphery of the islands differentiate into the smooth muscle and connective tissue to surround blood vessels. Lastly, multiple blood islands combine to form the primordial vascular plexus. Vasculogenesis first takes place outside of the embryo, in the yolk sac and chorion, followed by the development of an embryonal circulatory system approximately 2 days later. This developmental pattern means that the development of the circulatory system occurs in many regions simultaneously with vessels later merging; this is contrary to earlier-held notions that one central vessel or the heart develops first and then spreads outward. (Leach, Babawale et al. 2002, Semenza 2007, Aplin 2017).

Angiogenesis, on the other hand, is the process by which blood vessels are formed from preexisting vessels. This process involves dynamic and well-regulated changes to endothelial cells to generate new branches in the already established vascular network, followed by vascular remodeling into different calibers and differentiation into arteries, small capillaries or veins (Risau and Flamme 1995, Carmeliet 2000, Demir, Seval et al. 2007, Semenza 2007, Herr, Baal et al. 2009, Silvan, Diez-Torre et al. 2015). While vasculogenesis is mainly an embryonic event,

angiogenesis occurs in all stages of life and is part of multiple physiological processes such as uterine/ovarian reproductive cycling, placental development, muscle recovery post-exercise and wound healing. On the other hand, angiogenesis also plays a major role in pathological conditions such as ophthalmic and rheumatic diseases, chronic inflammatory diseases, and is a major contributor to cancerous tumor growth, metastasis, and survival. (Eisma, Spiro et al. 1997, Risau 1997, Aguayo, Manshouri et al. 2001, Ahlgren, Risberg et al. 2002, Ellis, Liu et al. 2002, Addicks and Giannis 2003, Takahashi and Shibuya 2005, Eichhorn, Kleespies et al. 2007, Ferreira, Barcelos et al. 2007, Chen, Zeng et al. 2010, Feige 2010, El Gehani, Al-Kikhia et al. 2011, Chen, Liu et al. 2013, Shibuya 2013, Chen and Lee 2014).

Two distinct processes can accomplish angiogenesis: endothelial sprouting also known as sprouting angiogenesis, and intussusceptive microvascular growth or intussusceptive angiogenesis. The regular mechanism for sprouting angiogenesis is composed of four classic steps; endothelial activation, cell migration, proliferation, and tube formation. In contrast, intussusceptive angiogenesis consist in splitting existing lumen of vessel with tissue folds and columns of interstitial tissue, leading eventually to the formation of two or more separate vessels (Poole and Coffin 1989, Patan 2004, De Spiegelaere, Casteleyn et al. 2012, Ackermann, Houdek et al. 2013, Belle, Ysasi et al. 2014, Mateos, Zamora et al. 2017).

1.4 Study of angiogenesis in the placenta

There are four main approaches to study placental angiogenesis *in vivo*: the first is the classical use of histological or morphologic methods for analysis, providing a descriptive analysis of the placenta. Techniques such as fixing tissue, sectioning and staining it for later evaluation under the microscope, use of confocal microscopy and florescence target detection linked to immunological methods, new computerized image analysis, and perfusion fixation of placental vessels, have increased the value and usefulness of in vivo studies (Takizawa, Eguchi et al. 2007, Huppertz 2008). A second approach is by using vascular casting along with digestion of tissue and later scanning of the remaining vascular cast with electron microscope (Leiser and Kohler 1983, Hafez, Caceci et al. 2007). The third approach is the use of noninvasive imaging tools such as MRI and Doppler ultrasound (Andescavage, du Plessis et al. 2015, Sato, Noguchi et al. 2016, Collins, Welsh et al. 2017, Lemery Magnin, Fitoussi et al. 2018, Salavati, Gordijn et al. 2018). Some disadvantages of using Doppler for placental blood flow are the variability between

different operators and interpretation and the need for special training and expertise to utilize the equipment (Grigsby 2016). Despite the limitations, this non-invasive technique offers great advantages to study placenta in humans and animals because they do not affect the course of the pregnancy.

The fourth approach is to use biochemical methodologies to quantify the relative levels of expression of genes known to regulate angiogenesis, or detect genes been highly expressed during angiogenesis of the placenta with the intention of detect novel angiogenic genes. (Bogic, Brace et al. 2001, Cho, Roh et al. 2003, Wang, Li et al. 2003, Viita, Markkanen et al. 2008, Xia, Zhen et al. 2017). Also, there is always the alternative of using cell or tissue models to study placental angiogenesis. Some the most utilized methods are trophoblastic cell lines or 2D-3D cell cultures, embryonic stem cell–derived embryoid bodies, culture of placental explants, three-dimensional spheroid models and more recently placental chips (Lee, Romero et al. 2016, Blundell, Yi et al. 2018). The list expands every year. These and other methodologies are well reviewed by Huckle (2017) and Herr, Baal et al. (2010).

Many of these technologies for study the placenta are being further developed by the Human Placenta Project from the National Institute for Child Health and Human Development, a collaborative research effort to understand the role of the placenta in health and disease. This project aims to develop new tools to study the placenta in real time to learn how it develops and functions throughout pregnancy. With the hope of finding treatments to pathologies like preeclampsia, fetal growth restriction, gestational hypertension and eclampsia. (Guttmacher and Spong 2015) This initiative exemplifies the comprehensive research and clinical efforts currently devoted to placental studies and reflects the impact of the placenta in human health.

1.5 Use of cat as a viable model for understanding angiogenesis in the placenta

The use of animal models for studying placenta and embryology date as old as Hippocrates in 400 B.C.E, who speculated about the nature of chicken embryonic development, then related these concepts to human development. Most likely, knowledge gained in the chicken egg lead him to believe that the human fetus gained nourishment by sucking blood from the placenta (Abbott 1936), Aristoteles, Galeno, da Vinci and Harvey follow, all of whom turned their attention to animals to understand human embryonic development and the placenta. Consequently, the use of animal models has been crucial since ancient times, and the greatest

portion of our current knowledge in placenta comes from comparative studies in animals (Longo and Reynolds 2010).

The need of animal models for studying the placenta arises due to the difficulties, risks, and ethical limitations attending study the human placenta directly, especially when invasive methodologies are required to examine its function in health and disease. Availability of human specimens is limited to 4 to 12 weeks of pregnancy (where in most countries it is legal to induce abortions of healthy pregnancies) or delivered human term placenta (36-40 weeks) (Herr, Baal et al. 2010, Benirschke, Burton et al. 2012). The placenta is one of the most evolutionarily diverse organs (Schmidt, Morales-Prieto et al. 2015), and each species has its own placental peculiarities. In fact, the human placenta has many characteristics that make it exceptional, as humans have primary interstitial implantation in a simplex uterus, yolk sac placentation is absent, and an allantoic stalk instead of an allantoic sac is formed (Carter 2007), making more difficult the selection of non-human placental models. Often researchers are obliged to base the choice of model on available expertise, facilities available, and cost rather than on purely scientific considerations. Therefore, it is important to recognize the strengths and limitations of each model in order to choose the most adequate for each type of research and for correct interpretation of the results. Despite these difficulties, many animal models to study placenta have been developed and have proven indispensable research tools for study placental biology, placental angiogenesis, and placental pathology. Additionally, models are valuable to establish preliminary or basic research that can be useful for planning meaningful human research, saving valuable resources in the long run and also reducing the risks.

In spite of the anatomical differences in placentas across species, key genes regulating development show high degrees evolutionary conservation (Cross, Baczyk et al. 2003, Rawn and Cross 2008, Benirschke, Burton et al. 2012). A study comparing mRNA and protein from mouse and human placenta at term found more than 7000 ortholog genes, 70% of which were expressed in both species, while 90% of placental proteins where shared between humans and mice (Cox, Kotlyar et al. 2009). These results provide foundational evidence for the scientific value of animal models. There are no similar studies comparing genomics and proteomics of placental expression in cats and humans, but multiple reports recognize a high homology between genomes of humans and cats (Murphy, Larkin et al. 2005, Pontius, Mullikin et al. 2007, O'Brien, Johnson et al. 2008). Thus, it is reasonable to expect that very similar mRNA and protein

expression patterns will occur between cats and human. In fact, some of the VEGF family members present in humans are better represented in cats than in mouse. This is the case with PLGF, where in mouse only one heparin binding isoform is expressed, whereas 3 different isoforms are predicted to be expressed in cats.

Some of the most common animal models for placenta include rodents, especially mice, rats and guinea pigs; other lab animals like rabbits; small ruminants such as sheep and finally, non-human primates from the new and old world. (Anthony, Scheaffer et al. 2003, Reynolds, Borowicz et al. 2005, Carter 2007, Barry and Anthony 2008, Barry, Rozance et al. 2008, McCarthy, Kingdom et al. 2011, Chaouat and Clark 2015, Grigsby 2016). Small lab animals like rodents and lagomorphs have the advantage of large liter size, short generation times, and being relatively economical to house and maintain. Another advantage of mice is the array of options for genetic manipulation and commercial availability of genetically modified lines. However, small size can be a disadvantage when studying already small structures, and makes difficult manipulation in some experimental settings like cannulation of friable blood vessels for vascular casting.

Sheep is another popular animal especially for study physiology of the placenta; it has a similar size compared to human placenta, and the weight of a newborn lamb is similar to that of a human newborn. Sheep are easy to handle and enable placement of intrauterine catheters for regular sampling throughout pregnancy. (Barry and Anthony 2008). Limitations of sheep include differences in the distribution and shape of the placental interface. Sheep form multiple placentomes distributed along the chorion in contrast to a single discoidal placenta as in humans. Additionally, humans have hemochorial placenta while the sheep placenta is endotheliochorial leaving the uterine epithelium intact. For some investigators, this characteristic is desirable for placental studies since maternal and fetal tissue can be easily distinguished, allowing for higher-resolution analysis (Ramsey 1982, Reynolds, Borowicz et al. 2005). However, these 'convenient' differences in microanatomy are reflective of functional and immunological differences in the ovine pregnancy compared to humans.

Non-human primates, especially the chimpanzee and gorilla, have placentation that is very similar to humans. We primates share longer gestational lengths than rodents and sheep, have similar endocrine functions especially regarding progesterone and other reproductive steroids, unicornuate uterus, and uterine contraction (Grigsby 2016). The main limitations of

non-human primate studies are the elevated cost, the use of endangered or vulnerable species and ethical concerns of animal cruelty. Owing to these limitations, only a precious few specialized facilities have access to this model.

For the purposes of the research described in this dissertation, we utilized the cat as a model to study angiogenesis in the placenta. We collected placental tissue directly after ovariohysterectomy of pregnant queens from a local spay and neuter clinic. Each spring, the number of feral or stray cats that become pregnant increases drastically. The presence of local sterilization programs for feral cat colonies, along with the multiparous nature of feline pregnancies, seasonally raises the probability that tissue discarded from these routine ovariohysterectomies will contain multiple feto-placental samples, collectively representing all stages of pregnancy. Some of the advantages of this approach are the utilization for research of tissue that normally will be discarded, giving value to an otherwise waste by-product of elective surgery. In addition, there is no need for housing or managing cat colonies, reducing the cost and ethical concerns related to pregnancy termination for research. Other advantages include the increasing level of detail and fidelity of the publically available sequence of the cat genome. Although the annotation of the domestic cat genome is not as thorough as that for human or mouse, it is been improving rapidly, becoming an extremely valuable and viable tool for gene expression and protein analysis. As noted above, the vast majority of genes and encoded proteins present in humans are also expressed in feline placenta. *Taken together, these considerations make the domestic cat placenta a suitable biological setting for our basic research, allowing exploration of the relationship between the dynamics of placental vascular microanatomy and expression of angiogenic genes throughout a healthy pregnancy*.

The discussion above notwithstanding, there are limitations of the feline placental model. We have neither control over environmental factors nor awareness of medical conditions affecting the queens during pregnancy, thus we may expect increased variability in our quantitative results. Additionally, there likely exists more genetic variation in outbred cat populations compared to lab mouse lines, although this is true of human populations as well. Other limitations include limited availability of research tools and reagents specifically designed to target cat gene sequences or proteins, for example pre-validated molecular probes or primary antibodies, thus complicating otherwise routine research protocols. Out-of-season availability of tissue is another concern, stemming from the nature of the feline estrus cycle, making access to

samples outside spring or early summer very limited in Virginia. A final concern is the difference in placental characteristics compared to humans, although except for the chimpanzee and the gorilla, most other well-established animal models to study placenta likewise differ to a degree in placentation type compared to humans. The factor of different anatomical details needs to be considered, but does not diminish the value of the domestic cat for placental research.

1.6 The Vascular Endothelial Growth Factor (VEGF) family

The VEGF family members are major regulators of angiogenesis, vasculogenesis, and lymphangiogenesis. VEGF family members are part of the platelet-derived growth factor superfamily, among the first proteins identified as potent mitogens and chemotactic agents with activity toward mesenchyme-derived cells. Members of this family include VEGF-A, VEGF-B, VEGF-C, VEGF-D and Placental Growth Factor (PLGF) (**Figure 1.4**). These factors interact with multiple tyrosine kinase receptors, including: Vascular endothelial growth factor receptor 1 (also known as VEGFR-1 or Flt-1) and its soluble/secreted variant sFlt-1, VEGFR-2 (Flk-1, KDR), VEGFR-3 (Flt-4), and the non-tyrosine kinase co-receptors neuropilin 1 and 2 (NRP-1, NRP-2) (Clark, Smith et al. 1998). VEGF-C and VEGF-D interact with VEGFR-2 and VEGF-R3 and are mainly positive regulators of lymphangiogenesis, with very limited angiogenic properties (Lohela, Saaristo et al. 2003). Many VEGF family members can also interact with heparin sulfate proteoglycans (Maruotti and Ribatti 2008, Pardali, Godfrey et al. 2013, Shibuya 2013). The interaction between ligands and tyrosine kinase receptors activates the protein phosphorylating catalytic activity of the latter and triggers complex pathways involving multiple intracellular proteins and other regulators of the cascade (Fan, Arif et al. 2012). Structurally, ligands of the VEGF family and their receptors are found as homodimers, although several can form heterodimers as well, adding complexity to their function. Another typical feature of VEGF members is the formation of multiple isoforms derived from the same gene by alternative mRNA splicing (Houck, Ferrara et al. 1991, Cao, Ji et al. 1997, Burchardt, Burchardt et al. 1999, He, Smith et al. 1999, Ladomery, Harper et al. 2007). This gives can result in the protein having a radically different function, as in the case of secreted versions of the receptor Flt-1. For the ligands, alternative transcript processing results in mRNAs encoding monomers of about 120 to 200 amino acids in length. With notable exceptions, the difference in function of splice isoforms is not so evident, and their detailed properties and functions remain unclear.

1.7 VEGF A

VEGF-A is perhaps the most thoroughly studied member of the VEGF family. It was first identified in ascites fluids and cell culture from guinea pigs with hepatocarcinoma. This molecule was noted to transiently increase vascular permeability in multiple tissues hence its original designation as a Vascular Permeability Factor (Senger, Galli et al. 1983). It was not until 1989, when the name VEGF was coined by Ferrara and Henzel (1989) after discovering and cloning a diffusible mitogenic 45 kDa heparin-binding protein from bovine pituitary follicular cells. At the same time Plouet *et al.* independently identify the same factor and name it Vasculotropin (Plouet, Schilling et al. 1989). Latter it was recognized that the molecules where in fact the same.

VEGF-A is a homodimeric glycoprotein that acts as a potent angiogenic agent promoting endothelial cells proliferation and vascular permeability in multiple tissues, including placenta, cornea and retina, bone, reproductive tissues, and multiple tumor types. VEGF-A is also crucial for developing and maintaining the vasculature in the embryo and the placenta (Abbott and Buckalew 2000, Ferrara, Gerber et al. 2003, Douglas, Tang et al. 2009). Knock out a single allele of the VEGF A gene in mice embryo produces early embryonic lethality due to failure to develop the cardiovascular system, and improper angiogenesis in the placenta. Deletion of both alleles of VEGF-A results in still more severe alterations in the development of the vasculature (Carmeliet and Collen 2000). Lethality due to heterozygous knockout is not common in mammals and reveals the extraordinary dependence upon VEGF-A in embryonic development. These findings also suggest that a tight regulation of VEGF-A levels and gradients in the embryo are needed to accomplish an adequate angiogenic homeostasis (Carmeliet, Ferreira et al. 1996).

Oxygen tension is one of the main determinants of VEGF-A expression, mediated by the transcription factor hypoxia-inducible factor (HIF-1). Low oxygen partial pressures inhibit the hydroxylation of HIF-1α protein, reducing its degradation and thereby increasing the levels of HIF-1. This way HIF-1 binds to specific DNA segments (hypoxia response elements) nearby or within the promoter of VEGF-A and other hypoxia-responsive genes. This activates the expression of VEGF-A, increasing vascular permeability and promoting angiogenesis into the hypoxic tissue. As a result of increased vascular perfusion, tissue oxygen levels can be

normalized (Fraisl, Mazzone et al. 2009, Yeom, Min et al. 2014). Other regulators of VEGF-A expression include epidermal growth factor, PDGF, estrogen, and multiple oncogenic mutations. VEGF-A induces its effects in cells by interacting with the VEGFR-II (KDR), VEGFR-1 (Flt-1) (de Vries, Escobedo et al. 1992). VEGF-A can also interact with the co-receptors NPL-1 and the soluble form of Flt-1 (sFlt-1), although this latter interaction does not transduce any cell signal and functions as a decoy.

The VEGF-A gene is recognized to contain eight exons and seven introns, and multiple isoforms of VEGF-A are produced by alternative spicing of VEGF transcripts. All VEGF-A isoforms contain exons 1-5, which encodes the receptor-binding domain (see Chapter 3). The isoforms are denoted by the amino acid count of the secreted polypeptide (minus the signal peptide). In humans, there are at least five major isoforms; VEGF 121, VEGF 145, VEGF 165, VEGF 189, and VEGF 206. The most prevalent isoform is VEGF 165 followed by VEGF 189 and 121. In mouse, the VEGF-A gene has similar splicing variant array as the human homolog, although mice VEGF-A isoforms contain one less amino acid owing to a shorter exon 2. Therefore the main isoforms in the mouse are VEGF-120, VEGF-164, and VEGF-188 (Vempati, Popel et al. 2014). Smaller isoforms such as VEGF 121 lacks exon 6 and 7 while intermediate isoforms like VEGF 165 lacks exon 6 but retain exon 7 (Houck, Ferrara et al. 1991, Tischer, Mitchell et al. 1991). Additional variants of VEGF-A can be generated by proteolytic cleavage of VEGF 165 at the carboxyl terminus. Recently, other anti-angiogenic slice variants of VEGF A have been described, although their significance is still controversial and remains to be clarified (Bates, MacMillan et al. 2006, Eswarappa and Fox 2015).

VEGF 121 is a relatively acidic polypeptide that, thanks to the absence of exons 6 and 7, do not contain a heparin-binding domain. As a result, this isoform is not thought to interact with heparan sulfate proteoglycans in the extracellular matrix and can more freely diffuse into the interstitial medium or through the extracellular matrix. Loss of the heparin binding domain has shown to reduce the mitogenic activities of this isoform (Houck, Leung et al. 1992, Keyt, Berleau et al. 1996). In contrast, VEGF 189 and VEGF 206 are highly basic, and interact with heparan sulfate proteoglycans and can be sequestered on membranes and heparan-containing extracellular matrix. Under the right conditions, this isoforms can be released into a diffusible form by plasminogen proteolytic cleavage at the C terminus or by displacement from heparan by the enzyme heparinase. Other metalloproteinases are described to have

similar effects (Kim, Im et al. 2014, Vempati, Popel et al. 2014). The proteolytic activity generates bioactive fragments capable of inducing angiogenesis (Houck, Leung et al. 1992). This demonstrates the effect of proteolytic enzymes for regulating VEGF-A bioavailability in tissue microenvironment, and suggest that larger isoforms can act as reservoirs for VEGF in the tissue. Furthermore, heparin-binding isoforms may play a role under tissue damage, inflammation or tumorigeneses, due to their rapid bioavailability in tissue, before smaller isoforms can be synthesized by cells, thus acting as a rapid tissue response after injury to promote cell survival and induce angiogenesis (Lee, Jilani et al. 2005). VEGF 165 has intermediate, balanced properties, can be secreted and diffuse to reach distant cells and can also be sequestered by the extracellular matrix giving this isoform more diverse function and arguably optimal angiogenic potency and bioavailability (Park, Keller et al. 1993).

1.8 Biology of VEGF-A variants

Mice expressing only VEGF 164 gives rise to offspring that appears normal (Carmeliet, 2000; Stalmans et al., 2002), suggesting that this isoform is capable of serving most of the functions of its smaller and larger variants. On the other hand, half of the transgenic mice expressing only VEGF 120 died during the first days after delivery and all mice died within two weeks of age (Carmeliet, Ng et al. 1999). These mice showed elevated levels of VEGF 120 compared to wild-type mice, suggesting a compensatory response to increase angiogenesis relying only in VEGF 121. This compensatory regulation was enough to produce term offspring in contrast to VEGF A null mice that die before 2 weeks of gestation. These results demonstrate that the different VEGF isoforms are not functionally equivalent and suggest an indispensable role of heparin binding domain isoforms for normal embryonic development and adult homeostasis. Interestingly, VEGF 120 mice showed reduced angiogenesis and especially diminished branching, as well as increased vascular permeability. In a similar fashion, mice only expressing VEGF-188 developed insufficient arterial outgrowth in the retina, dwarfism, and impaired bone growth (Maes, Stockmans et al. 2004). These changes appeared less severe and produce mice with longer survival times compared to their VEGF 120 counterparts, suggesting that VEGF 188 have a stronger compensation capability. It seems that the capability of interacting with the cell membrane and extracellular matrix by VEGF 188, and to

some degree by VEGF 164, might be required for developing and maintenance of vascular networks.

Another approach to investigating the function of VEGF isoforms is to evaluate angiogenesis in tumoral cells engineered to express isolated VEGF-A isoforms. Such tumors have shown different capacities for vascular recruitment and maintenance *in vivo*. Moreover, VEGF 188 overexpressing tumors are smaller, more stable, have denser capillary networks composed of vessels of smaller caliber, and induce less hemorrhage relative to tumors expressing smaller VEGF-A isoforms. In contrast, vessels in VEGF 120-overexpressing tumors are highly unstable and leaky; their capillaries are tortuous with a larger diameter and reveal fewer branch points relative to normal tumors or to VEGF 188 overexpressing tumors. On the other hand, tumor growth appears to be most rapid in tumors that express VEGF 164. (Cheng, Nagane et al. 1997, Grunstein, Masbad et al. 2000, Ruhrberg, Gerhardt et al. 2002, Yu, Rak et al. 2002, Küsters, de Waal et al. 2003, Tozer, Akerman et al. 2008). *Taken together, these results suggest that a mixture of different isoforms are needed for proper regulation of angiogenesis*. A 'panel' of different isoforms may help to maintain appropriate VEGF gradients that properly regulate angiogenesis (Álvarez-Aznar, Muhl et al. 2017). Mathematical modeling studies suggest that heparin-binding isoforms such as VEGF-A 189 produce a higher concentration in the periphery of the cell expressing it, thus producing a steep gradient. In contrast, diffusible isoforms, such as VEGF-A 121, can travel longer in the interstitial space and form shallower gradients compared to the larger isoforms. These gradients have an effect in vascular pattering due to its signaling to stalk and tip cells during angiogenesis (Grunstein, Masbad et al. 2000, Vempati, Popel et al. 2014).

In the cat, NCBI Gnome system predicts two isoforms from the chromosome B2 genomic sequence (NC_018727.3). One isoform, called variant X1 (XM_023253550.1), is predicted to encode a protein of 657AA, well above the typical size for any known VEGF. Further analysis of the sequence reveals a protein of 189AA located in the beginning of the predicted sequence. The second predicted isoform, called variant 1 (NM_001009854.1), is designated as provisional due to a report to NCBI of this sequence by Koga *et al.* (2001) from the University of Tokyo, encoding the same VEGF 189. These two isoforms have a signal peptide of 26AA in the 5'end, predicting a final product of 163AA that appears to be the heparin-binding equivalent of the VEGF 165 in humans. Another annotation of the feline genome by the company Ensamble

(ENSFCAG00000026462) predicts five different isoforms base on homology with other species and computational analysis; all predicted isoforms have more than 200AA and none of them have been corroborated by experimental data. It is clear that more research is needed to reveal the real quantity and characteristics of VEGF-A isoforms in the domestic cat. Comparative analysis of these isoforms in the cat placenta or in pathological conditions like tumor growth could help to understand the role of different VEGF A isoforms in health and disease.

1.9 The VEGF family in placental vascularization

VEGF A is one of the most important regulators of angiogenesis in the placenta. Following implantation, inadequate levels of VEGF-A and their receptors are been linked with pathologies of the pregnancy such as fetal growth restriction, preeclampsia and abortions. Accordingly, in mice, knockout of either VEGF A or its receptors results in multiple vascular malformations and consequently in embryonic lethality (Breier 2000). Expression of VEGF-A transcripts in humans, sheep and pigs have shown to be localized in fetal trophoblast cells and shown dynamic levels of expression dependent to the stage of pregnancy and are affected by multiple pathologies of the pregnancy (Winther, Ahmed et al. 1999, Bogic, Brace et al. 2001, Demir, Kayisli et al. 2004).

In humans, during the first trimester, *in-situ* hybridization revealed uneven distribution of Flt-1 and VEGF A mRNA around the villous cytotrophoblast (Clark, Smith et al. 1996), this location suggest a role in early vasculogenesis and blood vessel branching which is very active during early placentation. Additionally this VEGF A location could participate in placental invasion and attachment (Ahmed, Li et al. 1995, Vuckovic, Ponting et al. 1996). In human and ovine placenta, VEGF A expression increases gradually along the gestation in normal pregnancies (Cheung, 1997). Similarly, in the primate common marmoset, VEGF was immunolocalized in maternal decidual and cytotrophoblast cells and Flt 1 was only localized in the syncytial trophoblast with increased expression in placentas from 10 weeks to term. KDR expression was only present in mesenchymal cells during early placentation and endothelial cells of maternal and fetal origin in the third trimester (Wulff, Wilson et al. 2002). In the sheep, mRNA levels of VEGF A, flt 1 and KDR have been positively correlated to placental blood flow, placental vascularization and placental weight, suggesting that the VEGF-A and their receptors Flt-1 and KDR are critically involved in placental angiogenesis (Chen and Zheng

2014). VEGF A is expressed in large amounts at the caruncles and cotyledons during the third trimester. VEGF A, Flt-1 and KDR transcripts were also localized in placental vasculature, especially endothelial cells of maternal arteries. (Tsoi, Wen et al. 2002, Borowicz, Arnold et al. 2007). Localization of VEGF A and its receptors in the same cell types of previously mentioned animals suggest an autocrine cycle of VEGF A to stimulate endothelial cells and regulate placental angiogenesis, although other paracrine interactions between different cell types or even feto-maternal interactions have also been considered. For example, in humans during the second trimester, VEGF A localized in Hofbauer cells (macrophages of the placenta) is believed to play a key role in promoting angiogenesis by release near endothelial cells (Cooper, Sharkey et al. 1995, Demir, Seval et al. 2007, Bouwland-Both, Steegers et al. 2013, Loegl, Hiden et al. 2016).

VEGF A and Flt-1 co-localization in cytotrophoblast of multiple species may be relevant for placental proliferation and invasion on the placenta. Secretion of VEGF A from the cytotrophoblast during early implantation has been proposed to stimulate endothelial cells and trigger vasculogenesis and angiogenesis in a paracrine way (Demir, Seval et al. 2007). Alternately, VEGF-A localization in early trophoblastic cells may be implicated in the implantation of the placenta. Consistent with this function, levels of VEGF A were found in uterus during peri-implantation stages in different species such as rabbits, hamsters, rat, mouse, dogs, and humans. (Das, Chakraborty et al. 1997, Yi, Jiang et al. 1999, Rabbani and Rogers 2001, Sugino, Kashida et al. 2002, Schafer-Somi, Sabitzer et al. 2013). Additionally, VEGF A and estrogen increase uterine endometrial proliferation and microcirculation during the menstrual cycle in mouse, thus preparing the uterine environment for proper attachment of the embryo (Rockwell, Pillai et al. 2002). This early expression of VEGF A in maternal and fetal tissue is consistent with VEGF functioning to support fetal invasion, attachment and implantation into maternal tissue, increase proliferation of maternal endometrium and fetal trophoblast, contributing to vascular permeabilization and uterine edema, and developing the vasculature in the early placenta (Halder, Zhao et al. 2000, Ancelin, Buteau-Lozano et al. 2002).

In the cow, VEGF A was immunodetected in fetal and maternal endothelial cells early in the pregnancy, during implantation, and throughout the pregnancy until delivery. During early pregnancy, VEGF was localized in trophoblasts giant cells and in the endometrium, during mid pregnancy VEGF was localized in the stroma of maternal caruncular septa and decreased near term. Flt-1 was co-localized with VEGF A in trophoblast and uterine epithelium around

implantation. Later, in definitive placentomes, VEGFR-1 was localized in trophoblast giant cells and in maternal endothelial cells in the center of the placentome. KDR and Flt-1 were also co-localized in uterine epithelium and trophoblast as well as in blood vessel tissue and uterine glands.(Pfarrer, Ruziwa et al. 2006)

In the dog placenta, VEGF A transcripts were detected following implantation and rose during mid gestation in placental girdle and uterus but were downregulated close to parturition. Similarly, both receptors' mRNAs were gradually increased towards mid-gestation; Flt-1 was downregulated before delivery, whereas KDR remained at the same level from mid-pregnancy. Immunological detection of VEGF A in gravid uterus showed it at the luminal side of the endometrium, in epithelial cells well as in superficial and deep uterine glands and myometrium. (Gram, Hoffmann et al. 2015)

Despite the vital importance of VEGF A during pregnancy, limited research has been performed to study VEGF A isoforms in the placenta. One of the few reports available mouse detected VEGF 164, VEGF 120, and VEGF 188 using end-point PCR and northern blot in peri-implantation uterus. Semi-quantitative measurements from agarose gels after electrophoresis showed that VEGF 164 was the most abundant isoform followed by VEGF 121. Northern blot analysis localized VEGF 164 in endothelial cells of the decasualizing stroma cells during early pregnancy. Expression of VEGF 164 was more intense on days 6 to 8 at the mesometrial pole (Halder, Zhao et al. 2000). These results are consistent with panVEGF-A measurements in mouse and other species and with the previously mentioned functions of VEGF A in the placenta. Despite the great value of these results and its comparison with neuropilin 1 and KDR, a more compressive analysis with updated techniques and including other factors such as PLGF isoforms and Flt-1 is needed and would provide a wider understanding of the placental angiogenic process throughout the pregnancy. In cats, there is just one report of measurement of different placental isoforms, that was done by our laboratory (Weerakoon, Frydman et al. 2015). It is necessary to increase the efforts toward understanding the distinct function of VEGF and PLGF isoforms in placental development. This should provide a more complete view of the interactions between the VEGF family ligands and their receptors under different conditions, and how this affects vascular patterning during normal pregnancy. Knowledge from this type of research may be vital to understand and hopefully detect, prevent or treat pathologies of the pregnancy such as preeclampsia. In the same way, a better understanding of the angiogenic

process including the role of splicing variants of the VEGF family can be useful in understanding tumor angiogenesis and develop ways to control it.

1.10 Placental Growth Factor (PLGF)

Placental growth factor was the second member of the VEGF family to be discovered, characterized from human placenta (Maglione, Guerriero et al. 1991). In humans, PLGF is coded by chromosome 14q24 and in mice is located on chromosome 12, while in the cat it is located on chromosome B3. In cats, PLGF has a gene of 12.7kb with 7 exons separated by 6 relatively large introns. Like other members of the VEGF family, PLGF can be expressed in different isoforms thanks to alternative splicing of pre-mRNA transcripts. In humans, there are 4 isoforms of PLGF described, predicting proteins of 131, 152, 203, and 224 amino acids without counting the 18 AA signal peptide. As with the VEGF As, the main differences among these isoforms are their capability to interact with heparin. PLGF-2 and PLGF-4 can interact with the extracellular matrix through a heparin binding domain coded by the exon 6. On the contrary, isoforms PLGF-3 and PLGF-1 do not bind to heparin; hence, can be secreted and diffuse to interact with distant cell targets (Ferrara 2000, Yao, Yang et al. 2005, De Falco 2012). In mouse, there is only one PLGF-2 isoform described, and it is heparin binding (DiPalma, Tucci et al. 1996). In cats, there are three predicted isoforms based on genome wide sequencing analysis and homology with other species (NCBI gene ID:101095904), although there is yet no published experimental evidence to support this prediction.

PLGF is secreted as a glycosylated homodimer held together by two disulfide bonds. While in the extracellular matrix, PLGF exerts its activity directly through binding and activating the tyrosine kinase receptor Flt-1 (VEGFR-1), inducing its dimerization and phosphorylation. PLGF directly activates endothelial cells, macrophages, and hematopoietic progenitor cells. PLGF induces proliferation, increases permeability and enhances the sensitivity of the cell to VEGF-A. There seems to be conflicting evidence about the proangiogenic effect of PLGF. In some models PLGF promotes VEGF induced angiogenesis (Carmeliet, Moons et al. 2001), while in others antagonizes VEGF action. (Eriksson, Cao et al. 2002, Tarallo, Vesci et al. 2010). PLGF shares Flt-1 receptor with VEGF-A and VEGF-B but can interact with Flt-1 with much higher affinity than VEGF-A/B and therefore, it is able to displace VEGF-A/B from Flt-1. (Olofsson, Pajusola et al. 1996, Carmeliet, Moons et al. 2001, De Falco, Gigante et al. 2002, Ferrara, Gerber

et al. 2003). When VEGF-A is displaced from Flt-1, more VEGF-A is free to activate KDR, thus PLGF may act as an indirect activator of KDR (VEGFR-2), which has about 10 times greater mitogenic effect in the cells than Flt-1. In some cases, when the same cell is expressing VEGF A and PLGF, this cell may secrete VEGF A/PLGF heterodimers (DiSalvo, Bayne et al. 1995) which can activate with KDR or induce KDR/Flt-1 receptor heterodimers (Tarallo, Vesci et al. 2010). Another alternative path for KDR activation is possible when the same cell express both receptors (such as endothelial cells) and the activation of Flt-1 can trigger a near KDR by trans-phosphorylation and initiate a double kinase activation (Autiero, Waltenberger et al. 2003).

PLGF is highly expressed in the placenta during all stages of the human pregnancy, although specific isoform characterization remains obscure. In the placenta, PLGF stimulates trophoblast proliferation and migration into the maternal decidua (Burton, Charnock-Jones et al. 2009), and stimulates fetal and maternal angiogenesis, but the specific functions or regulation of different PLGF isoforms are not clear. In humans, PLGF was immunolocalized in the vasculosyncytial membrane, in the media of large blood vessels of the placental villi and in maternal decidua cells. In situ hybridization detected PLGF mRNA also in the vasculosyncytial membrane of villous trophoblast, suggesting that PLGF acts as a paracrine factor for vascular endothelial cells in placental angiogenesis and an autocrine mediator of trophoblast function (Khaliq, Li et al. 1996, Vuorela, Hatva et al. 1997). In mouse, PLGF is detected very early in embryonic development, with PLGF transcripts localized in trophoblastic giant cells in the yolk sac. PLGF has also been localized in different cell types including endothelial cells, fibroblast, lymphocytes, keratinocytes and in tumor-derived cell lines and in tumor-associated macrophages (Persico, Vincenti et al. 1999). This PLGF tissue and cellular distribution correlates with its role in early vasculogenesis and angiogenesis and in chronic inflammation as well as tumoral angiogenesis (Achen, Gad et al. 1997).

As for VEGF A, low oxygen tension seems to be the main stimulus for PLGF expression in tissue. Similarly to VEGF, PLGF responds to high levels of the transcription factor, hypoxia-inducible factor 1. HIF-1 interacts directly with the 3' promoter region of VEGF, but this mechanism is not present with PLGF. The exact pathway for HIF-1/PLGF interaction is not yet clear (Zimna and Kurpisz 2015, Wang, Gu et al. 2017). Other PLGF positive regulators include, but are not limited to, nitric oxide (Gigante, Morlino et al. 2006, Jaba, Zhuang et al. 2013), inflammatory cytokines like interleukin 1 and tumor necrosis factor-α. PLGF expression is also

stimulated by growth factors such as transforming growth factor-β1 (Yao, Yang et al. 2005) and multiple oncogenes (Viglietto, Maglione et al. 1995, Larcher, Franco et al. 2003). The long list of PLGF positive regulators highlights its role in multiple pathologies and in cancer.

In the skin, PLGF knock out ($PLGF^{-/-}$) mice exhibit impaired wound healing, while PLGF overexpression driven by transgene delivery increases skin vascularization without affecting lymphatic vessels, increases cutaneous hypersensitivity and accelerates wound healing (Cianfarani, Tommasi et al. 2006, Odorisio, Cianfarani et al. 2006). In solid tumor models, $PLGF^{-/-}$ mice have shown reductions in tumoral angiogenesis, growth, and metastasis (Fischer, Jonckx et al. 2007, Van de Veire, Stalmans et al. 2010). In contrast, when PLFG is overexpressed in skin tumors, tumor growth, angiogenesis, and metastasis increases (Marcellini, De Luca et al. 2006, Fischer, Jonckx et al. 2007). All these studies are supportive of the key function of PLGF in many diseases including cancer.

To study the biological function of PLGF, multiple studies of gain and loss of function have been performed in different species, tissues, and developmental stages (Carmeliet, Moons et al. 2001, Gigante, Morlino et al. 2006, Huang, He et al. 2015). $PLGF^{-/-}$ mice showed normal embryonic development, grew apparently healthy, and are fertile. In this case, the lack of PLGF did not appear to confer any negative impact on vascular development, making evident the dispensability of PLGF for normal homeostasis or suggesting that there may be redundancy in the PLGF function. However, when $PLGF^{-/-}$ mice were subjected to pathological conditions such as ischemia, inflammation, wound healing and cancer, these mice demonstrated impaired angiogenesis, reduce plasma extravasation and delayed vascular collateral growth after ischemia. $PLGF^{-/-}$ mice incapability to adapt and compensate to these pathological conditions highlights the role of PLGF mainly as an angiogenic regulator in pathological conditions (Carmeliet, Moons et al. 2001, Dewerchin and Carmeliet 2012, Ryan, McCarthy et al. 2018).

In a mouse model of preeclampsia, placental overexpression of PLGF showed a reduction in the symptoms of preeclampsia. Furthermore, overexpression of soluble Flt-1 in human pregnancies is correlated to decreased plasma levels of PLGF and other growth factors, and causes endothelial dysfunction in maternal tissue leading to preeclampsia. This evidence supports the important function of PLGF in placental angiogenesis especially under pathological conditions, which makes PLGF an ideal target for developing safe drugs treating angiogenesis (Luttun, Autiero et al. 2004, Suzuki, Ohkuchi et al. 2009, Furuya, Kurasawa et al. 2011). Serum

levels of PLGF during human pregnancy show a consistent rise through the second trimester of the pregnancy (Levine, Maynard et al. 2004, Lam, Lim et al. 2005). This period corresponds to the time of maximum vessel maturation and enlargement in the placenta (Pang, Bates et al. 2017), suggesting that PLGF could influence vascular patterning in the placenta helping to shift from increased branching in early pregnancy towards vessel enlargement, elongation and maturation during mid-pregnancy. Serum levels of PLGF decrease during the third trimester. Lower PLGF levels in maternal circulation have been linked to lower neonatal weight in normal pregnancies (Benton, Hu et al. 2012). Of the various isoforms of human PIGF, PIGF-2 is reported to enhance VEGF-induced permeability (Pang, Bates et al. 2017).

The distinct function of PLGF isoforms is not well characterized. Existing studies reveal that non-heparin binding isoforms under *in vitro* conditions are secreted into cell culture medium, and PlGF 3 (non-heparin binding) induces a relatively weak stimulatory activity over endothelial cells and does not stimulate bovine capillary endothelial cell proliferation. In contrast, PLGF-2 (heparin binding) induces a stronger mitogenic and vascular permeability activities than PLGF-1 (non-heparin binding) *in vitro*. Furthermore, perhaps due to its weak mitogenic activity, PLGF-1 can acts as an antiangiogenic molecule by forming VEGF-A/PLGF-1 heterodimers (Cao, Ji et al. 1997, Eriksson, Cao et al. 2002, Wang, Gu et al. 2017). Despite plausible evidence of PLGF importance in angiogenic pathologies, very little is known about the function of specific isoforms normal physiological conditions, including in the placental development. It is clear that more research is needed in order to clarify the role the PLGF isoforms may play in physiological and pathological conditions.

1.11 VEGF tyrosine kinase receptors, Flt-1 and KDR

There are three types of VEGFRs: the most important for angiogenesis are Flt-1 and KDR while VEGF R3 functions as a lymphangiogenesis receptor. Flt-1 and KDR are tyrosine kinases, essential and well-known regulators of angiogenesis during embryogenesis, placental development and in adult life. Tight regulation of these receptors is needed to maintain normal homeostasis. The Flt1 receptor was first identified in 1990 from a human placental cDNA library, as a sequence encoding a 180 kDa tyrosine kinase homodimer receptor with high affinity for VEGF A, VEGF B and PLGF (Olofsson, Korpelainen et al. 1998, Charnock-Jones 2002). The protein is very similar to KDR, encompasses seven extracellular immunoglobulin domains, a

transmembrane domain, and a cytoplasmic split kinase domain. Due to its resemblance to the Fms receptor, this new receptor was named Flt-1 (Fms-like tyrosine kinase-1) (Shibuya, Yamaguchi et al. 1990).

Flt-1 is expressed primarily in endothelial cells and secondarily in other cells such as monocytes and hematopoietic stem cells (Sawano, Iwai et al. 2001). In placenta, Flt-1 and KDR were founded in vasculogenic and angiogenic precursor cells as well as in endothelial cells (Demir, Kayisli et al. 2004). Despite extensive research in Flt-1, the precise function of this receptor is still under debate. Similarly to its ligand PLGF, the function of Flt-1 has been identified as a pro-angiogenic agent, although some studies describe an anti-angiogenic function. The function and signaling properties of Flt-1 may well depend on the target cell type, developmental stage, and levels of or ratios to other VEGF ligands (Ferrara, Gerber et al. 2003). Furthermore, there is evidence that Flt-1 activation by different ligands such as PLGF or VEGF A may trigger a dissimilar phosphorylation cascade and result in a different cellular response (Autiero, Waltenberger et al. 2003).

Activation of Flt-1 and KDR by their ligands induces a phosphorylation cascade with an activation of downstream PI3K-Akt and Ras pathways producing a strong signaling for cell proliferation (Heldin and Westermark 1999). The tyrosine kinase activity of Flt-1 is about 10 fold weaker than VEGFR-2 (Keyt, Nguyen et al. 1996, Sawano, Takahashi et al. 1996), although its affinity for PLGF and VEGF A is much higher than KDR, resulting in Flt-1 acting as a negative regulator of the availability of VEGF A to activate KDR. At the same time, when PLGF is increased in the tissue microenvironment, PLGF can displace VEGF-A from Flt-1, thus PLGF/Flt works in conjunction to regulate angiogenesis by limiting VEGF A bioavailability (Autiero, Waltenberger et al. 2003, Fischer, Mazzone et al. 2008, Moe, Heidecke et al. 2017). Nevertheless, the consequences of this interactions are not well understood, especially when different isoforms of PLGF/VEGF plus soluble Flt-1 and metalloproteinase are taken into consideration.

Alternative splicing of Flt-1 pre-mRNA produces at least two different isoforms in humans. A truncated variant maintains the integrity of six extracellular immunoglobulin domains but lacks the seventh, as well as the transmembrane helix and the intracellular kinase domain of the protein. This results in a secreted version of Flt-1 (sFlt-1) that does not trigger an intracellular signal and therefore acts as a VEGF A/PLGF trap, potentially reducing angiogenesis. High

serum levels of sFlt1 compared to PLGF are used as biomarkers for early detection and evaluation of preeclampsia in humans (Heydarian, McCaffrey et al. 2009, Bian, Shixia et al. 2015, Spiel, Salahuddin et al. 2017, Tantawy, Adly et al. 2017, Black and da Silva Costa 2018, Saleh, van den Meiracker et al. 2018, Tardif, Dumontet et al. 2018). This emphasizes the critical function VEGF family in the pregnancy.

VEGFR 2 or KDR ('Kinase-insert domain receptor') was identified from enriched primitive hematopoietic cells (Matthews, Jordan et al. 1991) and fetal liver cells in mice (Matthews, Jordan et al. 1991). KDR is the major angiogenic receptor and is critical in mediating a diverse set of endothelial cell responses in normal and pathological conditions, e.g. hematopoiesis, vasculogenesis, angiogenesis, and vascular permeability. Most of the VEGF A functions in endothelial cells are considered to be mediated by KDR signals. KDR is expressed in endothelial cells of blood vessels and lymphatics including their precursor cells and other cell types like megakaryocytes and in hematopoietic stem cells (Katoh, Tauchi et al. 1995). The levels of expression of KDR increases in tissue undergoing vasculogenesis, angiogenesis and pathological neovascularization such as macular degeneration of the eye. Furthermore, KDR is also considered one of the main players during pathological angiogenesis and tumor growth, metastasis, and survival. These characteristics have spurred an intense research effort to understand KDR's role in cancer biology and to develop both pro- and anti-angiogenic therapies (Millanta, Silvestri et al. 2006, Sun, Zhou et al. 2014, Lee, Pyun et al. 2015, Ravi, Sanford et al. 2016, Zhou, Wang et al. 2016).

In mice, KDR knockout produces early embryonic lethality due to insufficient vascular development. On the other hand, deletion of Flt1 produces endothelial overgrowth, tortuous and leaky blood vessels, and vascular disorganization leading to embryonic lethality (Fong, Rossant et al. 1995). This demonstrates that KDR is indispensable for embryonic angiogenesis while supporting the notion that Flt-1 may act as a VEGF regulator/decoy receptor during development. To investigate this hypothesis, Flt 1 and KDR transgenes carrying a mutation in their kinase domains were developed. Both receptors should retain their ability to capture their ligands, but not transduce signals to the cell. KDR-TK deficient mice die early in the development, resembling KDR null mice and VEGF A null mice. In contrast, Flt-1TK deficient mice were viable and showed normal blood vessel formation (Hiratsuka, Minowa et al. 1998). This provides strong evidence that Flt-1 main function during early embryonic development is to

regulate the bioavailability of VEGF A to interact with KDR. Additionally, this result suggests that the tyrosine kinase activity of KDR is vital to stimulate angiogenesis. These mice, however, displayed a reduction in macrophage migration, in accord with previously-observed expression of Flt-1 in macrophages and suggesting a role in macrophage function (Barleon, Sozzani et al. 1996, Clauss, Weich et al. 1996, Niida, Kondo et al. 2005). In addition, multiple studies comparing normal Flt-1 mice with Flt-TK deficient mice have shown an increase in angiogenesis in wild-type mice. This is especially evident in pathologies like carcinomas, glioblastoma (Kerber, Reiss et al. 2008), where Flt-TK deficient mice develop smaller tumors or less vascularize neoplasias and with smaller metastatic tumors (Hiratsuka, Nakamura et al. 2002, Schwartz, Rowinsky et al. 2010). Similar effects have been reported in multiple inflammatory diseases (Murakami, Iwai et al. 2006, Sato, Amano et al. 2013). These results indicate that Flt-1 can behave differently depending on the conditions and that despite its weak tyrosine kinase activity compared to KDR, that activity is still relevant, especially in pathological conditions. This makes Flt 1 receptor an interesting target for pathologies affecting angiogenesis, especially in cancer (Shibuya and Claesson-Welsh 2006).

1.12 Statement of hypothesis and aims

The review of literature above seeks to document the evidence that 1) development of an adequate placental vasculature, involving both maternal and fetal contributions, is crucial for normal gestation and fetal health and 2) the broadly-defined VEGF family, notably VEGF-A, PLGF, and their respective complements of isoforms derived from alternative pre-mRNA processing, are key regulators of endothelial cell proliferation and the establishment of vessel architecture. To gain a deeper understanding of the role(s) of the VEGF family in placental vascular development throughout gestation, we have undertaken a study of the dynamics of placental microanatomy across the duration of pregnancy in the domestic cat, with particular attention to changes in vascular structure (Chapter 2). *We hypothesize that these changes in placental vessel density or architecture can be better appreciated as consequences of dynamic expression of VEGF family members in the same tissues* (Chapter 3). A final study, described in Chapter 4, utilized our novel, cat-reactive molecular tools to study VEGF family expression in clinical specimens of feline squamous cell carcinoma.

1.13 References

Abbott, B. D. and A. R. Buckalew (2000). "Placental defects in ARNT-knockout conceptus correlate with localized decreases in VEGF-R2, Ang-1, and Tie-2." Dev Dyn **219**(4): 526-538.

Abbott, M. E. (1936). "A History of Embryology : (A Review)." Canadian Medical Association Journal **34**(1): 82-85.

Achen, M. G., J. M. Gad, S. A. Stacker and A. F. Wilks (1997). "Placenta growth factor and vascular endothelial growth factor are co-expressed during early embryonic development." Growth Factors **15**(1): 69-80.

Ackermann, M., J. P. Houdek, A. Ysasi, W. Wagner, J. Belle, A. Tsuda, S. J. Mentzer and M. A. Konerding (2013). "Sprouting and intussusceptive angiogenesis in post-pneumonectomy lung growth." International Journal of Molecular Medicine **32**: S76-S76.

Addicks, E. and A. Giannis (2003). "Angiogenesis inhibitors for cancer treatment." Nachrichten Aus Der Chemie **51**(2): 136-141.

Aguayo, A., T. Manshouri, S. O'Brien, M. Keating, M. Beran, C. Koller, H. Kantarjian, A. Rogers and M. Albitar (2001). "Clinical relevance of Flt1 and Tie1 angiogenesis receptors expression in B-cell chronic lymphocytic leukemia (CLL)." Leukemia Research **25**(4): 279-285.

Ahlgren, J., B. Risberg, K. Villman and J. Bergh (2002). "Angiogenesis in invasive breast carcinoma - a prospective study of tumour heterogeneity." European Journal of Cancer **38**(1): 64-69.

Ahmed, A., X. F. Li, C. Dunk, M. J. Whittle, D. I. Rushton and T. Rollason (1995). "Colocalisation of vascular endothelial growth factor and its Flt-1 receptor in human placenta." Growth Factors **12**(3): 235-243.

Álvarez-Aznar, A., L. Muhl and K. Gaengel (2017). VEGF receptor tyrosine kinases: key regulators of vascular function. Current topics in developmental biology, Elsevier. **123:** 433-482.

Ancelin, M., H. Buteau-Lozano, G. Meduri, M. Osborne-Pellegrin, S. Sordello, J. Plouët and M. Perrot-Applanat (2002). "A dynamic shift of VEGF isoforms with a transient and selective progesterone-induced expression of VEGF₁₈₉ regulates angiogenesis and vascular permeability in human uterus." Proceedings of the National Academy of Sciences **99**(9): 6023-6028.

Andescavage, N. N., A. du Plessis and C. Limperopoulos (2015). "Advanced MR imaging of the placenta: Exploring the in utero placenta-brain connection." Semin Perinatol **39**(2): 113-123.

Anthony, R. V., A. N. Scheaffer, C. D. Wright and T. R. Regnault (2003). "Ruminant models of prenatal growth restriction." Reprod Suppl **61**: 183-194.

Aplin, J. (2017). "Hematopoiesis Vasculogenesis and Angiogenesis in First Trimester Placenta." Placenta **57**: 235-235.

Autiero, M., J. Waltenberger, D. Communi, A. Kranz, L. Moons, D. Lambrechts, J. Kroll, S. Plaisance, M. De Mol, F. Bono, S. Kliche, G. Fellbrich, K. Ballmer-Hofer, D. Maglione, U. Mayr-Beyrle, M. Dewerchin, S. Dombrowski, D. Stanimirovic, P. Van Hummelen, C. Dehio, D. J. Hicklin, G. Persico, J.-M. Herbert, D. Communi, M. Shibuya, D. Collen, E. M. Conway and P. Carmeliet (2003). "Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1." Nature Medicine **9**: 936.

Barleon, B., S. Sozzani, D. Zhou, H. A. Weich, A. Mantovani and D. Marme (1996). "Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1." Blood **87**(8): 3336-3343.

Baron, M. H., J. Isern and S. T. Fraser (2012). "The embryonic origins of erythropoiesis in mammals." Blood **119**(21): 4828.

Barry, J. S. and R. V. Anthony (2008). "The pregnant sheep as a model for human pregnancy." Theriogenology **69**(1): 55-67.

Barry, J. S., P. J. Rozance and R. V. Anthony (2008). "An animal model of placental insufficiency-induced intrauterine growth restriction." Semin Perinatol **32**(3): 225-230.

Bates, D. O., P. P. MacMillan, J. G. Manjaly, Y. Qiu, S. J. Hudson, H. S. Bevan, A. J. Hunter, P. W. Soothill, M. Read, L. F. Donaldson and S. J. Harper (2006). "The endogenous anti-angiogenic family of splice variants of VEGF, VEGFxxxb, are down-regulated in pre-eclamptic placentae at term." Clin Sci (Lond) **110**(5): 575-585.

Belle, J., A. Ysasi, R. D. Bennett, N. Filipovic, M. I. Nejad, D. L. Trumper, M. Ackermann, W. Wagner, A. Tsuda, M. A. Konerding and S. J. Mentzer (2014). "Stretch-induced intussuceptive and sprouting angiogenesis in the chick chorioallantoic membrane." Microvascular Research **95**: 60-67.

Benirschke, K., G. J. Burton and R. N. Baergen (2012). Placental types. Pathology of the human placenta, Springer**:** 27-39.

Benton, S. J., Y. Hu, F. Xie, K. Kupfer, S. W. Lee, L. A. Magee and P. von Dadelszen (2012). "Can placental growth factor in maternal circulation identify fetuses with placental intrauterine growth restriction?" Am J Obstet Gynecol **206**(2): 163 e161-167.

Bian, Z., C. Shixia and T. Duan (2015). "First-Trimester Maternal Serum Levels of sFLT1, PGF and ADMA Predict Preeclampsia." PLoS One **10**(4): e0124684.

Black, C. and F. da Silva Costa (2018). "Biomarker Immunoassays in the Diagnosis of Preeclampsia: Calculating the sFlt1/PlGF Ratio Using the Cobas((R))e 411 Analyser." Methods Mol Biol **1710**: 9-26.

Blundell, C., Y. S. Yi, L. Ma, E. R. Tess, M. J. Farrell, A. Georgescu, L. M. Aleksunes and D. Huh (2018). "Placental Drug Transport-on-a-Chip: A Microengineered In Vitro Model of Transporter-Mediated Drug Efflux in the Human Placental Barrier." Adv Healthc Mater **7**(2).

Bogic, L. V., R. A. Brace and C. Y. Cheung (2001). "Developmental expression of vascular endothelial growth factor (VEGF) receptors and VEGF binding in ovine placenta and fetal membranes." Placenta **22**(4): 265-275.

Borowicz, P. P., D. R. Arnold, M. L. Johnson, A. T. Grazul-Bilska, D. A. Redmer and L. P. Reynolds (2007). "Placental growth throughout the last two thirds of pregnancy in sheep: vascular development and angiogenic factor expression." Biol Reprod **76**(2): 259-267.

Bouwland-Both, M. I., E. A. Steegers, J. Lindemans, H. Russcher, A. Hofman, A. J. Geurts-Moespot, F. C. Sweep, V. W. Jaddoe and R. P. Steegers-Theunissen (2013). "Maternal soluble fms-like tyrosine kinase-1, placental growth factor, plasminogen activator inhibitor-2, and folate concentrations and early fetal size: the Generation R study." Am J Obstet Gynecol **209**(2): 121 e121-111.

Breier, G. (2000). "Angiogenesis in embryonic development--a review." Placenta **21 Suppl A**: S11-15.

Burchardt, T., M. Burchardt, M. W. Chen, R. Buttyan, A. de la Taille, A. Shabsigh and R. Shabsigh (1999). "Expression of VEGF splice variants 144/145 and 205/206 in adult male tissues." IUBMB Life **48**(4): 405-408.

Burton, G. J., D. S. Charnock-Jones and E. Jauniaux (2009). "Regulation of vascular growth and function in the human placenta." Reproduction **138**(6): 895-902.
Cao, Y., W.-R. Ji, P. Qi, Å. Rosin and Y. Cao (1997). "Placenta growth factor: identification and characterization of a novel isoform generated by RNA alternative splicing." Biochemical and biophysical research communications **235**(3): 493-498.
Carmeliet, P. (2000). "Mechanisms of angiogenesis and arteriogenesis." Nature Medicine **6**(4): 389-395.
Carmeliet, P. and D. Collen (2000). "Transgenic mouse models in angiogenesis and cardiovascular disease." The Journal of Pathology **190**(3): 387-405.
Carmeliet, P., V. Ferreira, G. Breier, S. Pollefeyt, L. Kieckens, M. Gertsenstein, M. Fahrig, A. Vandenhoeck, K. Harpal and C. Eberhardt (1996). "Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele." Nature **380**(6573): 435.
Carmeliet, P., L. Moons, A. Luttun, V. Vincenti, V. Compernolle, M. De Mol, Y. Wu, F. Bon, L. Devy, H. Beck, D. Scholz, T. Acker, T. DiPalma, M. Dewerchin, A. Noel, I. Stalmans, A. Barra, S. Blacher, T. Vandendriessche, A. Ponten, U. Eriksson, K. H. Plate, J. M. Foidart, W. Schaper, D. S. Charnock-Jones, D. J. Hicklin, J. M. Herbert, D. Collen and M. G. Persico (2001). "Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions." Nature Medicine **7**(5): 575-583.
Carmeliet, P., L. Moons, A. Luttun, V. Vincenti, V. Compernolle, M. De Mol, Y. Wu, F. Bono, L. Devy and H. Beck (2001). "Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions." Nature medicine **7**(5): 575.
Carmeliet, P., Y.-S. Ng, D. Nuyens, G. Theilmeier, K. Brusselmans, I. Cornelissen, E. Ehler, V. V. Kakkar, I. Stalmans and V. Mattot (1999). "Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF 164 and VEGF 188." Nature medicine **5**(5): 495.
Carter, A. M. (2007). "Animal models of human placentation--a review." Placenta **28 Suppl A**: S41-47.
Carter, A. M. (2012). "Evolution of placental function in mammals: the molecular basis of gas and nutrient transfer, hormone secretion, and immune responses." Physiol Rev **92**(4): 1543-1576.
Carter, A. M. and A. C. Enders (2004). "Comparative aspects of trophoblast development and placentation." Reprod Biol Endocrinol **2**: 46.
Chaouat, G. and D. A. Clark (2015). "Are animal models useful or confusing in understanding the human feto-maternal relationship? A debate." J Reprod Immunol **108**: 56-64.
Charnock-Jones, D. S. (2002). "Soluble flt-1 and the angiopoietins in the development and regulation of placental vasculature." J Anat **200**(6): 607-615.
Chen, D. B. and J. Zheng (2014). "Regulation of placental angiogenesis." Microcirculation **21**(1): 15-25.
Chen, L. W., L. Y. Liu, L. Ye, A. Shen, Y. Q. Chen, T. J. Sferra and J. Peng (2013). "Patrinia scabiosaefolia inhibits colorectal cancer growth through suppression of tumor angiogenesis." Oncology Reports **30**(3): 1439-1443.
Chen, L. Y., X. Zeng, J. H. Wang, S. S. Briggs, E. O'Neill, J. L. Li, R. Leek, D. J. Kerr, A. L. Harris and S. J. Cai (2010). "Roles of Tetrahydrobiopterin in Promoting Tumor Angiogenesis." American Journal of Pathology **177**(5): 2671-2680.

Chen, M. C. and C. H. Lee (2014). "Connexin 43 suppresses tumor angiogenesis by downregulation of vascular endothelial growth factor." Cancer Research **74**(19).
Cheng, S.-Y., M. Nagane, H.-J. S. Huang and W. K. Cavenee (1997). "Intracerebral tumor-associated hemorrhage caused by overexpression of the vascular endothelial growth factor isoforms VEGF121 and VEGF165 but not VEGF189." Proceedings of the National Academy of Sciences **94**(22): 12081-12087.
Cho, G. J., G. S. Roh, H. J. Kim, Y. S. Kim, S. H. Cho, W. J. Choi, W. Y. Paik, S. S. Kang and W. S. Choi (2003). "Differential expression of placenta growth factors and their receptors in the normal and pregnancy-induced hypertensive human placentas." J Korean Med Sci **18**(3): 402-408.
Cianfarani, F., R. Tommasi, C. M. Failla, M. T. Viviano, G. Annessi, M. Papi, G. Zambruno and T. Odorisio (2006). "Granulocyte/macrophage colony-stimulating factor treatment of human chronic ulcers promotes angiogenesis associated with de novo vascular endothelial growth factor transcription in the ulcer bed." British Journal of Dermatology **154**(1): 34-41.
Clark, D., S. Smith, D. Licence, A. Evans and D. Charnock-Jones (1998). "Comparison of expression patterns for placenta growth factor, vascular endothelial growth factor (VEGF), VEGF-B and VEGF-C in the human placenta throughout gestation." Journal of Endocrinology **159**(3): 459-467.
Clark, D. E., S. K. Smith, A. M. Sharkey and D. S. Charnock-Jones (1996). "Localization of VEGF and expression of its receptors flt and KDR in human placenta throughout pregnancy." Hum Reprod **11**(5): 1090-1098.
Clauss, M., H. Weich, G. Breier, U. Knies, W. Rockl, J. Waltenberger and W. Risau (1996). "The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis." J Biol Chem **271**(30): 17629-17634.
Collins, S. L., A. W. Welsh, L. Impey, J. A. Noble and G. N. Stevenson (2017). "3D fractional moving blood volume (3D-FMBV) demonstrates decreased first trimester placental vascularity in pre-eclampsia but not the term, small for gestation age baby." PLoS One **12**(6): e0178675.
Cooper, J. C., A. M. Sharkey, J. McLaren, D. S. Charnock-Jones and S. K. Smith (1995). "Localization of vascular endothelial growth factor and its receptor, flt, in human placenta and decidua by immunohistochemistry." J Reprod Fertil **105**(2): 205-213.
Cox, B., M. Kotlyar, A. I. Evangelou, V. Ignatchenko, A. Ignatchenko, K. Whiteley, I. Jurisica, S. L. Adamson, J. Rossant and T. Kislinger (2009). "Comparative systems biology of human and mouse as a tool to guide the modeling of human placental pathology." Mol Syst Biol **5**: 279.
Cross, J., D. Baczyk, N. Dobric, M. Hemberger, M. Hughes, D. Simmons and H. Yamamoto (2003). "Genes, development and evolution of the placenta." Placenta **24**(2): 123-130.
Das, S. K., I. Chakraborty, J. Wang, S. K. Dey and L. H. Hoffman (1997). "Expression of Vascular Endothelial Growth Factor (VEGF) and VEGF-Receptor Messenger Ribonucleic Acids in the Peri-Implantation Rabbit Uterus1." Biology of Reproduction **56**(6): 1390-1399.
De Falco, S. (2012). "The discovery of placenta growth factor and its biological activity." Exp Mol Med **44**: 1-9.
De Falco, S., B. Gigante and M. G. Persico (2002). "Structure and function of placental growth factor." Trends in cardiovascular medicine **12**(6): 241-246.

De Spiegelaere, W., C. Casteleyn, W. Van den Broeck, J. Plendl, M. Bahramsoltani, P. Simoens, V. Djonov and P. Cornillie (2012). "Intussusceptive Angiogenesis: A Biologically Relevant Form of Angiogenesis." Journal of Vascular Research **49**(5): 390-404.

de Vries, C., J. A. Escobedo, H. Ueno, K. Houck, N. Ferrara and L. T. Williams (1992). "The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor." Science **255**(5047): 989-991.

Demir, R., U. A. Kayisli, Y. Seval, C. Celik-Ozenci, E. T. Korgun, A. Y. Demir-Weusten and B. Huppertz (2004). "Sequential expression of VEGF and its receptors in human placental villi during very early pregnancy: differences between placental vasculogenesis and angiogenesis." Placenta **25**(6): 560-572.

Demir, R., Y. Seval and B. Huppertz (2007). "Vasculogenesis and angiogenesis in the early human placenta." Acta Histochemica **109**(4): 257-265.

Dewerchin, M. and P. Carmeliet (2012). "PlGF: a multitasking cytokine with disease-restricted activity." Cold Spring Harbor perspectives in medicine: a011056.

DiPalma, T., M. Tucci, G. Russo, D. Maglione, C. Lago, A. Romano, S. Saccone, G. D. Valle, L. De Gregorio and T. Dragani (1996). "The placenta growth factor gene of the mouse." Mammalian Genome **7**(1): 6-12.

DiSalvo, J., M. L. Bayne, G. Conn, P. W. Kwok, P. G. Trivedi, D. D. Soderman, T. M. Palisi, K. A. Sullivan and K. A. Thomas (1995). "Purification and characterization of a naturally occurring vascular endothelial growth factor· placenta growth factor heterodimer." Journal of Biological Chemistry **270**(13): 7717-7723.

Douglas, N. C., H. Tang, R. Gomez, B. Pytowski, D. J. Hicklin, C. M. Sauer, J. Kitajewski, M. V. Sauer and R. C. Zimmermann (2009). "Vascular endothelial growth factor receptor 2 (VEGFR-2) functions to promote uterine decidual angiogenesis during early pregnancy in the mouse." Endocrinology **150**(8): 3845-3854.

Eichhorn, M. E., A. Kleespies, M. K. Angele, K. W. Jauch and C. J. Bruns (2007). "Angiogenesis in cancer: molecular mechanisms, clinical impact." Langenbecks Archives of Surgery **392**(3): 371-379.

Eisma, R. J., J. D. Spiro and D. L. Kreutzer (1997). "Vascular endothelial growth factor expression in head and neck squamous cell carcinoma." Am J Surg **174**(5): 513-517.

El Gehani, K., L. Al-Kikhia, N. Mansuri, K. Syrjanen, O. Al-Fituri and A. Elzagheid (2011). "Angiogenesis in urinary bladder carcinoma as defined by microvessel density (MVD) after immunohistochemical staining for Factor VIII and CD31." Libyan Journal of Medicine **6**.

Ellis, L. M., W. B. Liu, Y. Do Jung, N. Reinmuth, O. Stoeltzing, A. Takeda, M. Akagi, A. A. Parikh and S. Ahmad (2002). "Synopsis of angiogenesis inhibitors in oncology." Oncology-New York **16**(5): 14-22.

Enders, A. C. and A. M. Carter (2004). "What can comparative studies of placental structure tell us?--A review." Placenta **25 Suppl A**: S3-9.

Eriksson, A., R. Cao, R. Pawliuk, S. M. Berg, M. Tsang, D. Zhou, C. Fleet, K. Tritsaris, S. Dissing, P. Leboulch and Y. Cao (2002). "Placenta growth factor-1 antagonizes VEGF-induced angiogenesis and tumor growth by the formation of functionally inactive PlGF-1/VEGF heterodimers." Cancer Cell **1**(1): 99-108.

Eriksson, A., R. H. Cao, R. Pawliuk, S. M. Berg, M. Tsang, D. Zhou, C. Fleet, K. Tritsaris, S. Dissing, P. Leboulch and Y. H. Cao (2002). "Placenta growth factor-1 antagonizes VEGF-induced angiogenesis and tumor growth by the formation of functionally inactive PlGF-1/VEGF heterodimers." Cancer Cell **1**(1): 99-108.

Eswarappa, S. M. and P. L. Fox (2015). "Antiangiogenic VEGF-Ax: A New Participant in Tumor Angiogenesis." Cancer Res **75**(14): 2765-2769.
Fan, Y., A. Arif, Y. Q. Gong, J. Jia, S. M. Eswarappa, B. Willard, A. Horowitz, L. M. Graham, M. S. Penn and P. L. Fox (2012). "Stimulus-dependent phosphorylation of profilin-1 in angiogenesis." Nature Cell Biology **14**(10): 1046-+.
Feige, J. J. (2010). "Tumor angiogenesis: recent progress and remaining challenges." Bulletin Du Cancer **97**(11): 1305-1310.
Ferrara, N. (2000). "Vascular endothelial growth factor and the regulation of angiogenesis." Recent Progress in Hormone Research, Vol 55 **55**: 15-36.
Ferrara, N., H.-P. Gerber and J. LeCouter (2003). "The biology of VEGF and its receptors." Nature medicine **9**(6): 669.
Ferrara, N. and W. J. Henzel (1989). "Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells." Biochemical and biophysical research communications **161**(2): 851-858.
Ferreira, M. A. N. D., L. S. Barcelos, M. M. Teixeira, Y. S. Bakhle and S. P. Andrade (2007). "Tumor growth, angiogenesis and inflammation in mice lacking receptors for platelet activating factor (PAF)." Life Sciences **81**(3): 210-217.
Fischer, C., B. Jonckx, M. Mazzone, S. Zacchigna, S. Loges, L. Pattarini, E. Chorianopoulos, L. Liesenborghs, M. Koch and M. De Mol (2007). "Anti-PlGF inhibits growth of VEGF (R)-inhibitor-resistant tumors without affecting healthy vessels." Cell **131**(3): 463-475.
Fischer, C., B. Jonckx, M. Mazzone, S. Zacchigna, S. Loges, L. Pattarini, E. Chorianopoulos, L. Liesenborghs, M. Koch, M. De Mol, M. Autiero, S. Wyns, S. Plaisance, L. Moons, N. van Rooijen, M. Giacca, J. M. Stassen, M. Dewerchin, D. Collen and P. Carmeliet (2007). "Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels." Cell **131**(3): 463-475.
Fischer, C., M. Mazzone, B. Jonckx and P. Carmeliet (2008). "FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy?" Nat Rev Cancer **8**(12): 942-956.
Fong, G.-H., J. Rossant, M. Gertsenstein and M. L. Breitman (1995). "Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium." Nature **376**: 66.
Fraisl, P., M. Mazzone, T. Schmidt and P. Carmeliet (2009). "Regulation of angiogenesis by oxygen and metabolism." Developmental cell **16**(2): 167-179.
Furuya, M., K. Kurasawa, K. Nagahama, K. Kawachi, A. Nozawa, T. Takahashi and I. Aoki (2011). "Disrupted balance of angiogenic and antiangiogenic signalings in preeclampsia." Journal of pregnancy **2011**.
Gigante, B., G. Morlino, M. T. Gentile, M. G. Persico and S. De Falco (2006). "Plgf(-/-) eNos(-/-) mice show defective angiogenesis associated with increased oxidative stress in response to tissue ischemia." Faseb Journal **20**(7): 970-+.
Gram, A., B. Hoffmann, A. Boos and M. P. Kowalewski (2015). "Expression and localization of vascular endothelial growth factor A (VEGFA) and its two receptors (VEGFR1/FLT1 and VEGFR2/FLK1/KDR) in the canine corpus luteum and utero-placental compartments during pregnancy and at normal and induced parturition." Gen Comp Endocrinol **223**: 54-65.
Grigsby, P. L. (2016). "Animal Models to Study Placental Development and Function throughout Normal and Dysfunctional Human Pregnancy." Seminars in reproductive medicine **34**(1): 11-16.

Grunstein, J., J. J. Masbad, R. Hickey, F. Giordano and R. S. Johnson (2000). "Isoforms of Vascular Endothelial Growth Factor Act in a Coordinate Fashion To Recruit and Expand Tumor Vasculature." Molecular and Cellular Biology **20**(19): 7282-7291.

Guimaraes Filho, H. A., L. L. da Costa, E. Araujo Junior, L. M. Nardozza, P. M. Nowak, A. F. Moron, R. Mattar and C. R. Pires (2008). "Placenta: angiogenesis and vascular assessment through three-dimensional power Doppler ultrasonography." Arch Gynecol Obstet **277**(3): 195-200.

Guttmacher, A. E. and C. Y. Spong (2015). "The human placenta project: it's time for real time." Am J Obstet Gynecol **213**(4 Suppl): S3-5.

Haeger, J. D., N. Hambruch and C. Pfarrer (2016). "The bovine placenta in vivo and in vitro." Theriogenology **86**(1): 306-312.

Hafez, S. A., T. Caceci, L. E. Freeman and K. E. Panter (2007). "Angiogenesis in the caprine caruncles in non-pregnant and pregnant normal and swainsonine-treated does." Anat Rec (Hoboken) **290**(7): 761-769.

Halder, J. B., X. Zhao, S. Soker, B. C. Paria, M. Klagsbrun, S. K. Das and S. K. Dey (2000). "Differential expression of VEGF isoforms and VEGF164-specific receptor neuropilin-1 in the mouse uterus suggests a role for VEGF164 in vascular permeability and angiogenesis during implantation." genesis **26**(3): 213-224.

He, Y., S. K. Smith, K. A. Day, D. E. Clark, D. R. Licence and D. S. Charnock-Jones (1999). "Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity." Mol Endocrinol **13**(4): 537-545.

Heldin, C. H. and B. Westermark (1999). "Mechanism of action and in vivo role of platelet-derived growth factor." Physiol Rev **79**(4): 1283-1316.

Herr, F., N. Baal, R. Widmer-Teske, T. McKinnon and M. Zygmunt (2010). "How to study placental vascular development?" Theriogenology **73**(6): 817-827.

Herr, F., N. Baal and M. Zygmunt (2009). "Studies of placental vasculogenesis: a way to understand pregnancy pathology?" Z Geburtshilfe Neonatol **213**(3): 96-100.

Heydarian, M., T. McCaffrey, L. Florea, Z. Yang, M. M. Ross, W. Zhou and S. E. Maynard (2009). "Novel splice variants of sFlt1 are upregulated in preeclampsia." Placenta **30**(3): 250-255.

Hiratsuka, S., O. Minowa, J. Kuno, T. Noda and M. Shibuya (1998). "Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice." Proceedings of the National Academy of Sciences of the United States of America **95**(16): 9349-9354.

Hiratsuka, S., K. Nakamura, S. Iwai, M. Murakami, T. Itoh, H. Kijima, J. M. Shipley, R. M. Senior and M. Shibuya (2002). "MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis." Cancer Cell **2**(4): 289-300.

Houck, K. A., N. Ferrara, J. Winer, G. Cachianes, B. Li and D. W. Leung (1991). "The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA." Molecular endocrinology **5**(12): 1806-1814.

Houck, K. A., D. Leung, A. Rowland, J. Winer and N. Ferrara (1992). "Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms." Journal of Biological Chemistry **267**(36): 26031-26037.

Huang, H., J. He, D. Johnson, Y. Wei, Y. Liu, S. Wang, G. A. Lutty, E. J. Duh and R. D. Semba (2015). "Deletion of placental growth factor prevents diabetic retinopathy and is associated with Akt activation and HIF1alpha-VEGF pathway inhibition." Diabetes **64**(1): 200-212.

Huckle, W. R. (2017). "Cell- and Tissue-Based Models for Study of Placental Development." Prog Mol Biol Transl Sci **145**: 29-37.
Huppertz, B. (2008). "The anatomy of the normal placenta." J Clin Pathol **61**(12): 1296-1302.
Jaba, I. M., Z. W. Zhuang, N. Li, Y. F. Jiang, K. A. Martin, A. J. Sinusas, X. Papademetris, M. Simons, W. C. Sessa, L. H. Young and D. Tirziu (2013). "NO triggers RGS4 degradation to coordinate angiogenesis and cardiomyocyte growth." Journal of Clinical Investigation **123**(4): 1718-1731.
Katoh, O., H. Tauchi, K. Kawaishi, A. Kimura and Y. Satow (1995). "Expression of the vascular endothelial growth factor (VEGF) receptor gene, KDR, in hematopoietic cells and inhibitory effect of VEGF on apoptotic cell death caused by ionizing radiation." Cancer Research **55**(23): 5687-5692.
Kerber, M., Y. Reiss, A. Wickersheim, M. Jugold, F. Kiessling, M. Heil, V. Tchaikovski, J. Waltenberger, M. Shibuya, K. H. Plate and M. R. Machein (2008). "Flt-1 signaling in macrophages promotes glioma growth in vivo." Cancer Res **68**(18): 7342-7351.
Keyt, B. A., L. T. Berleau, H. V. Nguyen, H. Chen, H. Heinsohn, R. Vandlen and N. Ferrara (1996). "The carboxyl-terminal domain (111165) of vascular endothelial growth factor is critical for its mitogenic potency." Journal of Biological Chemistry **271**(13): 7788-7795.
Keyt, B. A., H. V. Nguyen, L. T. Berleau, C. M. Duarte, J. Park, H. Chen and N. Ferrara (1996). "Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors. Generation of receptor-selective VEGF variants by site-directed mutagenesis." J Biol Chem **271**(10): 5638-5646.
Khaliq, A., X. F. Li, M. Shams, P. Sisi, C. A. Acevedo, M. J. Whittle, H. Weigh and A. Ahmed (1996). "Localisation of placenta growth factor (PlGF) in human term placenta." Growth factors **13**(3-4): 243-250.
Kim, A., M. Im, N. H. Yim and J. Y. Ma (2014). "Reduction of metastatic and angiogenic potency of malignant cancer by Eupatorium fortunei via suppression of MMP-9 activity and VEGF production." Sci Rep **4**: 6994.
King, B. F. (1993). "Development and structure of the placenta and fetal membranes of nonhuman primates." J Exp Zool **266**(6): 528-540.
Küsters, B., R. M. W. de Waal, P. Wesseling, K. Verrijp, C. Maass, A. Heerschap, J. O. Barentsz, F. Sweep, D. J. Ruiter and W. P. J. Leenders (2003). "Differential Effects of Vascular Endothelial Growth Factor A Isoforms in a Mouse Brain Metastasis Model of Human Melanoma." Cancer Research **63**(17): 5408-5413.
Ladomery, M. R., S. J. Harper and D. O. Bates (2007). "Alternative splicing in angiogenesis: The vascular endothelial growth factor paradigm." Cancer Letters **249**(2): 133-142.
Lam, C., K. H. Lim and S. A. Karumanchi (2005). "Circulating angiogenic factors in the pathogenesis and prediction of preeclampsia." Hypertension **46**(5): 1077-1085.
Larcher, F., M. Franco, M. Bolontrade, M. Rodriguez-Puebla, L. Casanova, M. Navarro, G. Yancopoulos, J. L. Jorcano and C. J. Conti (2003). "Modulation of the angiogenesis response through Ha-ras control., placenta growth factor, and angiopoietin expression in mouse skin carcinogenesis." Molecular Carcinogenesis **37**(2): 83-90.
Leach, L., M. O. Babawale, M. Anderson and M. Lammiman (2002). "Vasculogenesis, angiogenesis and the molecular organisation of endothelial junctions in the early human placenta." Journal of Vascular Research **39**(3): 246-259.

Lee, J. S., R. Romero, Y. M. Han, H. C. Kim, C. J. Kim, J.-S. Hong and D. Huh (2016). "Placenta-on-a-chip: a novel platform to study the biology of the human placenta." The Journal of Maternal-Fetal & Neonatal Medicine **29**(7): 1046-1054.

Lee, S., S. M. Jilani, G. V. Nikolova, D. Carpizo and M. L. Iruela-Arispe (2005). "Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors." The Journal of Cell Biology **169**(4): 681-691.

Lee, W. S., B. J. Pyun, S. W. Kim, S. R. Shim, J. R. Nam, J. Y. Yoo, Y. Jin, J. Jin, Y. G. Kwon, C. O. Yun, D. H. Nam, K. Oh, D. S. Lee, S. H. Lee and J. S. Yoo (2015). "TTAC-0001, a human monoclonal antibody targeting VEGFR-2/KDR, blocks tumor angiogenesis." MAbs **7**(5): 957-968.

Leiser, R. (1981). "Development of the Trophoblast in the Early Carnivore Placenta as Exemplified by the Cat." Acta Anatomica **111**(1-2): 88-89.

Leiser, R. and P. Kaufmann (1994). "Placental structure: in a comparative aspect." Exp Clin Endocrinol **102**(3): 122-134.

Leiser, R. and T. Kohler (1983). "The Blood-Vessels of the Cat Girdle Placenta - Observations on Corrosion Casts, Scanning Electron Microscopical and Histological Studies .1. Maternal Vasculature." Anatomy and Embryology **167**(1): 85-93.

Leiser, R. and B. Koob (1993). "Development and characteristics of placentation in a carnivore, the domestic cat." J Exp Zool **266**(6): 642-656.

Lemery Magnin, M., V. Fitoussi, N. Siauve, L. Pidial, D. Balvay, G. Autret, C. A. Cuenod, O. Clement and L. J. Salomon (2018). "Assessment of Placental Perfusion in the Preeclampsia L-NAME Rat Model with High-Field Dynamic Contrast-Enhanced MRI." Fetal Diagn Ther: 1-8.

Levine, R. J., S. E. Maynard, C. Qian, K. H. Lim, L. J. England, K. F. Yu, E. F. Schisterman, R. Thadhani, B. P. Sachs, F. H. Epstein, B. M. Sibai, V. P. Sukhatme and S. A. Karumanchi (2004). "Circulating angiogenic factors and the risk of preeclampsia." N Engl J Med **350**(7): 672-683.

Lewitus, E. and C. Soligo (2011). "Life-History Correlates of Placental Structure in Eutherian Evolution." Evolutionary Biology **38**(3): 287.

Loegl, J., U. Hiden, E. Nussbaumer, C. Schliefsteiner, S. Cvitic, I. Lang, C. Wadsack, B. Huppertz and G. Desoye (2016). "Hofbauer cells of M2a, M2b and M2c polarization may regulate feto-placental angiogenesis." Reproduction **152**(5): 447-455.

Lohela, M., A. Saaristo, T. Veikkola and K. Alitalo (2003). "Lymphangiogenic growth factors, receptors and therapies." Thromb Haemost **90**(2): 167-184.

Longo, L. D. and L. P. Reynolds (2010). "Some historical aspects of understanding placental development, structure and function." Int J Dev Biol **54**(2-3): 237-255.

Luttun, A., M. Autiero, M. Tjwa and P. Carmeliet (2004). "Genetic dissection of tumor angiogenesis: are PlGF and VEGFR-1 novel anti-cancer targets?" Biochimica Et Biophysica Acta-Reviews on Cancer **1654**(1): 79-94.

Maes, C., I. Stockmans, K. Moermans, R. Van Looveren, N. Smets, P. Carmeliet, R. Bouillon and G. Carmeliet (2004). "Soluble VEGF isoforms are essential for establishingepiphyseal vascularization and regulating chondrocyte development and survival." The Journal of Clinical Investigation **113**(2): 188-199.

Maglione, D., V. Guerriero, G. Viglietto, P. Delli-Bovi and M. G. Persico (1991). "Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor." Proc Natl Acad Sci U S A **88**(20): 9267-9271.

Marcellini, M., N. De Luca, T. Riccioni, A. Ciucci, A. Orecchia, P. M. Lacal, F. Ruffini, M. Pesce, F. Cianfarani and G. Zambruno (2006). "Increased melanoma growth and metastasis spreading in mice overexpressing placenta growth factor." The American journal of pathology **169**(2): 643-654.
Maruotti, N. and D. Ribatti (2008). "The role of vascular endothelial growth factor in angiogenesis." Salud I Ciencia **16**(4): 383-386.
Mateos, C. D., C. B. Zamora, J. A. Lafuente and J. M. G. Aznar (2017). "Quantification of sprouting angiogenesis under the effect of different growth factors involved in the tumor microenvironment." Cancer Immunology Research **5**(3).
Matthews, W., C. T. Jordan, M. Gavin, N. A. Jenkins, N. G. Copeland and I. R. Lemischka (1991). "A receptor tyrosine kinase cDNA isolated from a population of enriched primitive hematopoietic cells and exhibiting close genetic linkage to c-kit." Proceedings of the National Academy of Sciences **88**(20): 9026-9030.
McCarthy, F. P., J. C. Kingdom, L. C. Kenny and S. K. Walsh (2011). "Animal models of preeclampsia; uses and limitations." Placenta **32**(6): 413-419.
Miglino, M. A., C. E. Ambrosio, D. dos Santos Martins, C. V. Wenceslau, C. Pfarrer and R. Leiser (2006). "The carnivore pregnancy: the development of the embryo and fetal membranes." Theriogenology **66**(6-7): 1699-1702.
Millanta, F., G. Silvestri, C. Vaselli, S. Citi, G. Pisani, D. Lorenzi and A. Poli (2006). "The role of vascular endothelial growth factor and its receptor Flk-1/KDR in promoting tumour angiogenesis in feline and canine mammary carcinomas: A preliminary study of autocrine and paracrine loops." Research in Veterinary Science **81**(3): 350-357.
Moe, K., H. Heidecke, R. Dechend and A. C. Staff (2017). "Dysregulation of circulating autoantibodies against VEGF-A, VEGFR-1 and PlGF in preeclampsia - A role in placental and vascular health?" Pregnancy Hypertens **10**: 83-89.
Murakami, M., S. Iwai, S. Hiratsuka, M. Yamauchi, K. Nakamura, Y. Iwakura and M. Shibuya (2006). "Signaling of vascular endothelial growth factor receptor-1 tyrosine kinase promotes rheumatoid arthritis through activation of monocytes/macrophages." Blood **108**(6): 1849-1856.
Murphy, W. J., D. M. Larkin, A. Everts-van der Wind, G. Bourque, G. Tesler, L. Auvil, J. E. Beever, B. P. Chowdhary, F. Galibert, L. Gatzke, C. Hitte, S. N. Meyers, D. Milan, E. A. Ostrander, G. Pape, H. G. Parker, T. Raudsepp, M. B. Rogatcheva, L. B. Schook, L. C. Skow, M. Welge, J. E. Womack, J. O'Brien S, P. A. Pevzner and H. A. Lewin (2005). "Dynamics of mammalian chromosome evolution inferred from multispecies comparative maps." Science **309**(5734): 613-617.
Niida, S., T. Kondo, S. Hiratsuka, S.-I. Hayashi, N. Amizuka, T. Noda, K. Ikeda and M. Shibuya (2005). "VEGF receptor 1 signaling is essential for osteoclast development and bone marrow formation in colony-stimulating factor 1-deficient mice." Proceedings of the National Academy of Sciences of the United States of America **102**(39): 14016-14021.
O'Brien, S. J., W. Johnson, C. Driscoll, J. Pontius, J. Pecon-Slattery and M. Menotti-Raymond (2008). "State of cat genomics." Trends in Genetics **24**(6): 268-279.
Odorisio, T., F. Cianfarani, C. M. Failla and G. Zambruno (2006). "The placenta growth factor in skin angiogenesis." Journal of Dermatological Science **41**(1): 11-19.
Olofsson, B., E. Korpelainen, M. S. Pepper, S. J. Mandriota, K. Aase, V. Kumar, Y. Gunji, M. M. Jeltsch, M. Shibuya, K. Alitalo and U. Eriksson (1998). "Vascular endothelial growth

factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells." Proceedings of the National Academy of Sciences **95**(20): 11709-11714.
Olofsson, B., K. Pajusola, A. Kaipainen, G. Von Euler, V. Joukov, O. Saksela, A. Orpana, R. F. Pettersson, K. Alitalo and U. Eriksson (1996). "Vascular endothelial growth factor B, a novel growth factor for endothelial cells." Proceedings of the National Academy of Sciences **93**(6): 2576-2581.
Pang, V., D. O. Bates and L. Leach (2017). "Regulation of human feto-placental endothelial barrier integrity by vascular endothelial growth factors: competitive interplay between VEGF-A165a, VEGF-A165b, PIGF and VE-cadherin." Clin Sci (Lond) **131**(23): 2763-2775.
Pardali, E., R. Godfrey and J. Waltenberger (2013). "VEGF Signaling in Normal and Tumor Angiogenesis." Tumor Angiogenesis Regulators: 1-36.
Park, J. E., G.-A. Keller and N. Ferrara (1993). "The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF." Molecular biology of the cell **4**(12): 1317-1326.
Patan, S. (2004). "Vasculogenesis and angiogenesis." Cancer Treat Res **117**: 3-32.
Persico, M., V. Vincenti and T. DiPalma (1999). Structure, expression and receptor-binding properties of placenta growth factor (PlGF). Vascular growth factors and angiogenesis, Springer**:** 31-40.
Pfarrer, C. D., S. D. Ruziwa, H. Winther, H. Callesen, R. Leiser, D. Schams and V. Dantzer (2006). "Localization of vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 and VEGFR-2 in bovine placentomes from implantation until term." Placenta **27**(8): 889-898.
Plouet, J., J. Schilling and D. Gospodarowicz (1989). "Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT□20 cells." The EMBO journal **8**(12): 3801-3806.
Pontius, J. U., J. C. Mullikin, D. R. Smith, K. Lindblad-Toh, S. Gnerre, M. Clamp, J. Chang, R. Stephens, B. Neelam, N. Volfovsky, A. A. Schaffer, R. Agarwala, K. Narfstrom, W. J. Murphy, U. Giger, A. L. Roca, A. Antunes, M. Menotti-Raymond, N. Yuhki, J. Pecon-Slattery, W. E. Johnson, G. Bourque, G. Tesler and S. J. O'Brien (2007). "Initial sequence and comparative analysis of the cat genome." Genome Res **17**(11): 1675-1689.
Poole, T. J. and J. D. Coffin (1989). "Vasculogenesis and angiogenesis: two distinct morphogenetic mechanisms establish embryonic vascular pattern." J Exp Zool **251**(2): 224-231.
Rabbani, M. L. and P. A. Rogers (2001). "Role of vascular endothelial growth factor in endometrial vascular events before implantation in rats." Reproduction **122**(1): 85-90.
Ramsey, E. M. (1982). The placenta : human and animal. New York, N.Y., Praeger.
Ravi, V., E. M. Sanford, W. L. Wang, J. S. Ross, N. Ramesh, A. Futreal, S. Patel, P. J. Stephens, V. A. Miller and S. M. Ali (2016). "Antitumor Response of VEGFR2- and VEGFR3-Amplified Angiosarcoma to Pazopanib." J Natl Compr Canc Netw **14**(5): 499-502.
Rawn, S. M. and J. C. Cross (2008). "The evolution, regulation, and function of placenta-specific genes." Annual review of cell and developmental biology **24**: 159-181.
Reynolds, L. P., P. P. Borowicz, J. S. Caton, K. A. Vonnahme, J. S. Luther, D. S. Buchanan, S. A. Hafez, A. T. Grazul-Bilska and D. A. Redmer (2010). "Uteroplacental vascular development and placental function: an update." Int J Dev Biol **54**(2-3): 355-366.

Reynolds, L. P., P. P. Borowicz, K. A. Vonnahme, M. L. Johnson, A. T. Grazul-Bilska, D. A. Redmer and J. S. Caton (2005). "Placental angiogenesis in sheep models of compromised pregnancy." J Physiol **565**(Pt 1): 43-58.
Reynolds, L. P., P. P. Borowicz, K. A. Vonnahme, M. L. Johnson, A. T. Grazul-Bilska, J. M. Wallace, J. S. Caton and D. A. Redmer (2005). "Animal models of placental angiogenesis." Placenta **26**(10): 689-708.
Reynolds, L. P. and D. A. Redmer (2001). "Angiogenesis in the Placenta." Biology of Reproduction **64**(4): 1033-1040.
Risau, W. (1997). "Mechanisms of angiogenesis." Nature **386**: 671.
Risau, W. and I. Flamme (1995). "Vasculogenesis." Annu Rev Cell Dev Biol **11**: 73-91.
Rockwell, L. C., S. Pillai, C. E. Olson and R. D. Koos (2002). "Inhibition of Vascular Endothelial Growth Factor/Vascular Permeability Factor Action Blocks Estrogen-Induced Uterine Edema and Implantation in Rodents1." Biology of Reproduction **67**(6): 1804-1810.
Roescher, A. M., A. Timmer, J. J. H. Erwich and A. F. Bos (2014). "Placental pathology, perinatal death, neonatal outcome, and neurological development: a systematic review." PloS one **9**(2): e89419.
Ruhrberg, C., H. Gerhardt, M. Golding, R. Watson, S. Ioannidou, H. Fujisawa, C. Betsholtz and D. T. Shima (2002). "Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis." Genes & development **16**(20): 2684-2698.
Ryan, D. H., F. P. McCarthy, K. O'Donoghue and L. C. Kenny (2018). "Placental Growth Factor: A review of literature and future applications." Pregnancy hypertension.
Salavati, N., S. J. Gordijn, U. Sovio, E. H. R. Zill, A. Gebril, D. S. Charnock-Jones, S. A. Scherjon and G. C. S. Smith (2018). "Birth weight to placenta weight ratio and its relationship to ultrasonic measurements, maternal and neonatal morbidity: A prospective cohort study of nulliparous women." Placenta **63**: 45-52.
Saleh, L., A. H. van den Meiracker, R. Geensen, A. Kaya, J. E. Roeters van Lennep, J. J. Duvekot, K. Verdonk, E. A. P. Steegers, H. Russcher, A. H. J. Danser and W. Visser (2018). "Soluble fms-like tyrosine kinase-1 and placental growth factor kinetics during and after pregnancy in women with suspected or confirmed pre-eclampsia." Ultrasound Obstet Gynecol **51**(6): 751-757.
Sammin, D., B. Markey, H. Bassett and D. Buxton (2009). "The ovine placenta and placentitis-A review." Vet Microbiol **135**(1-2): 90-97.
Sato, M., J. Noguchi, M. Mashima, H. Tanaka and T. Hata (2016). "3D power Doppler ultrasound assessment of placental perfusion during uterine contraction in labor." Placenta **45**: 32-36.
Sato, T., H. Amano, Y. Ito, K. Eshima, T. Minamino, T. Ae, C. Katada, T. Ohno, K. Hosono, T. Suzuki, M. Shibuya, W. Koizumi and M. Majima (2013). "NSAID, aspirin delays gastric ulcer healing with reduced accumulation of CXCR4(+)VEGFR1(+) cells to the ulcer granulation tissues." Biomed Pharmacother **67**(7): 607-613.
Satoshi, F., K. Yusuke and S. Akihiko (2014). "A Comparison of the Histological Structure of the Placenta in Experimental Animals." Journal of Toxicologic Pathology **27**(1): 11-18.
Sawano, A., S. Iwai, Y. Sakurai, M. Ito, K. Shitara, T. Nakahata and M. Shibuya (2001). "Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans." Blood **97**(3): 785-791.

Sawano, A., T. Takahashi, S. Yamaguchi, M. Aonuma and M. Shibuya (1996). "Flt-1 but not KDR/Flk-1 tyrosine kinase is a receptor for placenta growth factor, which is related to vascular endothelial growth factor." Cell Growth Differ **7**(2): 213-221.

Schafer-Somi, S., S. Sabitzer, D. Klein, E. Reinbacher, H. Kanca, H. B. Beceriklisoy, O. A. Aksoy, I. Kucukaslan, H. C. Macun and S. Aslan (2013). "Vascular endothelial (VEGF) and epithelial growth factor (EGF) as well as platelet-activating factor (PAF) and receptors are expressed in the early pregnant canine uterus." Reprod Domest Anim **48**(1): 20-26.

Schmidt, A., D. M. Morales-Prieto, J. Pastuschek, K. Frohlich and U. R. Markert (2015). "Only humans have human placentas: molecular differences between mice and humans." J Reprod Immunol.

Schwartz, J. D., E. K. Rowinsky, H. Youssoufian, B. Pytowski and Y. Wu (2010). "Vascular endothelial growth factor receptor-1 in human cancer: concise review and rationale for development of IMC-18F1 (Human antibody targeting vascular endothelial growth factor receptor-1)." Cancer **116**(4 Suppl): 1027-1032.

Semenza, G. L. (2007). "Vasculogenesis, angiogenesis, and arteriogenesis: Mechanisms of blood vessel formation and remodeling." Journal of Cellular Biochemistry **102**(4): 840-847.

Senger, D. R., S. J. Galli, A. M. Dvorak, C. A. Perruzzi, V. S. Harvey and H. F. Dvorak (1983). "Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid." Science **219**(4587): 983-985.

Shibuya, M. (2013). "Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases." Journal of Biochemistry **153**(1): 13-19.

Shibuya, M. and L. Claesson-Welsh (2006). "Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis." Exp Cell Res **312**(5): 549-560.

Shibuya, M., S. Yamaguchi, A. Yamane, T. Ikeda, A. Tojo, H. Matsushime and M. Sato (1990). "Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family." Oncogene **5**(4): 519-524.

Silvan, U., A. Diez-Torre, Z. Bonilla, P. Moreno, M. Diaz-Nunez and J. Arechaga (2015). "Vasculogenesis and angiogenesis in nonseminomatous testicular germ cell tumors." Urologic Oncology-Seminars and Original Investigations **33**(6).

Spiel, M., S. Salahuddin, E. Pernicone, Z. Zsengeller, A. Wang, A. M. Modest, S. A. Karumanchi and J. L. Hecht (2017). "Placental soluble fms-like tyrosine kinase expression in small for gestational age infants and risk for adverse outcomes." Placenta **52**: 10-16.

Sugino, N., S. Kashida, A. Karube-Harada, S. Takiguchi and H. Kato (2002). "Expression of vascular endothelial growth factor (VEGF) and its receptors in human endometrium throughout the menstrual cycle and in early pregnancy." Reproduction **123**(3): 379-387.

Sun, Q., J. Zhou, Z. Zhang, M. Guo, J. Liang, F. Zhou, J. Long, W. Zhang, F. Yin, H. Cai, H. Yang, W. Zhang, Y. Gu, L. Ni, Y. Sai, Y. Cui, M. Zhang, M. Hong, J. Sun, Z. Yang, W. Qing, W. Su and Y. Ren (2014). "Discovery of fruquintinib, a potent and highly selective small molecule inhibitor of VEGFR 1, 2, 3 tyrosine kinases for cancer therapy." Cancer Biol Ther **15**(12): 1635-1645.

Suzuki, H., A. Ohkuchi, S. Matsubara, Y. Takei, M. Murakami, M. Shibuya, M. Suzuki and Y. Sato (2009). "Effect of recombinant placental growth factor 2 on hypertension induced by full-length mouse soluble fms-like tyrosine kinase 1 adenoviral vector in pregnant mice." Hypertension **54**(5): 1129-1135.

Takahashi, H. and M. Shibuya (2005). "The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions." Clin Sci (Lond) **109**(3): 227-241.

Takizawa, T., H. Eguchi, S. Namimatsu, U. Jeschke, R. Fuchs and J. M. Robinson (2007). "Histochemistry for placenta research: theory and application." J Nippon Med Sch **74**(4): 268-273.

Tantawy, A. A. G., A. A. M. Adly, E. A. R. Ismail, O. I. Youssef and M. E. Ali (2017). "Soluble fms-Like Tyrosine Kinase 1 as a Link Between Angiogenesis and Endothelial Dysfunction in Pediatric Patients With beta-Thalassemia Intermedia." Clin Appl Thromb Hemost **23**(8): 943-950.

Tarallo, V., L. Vesci, O. Capasso, M. T. Esposito, T. Riccioni, L. Pastore, A. Orlandi, C. Pisano and S. De Falco (2010). "A placental growth factor variant unable to recognize vascular endothelial growth factor (VEGF) receptor-1 inhibits VEGF-dependent tumor angiogenesis via heterodimerization." Cancer research: 0008-5472. CAN-0009-2609.

Tardif, C., E. Dumontet, H. Caillon, E. Misbert, V. Dochez, D. Masson and N. Winer (2018). "Angiogenic factors sFlt-1 and PlGF in preeclampsia: Prediction of risk and prognosis in a high-risk obstetric population." J Gynecol Obstet Hum Reprod **47**(1): 17-21.

Tiedemann, K. (1977). "On the yolk sac of the cat." Cell and Tissue Research **183**(1): 71-89.

Tischer, E., R. Mitchell, T. Hartman, M. Silva, D. Gospodarowicz, J. Fiddes and J. Abraham (1991). "The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing." Journal of Biological Chemistry **266**(18): 11947-11954.

Tozer, G. M., S. Akerman, N. A. Cross, P. R. Barber, M. A. Björndahl, O. Greco, S. Harris, S. A. Hill, D. J. Honess and C. R. Ireson (2008). "Blood vessel maturation and response to vascular-disrupting therapy in single vascular endothelial growth factor-A isoform–producing tumors." Cancer research **68**(7): 2301-2311.

Tsoi, S. C., Y. Wen, J. Y. Chung, D. Chen, R. R. Magness and J. Zheng (2002). "Co-expression of vascular endothelial growth factor and neuropilin-1 in ovine feto-placental artery endothelial cells." Mol Cell Endocrinol **196**(1-2): 95-106.

Van de Veire, S., I. Stalmans, F. Heindryckx, H. Oura, A. Tijeras-Raballand, T. Schmidt, S. Loges, I. Albrecht, B. Jonckx and S. Vinckier (2010). "Further pharmacological and genetic evidence for the efficacy of PlGF inhibition in cancer and eye disease." Cell **141**(1): 178-190.

Vempati, P., A. S. Popel and F. Mac Gabhann (2014). "Extracellular regulation of VEGF: isoforms, proteolysis, and vascular patterning." Cytokine Growth Factor Rev **25**(1): 1-19.

Viglietto, G., D. Maglione, M. Rambaldi, J. Cerutti, A. Romano, F. Trapasso, M. Fedele, P. Ippolito, G. Chiappetta, G. Botti and et al. (1995). "Upregulation of vascular endothelial growth factor (VEGF) and downregulation of placenta growth factor (PlGF) associated with malignancy in human thyroid tumors and cell lines." Oncogene **11**(8): 1569-1579.

Viita, H., J. Markkanen, E. Eriksson, M. Nurminen, K. Kinnunen, M. Babu, T. Heikura, S. Turpeinen, S. Laidinen, T. Takalo and S. Yla-Herttuala (2008). "15-lipoxygenase-1 prevents vascular endothelial growth factor A- and placental growth factor-induced angiogenic effects in rabbit skeletal muscles via reduction in growth factor mRNA levels, NO bioactivity, and downregulation of VEGF receptor 2 expression." Circ Res **102**(2): 177-184.

Vuckovic, M., J. Ponting, B. I. Terman, V. Niketic, M. W. Seif and S. Kumar (1996). "Expression of the vascular endothelial growth factor receptor, KDR, in human placenta." J Anat **188 (Pt 2)**: 361-366.

Vuorela, P., E. Hatva, A. Lymboussaki, A. Kaipainen, V. Joukov, M. G. Persico, K. Alitalo and E. Halmesmäki (1997). "Expression of vascular endothelial growth factor and placenta growth factor in human placenta." Biology of reproduction **56**(2): 489-494.
Wang, H., Q. Li, H. Lin, X. Yu, D. Qian, J. Dai, E. Duan and C. Zhu (2003). "Expression of vascular endothelial growth factor and its receptors in the rhesus monkey (Macaca mulatta) endometrium and placenta during early pregnancy." Mol Reprod Dev **65**(2): 123-131.
Wang, W. J., Q. Gu, X. Xu and Y. Zheng (2017). "A PlGF-1 Derived Peptide Inhibits Angiogenesis via HIF-1 beta/VEGF Pathway." International Journal of Peptide Research and Therapeutics **23**(3): 343-355.
Wang, Y. and S. Zhao (2010). Integrated Systems Physiology: from Molecules to Function to Disease. Vascular Biology of the Placenta. San Rafael (CA), Morgan & Claypool Life Sciences
Copyright (c) 2010 by Morgan & Claypool Life Sciences.
Weerakoon, S., G. Frydman, C. Martin, K. Gudenschwager and W. Huckle (2015). "Isoforms of VEGF-A in the Developing Feline Placenta." The FASEB Journal **29**(1_supplement): LB622.
Winther, H., A. Ahmed and V. Dantzer (1999). "Immunohistochemical localization of vascular endothelial growth factor (VEGF) and its two specific receptors, Flt-1 and KDR, in the porcine placenta and non-pregnant uterus." Placenta **20**(1): 35-43.
Wooding, P. and G. Burton (2008). Endotheliochorial Placentation : Cat, Dog, Bat. Comparative Placentation: Structures, Functions and Evolution. P. Wooding and G. Burton. Berlin, Heidelberg, Springer Berlin Heidelberg**:** 169-183.
Wulff, C., H. Wilson, S. E. Dickson, S. J. Wiegand and H. M. Fraser (2002). "Hemochorial placentation in the primate: expression of vascular endothelial growth factor, angiopoietins, and their receptors throughout pregnancy." Biol Reprod **66**(3): 802-812.
Xia, S., Y. Zhen, H. Ma and A. Wang (2017). "Abnormal expression of microRNA-575 leads to missed abortion through regulating apoptosis and angiogenesis." Exp Ther Med **14**(5): 3993-4000.
Yao, Y.-G., H. S. Yang, Z. Cao, J. Danielsson and E. J. Duh (2005). "Upregulation of placental growth factor by vascular endothelial growth factor via a post-transcriptional mechanism." FEBS letters **579**(5): 1227-1234.
Yeom, G. G., S. Min and S. Y. Kim (2014). "2,3,5,6-Tetramethylpyrazine of Ephedra sinica regulates melanogenesis and inflammation in a UVA-induced melanoma/keratinocytes co-culture system." Int Immunopharmacol **18**(2): 262-269.
Yi, X. J., H. Y. Jiang, K. K. H. Lee, W. S. O, P. L. Tang and P. H. Chow (1999). "Expression of vascular endothelial growth factor (VEGF) and its receptors during embryonic implantation in the golden hamster (Mesocricetus auratus)." Cell and Tissue Research **296**(2): 339-349.
Yu, J. L., J. W. Rak, G. Klement and R. S. Kerbel (2002). "Vascular Endothelial Growth Factor Isoform Expression as a Determinant of Blood Vessel Patterning in Human Melanoma Xenografts." Cancer Research **62**(6): 1838-1846.
Zhou, J., H. Wang, H. Zhang, A. M. Lutz, L. Tian, D. Hristov and J. K. Willmann (2016). "VEGFR2-Targeted Three-Dimensional Ultrasound Imaging Can Predict Responses to Antiangiogenic Therapy in Preclinical Models of Colon Cancer." Cancer Res **76**(14): 4081-4089.

Zimna, A. and M. Kurpisz (2015). "Hypoxia-Inducible Factor-1 in Physiological and Pathophysiological Angiogenesis: Applications and Therapies." Biomed Research International.

Zygmunt, M., F. Herr, K. Munstedt, U. Lang and O. D. Liang (2003). "Angiogenesis and vasculogenesis in pregnancy." Eur J Obstet Gynecol Reprod Biol **110 Suppl 1**: S10-18.

1-14 Figures

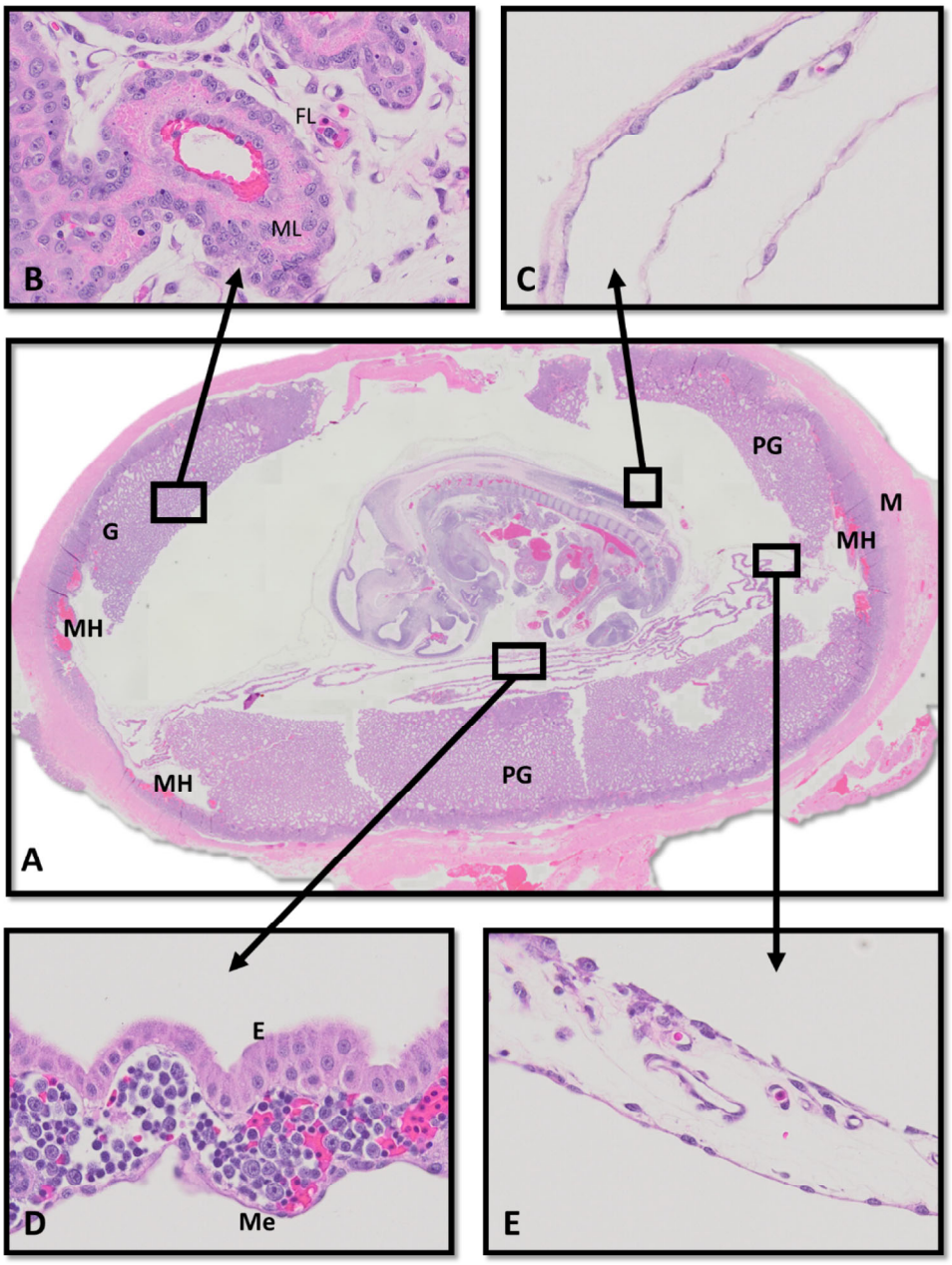


Figure 1.1 Morphologic characteristics of the placenta of the domestic cat of day 25. **A:** H&E stained whole conceptus composed of the uterus at the outside. Chorioallantoic placental girdle (**PG**) in from at back at the embryo. Note the area of the placental girdle outside the zonary placenta where lamellae are not formed. Between this area and the placenta is the marginal hematoma (**MH**), a maternal hemorrhagic zone present in carnivore placenta. The embryo is located at the center with the head at the right of the image. Even at very early age most of the organ are already distinguishable histologically. **B:** Detail of the lamellar zone. Fetal lamellae are clear with loose connective tissue; smaller blood vessel are visible. Maternal lamella has an eosinophilic color and bordered by syncytiotrophoblast cells with dark basophilic nuclei.

C: Detail of alantoamniotic membrane, a less vascularized double layer membrane in cats. The allantoic and amniotic membrane never appear to fuse. **D:** Detail of the yolk sac, composed of a thin squamous mesothelium (**Me**) The endodermal epithelium (**E**) is simple cuboidal. The intercellular spaces are distended with multiple fetal erythrocytes and other blood cell progenitors. **E:** Area of chorioallantoic membrane without placental formation.

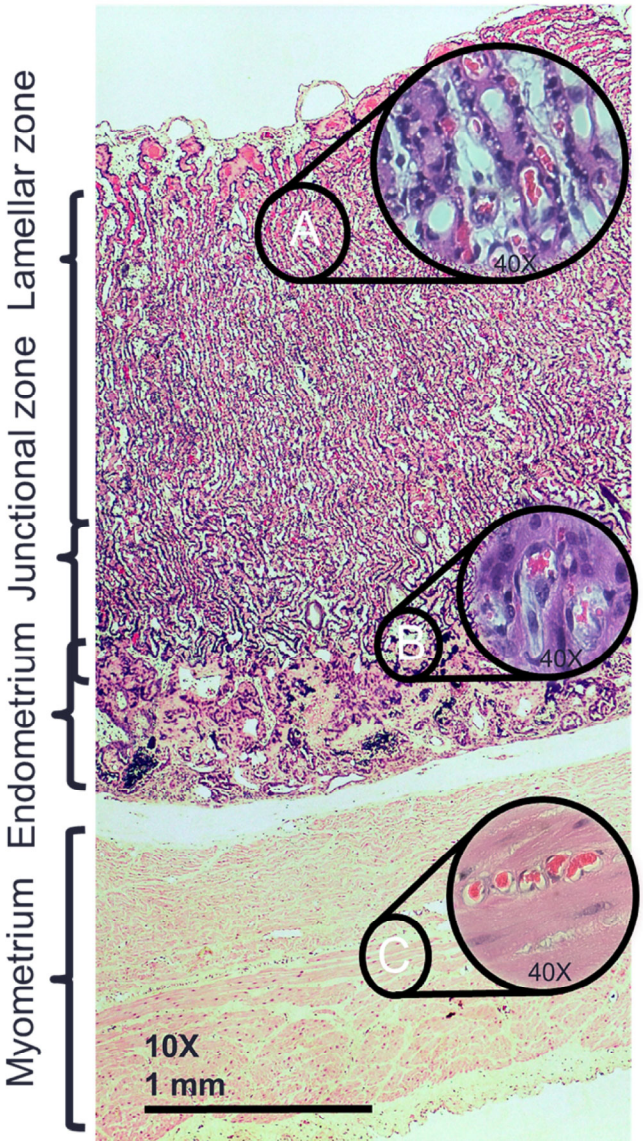


Figure 1.2. Histologic characteristics of the chorioallantoic placenta of the domestic cat at day 55. H&E staining of a transverse cut of the placenta. **A:** At the top is the lamellar zone, containing multiple villous structures called lamellae (see figure 1.3 for more detail). **B:** "Floor" of the placenta. This zone is called the junctional zone and delimitates the area of syncytiotrophoblast invasion into the endometrium. Maternal tissue past this zone into the fetus is called decidua and is detached from the uterus at parturition. The glandular zone is located between the junctional zone and the myometrium. It is composed by the bottom of uterine glands. **C:** The myometrium of the uterus is composed of smooth muscle in different orientations – an internal circular layer, a middle crisscrossing not seen in this image, and an external longitudinal layer. At the outside there is a serosal layer covering the uterus.

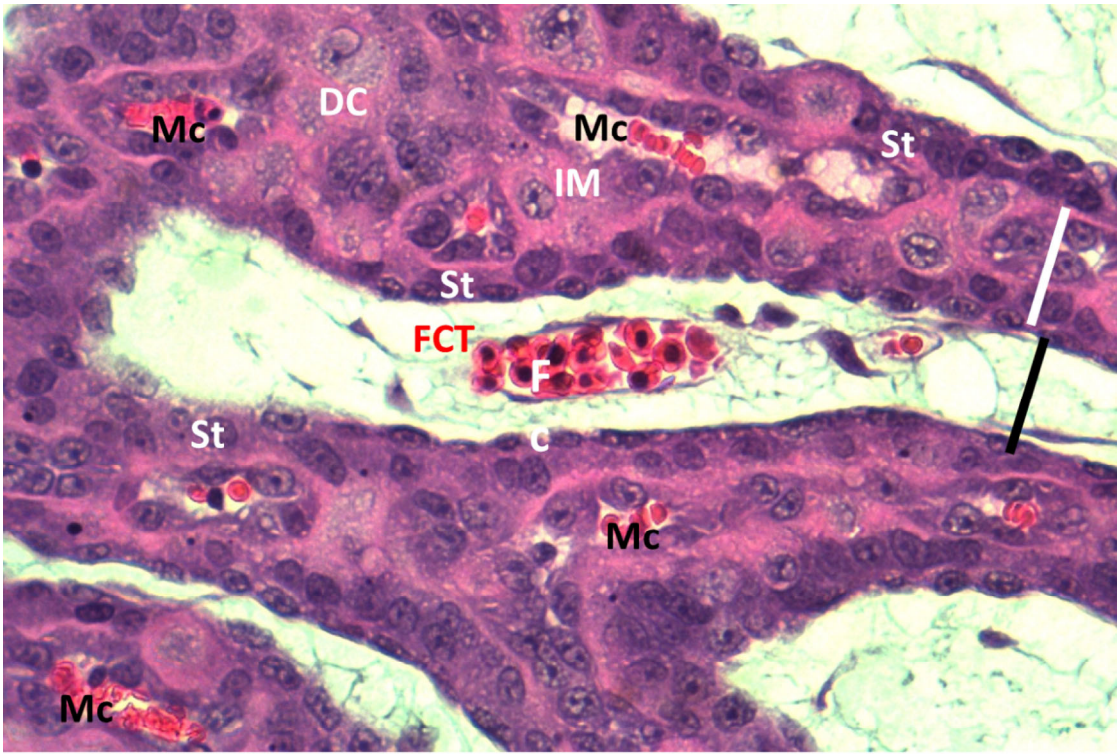


Fig. 1.2 Feto-maternal interface in the cat placenta: Cats have an endotheliochorial type of placentation. In this type of placenta, the trophoblasts, in particular the syncytiotrophoblasts (**St**), reach up to the maternal capillaries (**MC**) lined by maternal endothelium. White and black bars represent maternal and fetal lamellae respectively. **FCT**: fetal connective tissue; **DC**: decidual cell. **IM**, Interstitial membrane.

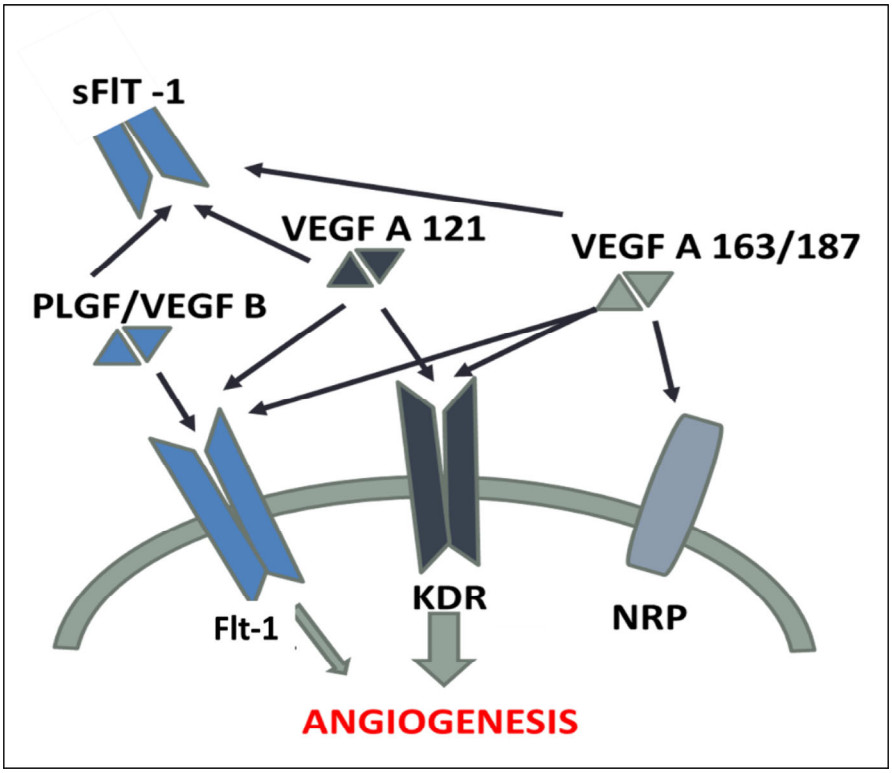


Figure 1.4. VEGF A family members and their receptors. Representation of the main VEGF A family members and their receptor selectivities. The main tyrosine kinase receptors are KDR (VEGFR-2) and Flt-1 (VEGFR-1). Neuropilin 1 and 2 can function as co-receptors in some cells. KDR has 10 times the mitogenic efficacy of Flt-1. VEGF A in all its isoforms can activate Flt-1 and KDR. PLGF can interact with higher affinity with Flt-1 but not with KDR and will displace VEGF A if bound to Flt-1. PLGF/VEGF-A heterodimer formation is possible although its functions are not clear. A truncated version of Flt-1, derived from alternative pre-mRNA splicing represents a soluble form of this receptor ("sFlt-1") that can act as an inhibitory trap or a reservoir for VEGF-A and PLGF.

Chapter 2
Microanatomy of feline placental development: Characterization of the feline placenta throughout pregnancy

2.1 Introduction

The placenta is a transitory organ formed by the interaction of maternal and fetal tissue to provide an adequate environment for normal embryonic and fetal development. The placenta is defined as the apposition or fusion of fetal membranes with the uterine mucosa or endometrium in the feto-maternal interface. (Benirschke, Burton, and Baergen 2012). An adequate establishment and growth of the placenta is key for a successful pregnancy, even determining the future health of the offspring (Reynolds and Redmer 2001; Guimaraes Filho et al. 2008). Throughout gestation, fetal vasculature of the placenta is formed by vasculogenesis and expanded through angiogenesis to provide adequate nutrients and gas exchange in accommodation of increasing fetal needs (Reynolds et al. 2010). Normal placental angiogenesis produces an adequate blood flow and is critical for the successful development of viable and healthy offspring (Reynolds and Redmer 2001; Guimaraes Filho et al. 2008). Furthermore, dysregulation in placental angiogenesis has been linked to pathologies of pregnancy and spontaneous abortions (Roescher et al. 2014).

In domestic cats, the formation of a transitory choriovitelline placenta starts approximately at day 12 post coitus (p.c.). (Leiser 1981). A definitive chorioallantoic placenta is established around day 20 p.c. and continues to grow around the equatorial region of the chorion, forming a girdle known as zonary placenta (Miglino et al. 2006). Here, chorioallantoic trophectoderm penetrates into the maternal endometrium reaching up to the maternal blood vessels, specifically to existing maternal endothelial cells occasionally surrounded by an interstitial layer. This forms the *endotheliochorial interhaemal membrane* (Anderson 1969; Barrau et al. 1975). The feline placental girdle has three histological zones. Deeper in maternal tissue is the *glandular zone*, mostly composed of the base of uterine endometrial glands. Closer to the fetus is the *junctional zone*, composed of syncytiotrophoblast cells invading into the remnants of the endometrium and decidua cells. Lastly is the *lamellar zone*, where the continuous growing of the membrane and proliferation of fetal and maternal placental tissues produces multiple thin intercalated layers of fetal and maternal tissue, organized in a complex

fashion typically arranged in parallel one to another (Leiser and Koob 1993). These structures are called *lamellae* and are the essential structures where feto-maternal exchange occurs (Leiser 1981, 1982; Leiser and Kohler 1984; Leiser and Koob 1993; Leiser 1979). The lamellar zone progressively enlarges during pregnancy and pushes the junctional zone deeper into the uterus, compacting the glandular zone (Leiser and Koob 1993). The feline placenta has been well studied in the past, with the abovementioned literature on its morphology providing a general description of the major structures of the feline placenta using electron microscopy and vasculature corrosion casting. While the value of these early reports in undeniable, most of them are more than 20 years old, and an updated revision seems appropriate. Additionally, other studies have not quantified changes in the vasculature or the rates of proliferation of different zones of the placenta at different stages of the pregnancy.

The present study aims to acquire baseline morphological parameters of feline placental growth during gestation, including the histological dynamics of the placental vasculature and its main anatomic structures. This information is vital to establish a detailed evaluation of angiogenic patterns and trophoblast/endometrial proliferation during pregnancy. Results from this research will be useful as a context for understanding changes in gene expression and immunohistochemical localization of VEGF family members in tissue, covered in Chapter 3.

2.2 Objectives

The main objective of this study is to acquire baseline measurements of feline placental growth during gestation, including the anatomical dynamics of the placental vasculature. Specifically, we will measure the thickness and estimate the microvascular density of the main layers of the placenta and uterus at different stages of the feline pregnancy. In addition, we will determine the number lamellae of the lamellar zone of the placenta. With these measurements, we seek to gain a deeper and quantitative understanding of how the placenta and its vasculature grow during gestation to accommodate the needs of the fetus. Knowledge of the anatomical dynamics will provide a basis against which to assess changes in expression of genes likely involved in vasculogenesis.

2.3 Methodology

As described in Chapter 1, the feline placenta was chosen as a system of study of normal placental development, in part owing to the ready availability of tissue across gestation, without the need to obtain or dedicate experimental animals. Moreover, gestation in domestic cats is of moderate duration (~63 days), allowing capture, across multiple samplings in a given breeding season, the full course of placentation. Finally, the size of the placental tissue and associated fetus lends itself to ready dissention and biochemical analysis.

2.3.1 Sample collection and preparation: Placental tissue, which is routinely discarded after ovariohysterectomy of pregnant cats at spay and neuter clinics (e.g., Mountain View Humane, Christiansburg, VA), was instead recovered immediately after surgery. (Our studies involving the use of discarded feline tissues was initiated with a protocol approved by the Virginia Tech Institutional Care and Use Committee, although in no instance did the tissue originate from university-owned animals, nor was any live animal used for this study.) Fetuses from a total of 49 whole reproductive tracts were removed from gravid uteri by dissection of the uterus and removal of the enclosing membranes. Fetuses were measured using a caliper to determine the occipital-sacral length and the biparietal diameter in order to estimate their gestational age according to formulae described in Zambelli et al. (2004); Zambelli et al. (2002). Samples of placenta, with or without adjacent maternal tissue, were placed in 10% formalin immediately after collection and fixed for at least 24 hrs at room temperature before trimming. To allow for lateral cross-sectioning, approximately 0.3 cm wide strips of placenta were placed in cassettes for paraffin embedding, 5 um cross-sectioning, and staining with Hematoxylin and Eosin (H&E), under standard histological protocols in Virginia Tech Animal Laboratory Services (ViTALS), the diagnostic lab affiliated with the College of Veterinary Medicine Teaching Hospital.

2.3.2 Tissue characterization. Slides were evaluated under an Olympus BX61VS microscope with Olympus XM 10 camera; images and measurements were recorded using Olympus VSI software. Multiple parameters were measured to evaluate placental growth and proliferation over the course of gestation. The thicknesses of the myometrium and the glandular, junctional, and lamellar zones were measured in six different sites in each the sample, and the averages were calculated (**Fig 2.1A-B**). Detailed characterization of the lamellar zone was performed by

measuring the thickness of the lamellae and the lamellar density. Thickness of lamellae was obtained by the average of 10 measurements per high powered field (400X; HPF); four different fields per slide were measured (**Fig 2.1C**). Lamellar density was assessed by measuring the number of lamellae crossed by an 800 um line perpendicular to the orientation of the lamellae (**Fig 2.1C**). To determine vascular density, the numbers of small or micro-vessels (arterioles, capillaries and venules) in different zones of the placenta and the myometrium were counted in four HPFs for each sample, each field corresponding to 196.2 um^2

2.3.3 Statistical analysis. Samples were categorized into four groups according to the estimated age of gestation. Statistical analysis of the data was performed using one-way ANOVA in the software JMP10. When a statistical difference was noted, Tukey's test was used to determine differences between groups; $p < 0.05$ was considered significant.

2.4 Results

2.4.1 Dimensions of placental zones and the uterus. We measured different anatomical zones of the placenta and the uterus in H&E stained sections **(Figure 2.1)** representing tissue throughout the course of feline pregnancy, to identify the placental zones with most dynamic structures. Dimensions and estimated gestational ages of the 49 samples collected are listed in **Table 2.1**.

2.4.2 Dynamics of placental dimensions. We identified the earliest evidence of placentation at approximately day 14 p.c. This temporary choriovitelline placenta was superficial and thinner than that which forms later in pregnancy. The choriovitelline placenta provides an early attachment to the endometrium but at this stage was avascular. (**Figure 2.2**) Choriovitelline placenta is developed from the extra embryonic ectoderm differentiated into trophoblasts and the vitelline sac membrane. Vasculature is present at this placenta around day 20 (not shown). From day 20 we identify the chorioallantoic placenta with three distinctive histological zones (**Figure 2.3A**). There is a clear increase in the overall thickness of the placenta, especially in the lamellar zone **(LZ).** Junctional zone **(JZ)** appears to maintain the same thickness together with the glandular zone **(GZ).** Quantification of zonal dimensions confirmed the gross histological impression. Placental thickness including the uterus increases from approximately 4mm in early

pregnancy to 6mm at days 46-50 (**Figure 2.3B**). After day 50, placental thickness is reduced, likely due to an effect of stretching and thinning of the myometrium as fetal size reached a maximum during late pregnancy. In contrast, dimensions of the lamellar zone in the placenta increases until term, increasing the volume of functional placenta (**Figure 2.4**). Lamellae of placentas from early pregnancy (day 20-25) were significantly smaller than all the other groups. Mid pregnancy group 26-35 days was statistically different from all older groups. During the second half of the pregnancy, the length of the LZ was statistically similar (Groups 36-45, 46-50 and 51-60). *Based on these results, it appears that the major increase in the LZ thickness occurs during mid pregnancy*.

2.4.3 Width of the lamellae. While the average thickness of the lamellar zone increases during pregnancy, the mean width of each lamella decreases steadily (**Figure 2.5**). Least-squares regression analysis of the relationship between the thicknesses of the lamella (TOL) and gestational age produces a line of fit with an R^2 of 0.74, suggesting a linear relationship between these two variables. Using this information, we developed a linear regression equation to predict the gestational age base of the average TOL; about 75% of the changes in TOL can be predicted by the day of the pregnancy. This formula could be useful to pathologists evaluating feline placental samples without history of the patient.

Day of pregnancy = 81.4 - 0.867 X TOL in um

Histologically, there are clear differences in the LZ from early pregnancy to late pregnancy (**Figure 2.5**). During early pregnancy, there are abundant spaces of loose connective tissue and cytotrophoblast around fetal capillaries; this space is reduced later in pregnancy. Additionally, fetal and maternal lamellae are thinned, and syncytiotrophoblast cells and endothelial cells have flattened and become reduced in the cytoplasm

2.4.4 Lamellar Density. Forty H&E stained sections were evaluated under the microscope to count the number of parallel lamellae crossed by an 800 um line. As expected based on the decreased mean lamellar thickness, we observed a steady increase in lamellar density throughout pregnancy (**Figure 2.6**), starting from an average of 6 lamellae per 800 um at day 20 and reaching 15 lamellae per 800 um at the end of pregnancy. Least squares regression analysis of

lamellar density in relation to the day of pregnancy produces a line of fit with an R^2 of 0.77, reflective of a strong correlation between the two variables. We created a linear equation to estimate the days of pregnancy from the density lamellar density in 800um.

Day of pregnancy = 2.684 + 3.482 x N of Lamellae per 800um

The increase in lamellar density is in agreement with the reduction in thickness of the lamellae, therefore packing more lamellae into the same area. Thus far we have confirmed that the feline placenta proliferate by increasing the thickness of the LZ, reducing the TOL and increasing the Lamellar density. Together, these changes bring maternal and fetal vessel into closer proximity, reducing the interhaemal barrier and likely increasing the exchange rates between fetal and maternal circulations.

2.4.5 Microvascular density. To estimate temporal and localization patterns of angiogenesis in the placenta, we estimate the microvascular density (MD), by counting capillaries, arterioles, and venules at 400X in different zones of the placenta and the myometrium throughout pregnancy, all data collected from the samples is organized in **Table 2.2**. The results, as expected, revealed that the most highly vascularized area of the placenta was the LZ (**Figure 2.7**), showing a constant increase in the MD, and apparently spike at the last portion of the pregnancy (days 55-60). On the other hand, in the GZ and the M the number of capillaries remains stable across pregnancy, except for the capillaries at the JZ/GZ at the end of the pregnancy (d-55-60) that were significantly different from the early stages in the same location. Statistical analysis indicated that MD during early pregnancy in the LZ was lower than late pregnancy (Days 40-50, 55-60), and the last stage of the pregnancy (days 55-60) had higher MD than any other stage. With these results, we can deduce that the LZ, over the course of the whole pregnancy and especially during the second half, have the higher rates of neovascularization in the placenta.

2.5 Discussion and Conclusions

We found that the placenta of the domestic cat is a very dynamic tissue, constantly changing to accommodate the needs of the fetus. We identify a non-vascular choriovitelline placenta at approximately day 12 of the pregnancy, in agreement with previous reports (Leiser

and Koob 1993; Wooding and Burton 2008). Vasculature to the yolk sac comes from a mesodermal layer, forming a bilaminar yolk sac around day 20. In the definitive placenta, the vasculature arises from the allantois. A similar process is also described for the dog placenta (Aralla et al. 2013). This means that the maternal villi and microvasculature in the endometrium are well established before fetal angiogenesis starts in the placenta. By doing so, endometrial glands in the endometrium serve as a guide from trophoblastic invasion.

By measuring the total thickness of the placenta with uterus, we detected an increase from approx. 4mm at day 20 to approx. 6mm at the end of the pregnancy (**Figure 2.5B**). This is similar to what other have found in the cat (Leiser 1982; Kehrer 1973b; Wooding and Burton 2008), this marked increase in the placental size is shared among mammals (Carter 2007). We were able to quantify this growth precisely in a large number of samples and perform a statistical analysis. We showed that the principal increase in placental proliferation occurs in the LZ around mid-pregnancy. There is agreement that the LZ of the placenta experiences the highest degree of growth overall, but Kerher (1973) suggested the higher growth rates occurred in late pregnancy instead. In contrast, Reynolds and Redmer (1995) described that the utero-placental growth rate, measured by weight, slows during the last half of the pregnancy in most mammals (except for cattle), despite the exponential growth of the fetus.

Based on the histological study of the zonary placenta, we notice that the expansion of the placenta occurred by proliferation of maternal and fetal villi including all its components, *i.e.* chorion, fetal vasculature, maternal vasculature, and endometrial glands. In this manner, the growing fetal side of the placenta expands mostly into the uterine lumen rather than relying only on the infiltration of the syncytium into the endometrium. This produce a superficial implantation, characteristic of the domestic cat, as has been pointed out by Kehrer 1973a, who describes that “formation of the zonary placental villi cannot just be based on erosion by the fetal syncytium and its advance into the endometrium, the final length of the villi is much too long for this. There has to be considerable expansion of the maternal blood capillary system” (Kehrer 1973a).

We described a constant decrease in the thickness of maternal and fetal lamellas during pregnancy, due to changes in the structure of cellular and connective tissue components of the placenta. As expected, a reduction in thickness is complemented by an increase in lamellar density that also occurs gradually across pregnancy. These two parameters suggests that

proliferation of cellular components of the LZ are maintained steadily during pregnancy, despite the fact that placental thickness, especially in the LZ, seems to be the same during the last trimester. A similar situation is described in the sheep placenta, here there is a stable increase in proliferation of fetal placental cells, measured by DNA concentration in tissue, despite the stable weights of the placenta during the last semester (Reynolds and Redmer 1995). Obviously, just weight of the placenta or its thickness alone does not properly describe its capacity to delivered sufficient nutrients and oxygen to the fetus, other factors that determine placental exchange rates are vascular perfusion, the distance between maternal and fetal capillaries, the nature of the interhaemal barrier, and expression of specific cell transporters. In this regard, we and others (Baur 1977; Carter 2009; Leiser and Kaufmann 1994) notice a reduction in the average distance of maternal and fetal capillaries of the lamellar zone as the pregnancy advances. Additionally, we estimated an increase in microvascular density of the LZ especially during the last trimester, a similar increase in vasculature has been described in mice placenta by micro-CT in mouse (Rennie et al. 2007). With this results, we can conclude that there is a constant remodeling of the stroma and vasculature of the placenta during pregnancy. Furthermore, during the last trimester, the placenta becomes more efficient by reducing the interhaemal distance, increasing blood flow and capillary density, and increasing in exchange surface. By doing this, the placenta of similar size compared to mid pregnancy, is able to withstand the increasing fetal needs during the last trimester as are reflected by the exponential weight gain at late pregnancy as stated by Zambelli et al. (2004). We can predict that the VEGF family has a great influence in the changes happening in the placenta during pregnancy, especially promoting angiogenesis in endothelial cells, inducing proliferation cellular components of the placenta, and increasing permeability of the vasculature. Although details of these mechanisms in the feline placenta remain to be elucidated.

Limitations of the study the early placentation in cats comes from the difficulty of tissue handling and orientation for histology. Another limiting factor is the resolution of light microscopy for identification of small features such as cell type differentiation or layers in the interhaemal membrane, and the 2D characteristics of histology that may not reflect changes or are difficult to interpret. Ideally, a better option for characterization of the vasculature patterns during pregnancy could be the use of microangiography techniques with a fluorescent contrast agent and subsequent 3D vascular casting; such technique have been described for other species

by Lacko *et al.* (2014) and (Rennie et al. 2007) The difficulties of tissue handling during cannulation and access to equipment ruled out this option for our study.

2.6 References

Anderson, J. W. 1969. 'Ultrastructure of the placenta and fetal membranes of the dog. I. The placental labyrinth', *Anat Rec*, 165: 15-35.

Aralla, M., D. Groppetti, L. Caldarini, F. Cremonesi, and S. Arrighi. 2013. 'Morphological evaluation of the placenta and fetal membranes during canine pregnancy from early implantation to term', *Res Vet Sci*, 95: 15-22.

Barrau, M. D., J. H. Abel, C. A. Torbit, and W. J. Tietz. 1975. 'Development of the implantation chamber in the pregnant bitch', *Am J Anat*, 143: 115-30.

Baur, R. 1977. 'Morphometry of the placental exchange area', *Adv Anat Embryol Cell Biol*, 53: 3-65.

Benirschke, Kurt, Graham J Burton, and Rebecca N Baergen. 2012. 'Placental types.' in, *Pathology of the human placenta* (Springer).

Carter, A. M. 2007. 'Animal models of human placentation--a review', *Placenta*, 28 Suppl A: S41-7.

———. 2009. 'Evolution of factors affecting placental oxygen transfer', *Placenta*, 30 Suppl A: S19-25.

Guimaraes Filho, H. A., L. L. da Costa, E. Araujo Junior, L. M. Nardozza, P. M. Nowak, A. F. Moron, R. Mattar, and C. R. Pires. 2008. 'Placenta: angiogenesis and vascular assessment through three-dimensional power Doppler ultrasonography', *Arch Gynecol Obstet*, 277: 195-200.

Kehrer, A. 1973a. 'Chorionic Development and Structure of Placenta Zonaria in Cat, Dog and Fox', *Zeitschrift Fur Anatomie Und Entwicklungsgeschichte*, 143: 25-42.

———. 1973b. '[Chorionic development and structure of the placenta zonaria in the cat, dog and fox (author's transl)]', *Z Anat Entwicklungsgesch*, 143: 25-42.

Lacko, L. A., M. Massimiani, J. L. Sones, R. Hurtado, S. Salvi, S. Ferrazzani, R. L. Davisson, L. Campagnolo, and H. Stuhlmann. 2014. 'Novel expression of EGFL7 in placental trophoblast and endothelial cells and its implication in preeclampsia', *Mech Dev*, 133: 163-76.

Leiser, R. 1979. '[Blastocyst implantation in the domestic cat. Light and electron microscopic study]', *Anat Histol Embryol*, 8: 79-96.

———. 1981. 'Development of the Trophoblast in the Early Carnivore Placenta as Exemplified by the Cat', *Acta Anatomica*, 111: 88-89.

———. 1982. 'Microvascularization of the Placenta of a Cat', *Acta Anatomica*, 112: 279-79.

Leiser, R., and P. Kaufmann. 1994. 'Placental structure: in a comparative aspect', *Exp Clin Endocrinol*, 102: 122-34.

Leiser, R., and T. Kohler. 1984. 'The Blood-Vessels of the Cat Girdle Placenta - Observations on Corrosion Casts, Scanning Electron Microscopical and Histological Studies .2. Fetal Vasculature', *Anatomy and Embryology*, 170: 209-16.

Leiser, R., and B. Koob. 1993. 'Development and characteristics of placentation in a carnivore, the domestic cat', *J Exp Zool*, 266: 642-56.

Miglino, M. A., C. E. Ambrosio, D. dos Santos Martins, C. V. Wenceslau, C. Pfarrer, and R. Leiser. 2006. 'The carnivore pregnancy: the development of the embryo and fetal membranes', *Theriogenology*, 66: 1699-702.

Rennie, M. Y., K. J. Whiteley, S. Kulandavelu, S. L. Adamson, and J. G. Sled. 2007. '3D visualisation and quantification by microcomputed tomography of late gestational

changes in the arterial and venous feto-placental vasculature of the mouse', *Placenta*, 28: 833-40.
Reynolds, L. P., P. P. Borowicz, J. S. Caton, K. A. Vonnahme, J. S. Luther, D. S. Buchanan, S. A. Hafez, A. T. Grazul-Bilska, and D. A. Redmer. 2010. 'Uteroplacental vascular development and placental function: an update', *Int J Dev Biol*, 54: 355-66.
Reynolds, Lawrence P, and Dale A Redmer. 1995. 'Utero-placental vascular development and placental function', *Journal of animal science*, 73: 1839-51.
Reynolds, Lawrence P., and Dale A. Redmer. 2001. 'Angiogenesis in the Placenta', *Biology of Reproduction*, 64: 1033-40.
Roescher, Annemiek M, Albert Timmer, Jan Jaap HM Erwich, and Arend F Bos. 2014. 'Placental pathology, perinatal death, neonatal outcome, and neurological development: a systematic review', *PLoS One*, 9: e89419.
Wooding, Peter, and Graham Burton. 2008. 'Endotheliochorial Placentation : Cat, Dog, Bat.' in Peter Wooding and Graham Burton (eds.), *Comparative Placentation: Structures, Functions and Evolution* (Springer Berlin Heidelberg: Berlin, Heidelberg).
Zambelli, D., C. Castagnetti, S. Belluzzi, and S. Bassi. 2002. 'Correlation between the age of the conceptus and various ultrasonographic measurements during the first 30 days of pregnancy in domestic cats (Felis catus)', *Theriogenology*, 57: 1981-7.
Zambelli, D., C. Castagnetti, S. Belluzzi, and C. Paladini. 2004. 'Correlation between fetal age and ultrasonographic measurements during the second half of pregnancy in domestic cats (Felis catus)', *Theriogenology*, 62: 1430-7.

2.7 Figures and Tables

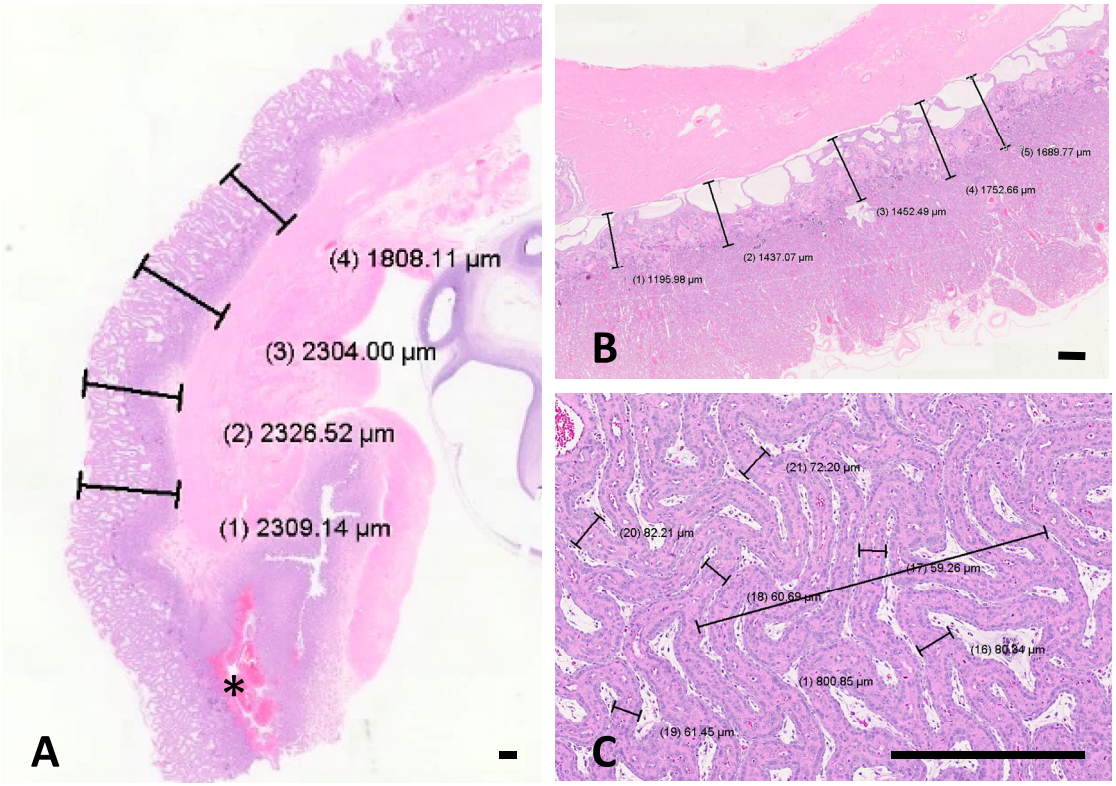


Figure 2.1: Example measurements of placental and uterine dimensions in the cat placenta. **A:** Measurements of the lamellar zone range from 1.8 to 2.7 mm in this sample. Note the presence of a marginal hematoma (*). **B:** multiple measurements of the glandular zone and Junctional zone were average per placental sample. **C:** Thickness of lamellae was obtained by averaging multiple measurements at four different fields per slide; Lamellar density was assessed by measuring the number of lamellae crossed by an 800 um line perpendicular to the orientation of the lamellae. Scale bar = 500um

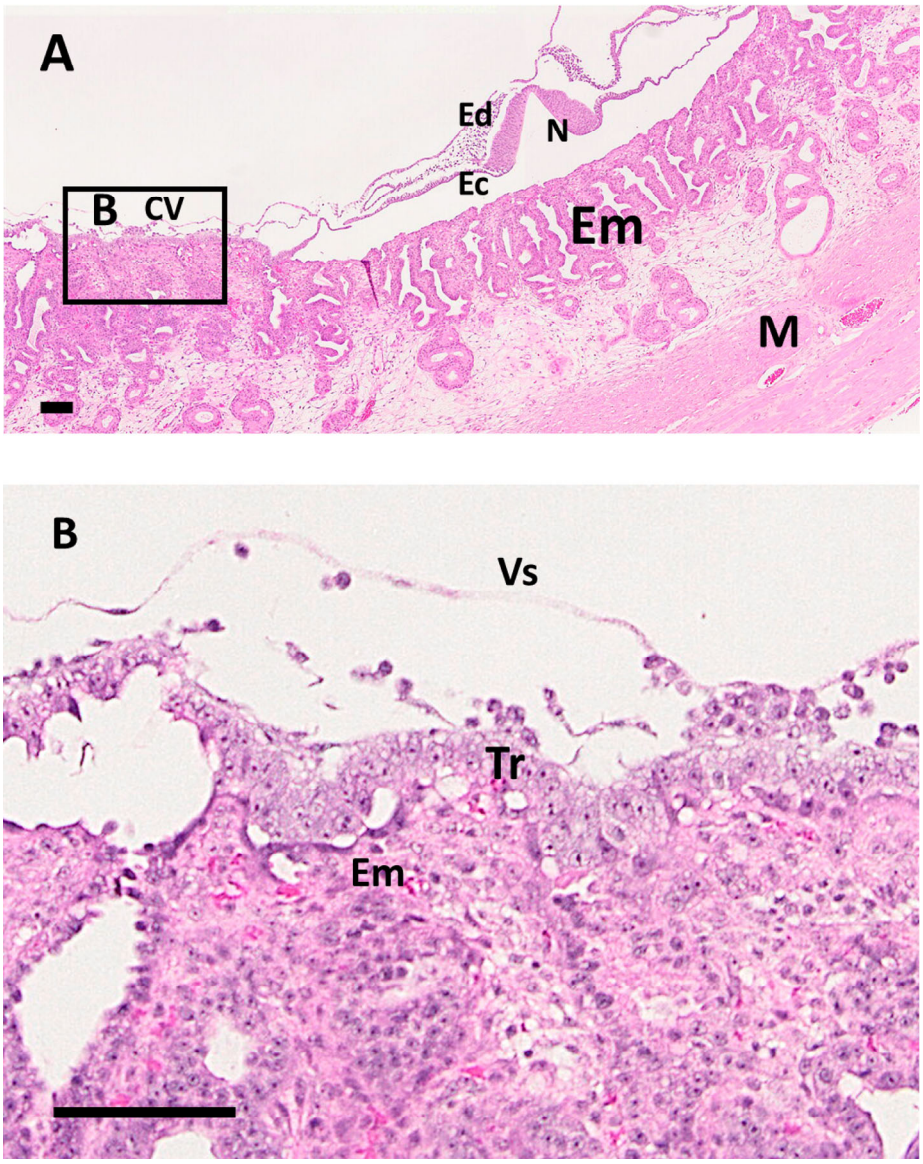


Figure 2.2: Choriovitelline placenta in the cat *circa* day 12. A: An early trilaminar embryo attached to the uterine wall by the early choriovitelline (**CV**) placenta. In the embryo, we distinguish in the ectoderm the neural grove (**N**) and, to each side, the neural crest. At the opposite side is the endoderm (**Ed**) and the mesoderm in between. Box in **Panel A** shows the area magnified in **panel B**. At each side of the embryo, the trophoblasts (Tr; from extra-embryonic ectoderm start to attach and penetrate into the endometrium (**Em**). In the luminal part, the endodermic vitelline membrane is attached to the trophoblast, forming the choriovitelline placenta. Note that this placentation is has no vasculature from the fetal side. Scale bar = 100 um.

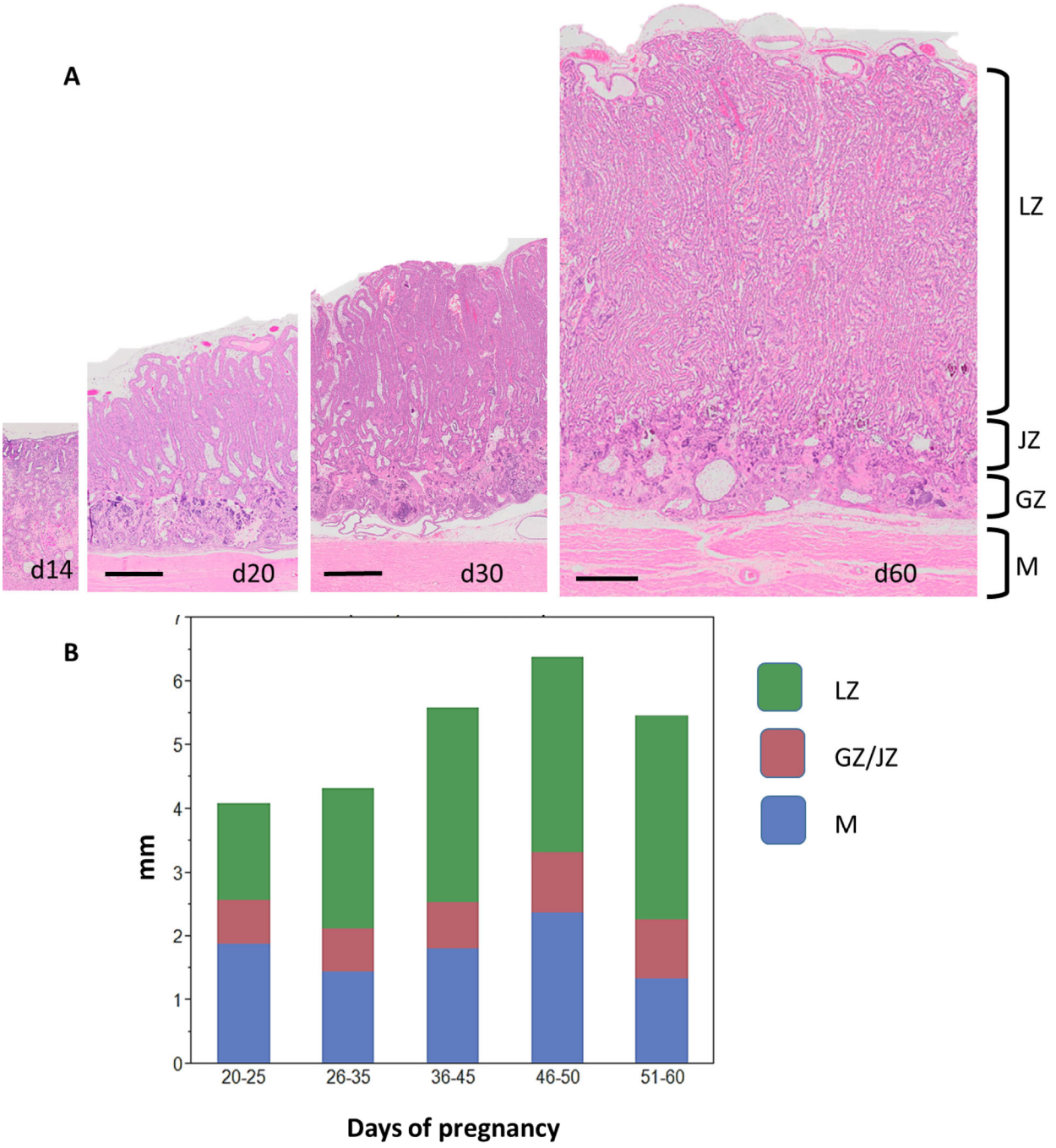


Figure 2.3: Dimensions of placenta and component structures across pregnancy. Continual growth of the placenta during pregnancy. **A:** Histological images comparing the formation of the placental formation (Chorioviteline placenta) **d14** approx. early pregnancy (**d20**) mid pregnancy (**d30**) and late pregnancy (**d60**)**.** All images have the same magnification (0.4X) The placental girdles of all stages except for day 14 have distinctive zones. There is a marked expansion of the lamellar zone (**LZ**)**,** specially during mid-pregnancy. Junctional zone (**JZ**) appears to have the same size together with the lamellar (**GZ**). **B:** Graphic representation of the means of measurements from 49 placental units**.** Mean total placental thickness ranged from 4mm at the beginning of the pregnancy to 6mm at the end. Scale bar = 500 um

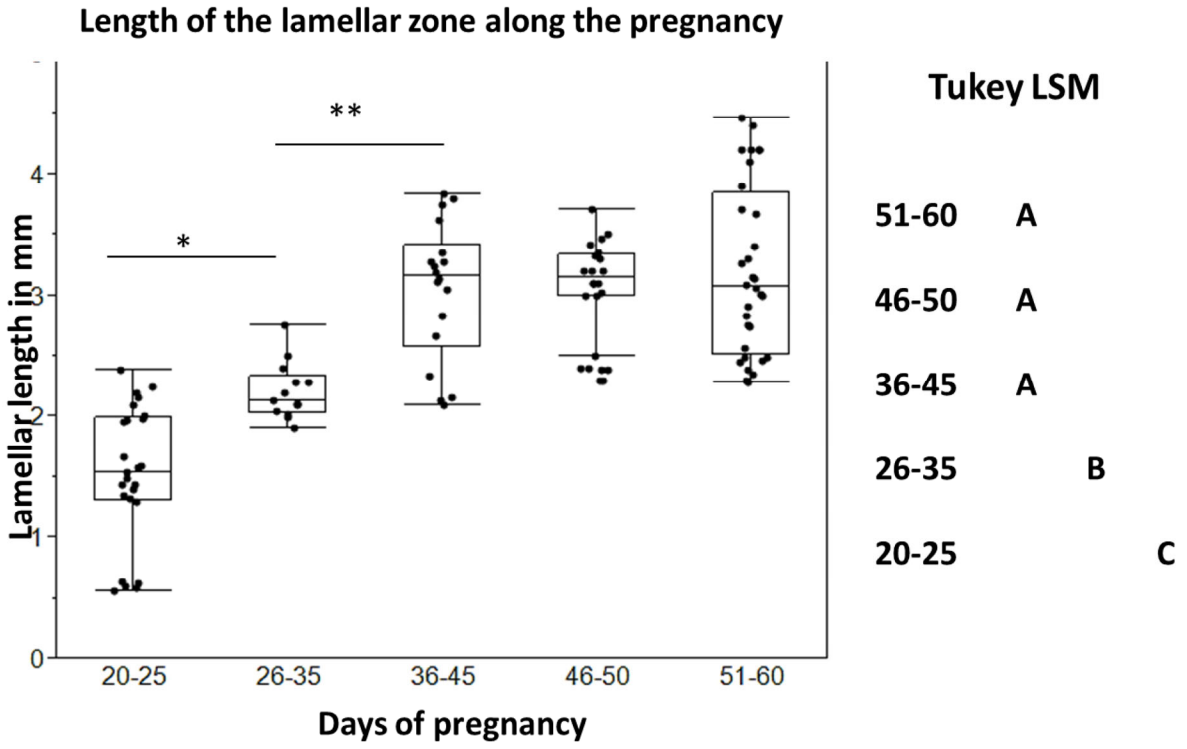


Figure 2.4: Lamellar length as a function of gestational age. Symbols represent mean measurements of lamellar length for each study placenta; box and whiskers indicate minima, first quartiles, medians, third quartiles, and maxima for each cluster of gestational ages. The length of the lamellar zone consistently increases during pregnancy. Lamellae of placentas from day 20-25 where statistically significantly smaller than all the other groups (*), Group 26-35 day was different from all older groups (**). Groups 36-45, 46-50 and 51-60 were statistically similar. Tukey LSM differences are included at the right of the graph for clarification. Thicknesses not connected by same letter are significantly different. Each data point represents an individual placenta.

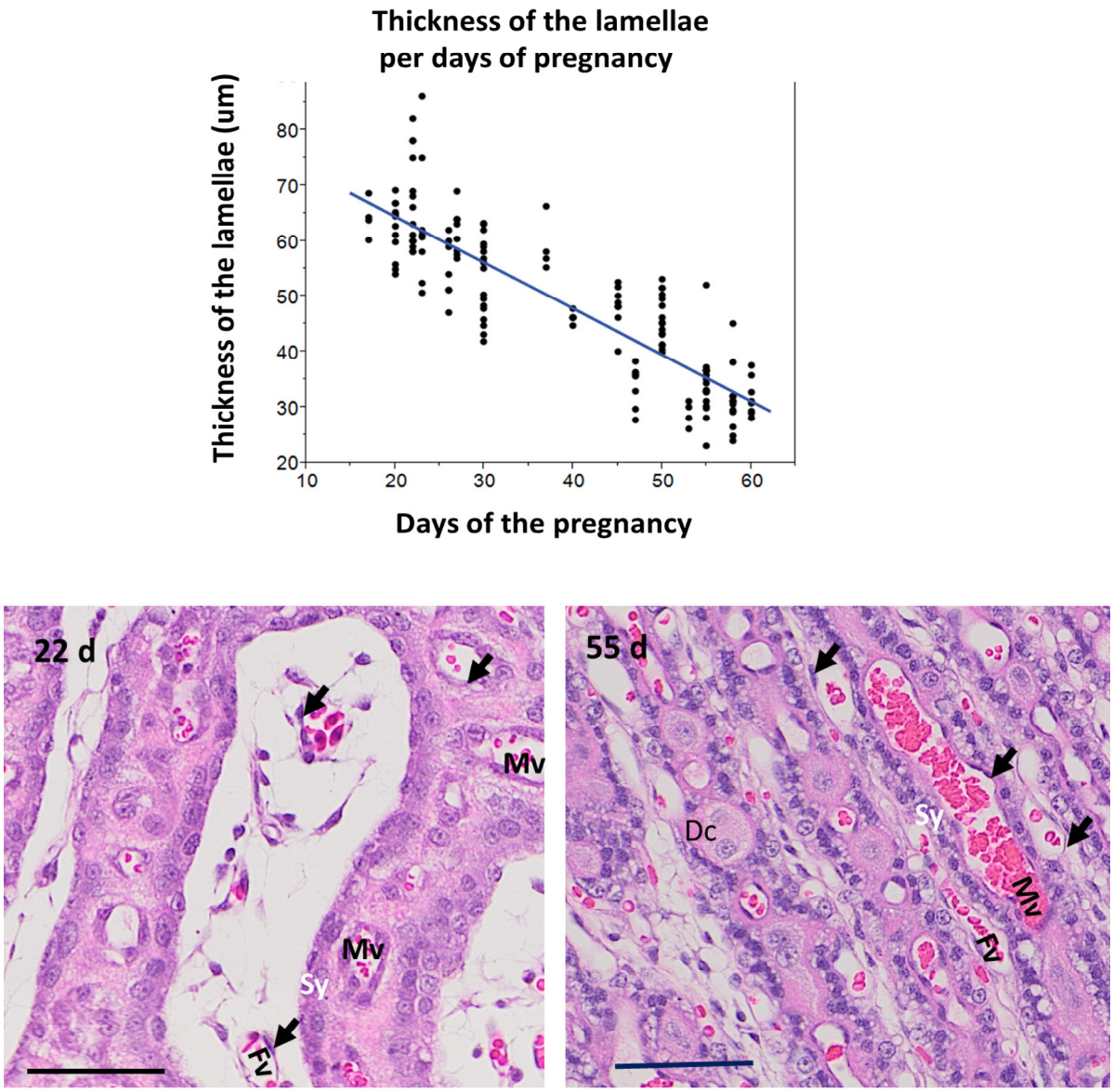


Figure 2.5: Lamellar width as a function of gestational age. Upper panel: Symbols represent measurements of lamellar width for each study placenta, plotted according to each litter's estimated gestational age. There is a steady reduction in the mean width of the lamellae as the pregnancy progress. **Lower panel:** Histological differences in the lamellar zone at day 22 and day 55. At day 55, fetal and maternal lamellae are thinned, syncytiotrophoblast cells (**Sy**) with dark nucleus and endothelial cells (Arrow) have flattened and are reduced its cytoplasm, bringing maternal (**Mv**) and fetal vessel (**Fv**) closer to each other and accommodating a greater density of vessels. Note the presence of decidual cells (**Dc**) as large, occasionally multinucleated cells with intracytoplasmatic vacuolations in the maternal lamellae. Nucleated red blood cells in fetal capillaries are present at day 22 but absent by day 22. Bar equals 50 um. Each data point represents the mean of 3-4 lamellar measurements in a single 20X field; 4 different fields were sampled for each slide (individual placenta).

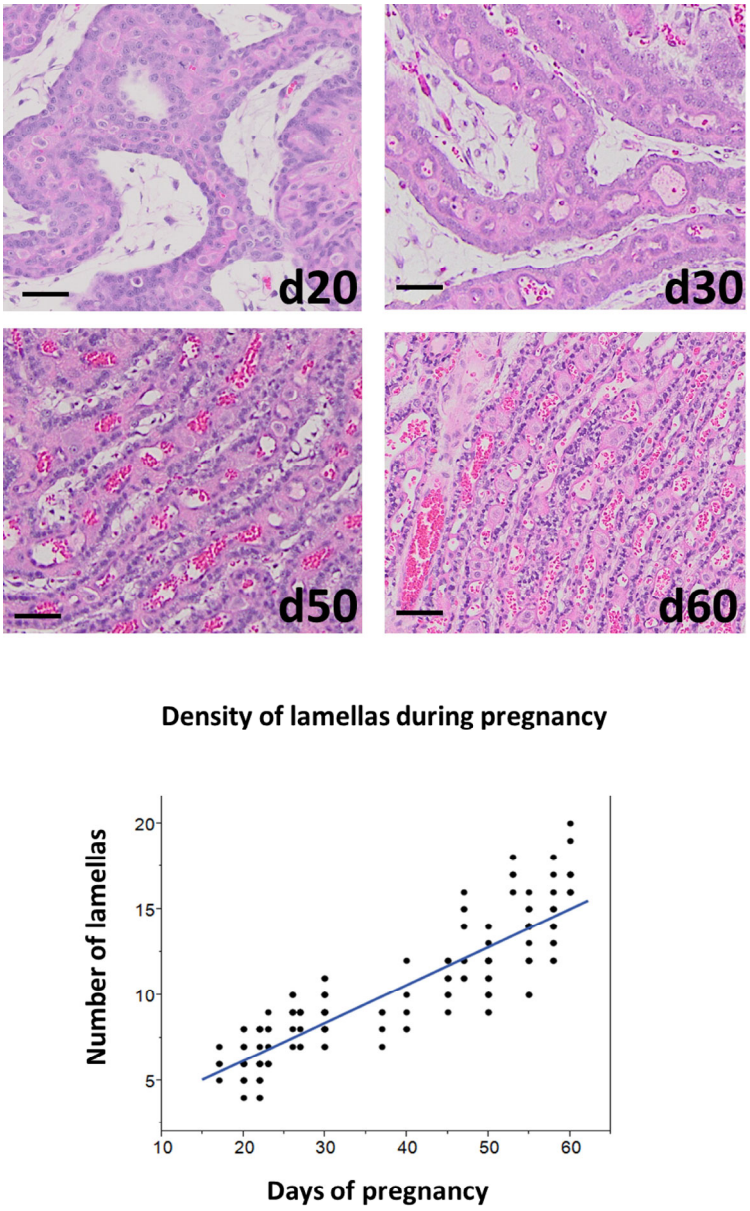


Figure 2.6: Lamellar density as function of gestational age. Lower panel: Symbols in the graph represent mean measurements of lamellar density (number of lamellae crossed by an 800 um line perpendicular to lamellae, measured at 4 different fields for each studied placenta), plotted according to each litter's estimated gestational age. There is a steady increase in the density of the lamellae during pregnancy. **Upper panel:** H&E light microscopy (10X) of the LZ at different stages of pregnancy (D20-30-50-60) showing increased organization of lamellae, reduction of thickness and increase in density along the pregnancy.

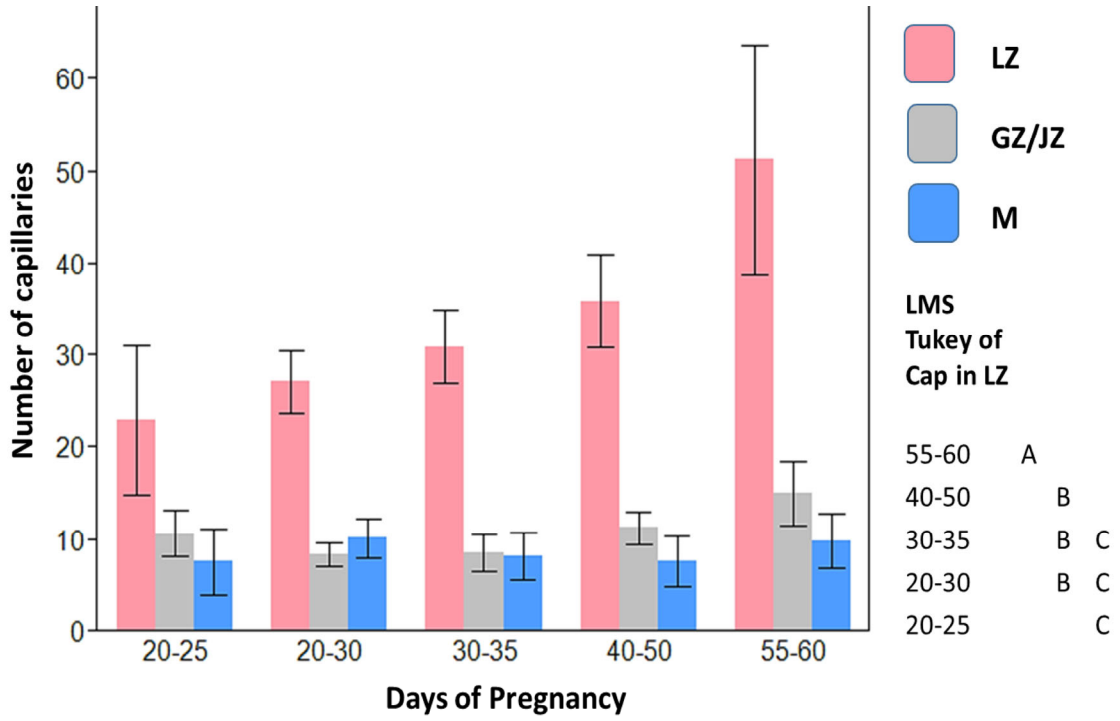


Figure 2.7: Microvascular density as function of gestational age. A sustained increase was observed in density of capillaries of the lamellar zone (**LZ**). In contrast, in the **GZ/JZ** and **M** zone, the number of capillaries generally remained stable across pregnancy, with the exception of capillaries at the **JZ/GZ** at the end of the pregnancy (day 55-60) that were significantly different compared to the early stages (day 20-15) at the same location. By this measure, the **LZ** is the most highly vascularized area of the placenta and increases its vasculature consistently during pregnancy, especially beyond day 50. Data used to generate means $\pm$ sds shown are the means of microvascular density measurements in 4 different 40X fields sampled from each slide (individual placenta).

Table 2.1 Dimensions and estimated gestational ages of study samples

Sample Name	Est. Day of Gestation	Desidua (um)	Myometrium (um)	Glandular & Junctional Zones (um)	Lamellar Zone (um)	Group (days)
F10P2	20	1.90		0.70	1.30	20-25
F10P3	20	1.10	1.70	0.50	0.60	20-25
F37P3	20	2.86		0.83	2.20	20-25
F42P2	20	2.50	1.82	0.78	1.59	20-25
F42P3	20	2.30		0.66	1.49	20-25
F45P5	20	3.64	0.65	0.57	3.32	20-25
F47P2	20	1.12	0.05	0.05	0.06	20-25
F47P3	20	1.61	0.94	0.75	0.64	20-25
F42P1	21	2.32	2.20	0.68	1.66	20-25
F42P4	21	2.66	1.60	0.66	1.95	20-25
F45P2	22	3.38	1.86	1.03	2.39	20-25
F45P3	22	2.89	1.95	0.72	1.96	20-25
F40P3	23	2.93		0.82	2.03	20-25
F46P4	26	2.90	0.90	0.65	2.11	26-35
F44P1	27	2.72		0.60	2.13	36-45
F44P2	27	2.33	2.90	0.80	1.54	20-25
F44P3	27	3.29	2.37	0.69	2.75	26-35
F46P1	27	3.90	2.30	0.70	2.20	26-35
F46P3	27	2.92	0.21	0.79	2.13	26-35
F9P1	28	3.10		0.60	2.40	26-35
F9P2	28	2.60		0.70	2.00	26-35
F9P3	28	2.80		0.70	2.10	26-35
F37P2	30	3.77	2.40	0.75	3.27	26-35
F39P2	40	3.19		0.86	2.33	36-45
F39P3	40	4.06	1.21	0.85	2.66	36-45
F4P1	43	4.00		0.90	3.20	36-45
F4P2	43	4.10		0.70	3.40	36-45
F4P3	43	3.90		0.90	3.00	36-45
F4P5	43	4.40		0.60	3.80	36-45
F31P2	46	4.10	2.40	1.10	3.10	46-50
F32P1	46	3.21	2.50	1.10	2.40	46-50
F52P2	47	4.10	2.16	0.95	3.02	46-50
F18P3	48	3.66	2.60	0.90	3.20	46-50
F51P2-3	49	4.27	1.62	0.85	3.33	46-50
F52P1	49	4.34	1.85	0.90	3.42	46-50
F52P5-7	49	3.80	2.30	0.90	3.35	46-50
F50P3-4	50	4.12	1.24	1.19	2.99	46-50
F52P3-4	50	4.22	2.00	1.08	3.46	46-50
F50P1-2	52	3.93	0.91	1.03	3.14	51-60

Sample Name	Est. Day of Gestation	Desidua (um)	Myometrium (um)	Glandular & Junctional Zones (um)	Lamellar Zone (um)	Group (days)
F38P2	53	4.27	1.06	0.60	3.70	51-60
F33P4	54	6.90	2.80	2.50	4.10	51-60
F19P2	55	3.10	0.80	0.70	2.50	51-60
F48P1	58	4.40	1.84	1.50	4.47	51-60
F34P2	59	5.00	1.20	0.77	3.80	51-60
F36P3	59	4.58	1.32	0.82	4.20	51-60
F3P2	60	3.60		0.60	3.10	51-60
F3P3	60	3.70		0.80	2.90	51-60
F3P4	60	4.00		0.70	3.30	51-60
F20P1	61	3.90	1.30	0.70	2.30	51-60

Table 2.2 Microvascular density per placental zone during gestation

Sample Name	Est. Day of Gestation	LZ Microvessel Count	GZ/JZ Microvessel Count	Mio Microvessel Count	Group (days)
F34P1	60	37	15	12	55-60
F34P1	60	35	21	10	55-60
F34P1	60	41	18	7	55-60
F34P1	60	39	17	5	55-60
F422	20	19	10	4	20-25
F422	20	20	11	8	20-25
F422	20	14	10	5	20-25
F422	20	15	13	4	20-25
F42P4	17	23	12	4	20-25
F42P4	17	18	10	16	20-25
F42P4	17	20	16	9	20-25
F42P4	17	16	12	8	20-25
F37P3	30	28	8	8	30-35
F37P3	30	29	6	7	30-35
F37P3	30	27	7	6	30-35
F37P3	30	26	8	11	30-35
F32	45	29	10	4	40-50
F32	45	35	14	12	40-50
F32	45	34	11	7	40-50
F32	45	33	9	8	40-50
39P2	40	40	11	9	40-50
39P2	40	46	10	6	40-50
39P2	40	36	13	5	40-50
39P2	40	35	13	11	40-50
F9P2	30	35	10		30-35
F9P2	30	33	9		30-35
F9P2	30	24	14		30-35
F9P2	30	37	9	13	30-35
F34P2	60	58	15	14	55-60
F34P2	60	55	17	11	55-60
F34P2	60	50	19	14	55-60
F34P2	60	68	15	6	55-60
F37P2	30	38	8	7	55-60
F37P2	30	31	9	7	30-35
F37P2	30	29	11	5	30-35
F37P2	30	35	6	9	30-35
42P3	20	30	8	10	20-30

Sample Name	Est. Day of Gestation	LZ Microvessel Count	GZ/JZ Microvessel Count	Mio Microvessel Count	Group (days)
42P3	20	26	10	13	20-30
42P3	20	23	7	10	20-30
42P3	20	30	9	8	20-30
F33	55	62	14	12	55-60
F33	55	50	10	9	55-60
F33	55	69	13	11	55-60
F33	55	65	15	12	55-60
F40P3	23	32	9	12	20-25
F40P3	23	39	6	7	20-25
F40P3	23	34	11	9	20-25
F40P3	23	26	9	6	20-25
36P3	58	55	13		55-60
36P3	58	60	12		55-60
36P3	58	63	12		55-60
36P3	58	59	9		55-60
44P1	37	36	10	9	30-35
44P1	37	29	9	6	30-35
44P1	37	32	7	7	30-35
44P1	37	34	7	12	30-35

Chapter 3
Gene expression dynamics of vascular endothelial growth factor family members in the feline placenta

3.1 Introduction

Angiogenesis, the formation of new blood vessels, is critical for development, reproduction, wound healing, and tissue regeneration, but is also a contributing factor with cancer and other diseases. During pregnancy, the vascular network in the placenta expands by two successive processes, vasculogenesis and angiogenesis, resulting in the formation of a dynamic vascular network competent to support fetal growth (Reynolds et al. 2010; Semenza 2007; Leach et al. 2002; Aplin 2017)**.** Angiogenesis is controlled by a variety of biochemical agents, notably the vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) families, and their receptors (Flt1; sFlt1and KDR). PLGF is a VEGF-related homodimeric glycoprotein expressed by the placenta during gestation. Abnormal placental vascularization is associated with pregnancy complications and birth defects (Breier 2000). In particular, an imbalance between PGF and sFlt-1 is associated with the onset of preeclampsia. In particular, an imbalance between PGF and sFlt-1 is associated with the onset of preeclampsia. (Bogic, Brace, and Cheung 2001; Demir et al. 2004; Winther, Ahmed, and Dantzer 1999).

In humans, alternative splicing of PLGF produces four different isoforms. As with the VEGF As, the main differences among these isoforms is their capability to interact with heparin, thanks to the presence of the exon 6 which codes for an heparin binding domain and can interact with the extracellular matrix (ECM). Smaller isoforms lack exon 6, thus can be secreted and diffuse to interact with distant cell targets (Yao et al. 2005; De Falco 2012; Ferrara 2000). There are no previous reports of PLGF in cats, but NCBI computational analysis of genome sequence predicts the existence of at least two feline mRNA variants (NCBI gene ID:101095904; **Figure 3.1**), The two variants of feline PLGF differing in length by 63 bp, corresponding to exon 6 being present in Var II but not Var I. This make the cat an appropriate model to study the difference between PLGF variants. Similarly, the VEGF-A gene can be translated into multiple isoforms by alternative spicing of VEGF transcripts. In humans, there are at least five major isoforms; The most common are VEGF 165, VEGF 189 and VEGF 121. Smaller isoforms such as VEGF 121 lack exons 6 and 7 while intermediate isoforms like VEGF 165 lacks exon 6 but

retain exon 7 (Houck et al. 1991; Tischer et al. 1991). As a result, VEGF 121 does not interact with heparan sulfate proteoglycans in the ECM and can more freely diffuse into the interstitial medium or through the ECM. VEGF 165 has intermediate, balanced properties, can be secreted and diffuse to reach distant cells but also can be sequestered by the ECM giving this isoform more diverse function and arguably optimal angiogenic potency and bioavailability (Park, Keller, and Ferrara 1993). In contrast, VEGF 189 is rapidly sequestered into the ECM. During inflammation, tumors growth or other situations, VEGF 189 can be released form this form of reservoir. There is limited data about the functions of PLGF and VEGF isoforms during angiogenesis and even less about their functions in the placenta during pregnancy. In the cat, NCBI Gnome system predicts two isoforms of VEGF (NC_018727.3), other bioinformatics services provide more predicted variants, but so far, no experimental data backs up these predictions.

Based on findings using tumor lines expressing selected VEGF A isoforms and variant-specific null mice, some authors suggest that different isoforms are critical for generating a microenvironmental gradient of angiogenic factors to guide endothelial cells during angiogenesis and vessel assembly. In this regard, larger isoforms have been related with increased vessel branching, producing denser vascular networks composed of vessels of smaller caliber. In contrast, predominance of smaller isoforms has been linked to formation of highly unstable, tortuous and leaky capillaries, with relatively larger diameter and fewer branch points. (Cheng et al. 1997; Grunstein et al. 2000; Ruhrberg et al. 2002; Tozer et al. 2008; Yu et al. 2002; Küsters et al. 2003).

During human pregnancy, circulating levels of PLGF show a consistent rise through the second trimester of the pregnancy (Lam, Lim, and Karumanchi 2005; Levine et al. 2004). This period corresponds to the time of maximum vessel maturation and enlargement in the placenta (Pang, Bates, and Leach 2017), suggesting that PLGF could influence vascular pattering in the placenta by mediating a shift from increased branching in early pregnancy towards vessel enlargement, elongation and maturation during mid-pregnancy. Human PLGF has been was immunolocalized in the vasculosyncytial membrane, in the media of large blood vessels of the placental villi and in maternal decidua cells, while *in situ* hybridization has detected its transcripts also in villous trophoblasts. Flt-1, principal receptor for PLGF, was also localized in syncytiotrophoblast and endothelial cells (Sawano et al. 2001). This localization suggest that

PLGF may induce autocrine trophoblast proliferation and migration into the maternal decidua and stimulate fetal and maternal angiogenesis, via paracrine activation on vascular endothelial cells (Burton, Charnock-Jones, and Jauniaux 2009; Khaliq et al. 1996; Vuorela et al. 1997).

Expression of VEGF-A transcripts in humans, bovine, canine and pigs has been localized in fetal trophoblast cells, placental macrophages, and endometrial glands. (Pfarrer et al. 2006; Gram et al. 2015; Bouwland-Both et al. 2013; Demir, Seval, and Huppertz 2007; Cooper et al. 1995; Loegl et al. 2016). VEGF A expression is dependent on the stage of pregnancy and is affected by multiple pathologies of the pregnancy (Bogic, Brace, and Cheung 2001; Demir et al. 2004; Winther, Ahmed, and Dantzer 1999). In humans, *in-situ* hybridization localized Flt-1 and VEGF A mRNA around the villous cytotrophoblast (Clark et al. 1996), KDR was located in vasculogenic and angiogenic precursor cells as well as in endothelial cells (Demir et al. 2004). VEGF A, KDR, and Flt-1 location patterns suggest roles in early vasculogenesis and blood vessel branching, very active processes during early placentation. Additionally this VEGF A location could participate in placental invasion and implantation (Ahmed et al. 1995; Vuckovic et al. 1996).

Despite the importance of VEGF A during pregnancy, limited research has been performed to study VEGF A isoforms in the placenta. It is necessary to increase the efforts toward understanding the distinct function of VEGF and PLGF isoforms in placental development. Such investigation should provide a more complete view of the interactions between the VEGF family ligands and their receptors under different conditions and how they affect vascular pattering during normal pregnancy. Knowledge gained may be vital to understand and hopefully detect, prevent, or treat pathologies of the pregnancy such as preeclampsia. In the same way, better understanding of the angiogenic process including the role of VEGF family splice variants can be useful to understanding cancer angiogenesis and develop ways to control it.

3.2 Objectives

The main objective of this study is to understand physiological angiogenesis using feline placentation as a model. We are interested in gaining insight into the role of different isoforms of VEGF family members in the formation and growth of the placenta during pregnancy. We hope that this study will produce valuable data for understanding the details of angiogenesis during

placentation and serve as a fundamental characterization of the VEGF family in the cat placenta. Under this broad umbrella, specific objectives of this study are to identify VEGF and PLGF isoforms expressed in the feline placenta, and then characterize the relative gene expression of feline VEGF A and PLGF isoforms and their receptors during the feline pregnancy. Alongside analysis of PLGF variant expression, we measured expression of the structurally- and functionally-related VEGF-A and VEGF-B family members and their shared receptors, as well as that of other genes associated with regulation of neovascularization, including Hypoxia-inducible factor 1 Alpha (HIF-1a), Angiopoietin-2, sFLT-1, and thrombospondin 1 (TSP 1). In addition, we attempt to detect protein levels of VEGF-A, PLGF, Flt-1 and KDR using western blot and immunohistochemistry to localize these angiogenic factors and their receptors in placental tissue.

3.3 Hypothesis

We hypothesize that the cat placenta express multiple isoforms of VEGF and PLGF and that gene expression patterns of these isoforms and their receptors varies across pregnancy in a manner consistent with the progression of development of the placenta and its vascularization, as described in Chapter 2. We predict that expression of longer (heparin-binding) VEGF isoforms is higher early in pregnancy when vessel branching occurs, and that shorter binding VEGF isoforms predominate later in support of vessel enlargement and maturation.

3.4 Methodology

3.4.1 Sample collection and preparation. A total of 86 placental specimens, derived from tissue that is otherwise routinely discarded after ovariohysterectomy of pregnant cats at spay and neuter clinics (e.g., Mountain View Humane, Christiansburg, VA), was instead recovered immediately after surgery as described in Chapter 2. Whole uteri were dissected, the placentas was detached from the uterine wall, and three to four samples from the central part of the placental girdle were collected using a 10mm punch biopsy (**Figure 3.2**). Fetuses were measured using a caliper to determine the occipital-sacral length and the biparietal diameter in order to estimate their gestational age according to formulae described in Zambelli *et al.* (Zambelli et al. 2002; Zambelli et al. 2004). Placental samples were rapidly placed in a 2 ml tube with RNAlater (Qiagen - Hilden, Germany) on ice for transport to the lab and stored refrigerated until processed

for RNA extraction. The remaining portion of the placenta with uterus was cut into approximately 1 cm^2 pieces and placed in 10% formalin for ≥24 hours for histological analysis.

3.4.2 RNA extraction. Placental samples (~60 mg) from each biopsy punch were transferred to 2ml polypropylene tubes with 1 ml of TRIzol (Invitrogen, Carlsbad, CA) on ice. Tissue was triturated using an electric tissue homogenizer drill (Tissuemiser – Fisher Scientific). Chloroform (200 ul) was added, and, after vortexing, samples were centrifuged for 15 min to separate 3 phases in the Trizol tube. Total RNA was extracted from the clear aqueous phase using a Zymo RNeasy mini kit (Zymo Reseach, Irvine, CA) according to the manufacturer's recommendations and including DNase 1 incubation to reduce carryover of genomic DNA. RNA concentration and quality was determined using a NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). All samples had 260/280 and 260/230 ratios over 1.90. RNA samples were stored at -20^{o}C until later use. Random-primed complementary DNA (cDNA) was synthesized using 1 μg of total RNA and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in 20uL reactions, following the manufacturer's instructions.

3.4.3 End point PCR. To detect the presence of cDNAs representing the various isoforms of agents of interest, we designed primers (**Table 3.1**) to amplify the complete coding sequence of VEGF-A (BH 621 – BH 657) and PLGF (BH-667 – BH-670), based on predicted NCBI sequences and homology with other species (**Figure 3.1**). PCR reactions were performed using 2 ul of cDNA template, 0.25 uM primers, and 1x PCR Taq polymerase mix (Qiagen - Hilden, Germany) in 50 ul reactions in the Hybaid SPRT 001 thermal cycler (Hybaid Ltd - Cambridge, United Kingdom). Cycle parameters were as follows:

Cycle no.	Denaturation (94^{o}C)	Annealing (55^{o}C)	Extension (72^{o}C)
1	2 min	1 min	2 min
2-29	1 min	1 min	2 min
30	1 min	1 min	10 min

PCR products were briefly stored at 4^{o}C and fractionated by agarose gel electrophoresis the same day.

3.4.4 Gel electrophoresis. To separate PCR products of the range of sizes anticipated based on the known multiple isoforms, we loaded PCR reactions onto 1.5 % arose gels in 1X Tris-Borate-EDTA for electrophoresis at 100 volts constant voltage (approximately 45 min on an 8 cm gel). Bands were detected on a UV light box in gels stained post-electrophoresis with 0.1% ethidium bromide. Agarose gel containing bands of interest were excised using a razor blade, and DNA products were recovered using QIAEX II gel extraction reagents (Qiagen - Hilden, Germany) following the manufacturer's instructions for the size of DNA fragments present (**Figure 3.3**).

3.4.5 Molecular Cloning. PCR amplicons were captured using a TA Cloning™ Kit, with a pCR 2.1 vector and One Shot TOP10 Chemically Competent *E. coli* (Invitrogen - Carlsbad, CA) following the manufacture's recommendations. Transformed bacteria were spread on LB agar plates supplemented with 100 ug/ml ampicillin and X-gal to select for likely transformants. Multiple white (positive) colonies of each targeted species were picked with sterile toothpicks into 1 ml LB/Amp cultures and incubated overnight at 37^{o}C with 200 rpm shaking. End-point PCR using 1 ul bacterial lysates as template and flanking M13For and M13Rev primers was used to screen for expected insert size; positive PCR amplicons were sequenced (Virginia Bioinformatics Institute Genomics Lab) to identify clones with non-mutated sequences and to ascertain insert orientation. Plasmid purification from selected clones was performed using the Perfect Prep Endofree maxi kit (5 Prime GmbH - Hilden Germany). Finally, purified plasmid DNA was sequenced to reconfirm presence of different isoforms and to compare with the predicted mRNA feline PLGF (XM003987840; XM003987841 and VEGF A sequences (XM_023253550.1; NM_001009854.1). In addition, **Figure 3.4** shows predicted amino acid sequences encoded by the cloned cDNAs. Purified plasmids were used as positive controls in the development of qRT-PCR assays specific for each variant of PLGF and VEGF transcripts. These full-length constructs were also used as a source of coding sequences for transient protein expression for western blotting positive controls.

3.4.6 Real Time qPCR. DNA Primers and Minor groove binding (MGB) DNA probes were designed in Primer Express 3.0.1. Custom TaqMan MGB probes and TaqMan Gene Expression Master Mix reagents were used (Applied Biosystems, Foster City, CA). Each detected isoform of feline PLGF and VEGF A was TA cloned into pCR-2.1 using sequence specific primers as

described above. Standard curve dilution series for each homogeneous cloned target were examined by real-time qPCR in order to document amplification efficiencies above 90% (**Figure 3.5**). Isoform specificities of the reagent sets were tested by preparing cross-reaction (off-target) standard curves. For example, primer and probes developed for VEGF A 119 were tested using VEGF 163 and VEGF A 186 homogeneous clones. Standard dilution of these cross reactions were made to assure that the primers and probes were only amplifying the intended VEGF/PLGF isoform (Figure 3.5). 18s rRNA was used as normalizer target with TaqMan VIC Ribosomal RNA control (Applied Biosystems, Foster City, CA, 4308329).

All real-time PCRs were run on a One-step Systems (Applied Biosystems) using regular 2hr cycles with triplicate for each sample and 20 ul reactions in a 96 wells plate. A total of 84 cDNA samples from placenta were used for relative expression analysis. A set of three 96 wells plates where used for each gene. Controls for each plate included, two negative control samples with ultrapure water to test for contamination, and two genomic (non-reverse transcribed) DNA controls to confirm cDNA-dependence of signal.

3.4.7 Western Blotting. As is the case for numerous domestic animal species, there are relatively few commercially-available antibodies prepared specifically for, or validated for use on, samples of feline origin. Thus we undertook the creation of protein standards, based on the known coding sequences for feline PLGF and VEGF A, to use as control reagents in the screening of candidate antibodies. Coding sequences for PLGF Var 1, 2 and VEGF-A 187 were directionally subcloned from pCR-2.1 (section **3.4.5** above**)** into pcDNAIntA (Huckle and Roche 2004) following digestion with Xhol and Xbal for pcDNAInta and Xhol – SpeI HF(NEB), for pCR-2.1, placing transcription under control of the constitutive CMV immediate-early promoter. Following plasmid purification and sequencing to confirm insert orientation and sequence fidelity, 5 ug of each construct was transfected into HEK293 cells in 60mm dishes using TransIT-293 reagents (Mirus - Madison, WI). Conditioned media were collected 48 hours after transfection, and PLGF and VEGF were adsorbed from media by overnight incubation at 4^{o}C in the presence of 350 ul heparin-agarose beads (Sigma - St. Louis, Missouri), with continuous end-over-end agitation. Following recovery of beads by centrifugation at 150xg, adsorbed proteins were solubilized by addition of 350 ul of 2X Laemmli sample buffer (BioRad, Hercules, CA) and fractionated on 12% polyacrylamide gels for antibody screening. Separated proteins were

transferred electrophoretically overnight at constant 30 mAmp. Proteins where blotted into nylon membranes (PVDF Immobilon – P, Millipore – Billerica, Massachusetts) which subsequently were blocked for 2 hours at 4^{o}C in 10 ml Odyssey blocking reagent (Licor) and incubated with primary antibodies (**Table 3.2**) diluted in 10 ml of 0.5X blocking buffer with 0.1% of tween 20 for 2 hours at room temperature over a rotamixer. After washing with PBS with 0.1 Tween 20 (PBST), immunoreactive protein species were located by 1 hour incubation in 0.5X blocking buffer / 0.1% of Tween 20 containing the appropriate anti-mouse, anti-rabbit, or anti-goat secondary antibodies labeled with infrared fluorophores 800CW or 680RD (Licor, Lincoln, NE). After washing in PBS/Tween-20, labeled protein species were imaged on an Odyssey Classic Infrared Imager. Membranes were probed subsequently with anti-beta-actin as a normalizing control.

3.4.8 Immunohistochemistry. Placental and uterine tissue, obtained as described under section 3.4.1, was fixed in 10% formalin for 24 hours and processed for paraffin embedding, 5 um sectioning, and staining with hematoxylin and eosin (H&E) in the Virginia Maryland College of Veterinary Medicine Diagnostic Lab (ViTALS). Selected tissue blocks were recut to 5 um sections, mounted, dried at 42°C overnight, and stored at room temperature for less than 3 days before staining. All steps were performed at room temperature unless otherwise specified using the ultraView Universal Alkaline Phosphatase Red Detection Kit from Ventana (#760-501) and manual conventional histology protocols. Briefly, sections were deparaffinized in xylene, rehydrated and washed with 1X reaction buffer (1X RB; Ventana cat. no. 950-300). Unmasking of antigens was performed in a polyethylene staining jar with Ventana cell conditioner 1 (950-124) for 60 min at 95 C. Sections were then rinsed in 1X RB and blocked with 125 uL of Ventana 760-050 for 8 min. Primary antibodies (Table 3.2) were diluted in Ventana incubation diluent (251-018); 125 uL of this dilution was used per slide. Slides were incubated for 1 hour and then washed with 1X RB. Negative control slides were exposed to incubation diluent without primary antibody. For secondary incubation, we used 125 uL of ultraView Universal Alkaline Phosphatase Red Detection Kit (Ventana 760-501), followed by 125 uL of UV red enhancer and incubated for 4 minutes. UV Fast Red A (100 ul) and 100 ul of UV red Naphthol was applied and incubated together for 8 min. Finally, 125 ul of UV Fast Red B was incubated for 8 min in rinsed with 1X RB. Slides were counterstained with hematoxylin for 45 sec, rinsed

and air dried for at least 30 min before the coverslip was applied. Samples were observed under a Nikon Eclipse Ci-S microscope; images were captured on a Nikon camera and analyzed using NIS-Elements Analysis D 5.01 image software.

3.5 Results

3.5.1 Detection of VEGF-A Isoforms and sequencing. Total RNA extracted from placental tissue of the domestic cat was subject to PCR using specific primers (**Table 3.1**) aiming to amplify all possible isoforms of PLGF (BH-667 – BH-670), and VEGF A (BH 644 – BH 646) based in predicted NCBI sequences and homology with other species (**Figure 3.1**). Gel electrophoresis of end point PCR product for PLGF detected bands of ~550 and ~600 bp, the presumptive isoforms 1 and 2 (**Figure 3.3A**). Gel electrophoresis of VEGF A PCR amplicons reveal 3 distinct bands of ~200, ~270 and ~300 bp. These Isoforms were present in the placenta at different stages of the pregnancy from day 20 to day 60 (**Figure 3.3B**). DNA was extracted from abovementioned bands, TA cloned into competent *E. coli*, and resulting plasmids were sequenced. Alignment of the sequences reveal three distinctive isoforms for VEGF A and two for PLGF. Using Translate tool from ExPASy, we predicted proteins of 187, 163 and 119 amino acids (excluding the predicted 26AA signal peptide). When compared to NCBI VEGF A predictions for cat and human VEGF A 165, there is a complete identity between the predicted 163 isoform and VEGF 163 isoform detected in placenta. Human and feline VEGF A proteins are highly conserved, although human VEGF 163 has two more AAs, in positions 32 and 141., (**Figure 3.4**) Based on homology with other human isoforms, this feline variants corresponded to the most common human VEGF A 149, 163 and 121. Differences in exon 6 and 7 are clear in smaller isoforms lacking AA 141 to 164 or 141 to 208 for VEGF 163 and 119 respectively. PLGF Isoforms detected were 170 and 149 AA equivalent to the human 131 and 152 isoforms (minus an 18AA signal peptide). This isoform has 100% identity with the predicted isoform in cat. In addition, there is a predicted PLGF 181 that we did not find in placenta.

3.5.2 Relative expression of VEGF A, VEGF B and PLGF isoforms in the placenta during pregnancy. Using qRT-PCR on 84 placental samples, we were able to quantify the relative expression of a combination of all isoforms, or "panVEGF-A," and the specific isoforms VEGF A 119, VEGF A 163, and VEGF A 187, in tissue from the placental girdle of the domestic cat.

All three isoforms were detected during pregnancy from groups 20-25 to 55-60 (**Figure 3.6**). With gestational age d20-25 as baseline, all VEGF A isoforms follow a similar pattern of expression, showing an upregulation at mid pregnancy (d30-35), followed by a steady decline toward term. Expression of VEGF A119, relative to VEGF A163 and VEGF A187, appears to rise early during pregnancy, with over 4-fold increases in expression compared to group d20-25 (**Figure 3.7**).

PLGF variant I and II were also detected in all stages of gestation measured. Both isoforms follow a similar pattern of expression, with constant expression during the first 2/3 of the pregnancy followed by a marked increase of over 4 times the expression at d20-25 (**Figure 3.8**). VEGF B, an angiogenic factor that shares the same receptors with PLGF, was constantly expressed during pregnancy. We measured a small increase in relative gene expression at late pregnancy, but no statistically significant differences were noted (**Figure 3.8**).

3.5.3 Relative expression of KDR, Flt-1 and sFlt-1 the placenta during pregnancy. Tyrosine kinase receptor Flt-1 constantly increases its expression during pregnancy and, by the end of the pregnancy (d55-60), reaches mean expression to 5 times the expression levels measured at d20-25 (**Figure 3.9**). Relative expression of KDR in the placenta, was stable during the whole pregnancy. Relative expression of the truncated soluble sFlt-1 was constant during pregnancy, with high individual variations (**Figure 3.9**).

3.5.4 Relative expression of other major angiogenic regulators in the placenta. HIF 1a is a transcription factor that couples tissue hypoxia to elevated VEGF A expression. In our study, no significant changes in expression of HIF 1a were measured during pregnancy (**Figure 3.10**). The anti angiogenic gene THBSP 1, showed a small down regulation during mid pregnancy, although no statistically significant differences were found. Expression of panVEGF-A was compared to THBS 1, revealing increasing ratios of panVEGF relative to THBS 1 during mid pregnancy. Another pro-angiogenic regulator, ANG 2, had a stable expression during pregnancy.

3.5.5 Immunolocalization of KDR and Flt-1 in placenta. Immunohistochemistry was used to localize VEGF A, PLGF, KDR and Flt-1 in 16 placental FFPE samples, representing a subset ranging from early pregnancy (day 14 p.c.) to near term (day 60). Controls used included non-

pregnant feline uterus, heart, kidney, liver (obtained from the ViTALS tissue block archive), as well as rat kidney, liver, heart, and spleen. These were used to assess the efficacy of IHC methodologies. For descriptive proposes, protein localization in placenta was separated in early pregnancy d14 to d25 and late pregnancy d45 to d60. All targeted proteins were detected in the placenta. KDR was abundantly immunolocalized from early pregnancy in maternal and fetal endothelial cells of the lamellar zone and in maternal lamella. KDR was also detected in uterine glands of the Glandular Zone (**Figure 3.11**). Late in pregnancy follows a similar distribution. The receptor was still immunolocalized in the maternal capillaries in the lamellae and occasionally in fetal capillaries, especially in the luminal side of the LZ, in epithelial cells of endometrial glands and capillaries in the uterine myometrium. Additionally, clusters of decidual cells in maternal lamella were immunoreactive (**Figure 3.12**).

The second tyrosine kinase receptor for VEGF family members, Flt-1, was immunolocalized during early pregnancy in the LZ on the placenta, specifically, in trophoblast cells of the fetal lamellae located at each side of the maternal lamellae. Positive trophoblast cells are apparently syncytiotrophoblast, although absolute determination based only in light microscopy is difficult. Flt-1-positive cells include endothelial cells in fetal capillaries and fetal fibroblast in the connective tissue. In addition, syncytiotrophoblasts around multinuclear cell clusters in the JZ showed Flt-1 immunoreactivity (**Figure 3.13**). At late pregnancy, Flt-1 follow the same localization pattern as earlier in pregnancy, immunolocalized in trophoblast cells at each side of the maternal lamellae, in maternal and fetal microvasculature, and in epithelial cells of endometrial glands (**Figure 3.14**)

3.5.6 Immunolocalization of PLGF in placenta. During early pregnancy, PLGF was immunolocalized in the LZ associated to maternal and fetal vasculature. (**Figure. 3.15**) During late pregnancy, PLGF was abundantly present in placenta, associated with maternal and fetal capillaries in the LZ and in endothelial cells of myometrium vasculature (**Figure 3.16**). This pattern of distribution was very similar to that of KDR.

3.5.7 Immunolocalization of VEGF A in placenta. VEGF was immunolocalized in clusters at the maternal lamellae early in the pregnancy (d20). Immunopositive cells appear to be decidua cells based on their distribution in the maternal lamellae and their morphology of. Although

further confirmation with other methodologies is needed. VEGA A also appeared to be localized in cells of the trophoblast attached to maternal lamellae, similarly to Flt-1. Distribution of VEGF A seems to vary from sample to sample and in different areas of the same slide (**Figure 3.17**). During late pregnancy, VEGF A had a similar distribution compared to early pregnancy, but appears to be localized increasingly in maternal endothelial cells in the lamella, and less prominently associated with trophoblast cells (**Figure 3.18)**.

We can summarize our IHC results to note that in the lamellae, KDR is located in endothelial cells of maternal capillaries in the center of the lamella, whereas Flt-1 is located in the trophoblast cells located at each side of the maternal lamellae, as well as in fetal vasculature (**Figure 3.19**). On the other hand, VEGF A immunolocalization varies from different structures of the placenta; in some areas VEGF A was localized in trophoblast cell while in others it was associated with endothelial cells. Finally, PLGF in the lamellae was immunodetected in maternal and fetal capillaries of the LZ.

3.5.8 Immunolocalization of VEGF A, PLGF, KDR and Flt-1 in fetal membranes.
We compared the protein localization of these main angiogenic regulators in the fetal membranes (**Figure 3.20**). We included chorioallantoid membrane in the equatorial region where the placental girdle is not developed. The advantage of looking at this area is the relatively simpler histology compared to the labyrinthine nature of the LZ. Allantoamnios and vitelline membrane were also included. KDR and PLGF were localized in endoderm of the yolk sack; KDR and Flt-1 also appear to co-localize in capillaries of the yolk sac. VEGF A and PLGF co-localize in blood cell precursors. In the chorioallantoic membrane at the paraplacenta, all proteins co-localized in the trophoblastic cells of the chorion. KDR was additionally detected in microvasculature related to the allantois and in the mesothelium of the allantois. In the allantoamnios, no protein was detected in amniotic membrane, although KDR and Flt-1 appear to co-localize in the mesothelium of the allantois that is partially attached to the amnions.

3.5.9 Immunodetection of VEGF A and Flt-1 protein in placenta. Tissue from 38 placental samples representing different times in pregnancy that had been stored in RNA later were processed for total protein extraction (**Table 3.4**). To assess quality and solubility of extracted proteins, we performed a sodium dodecyl sulfate polyacrylamide gel electrophoresis and

Coomassie blue staining. Sharp proteins bands of diverse sizes were seen in all samples and at different dilutions, suggestive of low degradation (**Figure 3.21A**) and encouraging their use for 'Western' immunoblotting studies. As a positive control for the western blotting methodology and as a normalizing protein control, we immunodetected beta-actin at different stages of the pregnancy. Clear bands of ~45kDa were seen in all stages of the pregnancy, confirming the correct use of western blotting technique (Figure 3.21B).

Due to the lack of commercially available antibodies for domestic cat, we sought to validate candidate antibodies using transiently expressed proteins in human HEK293 cells from previously cloned feline VEGF A 187 and canine Flt-1 constructs. Flt-1 was immunodetected using H-225 from Santa Cruz at approx. 124 kDa in the positive control of HEK293 cells (**Figure 3.22A**). VEGF A was detected with a band of approximately 18kDa, similarly to a human recombinant VEGF A 165 (R&D Systems)(**Figure 3.22B**). Based on the detection of recombinant proteins VEGF A187 and Flt-1 with the antibodies C1 and H-225, respectively, we continued with these antibodies to immunoblot VEGF-A and Flt-1 in placental samples. VEGF A was detected in placental tissue during early pregnancy with a positive band of ~20 KDa (**Figure 3.22C**). The receptor Flt-1 was immunolocalized in placental tissue as well as in aorta and heart. Despite multiple attempt to detect PLGF and KDR, using different antibodies and a variety of protein extraction protocols, incubation times and temperatures, as well as modifying conditions of electrophoresis (use of different reducing/alkylting agents) and blotting, we were not able to confidently detect PLGF or KDR in the placenta. Antibodies tested include Flk-1 (SC-F10), Flk-1 (SC-A3), Flt-1 (SC-D2), Flt-1(SC-C17), Flt-1 (N16), PLGF H90 (data not shown). Further studies are needed to identify alternative methods for detection of these proteins with western blotting techniques, although we consider the successful localization of protein by IHC in tissue sections, as presented earlier in this chapter to be more valuable.

3.6 Discussion and Conclusions

In this study, we provide evidence of differential spacio-temporal expression of angiogenic regulators of the VEGF family in the placenta. First, we identified placental expression of three isoforms of VEGF A (VEGF A119, VEGF A163, and VEGF A187) and two PLGF isoforms (PLGF I and PLGF II) (**Figure 3.3**). Based on homology with humans and computational analysis, we predict that these variants were homologues of the most common VEGF A and PLGF factors expressed in humans (**Figures 3.1 and 3.4**), and were very similar to mouse, dogs and other felids (data not shown). High degree of genomic conservation can be expected based on the critical functions of these proteins; as evolutionary genetic research has shown, small mutations in critical genes can have deleterious effects during development, such that selective pressure exists for conservation. (Cross et al. 2003; Rawn and Cross 2008; Benirschke, Burton, and Baergen 2012). Additionally, feline genome sequences projects have pointed out the prominent similarities between the human and feline genome (O'Brien et al. 2008; Pontius et al. 2007; Murphy et al. 2005).

The different variants detected differ in the presence of exons coding for heparin binding domains, thus different variants could have distinct proprieties and biological functions. Although their function and differences has not been extensively characterized, different isoform are believed to affect vascular patterning. Smaller diffusible isoforms without heparin binding domains like VEGF A119 have been linked to increase vascular perfusion, long tortuous vessels with large caliber and fewer branching. In contrast, longer heparin binding isoforms like VEGF A187 produce smaller, very dense capillary networks (Cheng et al. 1997; Grunstein et al. 2000; Ruhrberg et al. 2002; Tozer et al. 2008; Yu et al. 2002; Küsters et al. 2003; Carmeliet et al. 1999).

Relative gene expression analysis reveals that all isoforms were expressed in the placenta during pregnancy, and in general, all isoforms followed a similar pattern of expression, with relative increase in expression during mid-pregnancy following a gradual reduction of expression toward the end of the pregnancy (**Figure 3.6**). This is similar to measurements in the canine placenta, where VEGF A transcripts rose during mid-gestation in placental girdle and uterus but were downregulated close to parturition (Gram et al. 2015). In contrast, VEGF A in human ovine and pig pregnancies shows a different expression profile, where it gradually increases along gestation until term (Cheung 1997; Vonnahme, Wilson, and Ford 2001).

We measured placental levels of mRNA of the well-characterized transcription factor HIF 1a to evaluate if it was driving VEGF-A expression during pregnancy. We found that HIF 1a expression did not fluctuate measurably during the pregnancy (**Figure 3.10**) and most likely was not influencing the upregulation of VEGF A during mid-pregnancy. This uncoupling of HIF 1a and VEGF A expression has been documented before in mouse pregnancy (Daikoku et al. 2003). We hypothesize that in placenta, hormonal levels of progesterone or estrogen are stronger direct or indirect regulators of VEGF A and PLGF. Particularly in dogs and cats, plasma levels of progesterone rise during mid-pregnancy and then are reduced before delivery (Chakraborty 1987; Verhage, Beamer, and Brenner 1976), following similar pattern of expression VEGF A, whereas in humans, levels of progesterone increase constantly during pregnancy (Kumar and Magon 2012), following a similar pattern described for VEGF A expression as well. Estrogen (17-beta estradiol) also is increasingly expressed at term of pregnancy in humans. Nevertheless, there is conflicting evidence supporting the role of estrogen in uterine and placental angiogenesis. Some recognize estrogen as a VEGF A activator thought nuclear estradiol receptor (ER-beta) and via estrogen response elements at the VEGF A gene (Arnal et al. 2010; Kim and Bender 2009; Hyder et al. 2000). Others have shown that estrogen can stimulate uterine vascular permeability, but negatively regulates angiogenesis in the same tissue. In comparison, progesterone increases angiogenesis without affecting the permeability of the vessels (Ma et al. 2001) and can upregulate uterine VEGF A expression through activation of the nuclear progesterone receptor (Hyder et al. 2000; Goddard et al. 2014; Boroujeni, Boroujeni, and Gholami 2016; Kim et al. 2013). This evidence is consistent with the notion that in dogs and cats, progesterone may drive VEGF A expression in the placenta, although further studies are needed to evaluate this prediction.

Comparison between the relative changes in expression of the different isoforms during pregnancy reveal a higher upregulation of VEGF 119 at d30-35 relative to d20-25. (**Figure 3.7**). We can speculate that diffusible VEGF A119 could act as a signal from placental trophoblast to induce growth of maternal vasculature. Crossing the placental barrier in this case seems highly possible considering the low molecular weight of VEGF A protein (~45 kDa homodimer) plus the lack of heparin-binding domains. For example, equine chorionic gonodotrophin, a protein of similar size secreted by equine chorion, crosses the mare's thicker epitheliochorial placenta sufficiently to stimulate the corpus luteus and maintain progesterone levels and pregnancy

(Murphy and Martinuk 1991). Thus it is probable that VEGF A secreted by the fetus can cross the placental barrier and have an effect in maternal capillaries. The opposite scenario, where expression of maternal VEGF A could potentiate fetal angiogenesis is also possible. It would be interesting to localize different VEGF A isoforms in placental tissue, although regular immunological techniques are not suitable for such studies. Alternative methods, such as *in situ* hybridization using 'RNA scope' could be used to localize specific isoforms of VEGF A and PLGF in tissue to explore this possibility (Erben et al. 2018).

Relative increases in VEGF A119 during mid pregnancy could also be due to a role of VEGF A119 promoting elongation and increasing caliber of the vessels and at the same time increasing vascular permeability, in a period of time were increase in thickness of the LZ is at its peak and vascular and lamellar proliferation are high (**Figures 2.3-2.5**). This is contrary to the view that during the second trimester in humans there is increasing sprouting angiogenesis with extensive branching and anastomosis of the capillary network, while during the third trimester, elongation and increased caliber of existing branches are more prominent (Chen and Zheng 2014).

In order to investigate expression changes among relative VEGF A isoforms during pregnancy, we compared ratios between expression of VEGF A163/187, VEGF A165/119 and VEGF A 119/187, finding that all isoforms were expressed in similar ratios during pregnancy (**Figure 3.23**). In studies in mouse placenta, VEGF 164 was reported as the most prevalent, followed by VEGF A 120 (Halder et al., 2000), although these estimations were performed by semi-quantitative densitometric analysis of end point RT-PCR products. In addition, only placental samples until day 8 post coitus were evaluated. In humans, VEGF A121, 165 and 189 were measured using a similar semi-quantitative RT-PCR analysis to compare placental mRNA expression in normal pregnancies *vs* placentas with preeclampsia. All VEGF A isoforms' mRNAs increased expression at similar rates in preeclampsia; furthermore, in agreement with our findings, these investgators reported that mRNAs for all measured VEGF A isoforms were similarly expressed in normal placentas (Chung et al. 2004). Our detection of multiple isoform of VEGF A, those with and without heparin binding domains, in the cat placenta suggests that a mixture of isoforms is needed for adequate vascular proliferation. As proposed by others, a panel of different isoforms may help to maintain appropriate VEGF gradients that guides endothelial cells during angiogenesis (Álvarez-Aznar, Muhl, and Gaengel 2017).

We were able to detect VEGF A during early pregnancy using western blot and to localize VEGF A protein in placental tissue during early and late pregnancy using immunohistochemistry. VEGF A was detected on reducing/denaturing gels with a band of approximately 18 KDa at days 20 to 25 (**Figure 3.2**), supporting its role as an early promoter of placental angiogenesis. Additionally VEGF A was immunolocalized in clusters showing a patchy distribution in the maternal lamellae, possible associated to decidual cells. VEGF A was also localized in trophoblast cells of the LZ and in the para-placental region. Distribution of VEGF A seems to fluctuate in distribution from sample to sample and in different areas of the same slide. Interestingly, VEGF A has been associated previously with decidual cells and syncytiotrophoblasts in primates (Wulff et al. 2002) and in multinucleate cytotrophoblasts in cattle (Pfarrer et al. 2006). Additionally, in agreement with our localization and distribution of VEGF A, in humans this protein has been localized with an uneven distribution around the villous cytotrophoblast using *in situ* hybridization (Clark et al. 1996). VEGF A was also localized in maternal and fetal endothelial cells of larger vessels especially during late pregnancy and in the luminal side of the placenta. Similarly, others have found VEGF A associated with vasculature of the placenta in humans. (Tsoi et al. 2002; Borowicz et al. 2007) and bovine placenta (Pfarrer et al. 2006). These finsings support the indispensable role of VEGF A during placental angiogenesis.

Relative gene expression of PLGF var I and var II reveal that both isoforms are detected from early pregnancy with a stable expression during the first 2/3 of the pregnancy followed by a marked up-regulation at late pregnancy (**Figure 3.8**). Early expression of PLGF has been described in multiple species including mouse, human and ovine, supporting the crucial role PLGF to regulate placental angiogenesis (Persico, Vincenti, and DiPalma 1999; Grazul-Bilska et al. 2011; Wright et al. 2016). Late increased expression of PLGF has also been seen in rats (Choi et al. 2005). On the other hand, plasma levels of PLGF in humans peak at around 30 weeks, then progressively fall towards term (Sherrell et al. 2018; Saffer et al. 2013).

To determine differences in expression between the two isoforms, we calculated the ratios of expression of PLGF II relative to PLGF I. We found that ratio between the isoforms is steady during the first 2/3 of the pregnancy, while thereafter the PLGF II : PLGF I ratio increases during late pregnancy (**Figure 3.24**). In humans, measurements of maternal serum of PLGF concentrations during pregnancy reveal more than 3 times higher serum levels of PLGF 2 over

PLGF 1 during weeks 11 to 14 in pregnancy. Additionally PLGF VR1 was greatly reduced during pre-eclampsia, and both isoforms were reduced in pregnancies with trisomy of chromosome 21, 18 and 13 (Nucci et al. 2014).

Expression of PLGF during late pregnancy could induce maturation and stabilization of placental vasculature. This effect could be achieved by PLGF's described role to increase proliferation and recruitment of smooth muscle cells and supporting the proliferation of fibroblasts and pericytes around the vasculature (Yonekura et al. 1999; Bellik et al. 2005), although the effect of high levels of PLGF in the late pregnancy are not well understood. PLGF could have a pro-angiogenic effect by enhancing KDR kinase activation by VEGF A. In this situation, PLGF activation of Flt-1 has been shown to induce transphosphorylation of KDR and produce a synergetic mitogenic effect (Autiero et al. 2003). In another scenario, high concentrations of PLGF in the microvascular environment can displace VEGF A from Flt-1, thus increasing VEGF A bioavailability to interact with KDR and thus potentiate a angiogenic effect (Carmeliet and Collen 1999). This is consistent with the creation of conditions favorable for angiogenesis in the time of pregnancy that has high rates of proliferation in the placenta of the cat. Nevertheless, the specific function of PLGF Var II during this period remain to be clarified.

Immunolocalization of PLGF in the domestic cat placenta reveals a localization during early and late pregnancy in the LZ and myometrium, associated with maternal and fetal vasculature and in the trophoblast of the para-placental zone (**Figures 3.15-3.16**). Similarly, in humans and mouse, PLGF was localized in the vasculosyncytial membrane, in the media of large blood vessels of the placental villi, and in maternal decidua cells (Khaliq, Li, Shams, Sisi, Acevedo, Whittle, Weigh, et al. 1996; Vuorela et al. 1997). Due to the limitations of light microscopy, we could not differentiate between PLGF expression in endothelial cells from vasculosyncytial membrane, thus PLGF could be present in this location in cats as well. We did not find PLGF in the media of larger vessels but rather in the endothelium of larger vessels and arterioles and venules of the luminal side of the placental girdle. Other studies have been able to localize PLGF in endothelial cells (Persico, Vincenti, and DiPalma 1999).

Flt-1 vs Flt1 expression. qRT-PCR results of studies of Flt-1 expression showed a constant upregulation in expression during pregnancy up to parturition (**Figure 3.9**). At the same time, the soluble variant sFlt-1 was constantly expressed during pregnancy. In contrast, in the

dog Flt-1 is down regulated before pregnancy (Gram et al. 2015). Comparison of ratios of sFlt-1 relative to Flt-1 reveals a down regulation of sFlt-1 compared to Flt-1 (**Figure 3.25**). This is consistent with the creation of conditions favorable to angiogenesis in the placenta microenvironment, due to the reduction of the antiangiogenic effect of sFLT as a VEGF A decoy receptor. These conditions may be potentiated thanks to the increased PLGF mRNAs during the same period.

Flt-1 was immunodetected using western blot in placental tissue at d20 and d27, in agreement our gene expression analysis and with previous reports in human placenta (Khaliq, Li, Shams, Sisi, Acevedo, Whittle, Weich, et al. 1996; Helske et al. 2001; Hou et al. 2006). Western blot detection at later stages of the pregnancy was not successful as expected based in mRNA results. Flt-1 was immunolocalized during early and late pregnancy in trophoblast cells of the fetal lamellae located at each side of the maternal lamellae, in endothelial cells of fetal and maternal origin, and in fetal fibroblast in the connective tissue. Flt-1 was also localized in epithelial cells of endometrial glands. (**Figure 3.13-3.14**). Localization of placental Flt1 in endothelial cells and in different types of trophoblast has been described in multiple species, including mouse, dog, sheep and humans (Peters, De Vries, and Williams 1993; Cheung 1997; Clark et al. 1996; Tsoi et al. 2002; Borowicz et al. 2007). Based on colocalization of PLGF and Flt-1 in endothelial cells, we can predict that endothelial cells could have an autocrine stimulatory cycle in the placenta. In a similar fashion, Flt-1's location in trophoblast suggests a role during trophoblast proliferation, migration and invasion in response to PLGF stimulus from maternal or fetal cells. In addition, it is likely that maternal expression of VEGF A, particularly VEGF A119 from maternal decidual cells or fetal trophoblast, could stimulate fetal trophoblast in a paracrine or autocrine fashion. Currently, these mechanisms of placental stimulation are mainly speculative, but have been proposed by multiple authors in different species. For a comparative view of the different receptors and ligands immunolocalized in placenta, please view **Figure 3.19**.

Expression of KDR in the placenta was stable throughout pregnancy, a pattern of expression shared with the dog placenta (Gram et al. 2015). KDR was abundantly immunolocalized from early and late pregnancy in maternal and fetal endothelial cells of the LZ especially in the luminal side, in uterine glands of the GZ, in capillaries in the uterine myometrium, and clusters of decidual cells in maternal lamellae (**Figures 3.11-3.12**). Multiple

previous reports have localized KDR in maternal and fetal vasculature including human primates, mouse and humans, consistent with its localization in other tissues where KDR is a commonly-used marker endothelial cells (Katoh et al. 1995; Barleon et al. 1994; Vuckovic et al. 1996; Clauss et al. 1996; Sawano et al. 1996; Kumazaki et al. 2002; Wulff et al. 2002). KDR distribution in placental vasculature and trophoblast cells may produce similar paracrine and autocrine implications as previously described for Flt1.

During this study, our main goal was to use the feline placentation as model to understand physiological angiogenesis, with particular emphasis on describing the behavior of different isoforms and other main regulators of angiogenesis in relationship with vascular changes observed in the vasculature. We succeeded in identifying multiple isoforms of the main angiogenic regulators VEGF A and PLGF and suggest that, based on their differences in molecular structure, they should have differential temporal-spatial distributions in the placenta during pregnancy. Our study revealed differences in relative expression patterns of mRNAs of different isoforms of VEGF A and PLGF, suggesting a tightly regulated expression during pregnancy and consistent wut their vital roles during neovascularization in the placenta. We observed a distinct pattern of localization of angiogenic factors in feline placenta and identify multiple implications for placental angiogenesis and development. Based on these results, we can accept our hypothesis and conclude that the cat placenta express different isoforms of VEGF and PLGF, and those gene expression patterns PLGF and VEGF and their receptors vary across pregnancy in a manner consistent with the progression of development of the placenta and its vascularization. In view of these results, we postulate new questions and propose different ways in which different angiogenic factors could contribute to the proper vascularization of the placenta. We hope that these results could set the ground for continuing research in angiogenesis in domestic cat placenta and could be correlated to other species in a meaningful way.

3.7 References

Ahmed, A., X. F. Li, C. Dunk, M. J. Whittle, D. I. Rushton, and T. Rollason. 1995. 'Colocalisation of vascular endothelial growth factor and its Flt-1 receptor in human placenta', *Growth factors*, 12: 235-43.

Álvarez-Aznar, Alberto, Lars Muhl, and Konstantin Gaengel. 2017. 'VEGF receptor tyrosine kinases: key regulators of vascular function.' in, *Current topics in developmental biology* (Elsevier).

Aplin, J. 2017. 'Hematopoiesis Vasculogenesis and Angiogenesis in First Trimester Placenta', *Placenta*, 57: 235-35.

Arnal, J. F., C. Fontaine, A. Billon-Gales, J. Favre, H. Laurell, F. Lenfant, and P. Gourdy. 2010. 'Estrogen receptors and endothelium', *Arterioscler Thromb Vasc Biol*, 30: 1506-12.

Autiero, Monica, Johannes Waltenberger, Didier Communi, Andrea Kranz, Lieve Moons, Diether Lambrechts, Jens Kroll, Stephane Plaisance, Maria De Mol, Françoise Bono, Stefanie Kliche, Guido Fellbrich, Kurt Ballmer-Hofer, Domenico Maglione, Ulrike Mayr-Beyrle, Mieke Dewerchin, Saskia Dombrowski, Danica Stanimirovic, Paul Van Hummelen, Christoph Dehio, Daniel J. Hicklin, Graziella Persico, Jean-Marc Herbert, David Communi, Masabumi Shibuya, Désiré Collen, Edward M. Conway, and Peter Carmeliet. 2003. 'Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1', *Nat Med*, 9: 936.

Barleon, B., S. Hauser, C. Schollmann, K. Weindel, D. Marme, A. Yayon, and H. A. Weich. 1994. 'Differential expression of the two VEGF receptors flt and KDR in placenta and vascular endothelial cells', *J Cell Biochem*, 54: 56-66.

Bellik, L., M. C. Vinci, S. Filippi, F. Ledda, and A. Parenti. 2005. 'Intracellular pathways triggered by the selective FLT-1-agonist placental growth factor in vascular smooth muscle cells exposed to hypoxia', *Br J Pharmacol*, 146: 568-75.

Benirschke, Kurt, Graham J Burton, and Rebecca N Baergen. 2012. 'Placental types.' in, *Pathology of the human placenta* (Springer).

Bogic, L. V., R. A. Brace, and C. Y. Cheung. 2001. 'Developmental expression of vascular endothelial growth factor (VEGF) receptors and VEGF binding in ovine placenta and fetal membranes', *Placenta*, 22: 265-75.

Boroujeni, Mandana Beigi, Nasim Beigi Boroujeni, and Mohammadreza Gholami. 2016. 'The effect of progesterone treatment after ovarian induction on endometrial VEGF gene expression and its receptors in mice at pre-implantation time', *Iranian Journal of Basic Medical Sciences*, 19: 252-57.

Borowicz, P. P., D. R. Arnold, M. L. Johnson, A. T. Grazul-Bilska, D. A. Redmer, and L. P. Reynolds. 2007. 'Placental growth throughout the last two thirds of pregnancy in sheep: vascular development and angiogenic factor expression', *Biol Reprod*, 76: 259-67.

Bouwland-Both, M. I., E. A. Steegers, J. Lindemans, H. Russcher, A. Hofman, A. J. Geurts-Moespot, F. C. Sweep, V. W. Jaddoe, and R. P. Steegers-Theunissen. 2013. 'Maternal soluble fms-like tyrosine kinase-1, placental growth factor, plasminogen activator inhibitor-2, and folate concentrations and early fetal size: the Generation R study', *Am J Obstet Gynecol*, 209: 121 e1-11.

Breier, G. 2000. 'Angiogenesis in embryonic development--a review', *Placenta*, 21 Suppl A: S11-5.

Burton, G. J., D. S. Charnock-Jones, and E. Jauniaux. 2009. 'Regulation of vascular growth and function in the human placenta', *Reproduction*, 138: 895-902.
Carmeliet, P., and D. Collen. 1999. 'Role of vascular endothelial growth factor and vascular endothelial growth factor receptors in vascular development', *Curr Top Microbiol Immunol*, 237: 133-58.
Carmeliet, Peter, Yin-Shan Ng, Dieter Nuyens, Gregor Theilmeier, Koen Brusselmans, Ivo Cornelissen, Elisabeth Ehler, Vijay V Kakkar, Ingeborg Stalmans, and Virginie Mattot. 1999. 'Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF 164 and VEGF 188', *Nat Med*, 5: 495.
Chakraborty, P. K. 1987. 'Reproductive hormone concentrations during estrus, pregnancy, and pseudopregnancy in the Labrador bitch', *Theriogenology*, 27: 827-40.
Chen, D. B., and J. Zheng. 2014. 'Regulation of placental angiogenesis', *Microcirculation*, 21: 15-25.
Cheng, Shi-Yuan, Motoo Nagane, H-J Su Huang, and Webster K Cavenee. 1997. 'Intracerebral tumor-associated hemorrhage caused by overexpression of the vascular endothelial growth factor isoforms VEGF121 and VEGF165 but not VEGF189', *Proceedings of the National Academy of Sciences*, 94: 12081-87.
Cheung, Cecilia Y. 1997. 'Vascular Endothelial Growth Factor: Possible Role in Fetal Development and Placental Function', *Journal of the Society for Gynecologic Investigation*, 4: 169-77.
Choi, W. S., G. J. Cho, C. K. Won, and P. O. Koh. 2005. 'Expression of placenta growth factor mRNA in the rat placenta during mid-late pregnancy', *J Vet Sci*, 6: 179-83.
Chung, Jin-Young, Yang Song, Yuping Wang, Ronald R. Magness, and Jing Zheng. 2004. 'Differential Expression of VEGF, EG-VEGF, and VEGF Receptors in Human Placentas from Normal and Pre-eclamptic Pregnancies', *J Clin Endocrinol Metab*, 89: 2484-90.
Clark, D. E., S. K. Smith, A. M. Sharkey, and D. S. Charnock-Jones. 1996. 'Localization of VEGF and expression of its receptors flt and KDR in human placenta throughout pregnancy', *Hum Reprod*, 11: 1090-8.
Clauss, M., H. Weich, G. Breier, U. Knies, W. Rockl, J. Waltenberger, and W. Risau. 1996. 'The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis', *J Biol Chem*, 271: 17629-34.
Cooper, J. C., A. M. Sharkey, J. McLaren, D. S. Charnock-Jones, and S. K. Smith. 1995. 'Localization of vascular endothelial growth factor and its receptor, flt, in human placenta and decidua by immunohistochemistry', *J Reprod Fertil*, 105: 205-13.
Cross, JC, D Baczyk, N Dobric, M Hemberger, M Hughes, DG Simmons, and H Yamamoto. 2003. 'Genes, development and evolution of the placenta', *Placenta*, 24: 123-30.
Daikoku, Takiko, Hiromichi Matsumoto, Rajnish A. Gupta, Sanjoy K. Das, Max Gassmann, Raymond N. DuBois, and Sudhansu K. Dey. 2003. 'Expression of Hypoxia-inducible Factors in the Peri-implantation Mouse Uterus Is Regulated in a Cell-specific and Ovarian Steroid Hormone-dependent Manner: EVIDENCE FOR DIFFERENTIAL FUNCTION OF HIFs DURING EARLY PREGNANCY', *Journal of Biological Chemistry*, 278: 7683-91.
De Falco, Sandro. 2012. 'The discovery of placenta growth factor and its biological activity', *Exp Mol Med*, 44: 1-9.

Demir, R., U. A. Kayisli, Y. Seval, C. Celik-Ozenci, E. T. Korgun, A. Y. Demir-Weusten, and B. Huppertz. 2004. 'Sequential expression of VEGF and its receptors in human placental villi during very early pregnancy: differences between placental vasculogenesis and angiogenesis', *Placenta*, 25: 560-72.

Demir, R., Y. Seval, and B. Huppertz. 2007. 'Vasculogenesis and angiogenesis in the early human placenta', *Acta Histochemica*, 109: 257-65.

Erben, L., M. X. He, A. Laeremans, E. Park, and A. Buonanno. 2018. 'A Novel Ultrasensitive In Situ Hybridization Approach to Detect Short Sequences and Splice Variants with Cellular Resolution', *Mol Neurobiol*, 55: 6169-81.

Ferrara, N. 2000. 'Vascular endothelial growth factor and the regulation of angiogenesis', *Recent Progress in Hormone Research, Vol 55*, 55: 15-36.

Goddard, Lauren M., Thomas J. Murphy, Tönis Org, Josephine M. Enciso, Minako K. Hashimoto-Partyka, Carmen M. Warren, Courtney K. Domigan, Austin McDonald, Huanhuan He, Lauren A. Sanchez, Nancy C. Allen, Fabrizio Orsenigo, Lily C. Chao, Elisabetta Dejana, Peter Tontonoz, Hanna K. A. Mikkola, and M. Luisa Iruela-Arispe. 2014. 'Progesterone Receptor in the Vascular Endothelium Triggers Physiological Uterine Permeability Pre-implantation', *Cell*, 156: 549-62.

Gram, A., B. Hoffmann, A. Boos, and M. P. Kowalewski. 2015. 'Expression and localization of vascular endothelial growth factor A (VEGFA) and its two receptors (VEGFR1/FLT1 and VEGFR2/FLK1/KDR) in the canine corpus luteum and utero-placental compartments during pregnancy and at normal and induced parturition', *Gen Comp Endocrinol*, 223: 54-65.

Grazul-Bilska, Anna, Mary Johnson, Pawel Borowicz, Megan Minten, Jerzy J Bilski, Robert Wroblewski, Mila Velimirovich, Lindsey R Coupe, Dale A Redmer, and Lawrence Reynolds. 2011. *Placental development during early pregnancy in sheep: Cell proliferation, global methylation, and angiogenesis in the fetal placenta*.

Grunstein, Jeremy, Joseph J. Masbad, Reed Hickey, Frank Giordano, and Randall S. Johnson. 2000. 'Isoforms of Vascular Endothelial Growth Factor Act in a Coordinate Fashion To Recruit and Expand Tumor Vasculature', *Molecular and Cellular Biology*, 20: 7282-91.

Helske, S., P. Vuorela, O. Carpen, C. Hornig, H. Weich, and E. Halmesmaki. 2001. 'Expression of vascular endothelial growth factor receptors 1, 2 and 3 in placentas from normal and complicated pregnancies', *Mol Hum Reprod*, 7: 205-10.

Hou, X. H., D. H. Li, Y. Li, H. X. Zhao, C. L. Zhang, L. Huang, and Y. Q. Yao. 2006. '[Expression of fms-like tyrosine kinase receptor 1 in placenta of pre-eclampsia]', *Zhonghua Fu Chan Ke Za Zhi*, 41: 736-9.

Houck, Keith A, Napoleone Ferrara, Jane Winer, George Cachianes, Bing Li, and David W Leung. 1991. 'The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA', *Molecular endocrinology*, 5: 1806-14.

Huckle, W. R., and R. I. Roche. 2004. 'Post-transcriptional control of expression of sFlt-1, an endogenous inhibitor of vascular endothelial growth factor', *J Cell Biochem*, 93: 120-32.

Hyder, S. M., Z. Nawaz, C. Chiappetta, and G. M. Stancel. 2000. 'Identification of functional estrogen response elements in the gene coding for the potent angiogenic factor vascular endothelial growth factor', *Cancer Res*, 60: 3183-90.

Katoh, Osamu, Hiroshi Tauchi, Kuniko Kawaishi, Akiro Kimura, and Yukio Satow. 1995. 'Expression of the vascular endothelial growth factor (VEGF) receptor gene, KDR, in hematopoietic cells and inhibitory effect of VEGF on apoptotic cell death caused by ionizing radiation', *Cancer Res*, 55: 5687-92.

Khaliq, A., X. F. Li, M. Shams, P. Sisi, C. A. Acevedo, M. J. Whittle, H. Weich, and A. Ahmed. 1996. 'Localisation of placenta growth factor (PIGF) in human term placenta', *Growth factors*, 13: 243-50,color plates I-II,pre bk cov.

Khaliq, Asud, Xiao F Li, Munjiba Shams, Paraskevi Sisi, Carmen A Acevedo, Martin J Whittle, Herbert Weigh, and Asif Ahmed. 1996. 'Localisation of placenta growth factor (PlGF) in human term placenta', *Growth factors*, 13: 243-50.

Kim, K. H., and J. R. Bender. 2009. 'Membrane-initiated actions of estrogen on the endothelium', *Mol Cell Endocrinol*, 308: 3-8.

Kim, Minah, Hyeung Ju Park, Jae Won Seol, Jeon Yeob Jang, Young-Suk Cho, Kyu Rae Kim, Youngsok Choi, John P. Lydon, Francesco J. DeMayo, Masabumi Shibuya, Napoleone Ferrara, Hoon-Ki Sung, Andras Nagy, Kari Alitalo, and Gou Young Koh. 2013. 'VEGF-A regulated by progesterone governs uterine angiogenesis and vascular remodelling during pregnancy', *EMBO Molecular Medicine*, 5: 1415-30.

Kumar, Pratap, and Navneet Magon. 2012. 'Hormones in pregnancy', *Nigerian Medical Journal : Journal of the Nigeria Medical Association*, 53: 179-83.

Kumazaki, K., M. Nakayama, N. Suehara, and Y. Wada. 2002. 'Expression of vascular endothelial growth factor, placental growth factor, and their receptors Flt-1 and KDR in human placenta under pathologic conditions', *Hum Pathol*, 33: 1069-77.

Küsters, Benno, Robert M. W. de Waal, Pieter Wesseling, Kiek Verrijp, Cathy Maass, Arend Heerschap, Jelle O. Barentsz, Fred Sweep, Dirk J. Ruiter, and William P. J. Leenders. 2003. 'Differential Effects of Vascular Endothelial Growth Factor A Isoforms in a Mouse Brain Metastasis Model of Human Melanoma', *Cancer Res*, 63: 5408-13.

Lam, C., K. H. Lim, and S. A. Karumanchi. 2005. 'Circulating angiogenic factors in the pathogenesis and prediction of preeclampsia', *Hypertension*, 46: 1077-85.

Leach, L., M. O. Babawale, M. Anderson, and M. Lammiman. 2002. 'Vasculogenesis, angiogenesis and the molecular organisation of endothelial junctions in the early human placenta', *Journal of Vascular Research*, 39: 246-59.

Levine, R. J., S. E. Maynard, C. Qian, K. H. Lim, L. J. England, K. F. Yu, E. F. Schisterman, R. Thadhani, B. P. Sachs, F. H. Epstein, B. M. Sibai, V. P. Sukhatme, and S. A. Karumanchi. 2004. 'Circulating angiogenic factors and the risk of preeclampsia', *N Engl J Med*, 350: 672-83.

Loegl, J., U. Hiden, E. Nussbaumer, C. Schliefsteiner, S. Cvitic, I. Lang, C. Wadsack, B. Huppertz, and G. Desoye. 2016. 'Hofbauer cells of M2a, M2b and M2c polarization may regulate feto-placental angiogenesis', *Reproduction*, 152: 447-55.

Ma, W., J. Tan, H. Matsumoto, B. Robert, D. R. Abrahamson, S. K. Das, and S. K. Dey. 2001. 'Adult tissue angiogenesis: evidence for negative regulation by estrogen in the uterus', *Mol Endocrinol*, 15: 1983-92.

Murphy, B. D., and S. D. Martinuk. 1991. 'Equine chorionic gonadotropin', *Endocr Rev*, 12: 27-44.

Murphy, W. J., D. M. Larkin, A. Everts-van der Wind, G. Bourque, G. Tesler, L. Auvil, J. E. Beever, B. P. Chowdhary, F. Galibert, L. Gatzke, C. Hitte, S. N. Meyers, D. Milan, E. A. Ostrander, G. Pape, H. G. Parker, T. Raudsepp, M. B. Rogatcheva, L. B. Schook, L. C.

Skow, M. Welge, J. E. Womack, J. O'Brien S, P. A. Pevzner, and H. A. Lewin. 2005. 'Dynamics of mammalian chromosome evolution inferred from multispecies comparative maps', *Science*, 309: 613-7.

Nucci, M., L. C. Poon, G. Demirdjian, B. Darbouret, and K. H. Nicolaides. 2014. 'Maternal Serum Placental Growth Factor (PlGF) Isoforms 1 and 2 at 11-13 Weeks' Gestation in Normal and Pathological Pregnancies', *Fetal Diagnosis and Therapy*, 36: 106-16.

O'Brien, Stephen J., Warren Johnson, Carlos Driscoll, Joan Pontius, Jill Pecon-Slattery, and Marilyn Menotti-Raymond. 2008. 'State of cat genomics', *Trends in Genetics*, 24: 268-79.

Pang, V., D. O. Bates, and L. Leach. 2017. 'Regulation of human feto-placental endothelial barrier integrity by vascular endothelial growth factors: competitive interplay between VEGF-A165a, VEGF-A165b, PIGF and VE-cadherin', *Clin Sci (Lond)*, 131: 2763-75.

Park, John E, Gilbert-A Keller, and Napoleone Ferrara. 1993. 'The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF', *Molecular biology of the cell*, 4: 1317-26.

Persico, MG, V Vincenti, and T DiPalma. 1999. 'Structure, expression and receptor-binding properties of placenta growth factor (PlGF).' in, *Vascular growth factors and angiogenesis* (Springer).

Peters, K. G., C. De Vries, and L. T. Williams. 1993. 'Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth', *Proc Natl Acad Sci U S A*, 90: 8915-19.

Pfarrer, C. D., S. D. Ruziwa, H. Winther, H. Callesen, R. Leiser, D. Schams, and V. Dantzer. 2006. 'Localization of vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 and VEGFR-2 in bovine placentomes from implantation until term', *Placenta*, 27: 889-98.

Pontius, J. U., J. C. Mullikin, D. R. Smith, K. Lindblad-Toh, S. Gnerre, M. Clamp, J. Chang, R. Stephens, B. Neelam, N. Volfovsky, A. A. Schaffer, R. Agarwala, K. Narfstrom, W. J. Murphy, U. Giger, A. L. Roca, A. Antunes, M. Menotti-Raymond, N. Yuhki, J. Pecon-Slattery, W. E. Johnson, G. Bourque, G. Tesler, and S. J. O'Brien. 2007. 'Initial sequence and comparative analysis of the cat genome', *Genome Res*, 17: 1675-89.

Rawn, Saara M, and James C Cross. 2008. 'The evolution, regulation, and function of placenta-specific genes', *Annu Rev Cell Dev Biol*, 24: 159-81.

Reynolds, L. P., P. P. Borowicz, J. S. Caton, K. A. Vonnahme, J. S. Luther, D. S. Buchanan, S. A. Hafez, A. T. Grazul-Bilska, and D. A. Redmer. 2010. 'Uteroplacental vascular development and placental function: an update', *Int J Dev Biol*, 54: 355-66.

Ruhrberg, Christiana, Holger Gerhardt, Matthew Golding, Rose Watson, Sofia Ioannidou, Hajime Fujisawa, Christer Betsholtz, and David T Shima. 2002. 'Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis', *Genes & development*, 16: 2684-98.

Saffer, Craig, Gayle Olson, Kim A. Boggess, Richard Beyerlein, Charles Eubank, and Baha M. Sibai. 2013. 'Determination of placental growth factor (PlGF) levels in healthy pregnant women without signs or symptoms of preeclampsia', *Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health*, 3: 124-32.

Sawano, A., T. Takahashi, S. Yamaguchi, M. Aonuma, and M. Shibuya. 1996. 'Flt-1 but not KDR/Flk-1 tyrosine kinase is a receptor for placenta growth factor, which is related to vascular endothelial growth factor', *Cell Growth Differ*, 7: 213-21.

Sawano, Asako, Shinobu Iwai, Yoshiko Sakurai, Mikito Ito, Kenya Shitara, Tatsutoshi Nakahata, and Masabumi Shibuya. 2001. 'Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans', *Blood*, 97: 785-91.

Semenza, G. L. 2007. 'Vasculogenesis, angiogenesis, and arteriogenesis: Mechanisms of blood vessel formation and remodeling', *J Cell Biochem*, 102: 840-47.

Sherrell, Helen, Liam Dunn, Vicki Clifton, and Sailesh Kumar. 2018. 'Systematic review of maternal Placental Growth Factor levels in late pregnancy as a predictor of adverse intrapartum and perinatal outcomes', *European Journal of Obstetrics & Gynecology and Reproductive Biology*, 225: 26-34.

Tischer, Edmund, R Mitchell, T Hartman, M Silva, D Gospodarowicz, JC Fiddes, and JA Abraham. 1991. 'The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing', *Journal of Biological Chemistry*, 266: 11947-54.

Tozer, Gillian M, Simon Akerman, Neil A Cross, Paul R Barber, Meit A Björndahl, Olga Greco, Sheila Harris, Sally A Hill, Davina J Honess, and Christopher R Ireson. 2008. 'Blood vessel maturation and response to vascular-disrupting therapy in single vascular endothelial growth factor-A isoform–producing tumors', *Cancer Res*, 68: 2301-11.

Tsoi, S. C., Y. Wen, J. Y. Chung, D. Chen, R. R. Magness, and J. Zheng. 2002. 'Co-expression of vascular endothelial growth factor and neuropilin-1 in ovine feto-placental artery endothelial cells', *Mol Cell Endocrinol*, 196: 95-106.

Verhage, H. G., N. B. Beamer, and R. M. Brenner. 1976. 'Plasma Levels of Estradiol and Progesterone in the Cat During Polyestrus, Pregnancy and Pseudopregnancy', *Biology of Reproduction*, 14: 579-85.

Vonnahme, K. A., M. E. Wilson, and S. P. Ford. 2001. 'Relationship between placental vascular endothelial growth factor expression and placental/endometrial vascularity in the pig', *Biol Reprod*, 64: 1821-5.

Vuckovic, M., J. Ponting, B. I. Terman, V. Niketic, M. W. Seif, and S. Kumar. 1996. 'Expression of the vascular endothelial growth factor receptor, KDR, in human placenta', *J Anat*, 188 (Pt 2): 361-6.

Vuorela, Piia, Erika Hatva, Athina Lymboussaki, Arja Kaipainen, Vladimir Joukov, Maria Graziella Persico, Kari Alitalo, and Erja Halmesmäki. 1997. 'Expression of vascular endothelial growth factor and placenta growth factor in human placenta', *Biology of Reproduction*, 56: 489-94.

Winther, H., A. Ahmed, and V. Dantzer. 1999. 'Immunohistochemical localization of vascular endothelial growth factor (VEGF) and its two specific receptors, Flt-1 and KDR, in the porcine placenta and non-pregnant uterus', *Placenta*, 20: 35-43.

Wright, D., D. M. Gallo, S. Gil Pugliese, C. Casanova, and K. H. Nicolaides. 2016. 'Contingent screening for preterm pre-eclampsia', *Ultrasound Obstet Gynecol*, 47: 554-9.

Wulff, C., H. Wilson, S. E. Dickson, S. J. Wiegand, and H. M. Fraser. 2002. 'Hemochorial placentation in the primate: expression of vascular endothelial growth factor, angiopoietins, and their receptors throughout pregnancy', *Biol Reprod*, 66: 802-12.

Yao, Yong-Gang, Hoseong S Yang, Zhiming Cao, Jennifer Danielsson, and Elia J Duh. 2005. 'Upregulation of placental growth factor by vascular endothelial growth factor via a post-transcriptional mechanism', *FEBS letters*, 579: 1227-34.

Yonekura, H., S. Sakurai, X. Liu, H. Migita, H. Wang, S. Yamagishi, M. Nomura, M. J. Abedin, H. Unoki, Y. Yamamoto, and H. Yamamoto. 1999. 'Placenta growth factor and vascular endothelial growth factor B and C expression in microvascular endothelial cells and pericytes. Implication in autocrine and paracrine regulation of angiogenesis', *J Biol Chem*, 274: 35172-8.

Yu, Joanne L., Janusz W. Rak, Giannoula Klement, and Robert S. Kerbel. 2002. 'Vascular Endothelial Growth Factor Isoform Expression as a Determinant of Blood Vessel Patterning in Human Melanoma Xenografts', *Cancer Res*, 62: 1838-46.

Zambelli, D., C. Castagnetti, S. Belluzzi, and S. Bassi. 2002. 'Correlation between the age of the conceptus and various ultrasonographic measurements during the first 30 days of pregnancy in domestic cats (Felis catus)', *Theriogenology*, 57: 1981-7.

Zambelli, Daniele, Carolina Castagnetti, Stefano Belluzzi, and Cosimo Paladini. 2004. *Correlation between fetal age and ultrasonographic measurements during the second half of pregnancy in domestic cats (Felis catus)*.

3.8 Figures and Tables

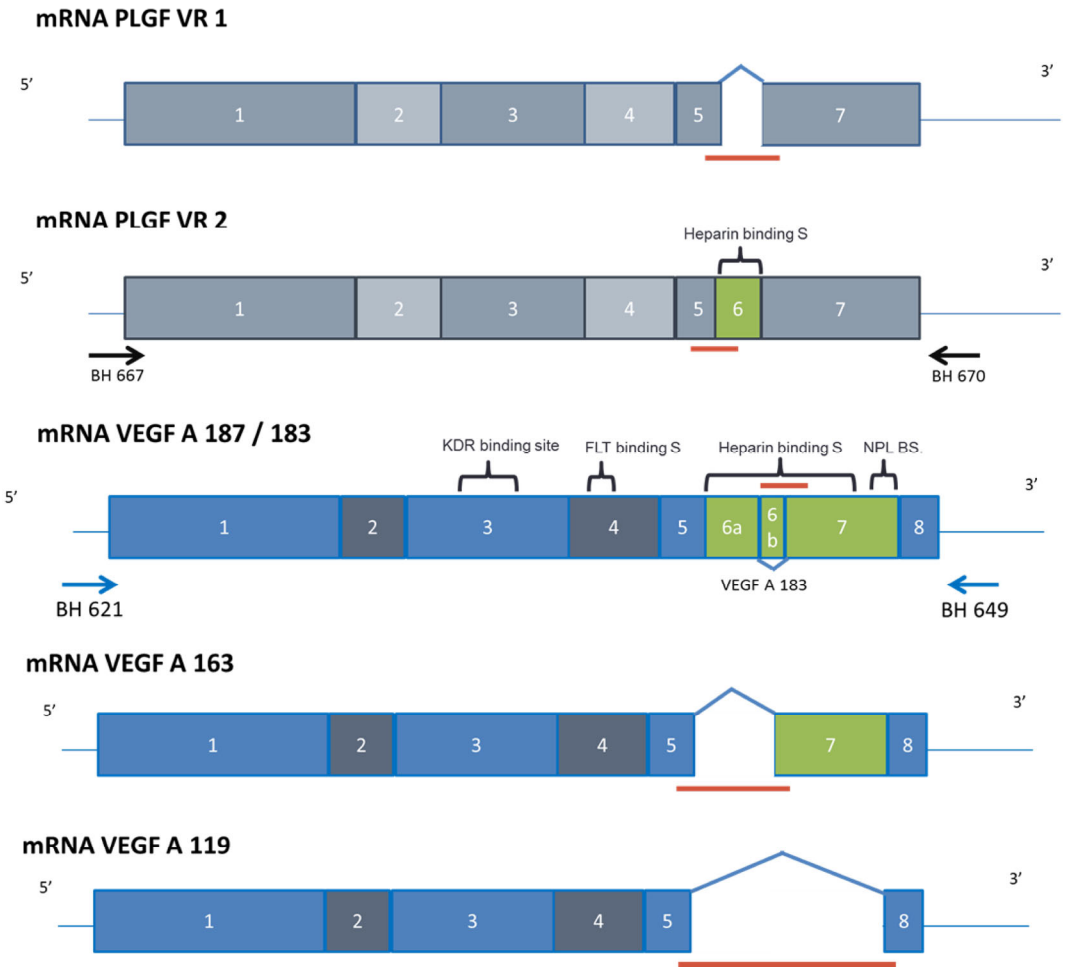


Figure 3.1. PLGF and VEGF A Variants. PLGF and VEGF A occur in multiple isoforms that differ in their biochemical and biological characteristics. In multiple species, alternative splicing of pre-mRNAs produces at least three isoforms of VEGF A and two isoforms of PLGF. Exon 1-5 are present in all isoforms and code for KDR- and Flt-1-binding domains in VEGF A and to Flt-1-binding domains in PLGF. Based on the similarities to human sequences, we can predict that these isoforms differ in biological properties such as their ability to bind to cell-surface heparin-sulfate proteoglycans. Smaller isoforms can be secreted and diffuse further into the cell microenvironment. Larger isoforms are sequestered in the ECM very close to the cell of origin.

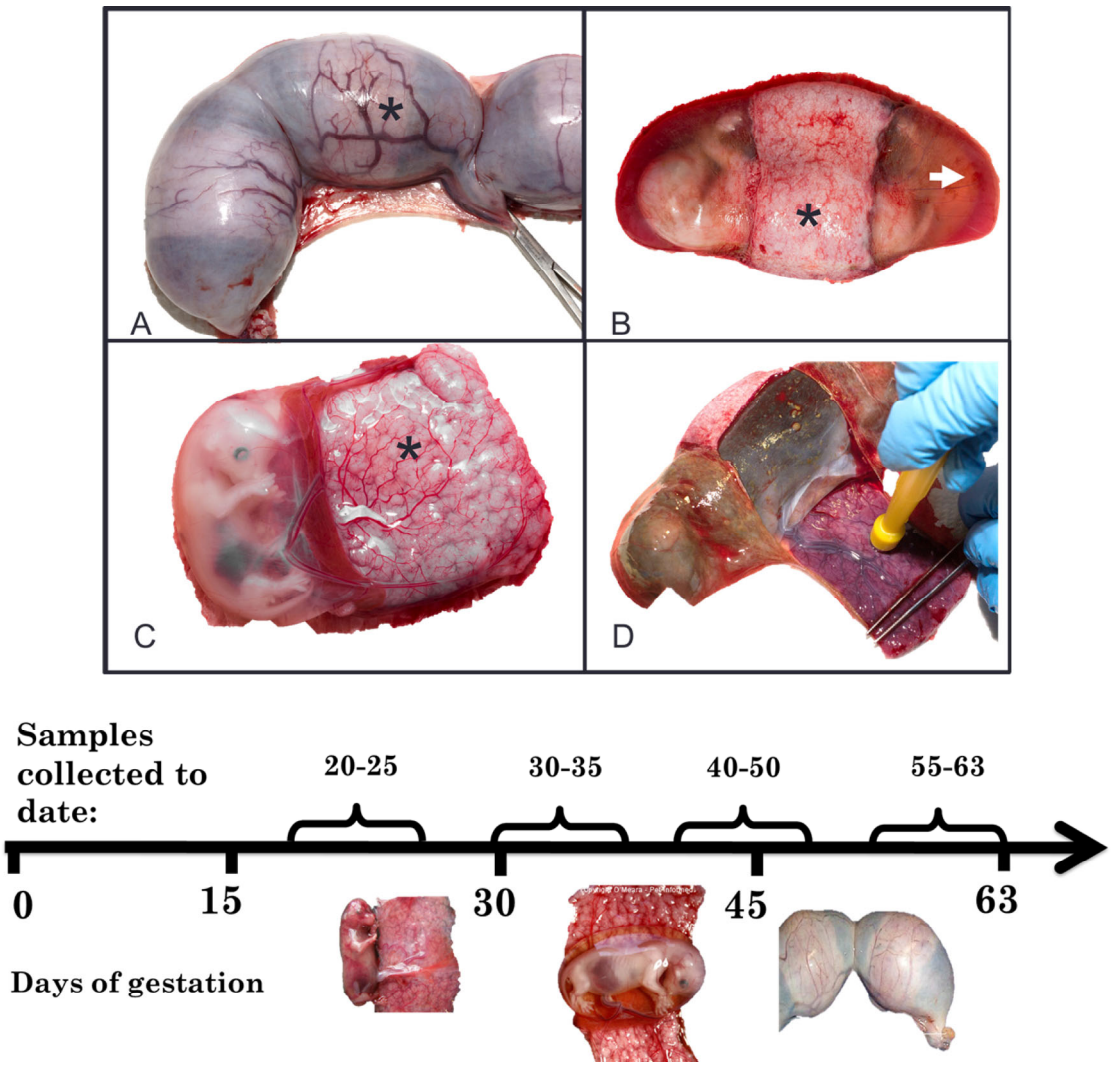


Figure 3.2 Sample collection from the placental girdle. **A**) Gravid uterus resected after OVH showing 2 conceptuses in one uterine horn. The serosa of the uterus contains a well developed vascular network (*). **B**) Whole conceptus after dissected the uterus, exposing the maternal side of the placental girdle (*). The fetus is surrounded by the chorioallantoic membrane (arrow). **C**) Dissecting the chorioallantoic membrane exposes the clear amniotic membrane surroundng the fetus and the fetal side of the chorioallantoic membrane. **D**) Routine procedure for collecting placental tissue samples from the chorioallantoic membrane in the placental girdle using a biopsy punch. **Lower panel:** For gene expression analysis, samples were grouped based on their gestational age estimated according to the crown-rump length and interparietal diameter. Samples were clustered in 4 groups (days 20-25; 30-35; 40-50; 55-63).

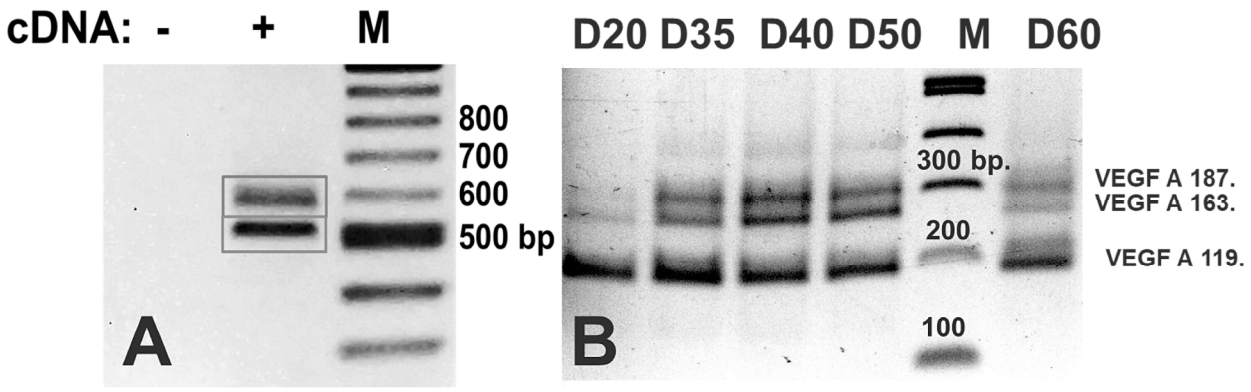
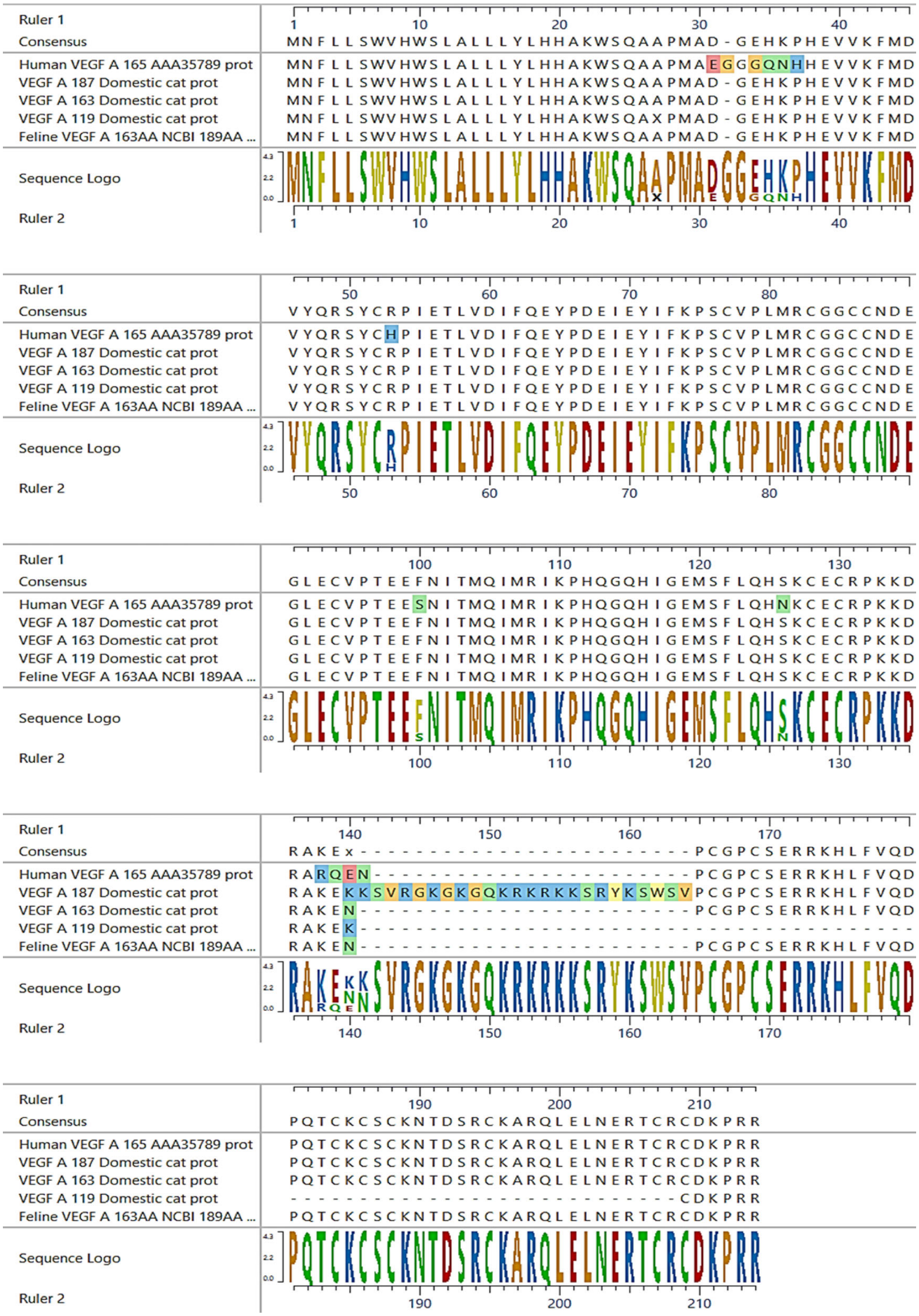


Figure 3.3: Detection and capture of PLGF and VEGF A isoforms: A) PCR and gel electrophoresis analysis of placental tissue reveals the presence of two bands of ~550 and ~600 bp. Purification and sequencing of these amplicons show identity with the PLGF Var I and II NCBI predicted sequences. For VEGF A we identify the sequence of the human homologous variants 119, 163 and 187. These isoforms were present in the placenta at different stages of the pregnancy from day 20 (D20) to day 60 (D60)

VEGF A Isoforms Alignment



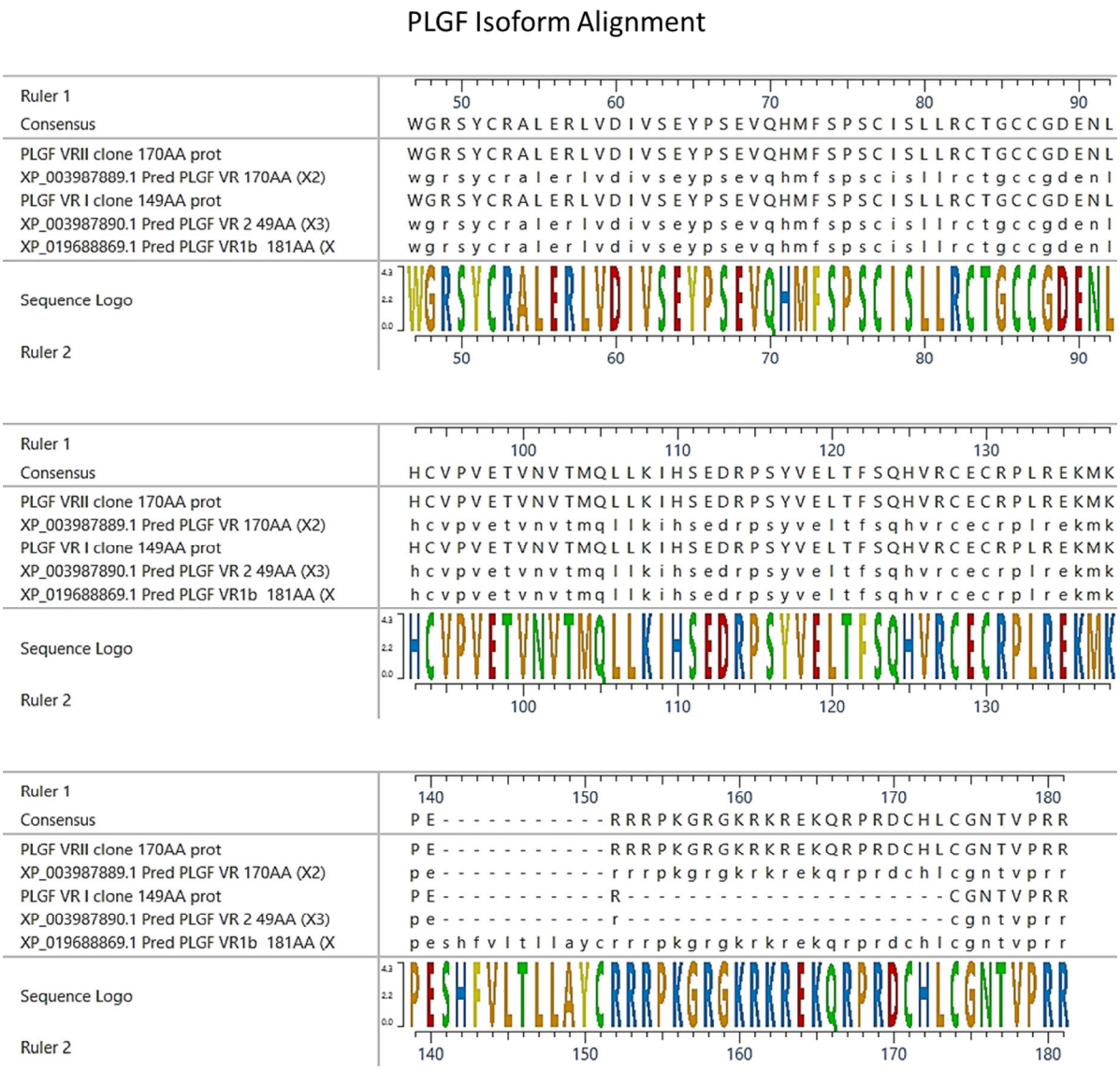


Figure 3.4. Alignment of detected PLGF and VEGF A polypeptides. Sequencing of cloned feline cDNAs and alignment reveal three distinctive isoforms for VEGF A and two for PLGF. Using the Translate tool from Expasy, predicted proteins of 187, 163 and 119 amino acids were aligned and compared to predicted NCBI VEGF A for cat and also human VEGF A 165. Complete identity was found between the predicted 163 isoform and 163 isoform detected in placenta. Human VEGF 163 has two additional residues, at positions 32 and 141). Human and feline proteins are highly conserved. Based on homology with other human isoforms, the feline variants correspond to the most common human VEGF A 189, 165 and 121 (alignment not shown for 189 and 121). PLGF isoforms detected were 170 and 149 AA, equivalent to the human 131 and 152 isoforms (minus the 18AA signal peptide), in agreement with the feline isoforms predicted by NCBI bioinformatics. In addition there is a longer PLGF 181 predicted that we did not find in placenta.

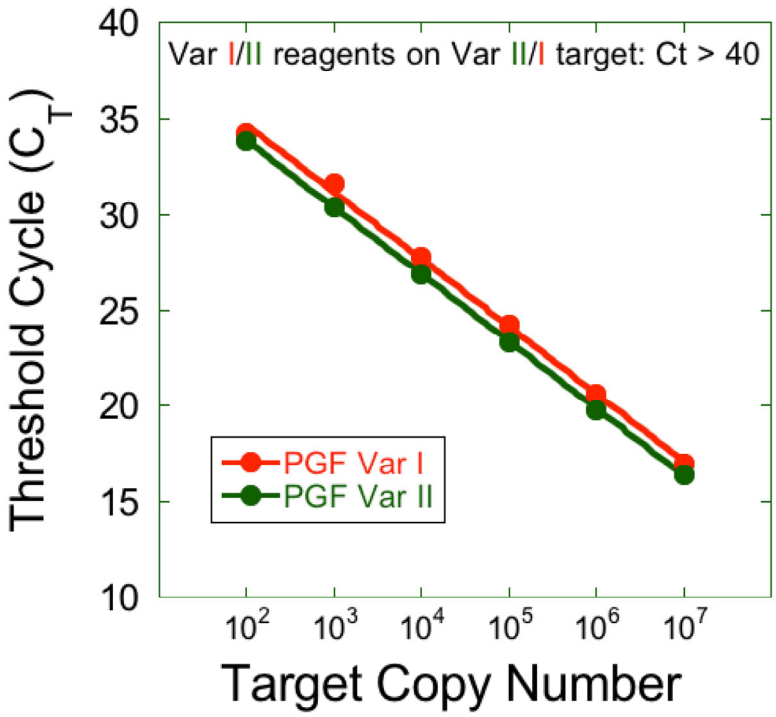


Figure 3.5: **PCR efficiency curves and specificity confirmation.** Satisfactory efficiencies of amplification and desired isoform specificity were confirmed using purified Var I and II plasmids as positive control targets. Real-time RT-PCR assays were designed to selectively detect cDNAs derived from feline PLGF Var I and II, using TaqMan-MGB probes targeting the unique exon junctions in Var II (exons 5/6) and Var I (exons 5/7). Isoform selectivity of the assays was confirmed by absence of detectable amplification after 40 cycles when, for example, the Var I directed reagents where used with the homogeneous cloned Var II plasmid standard. Cross-reaction assays and efficiency reactions were performed in the same way for all VEGF-A isoforms. Amplification efficiencies, reflected by the slope of the C_T *vs* log [Target] curves, were optimized for all qRT-PCR targets to reach efficiencies of 95 to 103 %.

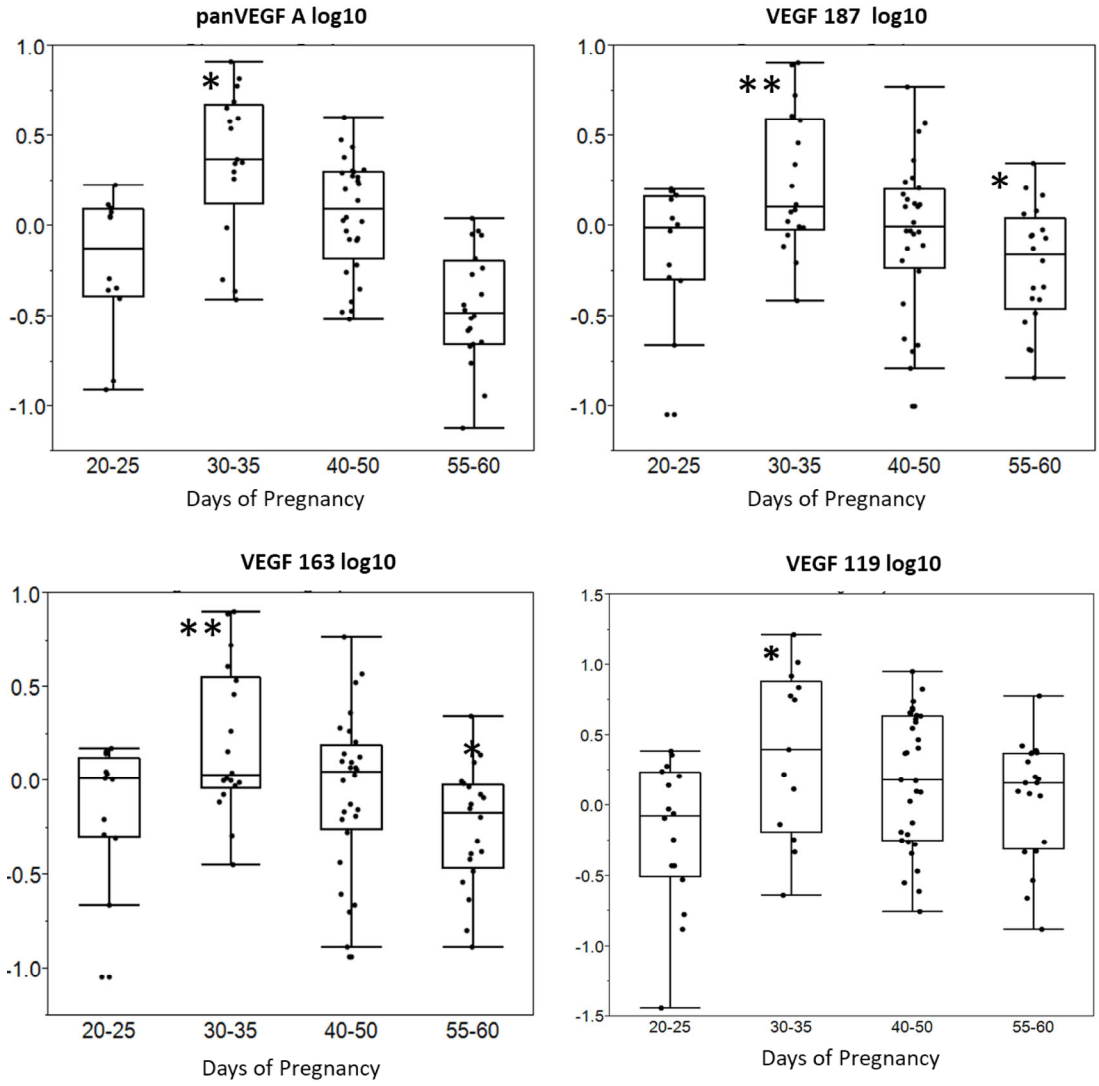


Figure 3.6: Placental VEGF A isoform expression across pregnancy. All forms of VEGF A mRNAs measured follow a similar pattern of expression during pregnancy. With the gestational d20-25 group as a baseline, qRT-PCR revealed upregulation of panVEGF-A and the specific isoforms VEGF -A119, VEGF-A163 and VEGF-A187 at d30-35, followed by a steady decline toward term. Results are expressed as the $\log_{10}$ of 2^DCT (where DCT = $CT_{VEGF} - CT_{18S\ rRNA}$). *, p=###; **, p=###. Each data point represents measurements from individual placentas.

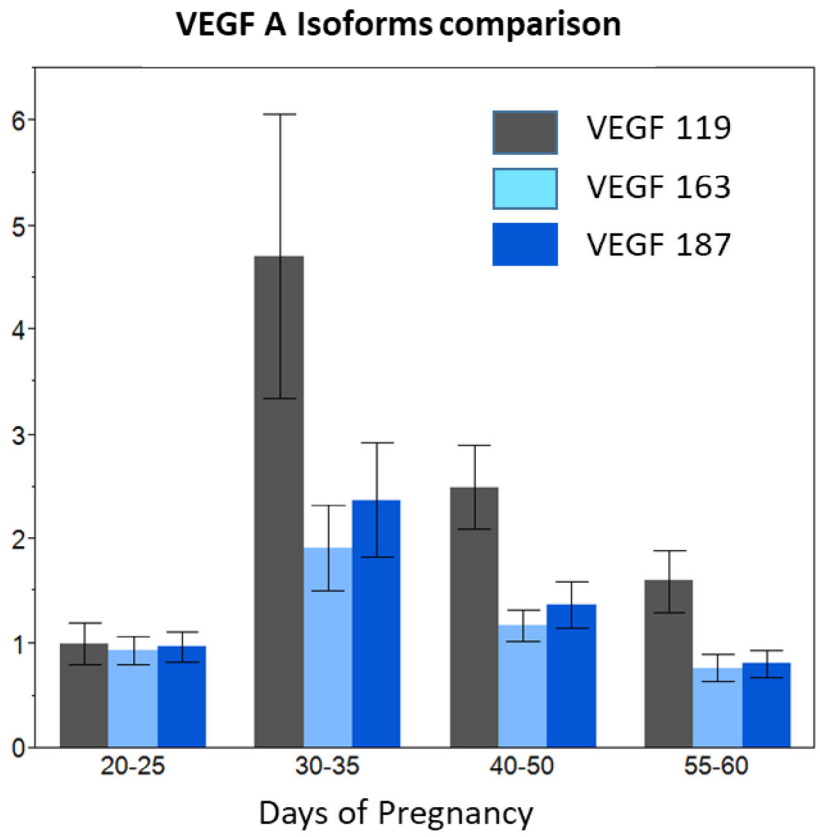


Figure 3.7: Consolidated comparison of placental VEGF A isoform expression. Expression of each VEGF A isoform is indexed to mean expression of the same isoform in the d20-25 group. VEGF A119 appears to rise to a greater degree than VEGF A163 or VEGF A187 early in pregnancy, consistent with a putative role for VEGF A119 in vascular extension during Lamellar Zone proliferation. Results shown are mean $\pm$ standard error.

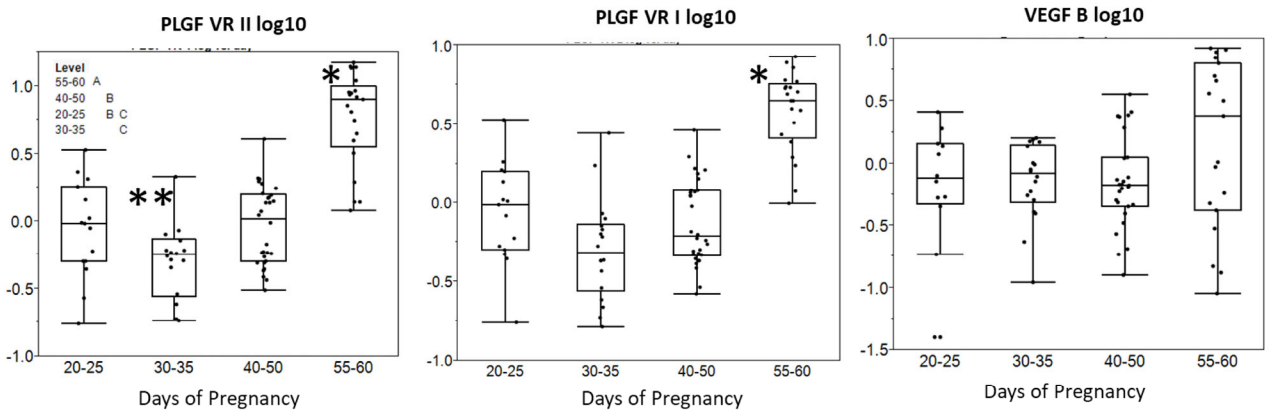


Figure 3.8: PLGF and VEGF B expression across pregnancy. PLGF Var 1 and 2 showed stable expression during the first 2/3 of pregnancy, followed by a marked increase toward term. Similar patterns of expression were seen with both variants. Increased ratio of PLGF1:panVEGF-A expression late in gestation may affect VEGF-A bioavailability, since PlGF can displace VEGF from FLT1, freeing it to signal through KDR. Each data point represents measurements from individual placentas.

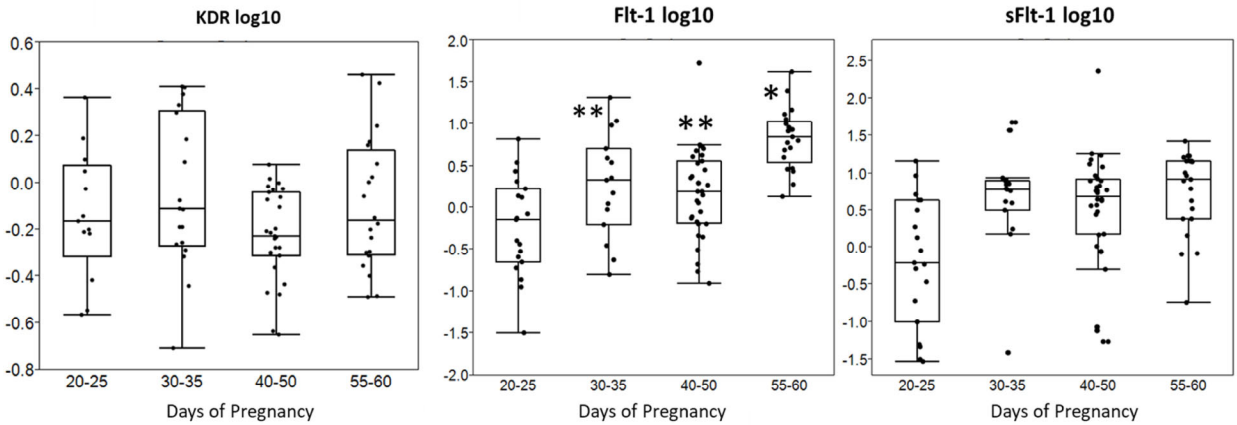


Figure 3.9: KDR and Flt-1 expression across pregnancy. Relative expression of KDR in the placenta was stable during the whole pregnancy. In contrast, expression of full-length Flt-1 mRNA relative to early pregnancy (d20-25) was increased, whereas relative expression of the truncated soluble sFlt-1 was constant during pregnancy. Each data point represents measurements from individual placentas.

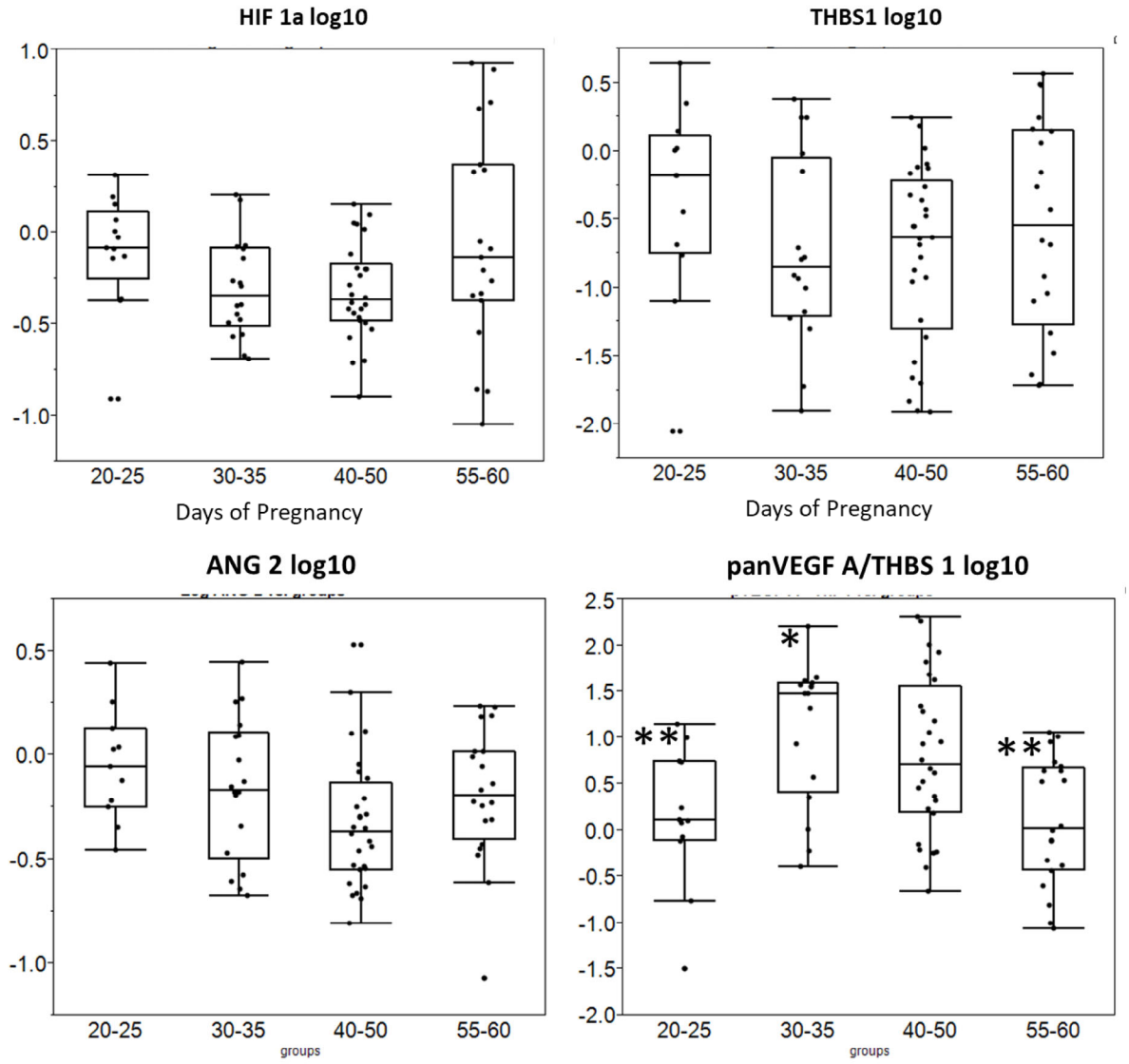


Figure 3.10: Relative expression of other major angiogenic regulators in the placenta. There were no significant changes in HIF1a expression in placenta during pregnancy. The anti angiogenic gene THBSP 1 showed a small down regulation during mid-pregnancy, although no significant differences were found. Another pro-angiogenic regulator, ANG 2, had a stable expression during pregnancy. Relative expression of PanVEGF-A was compared to THBS 1, revealing increasing ratios of panVEGF relative to THBS 1 during mid pregnancy. This is consistent with the creation of conditions favorable for angiogenesis in the time of pregnancy that has higher rates of Lamellar Zone proliferation. Each data point represents measurements from individual placentas.

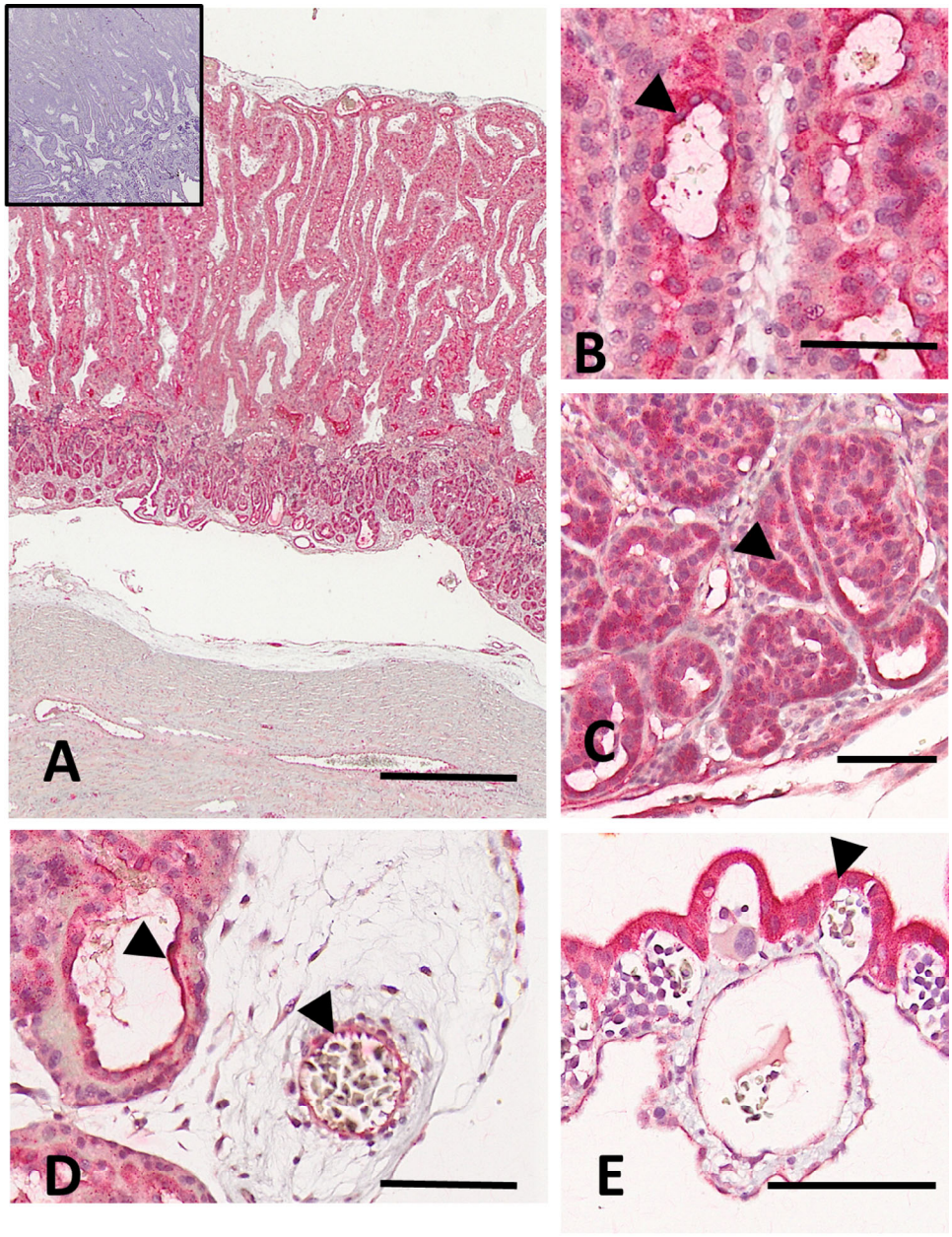


Figure 3.11: Immunolocalization of KDR in placenta during early pregnancy. KDR immunoreacitivty was abundant in placental sections from early pregnancy in maternal and fetal endothelial cells of the Lamellar Zone (arrow heads **in panels B** and **D**) and in maternal lamella (**B**). KDR was also detected in uterine glands of the Glandular Zone (**C**). **Panel E**: KDR was detected in the endoderm of the yolk sac. Capillaries and venules of myometrium were also positive. **Top left**: Negative control without primary antibody. **A**: scale bar = 500 um. **B,C,D,E**: scale bar = 70 um.

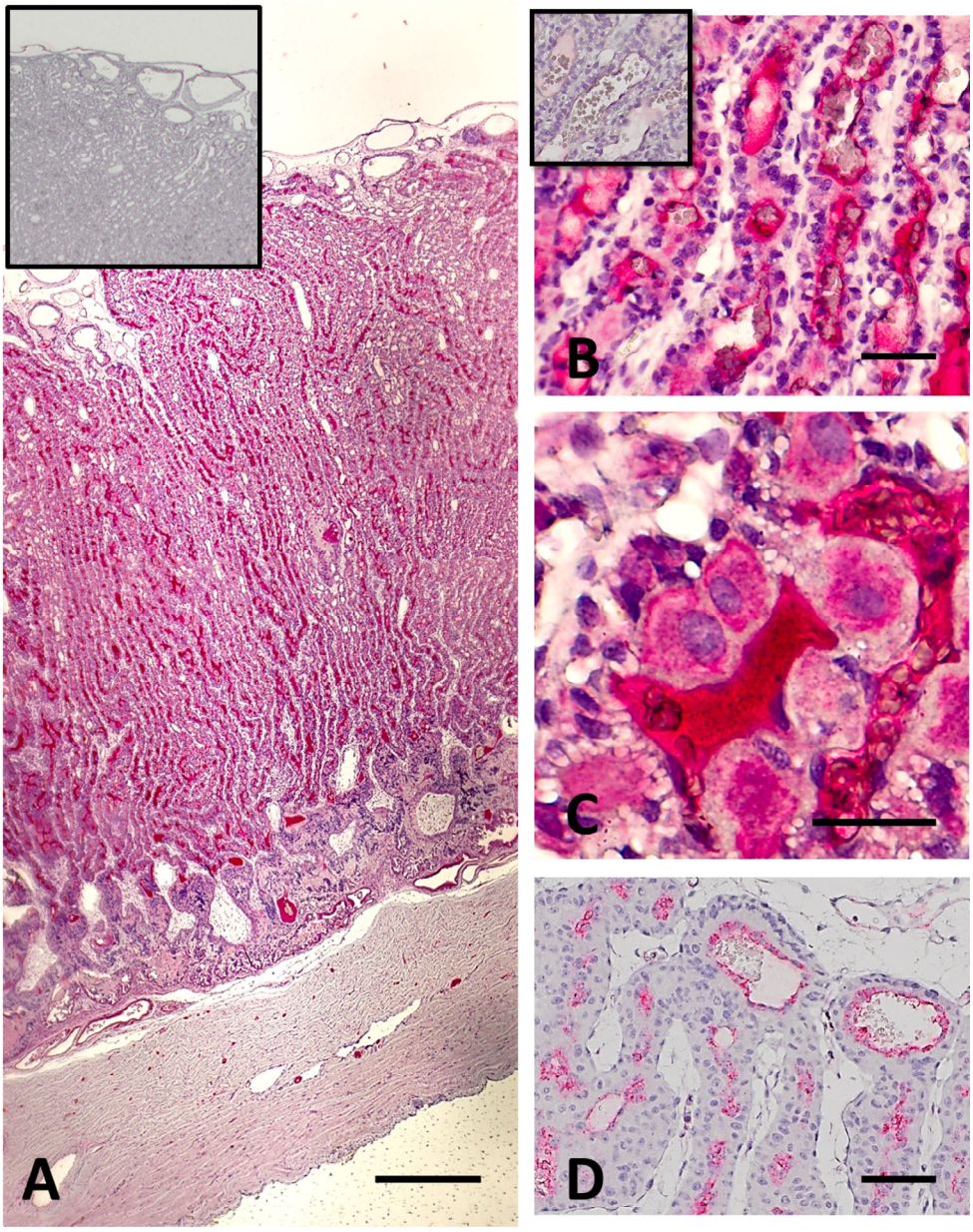


Fig 3.12 Immunolocalization of KDR in placenta during late pregnancy. Localization of the receptor in the maternal capillaries in the lamellae, some fetal capillaries are also positive specially in the top of the placenta. Epithelial cells of endometrial glands and Capillaries in the uterine myometrium are also positive. Clusters of decidua cells in maternal lamella are immunoreactive. Maternal capillaries Flt-1 positive pattern is similar to factor VIII, a marker for endothelial cells. Negative controls in the top left of A and B. Scale bar A; 500 um. Scale bar B, D; 50 um. Scale Bar C; 20 um

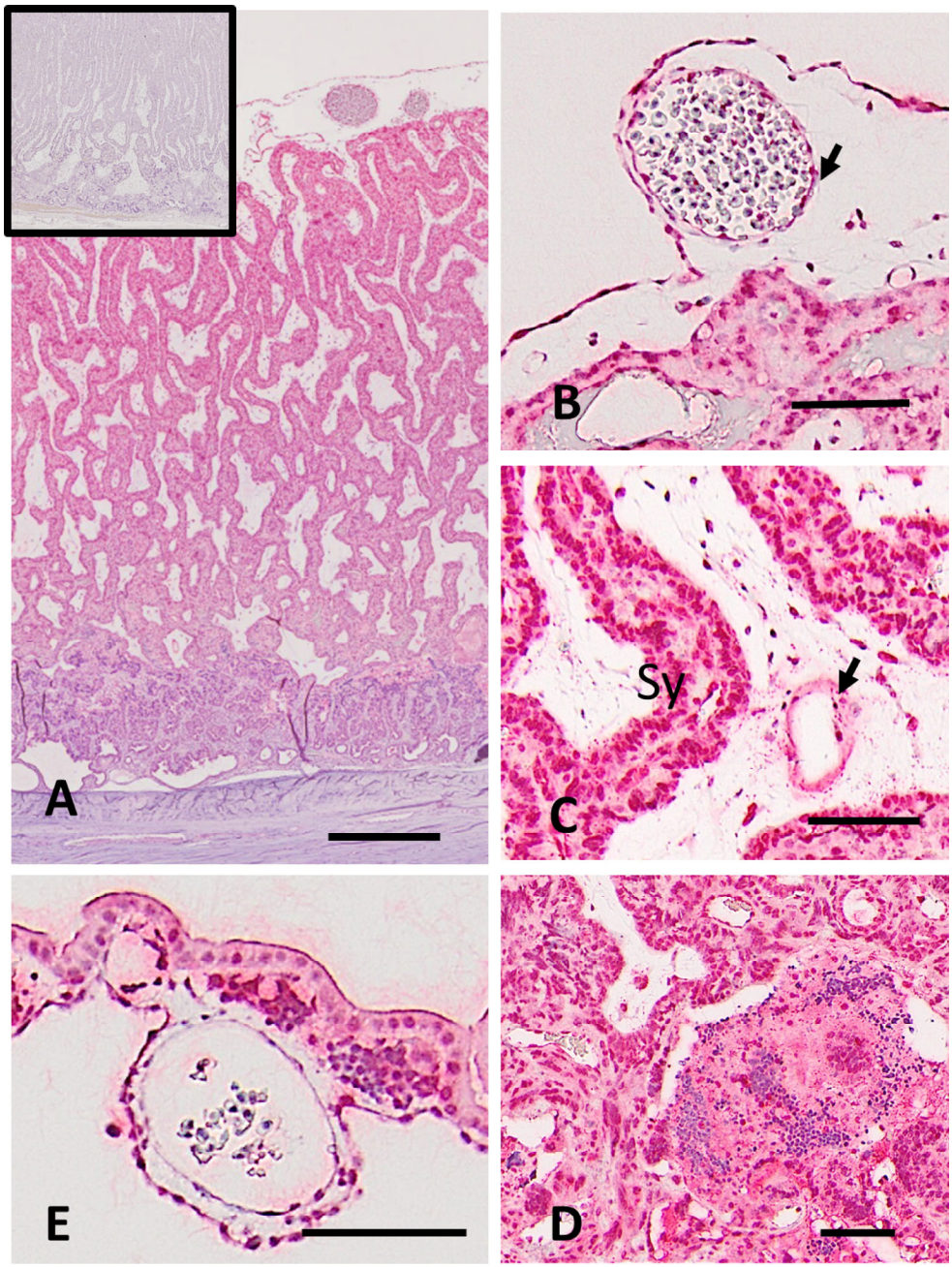


Figure 3.13: Immunolocalization of Flt-1 in placenta during early pregnancy. A: Flt-1 is expressed in the LZ on the placenta at d20. **C:** Flt-1 was localized in trophoblast cells (syncytiotrophoblast; Sy). Endothelial cells in fetal capillaries (Arrow in **B** and **C**). Fetal connective tissue (fibroblasts) of the fetal lamellae are also positive (**C**). Cytotrophoblasts around multinuclear cell clusters in the JZ showed Flt-1 positivity (**D**). In the yolk sack at day 20, mesothelium and blood cell precursors between mesothelium and the endoderm were also positive. Scale bar **A** = 500 um. Scale bar **B,C,D,E** = 70 um.

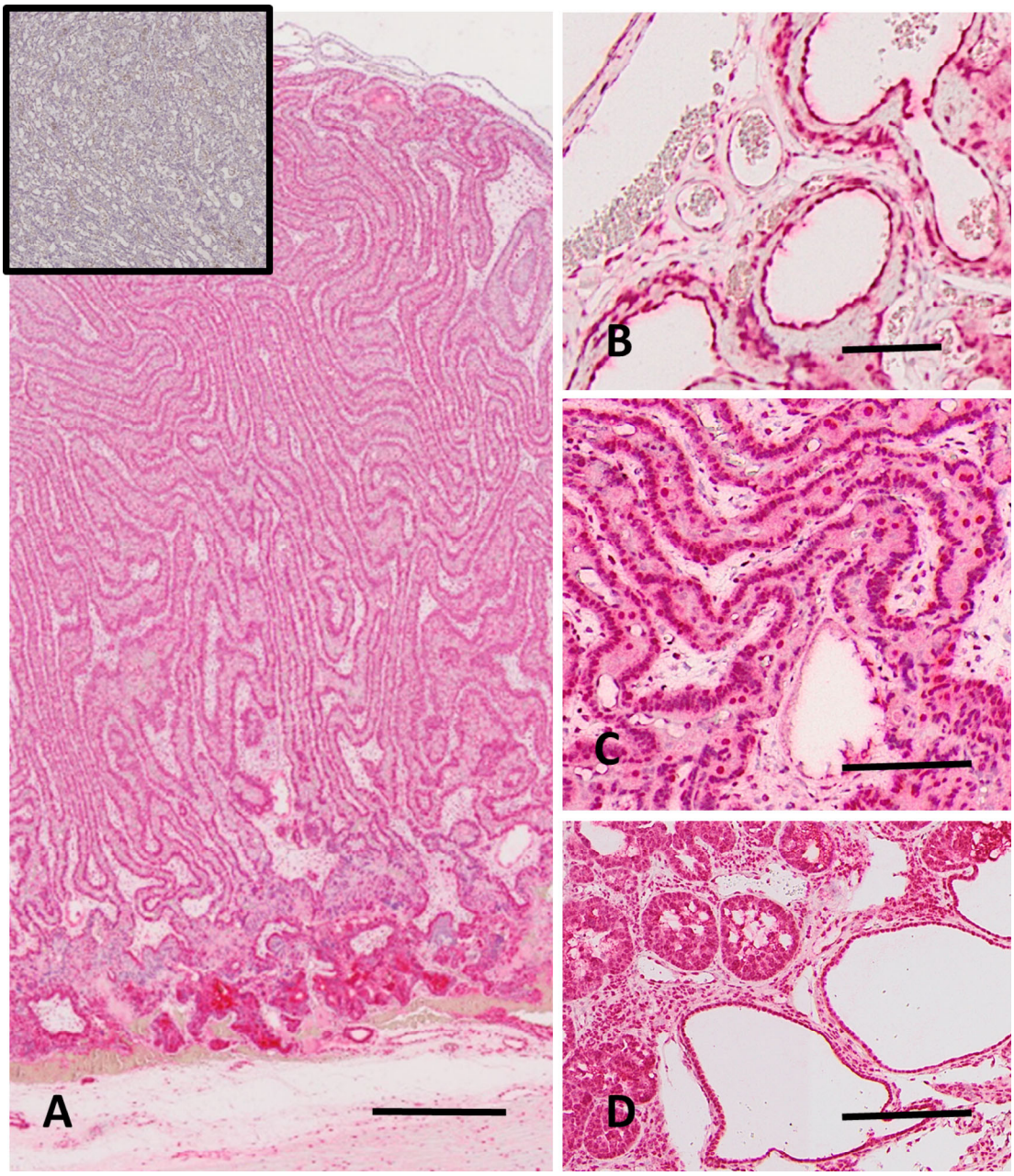


Figure 3.14: Immunolocalization of Flt-1 in placenta during late pregnancy. Flt 1 was immunolocalized in trophoblast cells at each side of the maternal lamellae, and in maternal and fetal microvasculature especially in the luminal side. Epithelial cells of endometrial glands were also immunopositive. Scale bar in **A** = 500 um. **B,C,D** = 100 um.

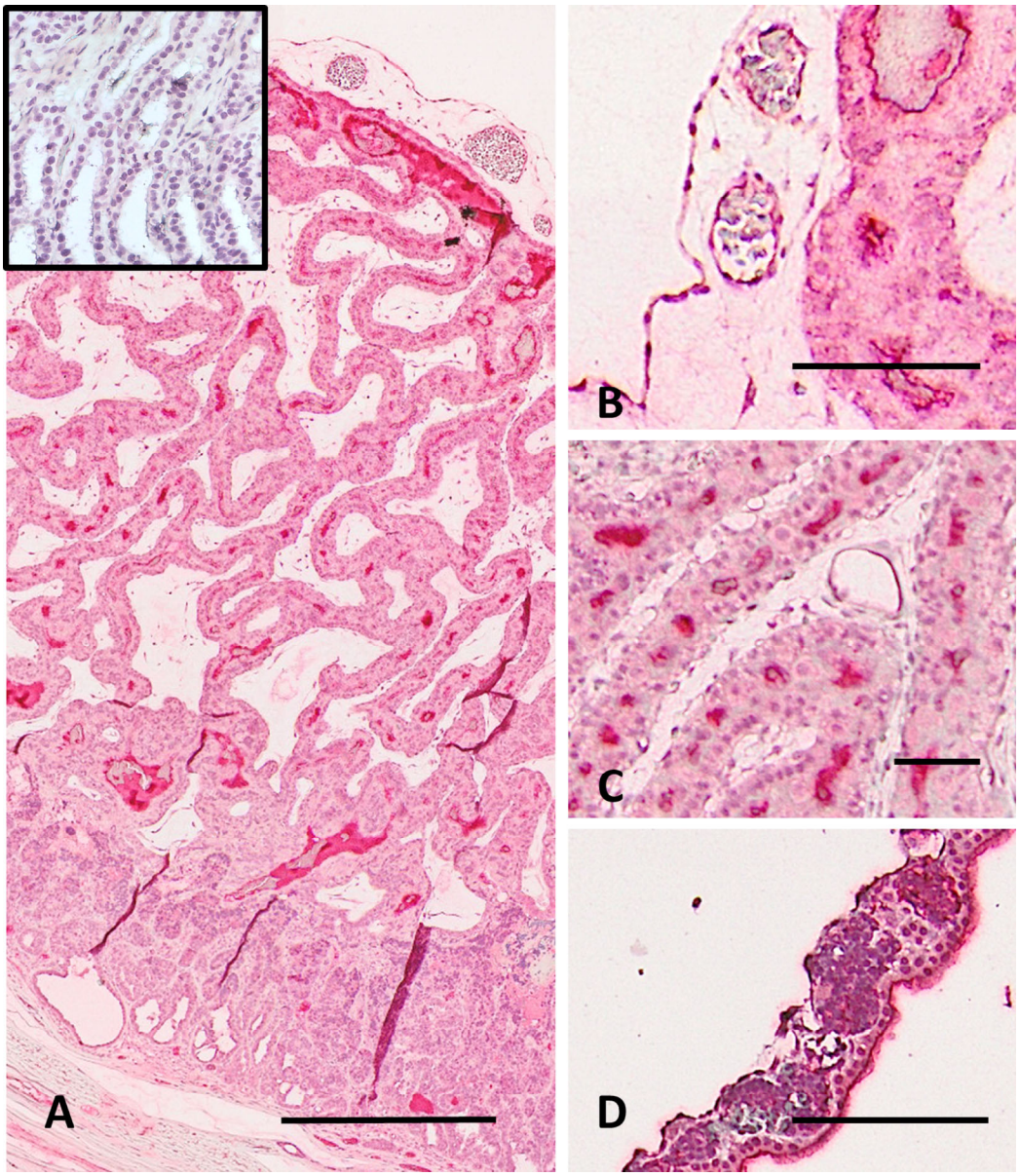


Figure 3.15: Immunolocalization of PLGF in placenta during early pregnancy. PGF was detected in early stages of Placental development d20, associated to maternal and fetal vasculature of the LZ. In the vitelline sac, PLGF was detected in endoderm and apparently in blood vessels precursors. Scale bar A; 500 um. Sacale bar B,C,D; 50 um.

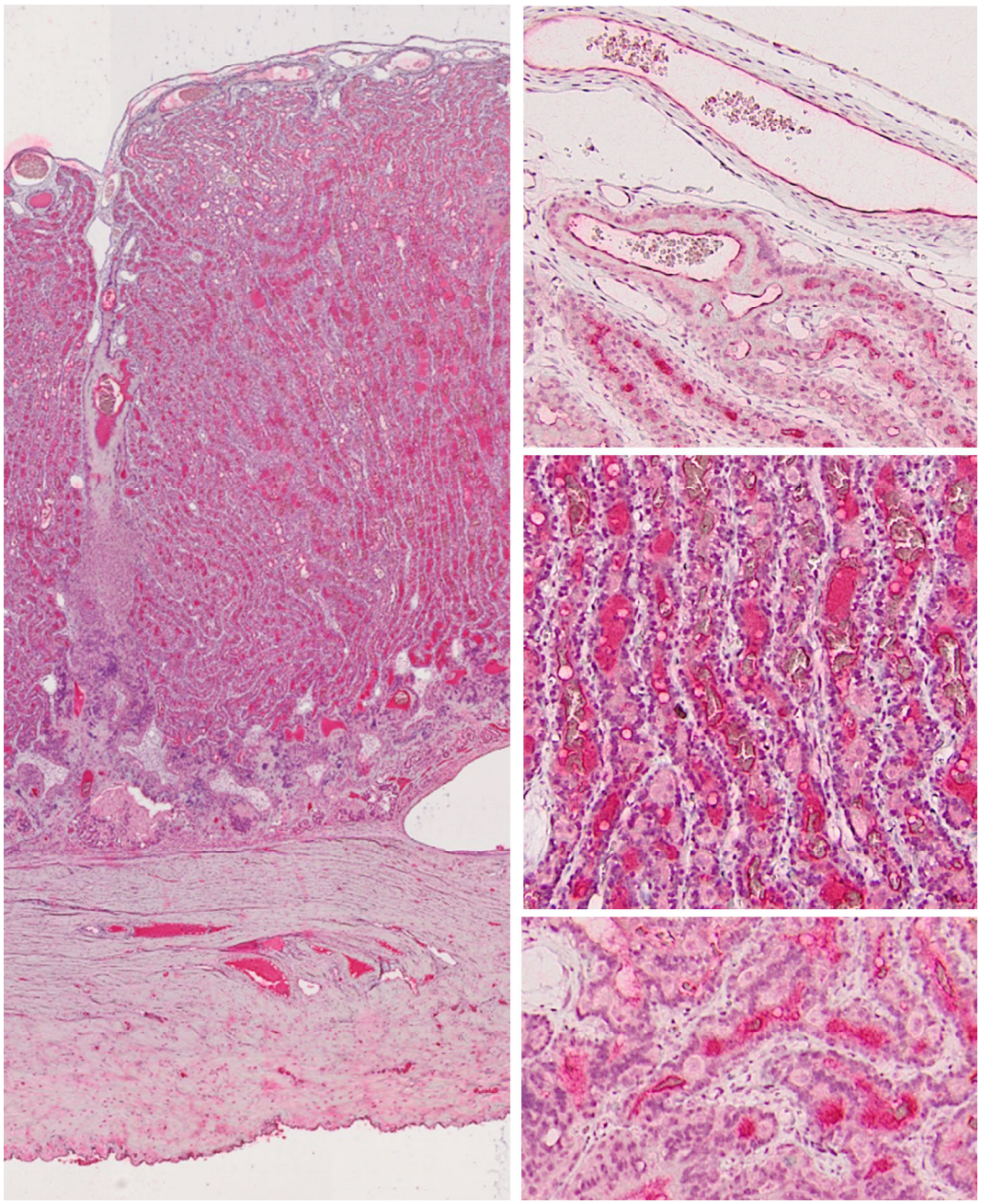


Figure 3.16: Immunolocalization of PLGF in placenta during late pregnancy. PLGF immunoreactivity was abundantly present in the placenta at near-term of gestation (d55), associated with maternal and fetal capillaries in the LZ in and the myometrium

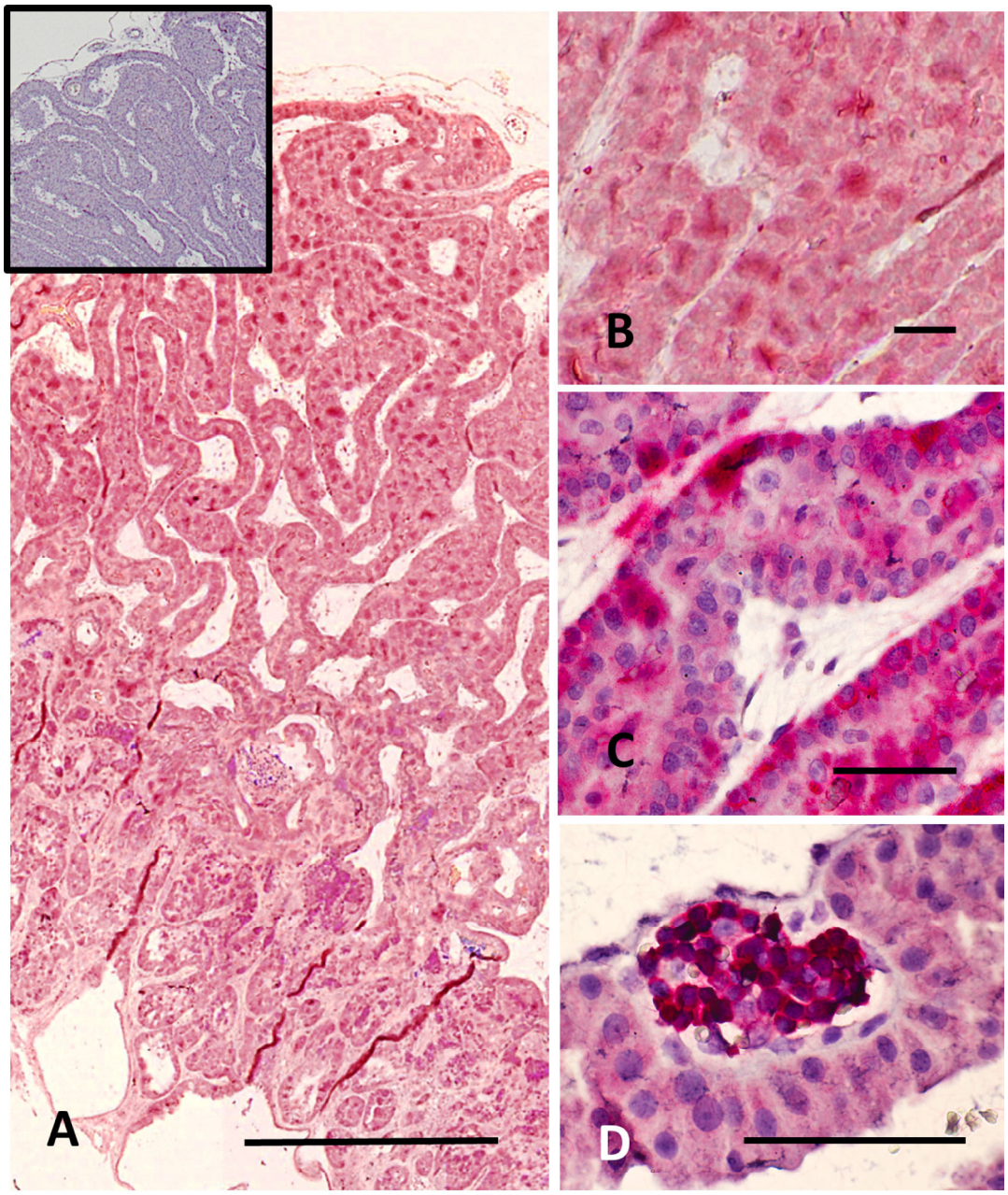


Figure 3.17: Immunolocalization of VEGF-A in placenta during early pregnancy. VEGF immunoreactivity was localized in clusters at the maternal lamellae and appears to be associated with decidual cells. VEGA-A was also localized in cells of the trophoblast attached to maternal lamellae. Distribution of VEGF A is variable from sample to sample and in different areas of the same slide. In the yolk sac, VEGF A was immunolocalized in blood cell precursors. Scale bar **A** = 500 um, **B,C,D** = 25 um.

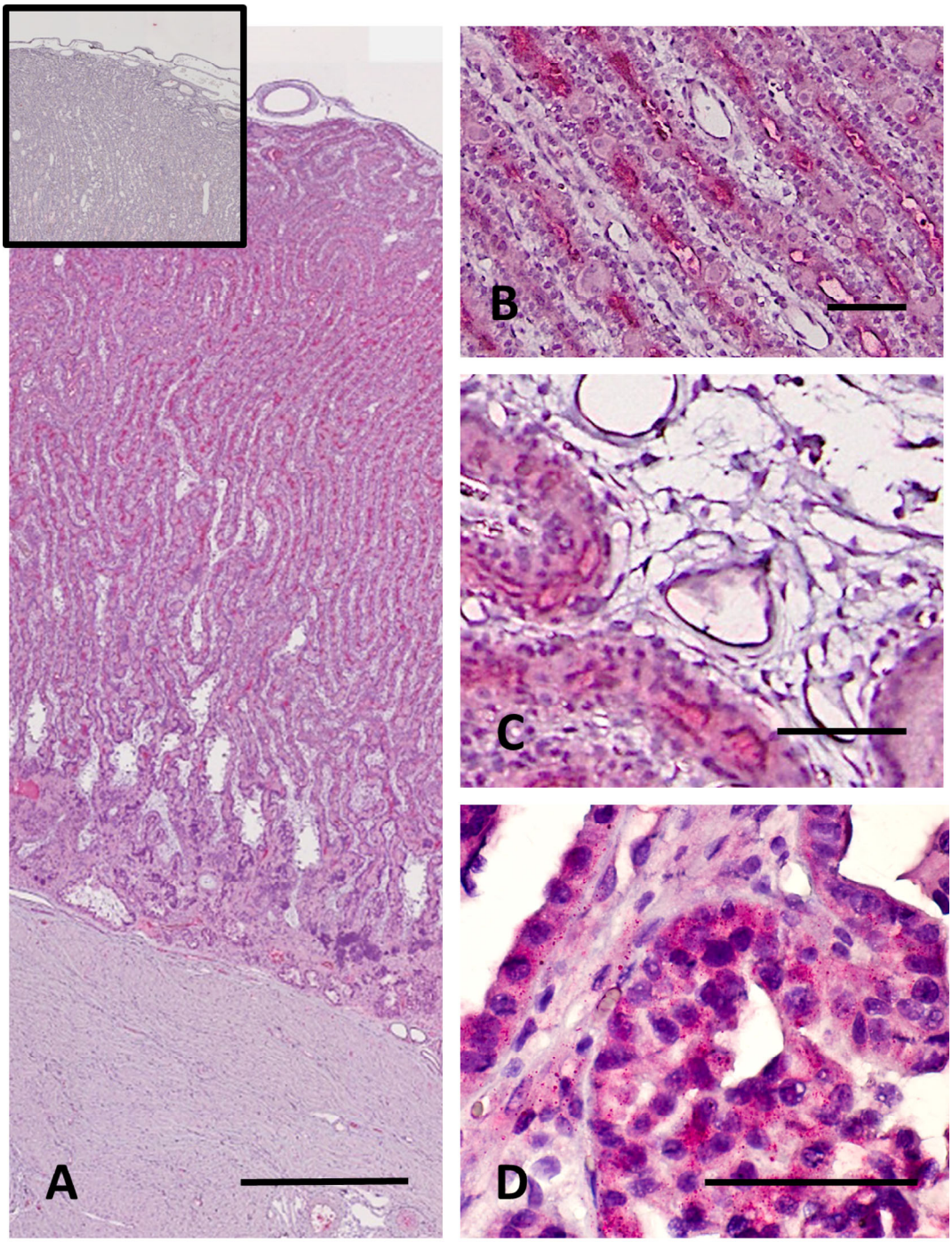


Figure 3.18: Immunolocalization of VEGF-A in placenta during late pregnancy. Late in pregnancy VEGF A, was immunodetected in the LZ, specifically associated with maternal capillaries and fetal vasculature of the luminal side. VEGF A was also detected in trophoblast cells of the LZ. Scale bar **A** = 500 um; **B,C,D** = 25 um.

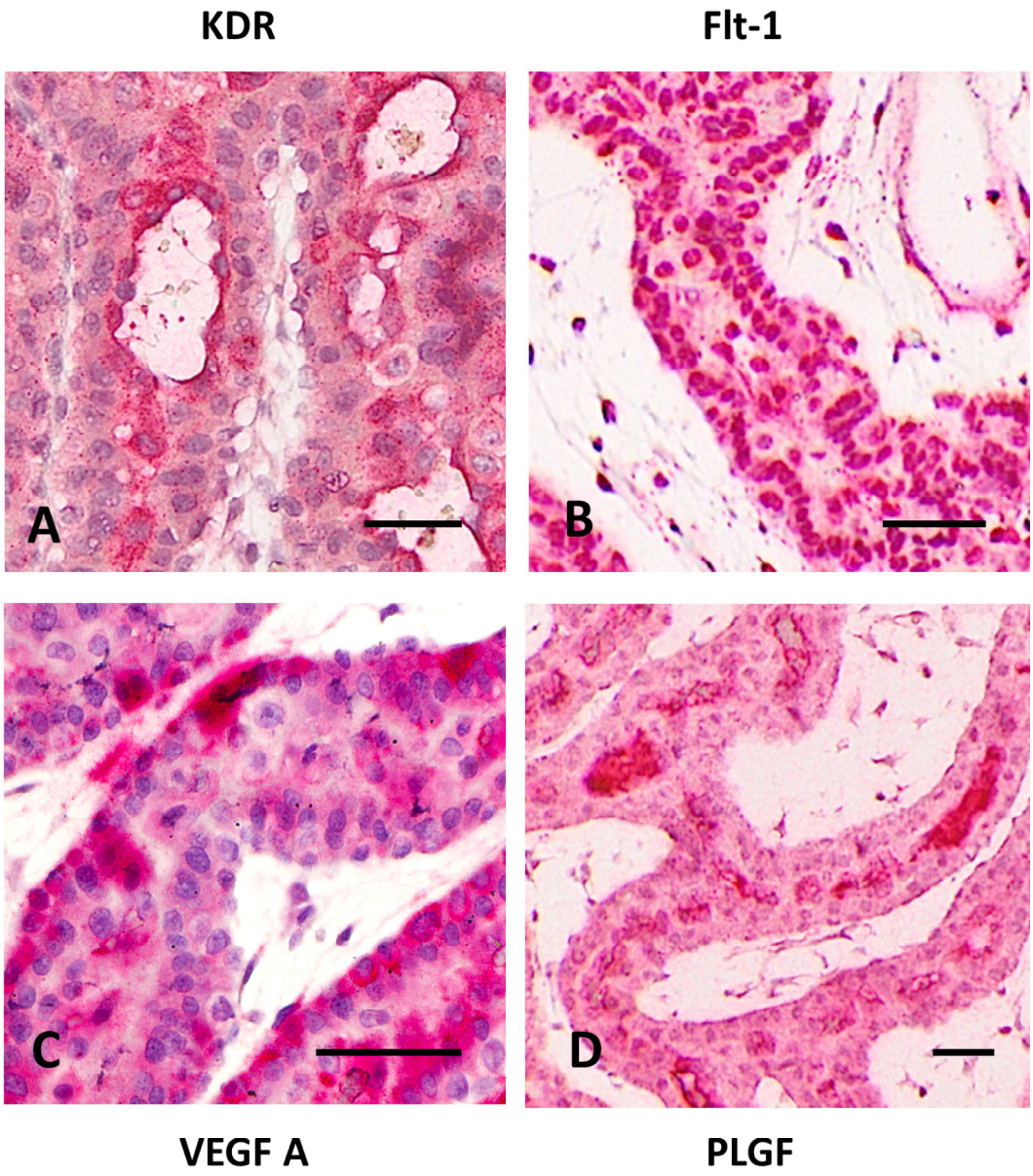


Figure 3.19: Comparison of immunolocalization of VEGF family and receptors in the lamella. KDR is detected in endothelial cells of maternal capillaries in the center of the lamella. Flt-1 is located in the trophoblasts located at each side of the maternal lamellae; Flt-1 was also localized in the fetal vasculature. VEGF A immunolocalization varies in different areas of the placenta; in some areas, VEGF A was localized in trophoblast cells and associated with endothelial cells. PLGF was immunodetected in maternal and fetal capillaries. Scale bar = 50 um.

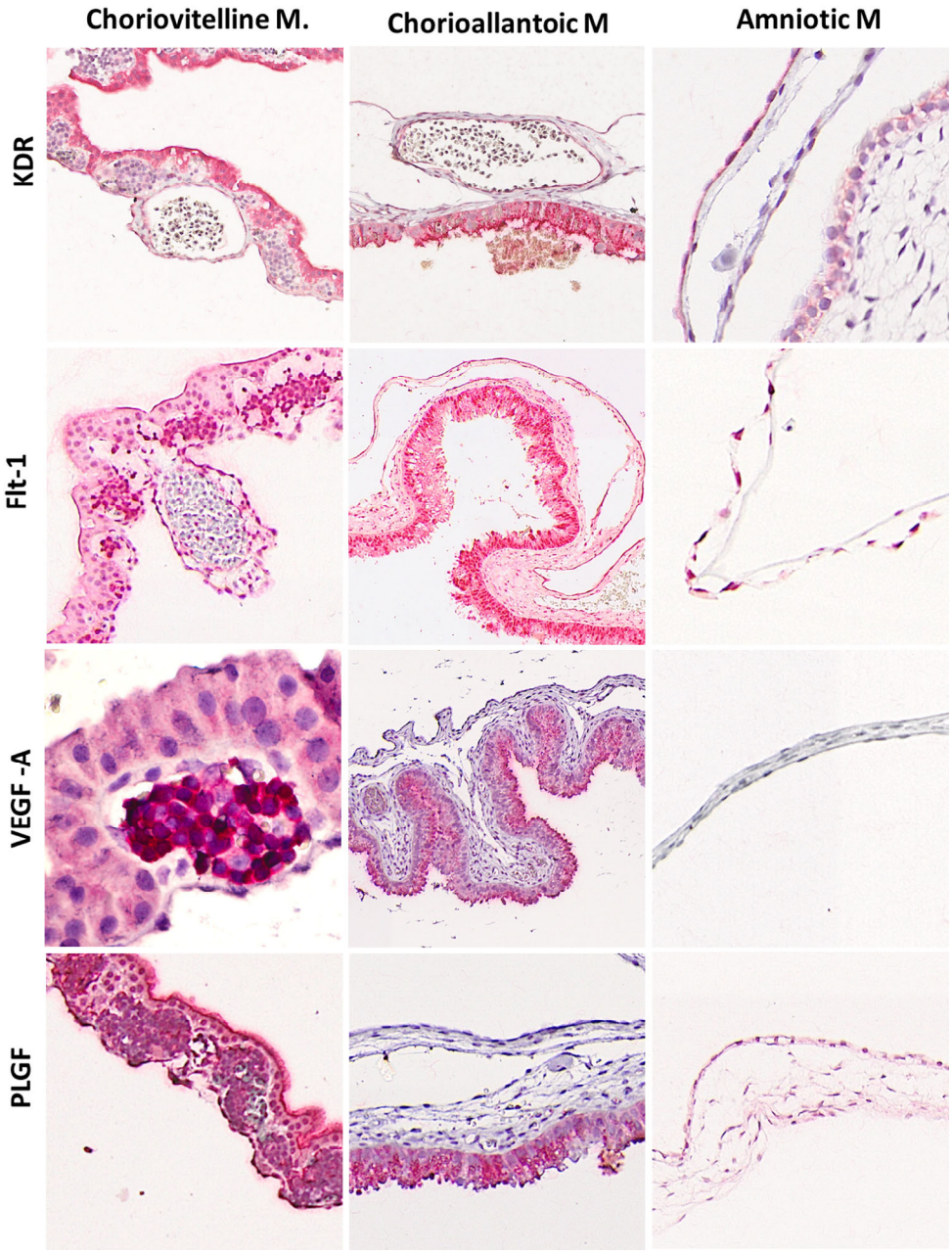


Figure 3.20: Comparison of VEGF family immunodetection in fetal membranes. KDR and PLGF were localized in endoderm of the yolk sack. KDR and Flt-1 also appear to co-localize in capillaries of the **yolk sac**. VEGF A and PLGF co-localize in blood cell precursors in the **chorioallantoic membrane** at the paraplacenta (outside zonary girdle). All proteins immuno-co-localized in the trophoblastic cells of the **chorion**. KDR was also detected in microvasculature related to the **allantois** and in the allantoic mesothelium. In the **allantoamnios**, KDR and Flt-1 appear to co-localize in the mesothelium of the allantois component of the amniotic sac but not in the amniotic membrane. Scale Bar = 25 um.

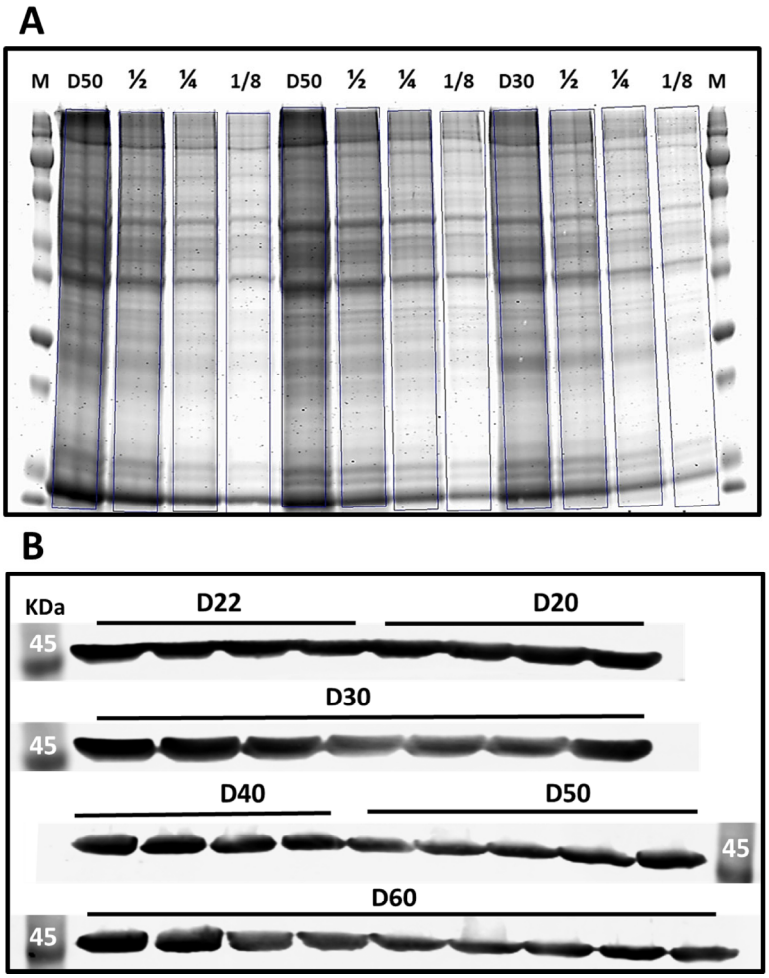


Figure 3.21: Quality assessment of protein samples and approach to Western Blot. **A**: Electrophoresis of extracted placenta protein samples stained with Coomassie blue. Sharp protein bars at broad range of sizes reveal its solubility and correct extraction. Four serial 1:1 dilutions of placental samples at d50 and d30 were made to establish optimal protein loading of electrophoretic gels for western blotting. **B**: Immunodetection of β-actin (c. 45 kDa) at different stages of the pregnancy to be used as a normalizing protein.

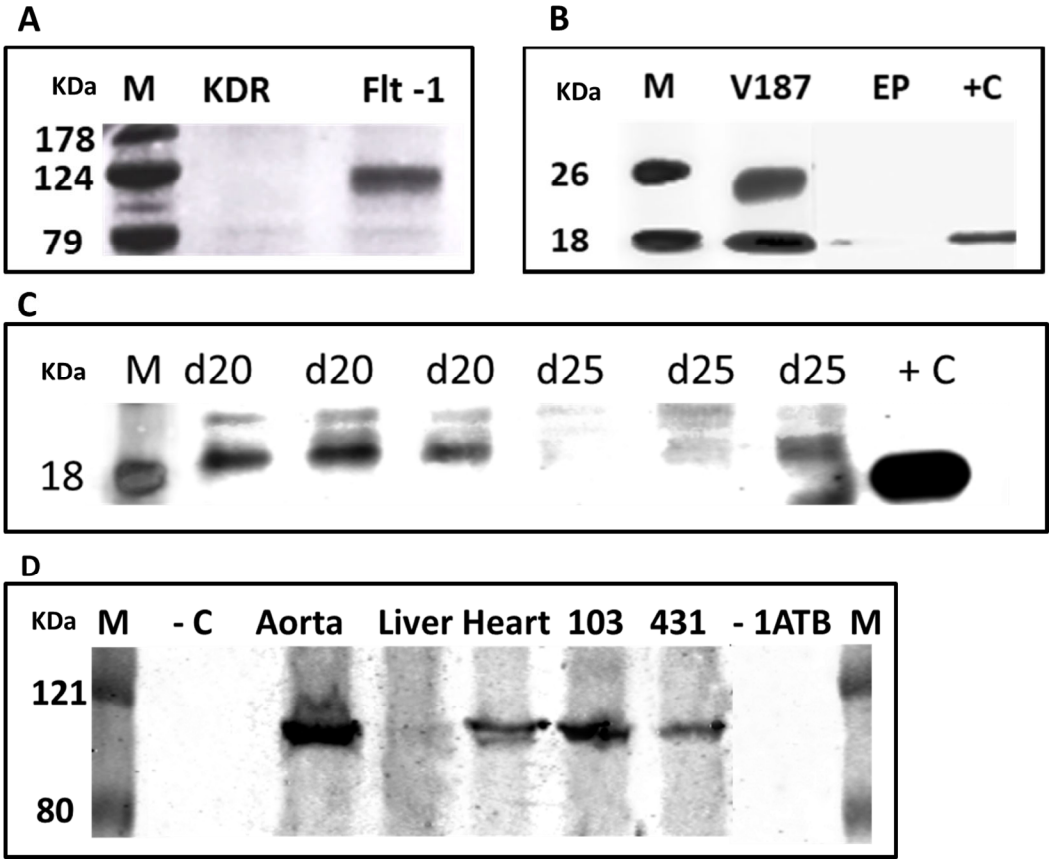


Figure 3.22: Validation of antibodies and immunodetection of VEGF A and Flt-1 in placenta. Owing to the lack of commercially available antibodies for domestic cat, we attempted to validate candidate antibodies using cloned feline and canine proteins expressed in HEK293 cells. Transient expression of feline VEGF A 187 (V187) and Flt-1 were used as positive control. **A**: Flt-1 (c. 125 kDa) was immunodetected using antibody H-225 from Santa Cruz. **B**: VEGF A was immunolocalized with 2 bands of approximately 18 and 26 KDa. "**EP**", empty plasmid; "**+C",** positive control with mouse recombinant VEGF A. **C:** VEGF A was detected in placental tissue during early pregnancy with a positive band of ~20 KDa, +C used was human recombinant VEGF A. **D:** The receptor Flt-1 was immunolocalized in placental tissue (sample f102 at d20, and f431 at d27). The receptor was also localized in multiple feline tissues. "**–C"**, negative control using only Laemmli buffer diluted in water. "**– 1ATB**", lane without primary antibody incubation.

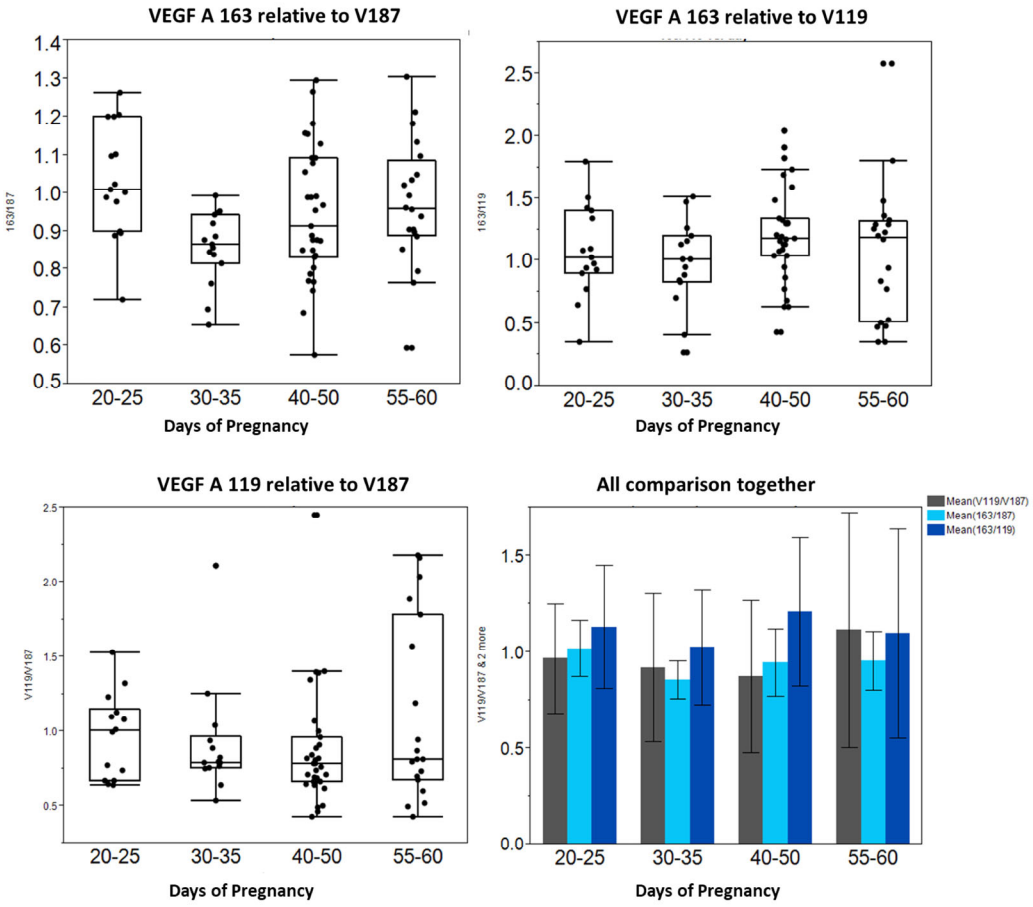


Figure 3.23: Comparisons between VEGF isoforms expressed in the placenta. We compared the ratios between different isoform of VEGF A in all possible combinations. All isoforms had similar expression levels during pregnancy. Each data point represents measurements from individual placentas.

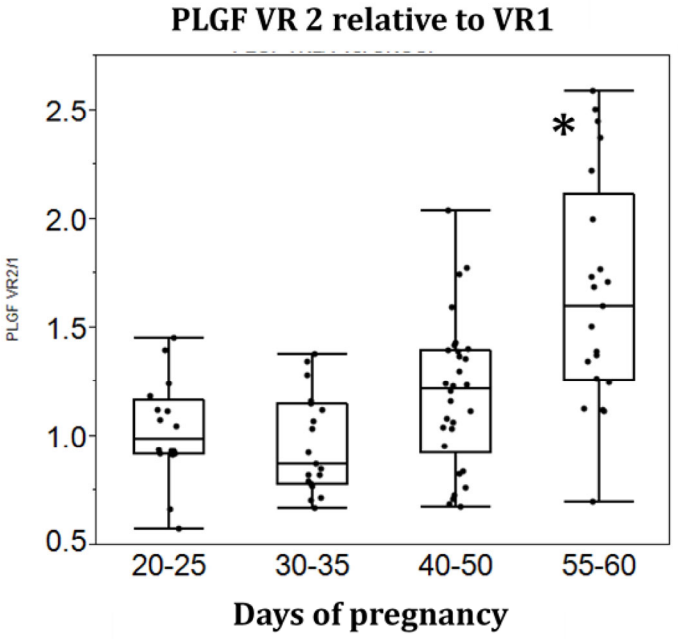


Figure 3.24: PLGF VR2:VR1 increases during late pregnancy. Comparison of expression of PLGF VR2 relative to PLGF VR1: There are similar expression ratios of PLGF 1 and 2 during early pregnancy and mid pregnancy, but there is a clear increase (~50%) in PLGF VR2:VR1 at late pregnancy. *p <0.0001. Each data point represents measurements from individual placentas.

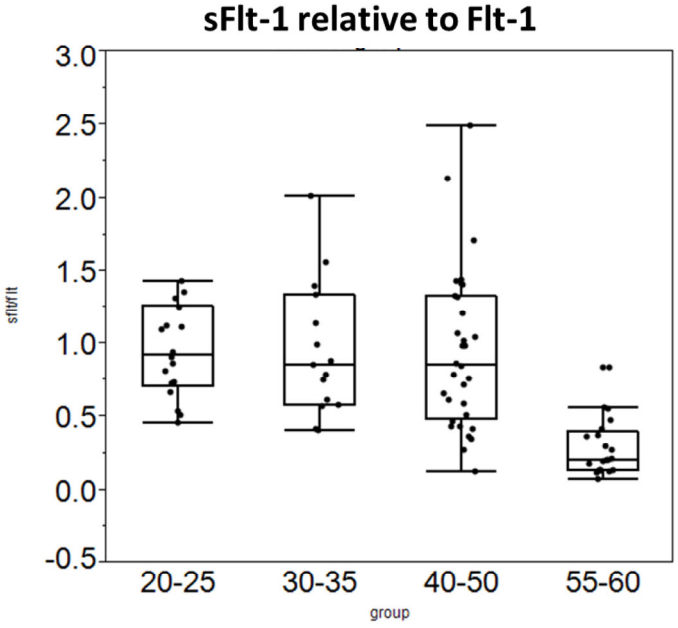


Figure 3.25: sFlt1:Flt1 is reduced at late pregnancy. Comparison of expression of Flt1 relative to sFlt-1 showed similar expression ratios of Flt-1/sFlt-1 during the first 2/3 of the pregnancy. After this, sFlt-1 is reduced compared to Flt-1 (**p <0.0001). This finding is consistent with generation of a pro-angiogenic environment during late placentation. Each data point represents measurements from individual placentas.

Table 3.1A Primers and probes for real-time PCR

Feline Gene Target	Oligonucleotide	S/AS/P	Sequence, 5'→3'
Pan VEGF A (exons 3/4)	BH-745	S	CCCACGGAGGAGTTCAACAT
	BH-746	AS	TGGCCTTGATGAGGTTTGATC
	BHTP-33	P	FAM-ACCATGCAGATTATGC-NFPMGB
VEGF A 119 (exons 4/5/8)	BH-790	S	ATGTGAATGCAGACCAAAGAAAGA
	BH-791	AS	CACCGCCTGGGCTTGT
	BHTP-39	P	FAM-AGCGAAAGAAAAATGTGACAAG-NFPMGB
VEGF A 163 (exons 4/5/7a/7b)	BH-711	S	AGCAAATGTGAATGCAGACCAA
	BH-712	AS	TGCAAGTACGTTCGTTTAACTCAAG
	BHTP-31	P	6FAM-AAAGAAAATCCCTGTGGGC-MGBNFQ
VEGF A 187 (exons 6s/7)	BH-713	S	AATCAGTTCGAGGAAAGGGAAAG
	BH-714	AS	TGCGGATCTTGTACAAACAAATG
	BHTP-32	P	VIC-CAAGAAATCCCGTCCCTGT-MGBNFQ
VEGF-B (exons 3/4)	BH-757	S	CCCGTTTCCCAGCCTGAT
	BH-758	AS	CGGGCATACACGTCTATCCA
	BHTP-38	P	VIC-ACCAGAAGAAAGTGGTGTC-NFPMGB
PLGF VR 1 (exons 5/6)	BH-676	S	TCCTACGTGGAGCTGACATTCTC
	BH-674	AS	CCCTTGGGTCTCCTCCTTTC
	BHTP-27	P	FAM-CAGAAAGGAGGAGACCCA-MGB
PLGF VR 2 (exons 5/7)	BH-676	S	TCCTACGTGGAGCTGACATTCTC
	BH-679	AS	GTGGGGGTTGTCTCTCTTCTGA
	BHTP-28	P	VIC-AGAAAGGTGCGGCAAT-MGB
Flt1 (exons 14/15)	BH-792	S	GGTTTAAAAACAACCACCAAATACAG
	BH-793	AS	TGCTGCTTCCCGGTCCTA
	BHTP-40	P	FAM-AACCCGGAATTATC-NFPMGB
sFlt1 (exon 13/intron 13)	BH-794	S	CTGCAGAGCCAGGAACATATACA
	BH-795	AS	GATCCGAGAGAAAACAGCCTTTT
	BHTP-41	P	FAM-ACAATTAGAGGTGAGCACTG-NFPMGB
KDR (exons 3/4)	BH-751	S	CGTAGCCTCGGTCATTTATGTCT
	BH-752	AS	TGTTGGTCGCTAACAGAAGCA
	BHTP-36	P	FAM-TGTTCAAGATTACAGGTCTC-NFPMGB
Thrombospondin-1 (exons 7/8)	BH-749	S	GTTCCCGATGGAGAATGCTG
	BH-750	AS	ACCAGCCATCATCCGCA
	BHTP-35	P	VIC-CGGTGTTGGCCCAGC-NFPMGB
Angiopoietin-2 (exons 7/8)	BH-747	S	GGAATGAAGCGTACTCACTGTATGA
	BH-748	AS	GCCGGCTGTCCCTGTAAGT
	BHTP-34	P	FAM-AAGAATTCAATTACAGGATTCAC-NFPMGB
HIF-1alpha (exons 8/9)	BH-755	S	TCTCAACCACAGTGCATTGTATGT
	BH-756	AS	GGAGAAAATCAAGTCGTGCTGAA
	BHTP-37	P	FAM-TAAATTACGTTGTGAGTGGTATT-NFPMGB

Table 3.1B: Primers end-point PCR and sequencing

Feline Gene Target	Oligonucleotide	S/AS	Sequence, 5'→3'
VEGF-A	BH-621	AS	CCATGAACTTTCTGCTCTCTTG
VEGF-A	BH-622	AS	GTTTGATCCGCATAATCTGCATG
Flt1 ex13	BH-623	S	ACGTGCCCAACGGATTTCACG
Flt1 ex13	BH-624	S	AATGCCGACGGAAGGAGAG
Flt1 ex13	BH-625	AS	GGAGGAGCATCTCCTCTGAG
Flt1 ex15	BH-626	AS	CTCTTCTGTGACTCTTTCAATAAACAG
Flt1 ex15	BH-627	AS	CCTTCTGGTTGGTGGCTTTGCA
Pred PlGF	BH-633	S	CCATGAGGCTGTTCACTTGCTTC
Pred PlGF	BH-634	AS	GGATCATCAGGAGCTGCATGGTGA
Pred KDR	BH-635	S	CTCTGGTTGTGAATGTCCCGCC
Pred KDR	BH-636	AS	GCACACATAGTCTCCTTGGTCCTGC
Pred VEGF-B	BH-639	S	CCAGAAGAAAGTGGTGTCATGGATAGA
Pred VEGF-B	BH-640	AS	CAGGTGTCTGGGTTGAGCTCTA
R/C of BH-622 VEGF-A	BH-643	S	CATGCAGATTATGCGGATCAAAC
VEGF-A exon 4-5	BH-644	S	CAAATGTGAATGCAGACCAAAGAAAG
VEGF-A exon 8a	BH-646	AS	CGCCTGGGCTTGTCACAT
VEGF-A exon 7b	BH-656	S	CCGCAGACGTGTAAATGTTCCTG
VEGF-A exon 8a/8b	BH-657	AS	TCTTCCTTCATTTCAGGTTTCTGGAT
PLGF exon 5	BH-664	S	GCCTCTGCGGGAGAAGATGAA
PLGF exon 1	BH-665	S	CCTACCTGCGGACTTCAGGCTC
PLGF exon 7?	BH-666	AS	TTCTGTCTCCTGCTCCCGGG
PLGF exon #__	BH-667	S	CCTTGGAACCTCCTGGCTTGG
PLGF exon 7?	BH-668	AS	TTACCTCCGGGGAACAGTATTGC
PLGF exon 6	BH-669	S	CAAGGGCAGGGGGAAGAGGA
PLGF exon 7?	BH-670	AS	AGGAGCCGGGTGGGGGTT
KDR Exon 9/10	BH-753	S	CTCTGGTCGTGAATGTCCCACC
KDR Exon 13	BH-754	AS	GCACACATAATCTCCTTGGTCCTGC

Table 3.2 Antibodies used for western blotting and immunohistochemistry

Protein Target	Antibody name	Antibody details	Dilution
panVEGF A	C-1 (sc-7269) Santa Cruz	Mouse monoclonal IgG	1/100
PLGF	H-90 (sc-20714) Santa Cruz	Rabbit polyclonal IgG	1/100
KDR	F-10 (sc-393179) Santa Cruz	Mouse monoclonal	1/200
FLT 1	H-225 (sc-9029) Santa Cruz	Rabbit polyclonal IgG	1/200
beta Actin	C-4 (sc-47778)	Mouse monoclonal	1/500
Mouse IgG	IRDye 800CW green Licor	Goat polyclonal	1/5000
Mouse IgG	IRDye 680 RD red Licor	Donkey polyclonal	1/5000
Goat IgG	IRDye 680 RD red Licor	Donkey polyclonal	1/5000
Goat IgG	IRDye 800CW green Licor	Donkey polyclonal	1/5000
Rabbit IgG	IRDye 800CW green Licor	Goat polyclonal	1/5000

Table 3.3 Placental samples and non-pregnant uterus collected with gestational age.

N	Sample Name	Length (C-R) mm	Age based on length	BPD Skull	Age based on skull	Definitive estimated age
152	F1 P1	120	75	20	59	59
165	F1 P2	115	72	23	61	61
168	F1 P3	121	75	24	62	62
169	F1 P3 F	121	75	24	62	62
170	F1 P3 MF	121	75	24	62	62
171	F1 P4	114	72	24	62	62
164	F1 P5	114	72	22	60	60
8	F10P1	8	20	2	28	20
9	F10P2	8	20	2	28	20
10	F10P3	8	20	2	28	20
12	F10P4	9	20	2	28	20
11	F10P5	8	20	2	28	20
7	F10P6	8	19	2	28	19
64	F11P1	40	35	7	42	42
57	F11P2	39	35	6	40	40
68	F11P3	40	35	8	44	44
67	F11P4	40	16	8	44	44
127	F13P1	107	68	17	56	56
159	F13P2	115	72	21	59	59
132	F13P3	106	68	18	57	57
27	F14P1	13	22	3	29	22
18	F14P2	12	22	3	29	22
19	F14P3	12	22	3	29	22
20	F14P4	12	22	3	29	22
21	F14P5	12	22	3	29	22
22	F14P6	12	22	3	29	22
58	F15P1	29	30	6	40	40
50	F15P2	28	29	6	38	29
51	F15P3	28	29	6	38	29
49	F15P4	27	29	5	37	29
48	F15P5	26	28	5	37	28
173	f16p1	110	70	27	63	63
39	f17p1	19	25	4	33	25
37	f17p2	18	25	5	35	25
38	f17p3	18	25	5	35	25
40	f17p4	19	25	4	33	25
81	f18p1	52	41	10	48	48
82	f18p2	54	42	10	48	48
83	f18p3	53	42	10	48	48
128	F19p1	96	63	17	56	56
129	F19p2	96	63	17	56	56
126	F19p3	96	63	16	55	55

N	Sample Name	Length (C-R) mm	Age based on length	BPD Skull	Age based on skull	Definitive estimated age
28	F2 P1	13	22			22
23	F2 P2	12	22			22
24	F2 P3	12	22			22
172	F20P1	95		24	62	62
166	F20P2	95		23	61	61
167	F20P3	94		23	61	61
91	F3 P1	52	41	11	49	49
84	F3 P2	50	40	10	48	48
92	F3 P3	55	43	11	49	49
93	F3 P4	55	43	11	49	49
151	F30P1	93	61	20	58	58
133	F30P1 Mat	90	60	19	58	58
134	F30P2	91	60	19	58	58
135	F30P2 sid	91	60	19	58	58
136	F30P3	90	60	19	58	58
137	F30P3 AM	90	60	19	58	58
138	F30P3 sid	90	60	19	58	58
139	F30P4	90	60	19	58	58
140	F30P4 AM	90	60	19	58	58
141	F30P4 Mat	90	60	19	58	58
150	F30P5	90	60	19	58	58
142	F30P5 AM	90	60	19	58	58
143	F30P5 Mat	90	60	19	58	58
144	F30P5 sid	90	60	19	58	58
70	F31P1	30	30	9	46	46
71	F31P1 YS	30	30	9	46	46
72	F31P2	31	31	9	46	46
73	F31P2 YS	31	31	9	46	46
74	F31P3	32	31	9	46	46
75	F31P3 YS	32	31	9	46	46
76	F31P4	31	31	9	46	46
69	F32P1	47	39	8	44	44
77	F32P2	41	36	9	46	46
78	F32P3	45	38	9	46	46
79	F32P4	45	38	9	46	46
114	F33P1	74	52	14	53	53
122	F33P2	71	51	15	54	54
123	F33P3	74	52	15	54	54
124	F33P4	73	52	15	54	54
153	F34P1	97	63	20	59	59
154	F34P2	98	64	20	59	59
160	F34P3	97	63	21	59	59
155	F34P4	97	63	20	59	59
94	F35P1	61	46	11	49	49

N	Sample Name	Length (C-R) mm	Age based on length	BPD Skull	Age based on skull	Definitive estimated age
95	F35P2	59	45	11	49	49
96	F35P3	61	46	11	49	49
97	F35P4	62	46	11	49	49
145	F36P1	95	62	19	58	58
146	F36P2	99	64	19	58	58
156	F36P3	105	67	20	59	59
55	F37P1	30	30	7	42	30
52	F37P2	29	30	6	40	30
53	F37P3	29	30	6	40	30
113	F38P1	73	52	14	52	52
115	F38P2	75	52	14	53	53
116	F38P3	76	53	14	53	53
121	F38P4	76	53	15	54	54
56	F39P1	42	36	6	39	39
59	F39P2	37	34	6	40	40
60	F39P3	38	34	6	40	40
61	F39P4	41	36	6	40	40
147	F4 P1	115	72	19	58	58
161	F4 P2	97	63	21	59	59
29	F40P1	13	22	4	33	22
36	F40P2	16	24	4	33	24
33	F40P3	14	23	4	31	23
35	F40P4	15	23	4	31	23
117	F41P1	78	54	14	53	53
125	F41P2	73	52	15	54	54
118	F41P3	75	52	14	53	53
119	F41P4	74	52	14	53	53
25	F42P1	12	22			22
26	F42P1 MAT	12	22			22
13	F42P2	10	21	2	28	21
15	F42P3	11	21	2	28	21
6	F42P4	3	17	2	28	17
16	F42P5	11	21	2	28	21
14	F42P6	10	21	2	28	21
54	F43P1	29	30	8	43	30
42	F44P1	22	27			27
47	F44P2	24	28			28
45	F44P3	23	27			27
30	F45P1	13	22			22
32	F45P2	14	22			22
31	F45P3	13	22			22
34	F45P4	14	23			23
43	F46P1	22	27			27
44	F46P2	22	27			27

N	Sample Name	Length (C-R) mm	Age based on length	BPD Skull	Age based on skull	Definitive estimated age
46	F46P3	23	27			27
41	F46P4	21	26			26
157	F48P1			20	59	59
162	F48P2			21	59	59
158	F48P3			20	59	59
163	F48P4			21	59	59
174	F49P1	<15d				
175	F49P2	<15d	16			
176	F49P3	<15d	16			
177	F49P4	<15d	16			
98	F5 P1	55	43	11	49	49
99	F5 P2	56	43	11	49	49
100	F5 P3	57	44	11	49	49
101	F5 P4	57	44	11	49	49
111	F50P1	66	48	13	52	52
120	F50P2	66	48	14	53	53
108	F50P3	66	48	12	51	51
112	F50P4	66	48	13	52	52
102	F51P1	61	46	11	49	49
103	F51P2	61	46	11	49	49
85	F51P3	61	46	10	48	48
86	F51P4	61	46	10	48	48
104	F52P1	58	44	11	49	49
87	F52P2	57	44	10	48	48
105	F52P3	57	44	11	49	49
106	F52P4	57	44	11	49	49
109	F52P5	60	45	12	51	51
110	F52P6	61	46	12	51	51
107	F52P7	60	45	11	49	49
148	F6 P1	109	69	19	58	58
149	F6 P2	110	70	19	58	58
130	F6 P3	108	69	17	56	56
131	F6 P4	110	70	17	56	56
80	F7P1	54	42	9	46	46
88	F7P2	52	41	10	48	48
89	F7P3	52	41	10	48	48
90	F7P4	49	40	10	48	48
62	F8P1	36	33	6	40	40
63	F8P2	38	34	6	40	40
65	F8P3	39	35	7	42	42
66	F8P4	41	36	8	43	43
17	F9P1	11	21	2	28	21
1	NP1 U					0
2	NP2 O					0

N	Sample Name	Length (C-R) mm	Age based on length	BPD Skull	Age based on skull	Definitive estimated age
3	NP2 U					0
4	NP3 U					0
5	NP4 U					0

Table 3.4: Protein samples of placenta at different stages of pregnancy used for immunoblot studies

N	Age	Name	protein ug/ml
1	17	425	1480
2	21	422	1273
3	21	423	1873
4	22	F45P1 F	1362.3
5	22	F45P2 F	1175.9
6	22	F45P1	1194.4
7	22	F45P2 RL	1302.7
8	22	421	1465
9	22	453	1474
10	22	454	1915
11	26	463	1488
12	27	441	1346
13	27	461	1496
14	27	462	1302
15	30	371	1372
16	30	372	1363
17	30	373	1627
18	39	391	1428
19	40	392	1652
20	40	393	1539
21	40	394	1747
22	47	521	1726
23	49	351	1225
24	49	352	1108
25	49	353	1574
26	49	354	1576
27	49	523	1577
28	49	524	1432
29	50	526	1605
30	58	F48P3 F	1816.4
31	58	F48P3 RL	1275.5
32	58	F48P4 RL	1309.9
33	58	361	1170
34	58	362	1533
35	59	F48P4 F	1545.9
36	59	363	1202
37	59	481	1784
38	59	482	1475

Table 3.5 Histologic sections used for IHC

Sample Name	Gestational age (days)	Short description
49 3-4	15	2 conceptus with early placental development in utero and ovary.
45 P5-P6	22	1 conceptus intact
45 P2	22	3 placenta sections and 1 whole embryo.
NP 1-2	0	Non pregnant uterus. 5 cross sections and 1 ovary.
47 P2	Day 15-20	Two conceptus and one cross section of early placental dev.
48 3-4	Day 60	Two sections of placenta with utero.
50 1-2	Day 50	4 sections of placenta with utero.
51 1-2	Day 50	Two sections of placenta with utero.
52 -1-2	Day 50	Two sections of placenta with utero.
50 F1	Day 50	Thorax fetus
48 1-2	Day 60	Two sections of placenta with utero.
47 P3	Day 15-20	One conceptus and two cross section of early placental dev and 1 ovary
F46p3	Day 26	Whole embryo and 1 section of placenta.
F46p1	Day 26	Whole embryo and 2 section of placenta.

Chapter 4
Characterization of the expression of angiogenic factors in cutaneous squamous cell carcinoma of domestic cats.

4.1 Introduction.

Squamous cell carcinoma (SCC) is a highly malignant neoplasia that arises from epidermal cells, inducing differentiation into keratinocytes usually producing keratin (Goldschmidt and Goldschmidt 2017; Baba and Câtoi 2007). SCC can be classified, based on the location of origin, as oral SCC, ocular SCC or cutaneous SCC (CSCC). The last is among the most common cancers in domestic animals and the second most common cancer in white humans (Johnson et al. 1992), with estimated human incidence having increased between 50-200% in the United States and Canada during the last two decades (Gallagher et al. 1995; Gallagher et al. 1990; Gray et al. 1997; Alam and Ratner 2001). This neoplasia produces a large medical and economic impact due to its local invasiveness, limited treatment approaches, and tendency to recur (Kim et al. 2011; De Souza et al. 2014). Prolonged solar radiation exposure (producing actinic keratosis), lack of skin pigmentation, sparse hair cover, or papillomavirus infection are the main factors inducing tumor progression. Similarly to other cancers, progression of actinic keratosis into SCC has been related to mutations in key genes involved cellular pathways that control DNA repair, cell growth, survival, and motility (Hanahan and Weinberg 2000; Liotta and Kohn 2001). For example, a mutation from ultraviolet radiation exposure in the P53 gene has been identified in more than 90% of CSCC in humans and has been detected in bovine, canine, feline and equine SCC as well (Ziegler et al. 1994; Carvalho et al. 2005; Teifke and Löhr 1996). This mutation has been correlated with upregulation of the angiogenic factor VEGF A in SCC (Frank Riedel 2000).

Progression of SCC by the abovementioned mechanisms can be manifested in tissue as marked increases in cell proliferation rates, reduction in cell death of tumoral keratinocytes, deregulated cell differentiation, and increased production of inflammatory and angiogenic cytokines. These progressive changes facilitate tumor growth, invasion, and metastasis (Yuspa et al. 1994; Chen et al. 1997; Smith et al. 1998). It is vital to understand the basic cell biology of this neoplasia, including its degree of reliance on angiogenesis for growth and metastasis. In this

regard, naturally occurring CSCC in cats can represent a good alternative model to study CSCC in humans, including the more aggressive types such as head and neck carcinoma (Wypij 2013).

Histologically, CSCC is characterized by islands, cords, and trabeculae of unorganized epidermal keratinocytes, invading and trespassing across the basal layer of the epidermis into the dermis. Often, neoplastic keratinocytes form islands secrete concentric eosinophilic keratin fibers in the center, forming keratin pearls that are useful for diagnosing SCC. Tumoral cells are normally large with an oval shape and have large hyperchromatic prominent nuclei, although large degrees anisocytosis and nuclear pleomorphism are described for less differentiated high-grade SCC. Tumoral cells can have multiple desmosomes in the membrane that are seen as bridges between cells (Baba and Câtoi 2007). CSCC locally invade the dermis and can reach bone and cartilage of affected areas with serious consequences for the patient. SCC induces an intense fibrous and inflammatory response from the surrounding tissue with pronounced angiogenic activity.

Angiogenesis is a major contributor to cancerous tumor growth, metastasis, and survival (Folkman 1995). Solid tumors rely on vascular perfusion to growth and migrate; otherwise they are limited to 1 to 2 mm before hypoxia in the center of the tumor induces necrosis (Cullen and Breen 2016). Furthermore, angiogenesis, estimated as microvascular density in tumors, can be used as a prognostic indicator for overall survival for lung and breast cancer, hepatic and gastric carcinoma, skin melanoma and glioblastomas in humans (Horak et al. 1992; Srivastava et al. 1988; Page and Jensen 1995; Tanigawa et al. 1997; Behrem et al. 2005; Kumar et al. 1999; Yao et al. 2007). Angiogenesis in normal and tumoral tissue is stimulated by secreted peptides from tumoral or adjacent stromal cells (Klagsbrun 1991). Among these angiogenic factors, the vascular endothelial growth factor (VEGF) family and their receptors are the most important angiogenic promoters of physiological and tumoral angiogenesis. (Carmeliet 2000; Demir, Seval, and Huppertz 2007; Herr, Baal, and Zygmunt 2009; Risau and Flamme 1995; Semenza 2007; Silvan et al. 2015; Hicklin and Ellis 2005; Dong et al. 2001; Nakopoulou et al. 2002; Fischer et al. 2008). Elevated expression of VEGF A and their receptors have been linked with histopathology grading, tumor progression and prognosis of multiple human tumors (Du et al. 2003; Tse et al. 2004). Furthermore, studies in a murine SCC model stimulated by hepatocyte growth factor showed over-expression of pro-angiogenic factors, such as the C-X-C chemokine, Gro-1/KC, IL-8, and VEGF A (Dong et al. 2004). Similarly, PLGF has been

related to tumoral angiogenesis and carcinogenesis. Higher PLGF mRNA or proteins levels correlates with pathological angiogenesis (Fischer et al. 2008), tumor size, metastasis, clinical stage, recurrence and poor prognosis of multiple types of cancers (Marcellini et al. 2006; Lacal et al. 2000; Parr et al. 2005; Zhang et al. 2005; Wei et al. 2005; Cheng et al. 2010).

In human oral and eyelid SCC, intra-tumoral microvascular density was studied using endoglin (CD105) in endothelial cells to quantify active angiogenesis. Results revealed higher microvascular density in SCC compared to normal oral mucosa or normal eyelid skin. (Schimming and Marmé 2002; Tzoutzos et al. 2014). In other studies, transcripts of PLGF were upregulated in human oral SCC compared to normal tissue. Furthermore, PLGF immunolocalization in tumors and PLGF serum levels measured by ELISA were significantly correlated with advanced progression and poorer prognosis of oral SCC (Cheng et al. 2013; Cheng et al. 2010). Research in canine SCC has immunolocalized VEGF A in all samples studied, and elevated proteins levels were detected in SCC localized in the toe, a location where SCC is typically more malignant and has higher index of metastasis, but was not elevated in other non-malignant neoplasias of the skin. These results suggest that VEGF A could be useful for evaluating malignancy in skin tumors of the dog (Maiolino, De Vico, and Restucci 2000). In addition, another study in dogs detected a significant increase in intra tumor microvascular density, and moderate correlation between immunolocalization of VEGF A and its receptor KDR in CSCC compared with a benign skin neoplasia (trichoepitheliomas) (Al-Dissi et al. 2007). In feline squamous cell carcinoma, there is very little information about how VEGF family may influence tumor progression and tumoral angiogenesis. One study was able to quantify the microvascular density of oral SCC, where higher microvascular densities were found in SCC located on the tongue, potentially explaining the clinical differences of poor clinical outcome of oral SCC in this location (Yoshikawa et al. 2012).

In cats, alternative pre-mRNA splicing produces three isoforms of VEGF and two isoforms of placental growth factor (PLGF) (unpublished data from our laboratory; Chapter 3). Sequence homology with mouse and human suggest that different isoforms have different biological behavior, although details of distinct function, properties, and regulation remain elusive. In general, the larger VEGF and PLGF isoforms are less soluble in the extracellular space and, owing to the presence of heparin-binding domains, accumulate in the extracellular matrix making this a reservoir of VEGF or PLGF that can be mobilized via proteolysis, whereas

smaller, relatively mobile isoforms have potential paracrine angiogenic actions on distant endothelial cells (Park, Keller, and Ferrara 1993; Lee et al. 2005; Vempati, Popel, and Mac Gabhann 2014; Kim et al. 2014; Houck et al. 1991; Tischer et al. 1991; Yao et al. 2005; De Falco 2012; Ferrara 2000).

As mentioned above, there are multiple reports supporting the importance of the VEGF family in CSCC in humans and other species, but very little is known about the role that specific variants of VEGF A and PLGF may have in CSCC progression. Additionally, the role of VEGF A, PLGF and the receptors KDR and Flt-1 is still unclear in feline CSCC. This information could be vital for understanding tumoral angiogenesis in cats, and could help potentiate the use of normally occurring CSCC in cat as a model to study SCC in humans.

We hypothesize that pro-angiogenic factors are present in elevated levels in CSCC and may therefore play a role in promoting tumorigenesis, Knowledge of the regulation of splice variant expression could bring valuable new information for understanding tumoral angiogenesis and develop targeted therapies.

4.2 Objectives

In the current study, we aimed to characterize the expression of three isoforms of VEGF A (186, 163, 119 amino acid forms); two isoforms of PLGF (I, II); and three receptors (FLT; sFLT; KDR) in cat CSCC biopsies in comparison to expression in normal haired skin (NHS) (**Figure 4.1**). We predict that pro-angiogenic factors are present in elevated levels in CSCC compared to normal skin and therefore may play a role in promoting tumor growth. Further, altered ratios of expression of VEGF or PLGF isoforms may provide clues regarding the vascular pathologies of this neoplasia.

4.3 Methodology

4.3.1 Sample collection selection and preparation: Samples of CSCC were selected from the tissue archives of the Virginia Maryland College of Veterinary Medicine Diagnostic Lab (ViTALS), stored as formalin-fixed, paraffin-embedded (FFPE) blocks. Criteria of selection include cat bread, sex, the location of the tumor, and quality of the samples in terms of the size of the tumor, the relative area of tumor compared to normal tissue, time of storage and good conservation of tissue. All cases selected were domestic shorthair cats, spayed female or

neutered males having CSCC located in the pinna. H&E stained 5 um sections were evaluated with a board-certified veterinary anatomic pathologist to assess the quality of the samples, evaluate the characteristics of the tumor, and rule out the presence of other pathologies of the skin, such as excessive dermatitis or necrosis of the tissue, the presence of parasites of fungi, that could confound our study.

After careful examination, fourteen cases dating from 2014 to 2018 were selected. (**Figure 4.2**). Samples from normal skin to be used as controls were obtained from a local veterinary clinic, in the form of freshly discarded tips of pinna routinely excised to mark feral cats that had undergone surgical sterilization. Control skin tissue destined for histology was immediately placed in 10% formalin for 48 hrs and embedded in paraffin using as described in Chapter 2. H&E stained 5 um sections of normal skin were evaluated by a pathologist to reconfirm the absence of dermal pathologies. Positive control tissue (feline placenta; Chapter 3) destined for gene expression analysis was stored in RNA Later immediately after collection. RNA was extracted using TRIsol reagent (Invitrogen) and quick RNA miniprep columns (Zymo) as described in Chapter 3.

4.3.2 RNA purification and cDNA synthesis. Total RNA was extracted from selected CSCC and normal skin FFPE blocks using the Quick-RNA FFPE Kit (Zymo Research, Irvine, CA) following the manufacturer's instructions. Briefly, 12 paraffin scrolls with a thickness of 5 um each were cut from individual blocks and placed in 1.5 ml Eppendorf tubes until further processing. Total RNA of positive control samples was extracted from feline placental tissue as described in Chapter 3 using Trizol reagents (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with an additional DNase digestion using the Quick RNA mini prep kit (Zymo Reseach, Irvine, CA). RNA concentrations were determined with the NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Additionally, RNA quality was assessed in a representative group of samples using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) (**Figure 4.3**). Random-primed cDNA was produced from 1 ug of RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in a 20 uL reaction according to the manufacturer's instructions. cDNA was not diluted prior to use for PCR.

4.3.3 Real-Time Quantitative PCR. As described in Chapter 3, DNA primers and minor groove binding (MGB) TaqMan DNA probes were designed in Primer Express 3.0.1 (Applied Biosystems, Foster City, CA) to target the exon-exon junctions unique to the respective VEGF and PLGF isoforms of interest. Custom probes (Applied Biosystems) and primers (Operon, Huntsville, AL) were used together with TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, 4369016). All real-time PCRs were run on a One-Step System (Applied Biosystems) in 10 ul triplicate reactions for each sample in 96 well plates. To verify the selectivity of isoform-directed qPCR assays, each isoform of feline PLGF and VEGF A was cloned using sequence-specific primers and the pCR™2.1-TOPO System (Thermofisher Scientific, Waltham, MA). Standard curves were generated to assure efficiencies above 90% for each target's qPCR reagent set. Specificity of the reactions was tested by cross-reaction standard curve. For example, primers and probe developed for VEGF A 119 were tested for their ability to detect homogeneous plasmid clones of VEGF 163 and VEGF A 186. Standard dilution of these cross reactions were made to assure that the primers and probes were only amplifying the intended VEGF isoform. 18S rRNA was used as normalizer gene with TaqMan VIC Ribosomal RNA control (Applied Biosystems, Foster City, CA) Two cDNA positive controls from feline placental tissue were used. Two negative control samples were used with ultrapure water to test for contamination, and two genomic (non-reverse transcribed) DNA controls were used in each qPCR run to confirm cDNA-dependence of signal.

4.3.4 Immunohistochemistry (IHC). Tissue sections from CSCC and NHS were cut at 5 μm, mounted, dried at 42 °C overnight, and stored at room temperature for less than 3 days before staining. All steps were performed at room temperature unless otherwise specified using the ultraView Universal Alkaline Phosphatase Red Detection Kit from Ventana (#760-501) and manual conventional histology protocols. Briefly, sections were deparaffinized in xylene, rehydrated and washed with 1X reaction buffer (1X RB; Ventana cat. no. 950-300). Unmasking of antigens was performed in a polyethylene staining jar with Ventana cell conditioner 1 (950-124) for 60 min at 95 C. Sections were then rinsed in 1X RB and blocked with 125 uL of Ventana 760-050 for 8 min. Primary antibody (see Table 3.2) was diluted in Ventana incubation diluent (251-018); 125 uL of this dilution was used per slide. Slides were incubated for 1 hour and then washed with 1X RB. Negative control slides were incubated without primary antibody

and using only incubation diluent. For secondary incubation, we used 125 uL of ultraView Universal Alkaline Phosphatase Red Detection Kit (Ventana 760-501), followed by 125 uL of UV red enhancer and incubated for 4 minutes. 100 ul of UV Fast Red A and 100 ul of UV red Naphthol was applied and incubated together for 8 min. Finally, 125 ul of UV Fast Red B was incubated for 8 min in rinsed with 1X RB. Slides were counterstained with hematoxylin for 45 sec, rinsed and air dried for at least 30 min before the coverslip was applied. Samples were observed under a Nikon Eclipse Ci-S microscope; images were captured on a Nikon camera and analyzed using NIS-Elements Analysis D 5.01 image software.

4.3.4.1 Quantification of IHC staining. Microphotographs of 10X images of normal skin pinna from two cats and cutaneous Squamous cell carcinoma (CSCC) from the pinna of 3 cats were analyzed using the NIH software ImageJ 1.5j8 to determine the surface area of positive immunoreactivity on each image, allowing comparison of normal skin to CSCC. Specifically, a selection of red chromogen staining pixels, indicative of positive immunostaining, were located and selected using the color threshold tool. Specific ranges of hue 225-255, Saturation 95-120 and brightness of 190-230 were used for all images. The selected pixels where compared to the total number of pixels in the image to obtain the percentage of selected pixels. Each sample was evaluated in three different areas. This methodology of IHQ using threshold for IHQ has been described by Jensen (2013) and multiple similar approaches for quantification of IHQ using Image J can be found in the literature (Varghese et al. 2014; Fu et al. 2015; Di Cataldo et al. 2010).

4.3.5 Statistical Analysis. One-way ANOVA was used to evaluate differences in IHC parameters between CSSC and normal skin. Alpha = 0.05

4.4 Results

For this study, 14 formalin-fixed paraffin-embedded (FFPE) samples of CSCC from the archive of the VMCVM Teaching Hospital and 10 NHS FFPE biopsies were selected. Total RNA was extracted from paraffin blocks, and cDNA was subjected to qPCR using cat-directed primers and probes. Protein localization in tissue was performed by immunohistochemical

analysis using antibodies directed against VEGF A, PLGF, FLT and KDR in 3 FFPE tissue sections of CSCC and compared against normal skin.

4.4.1 Gene expression analysis After normalization to expression of 18S rRNA internal marker, qRT-PCR analysis revealed a reduction ($p<0.05$) in relative expression of VEGF A 119, 163, and panVEGF A in CSCC compared to NHS, but significant differences in expression of VEGF A isoform 187 ($p=0.4$) were not detected (**Figure 4.4**). Relative expression of PLGF isoforms in CSCC and NHS, showed a down regulation of PLGF variants in CSCC, although no significant changes in the ratio PLGF VR II relative to VR I were noted (**Figure 4.5**). KDR and Flt-1 mRNAs were down regulated in CSCC compared to NHS. In contrast, sFLT 1 was upregulated in tumoral samples. When ratios between sFlt-1 and Flt-1 were compared, we detected abundant sFlt-1 relative to Flt-1 in CSCC (**Figure 4.6**) , potentially reflecting the presence of normal tissue at the tumor periphery. Moreover, we detected differences in ligand: receptor expression ratios (Figure 4.6), as the expression of VEGF A relative to its receptor KDR was higher in CSCC, consistent with our hypothesis.

4.4.2 Immunohistochemistry results. IHC results revealed that VEGF in normal pinna is localized to blood vessels, apocrine glands, and keratinocytes (**Figure 4.7**). Compared to CSCC samples, VEGF is apparently overexpressed in neoplastic cells undergoing hyperplastic and dysplastic keratinization. VEGF is also abundant in the vasculature around and inside the tumoral cells. It is important to notice that the localization of VEGF expression appears to change between samples and even within the same section, in concordance with reports from other authors (Margaritescu et al. 2009; Hanauer et al. 2002). PLGF immunoreactivity was detected in the endothelium of blood vessels in the normal pinna. In CSCC samples, there is an apparent increase in expression of PLGF in neoplastic keratinocytes of the stratum basale and in the abundant vasculature in the periphery of the tumor. KDR was clearly detected in blood vessel endothelium and in SCC tumoral cells. There is an apparent marked increase in expression of KDR in CSCC compared to the normal skin of the ear. FLT1 shows an immunolocalization pattern similar to PLGF (**Figure 4.8**). PLGF was localized in tumoral cells, especially in stratum basale of the dysplastic epidermis and in the periphery of the invaginations of tumoral keratinocytes. PLGF was also found in apocrine glands and epithelium of blood vessels.

To quantify the IHC signals in immunorective sites, Image J software was used to estimate the relative adundance of positive areas in histological images (**Figure 4.9**). Positive area per image is expressed relative to the total area of the frame, or percent of positive area. All proteins measured showed an increase in percent positive area in CSCC compared with normal skin of the pinna, although the highly-variable VEGF A immunoreactivity changes did not reach statistical significance ($p=0.054$).

4.5 Discussion and Conclusions

From these results, we may surmise that the abundance of transcripts encoding smaller isoforms of VEGF A and PLGF, as well as those of their receptors KDR and FLT1, were reduced in CSCC compared to normal skin; sFLT and VEGF A relative to KDR were increased in CSCC. The reduction of VEGF A and PLGF variants was unexpected, although an absence of difference in VEGF A mRNA expression in human oral SCC compared to epithelial dysplasia or normal gingiva has been reported (Carlile et al. 2001; Salven et al. 1997). Other studies reported a reduction of VEGF A immunostaining in cases of undifferentiated SCC compared to low grade differentiated oral SCC (Margaritescu et al. 2010). Furthermore, in agreement with our results, a previos investigation found that VEGF A was downregulated in head and neck SCC compared to precancerous lesions and to normal skin (Tae et al. 2000). Margaritescu et al. (2009) hypothesize that VEGF A expression could participate in regulation of physiological functions in non-neoplastic tissue but could be interrupted during neoplastic progression. In contrast, our gene expression results are at odds with studies showing VEGF A mRNA and PLGF upregulation in oral SCC (Cheng et al. 2013; Cheng et al. 2010; Nayak et al. 2012; Ko et al. 2015; Kim, Park, and Kim 2015) and canine CSCC (Maiolino, De Vico, and Restucci 2000; Al-Dissi et al. 2007), although we report higher ratios of panVEGF A relative to KDR in CSCC samples.

We believe that the presence of normal tissue around the tumor could influence mRNA results and explain, for example, the relative increase in sFLT-1 in SCC. To reduce this effect, we propose to extract RNA after manual trimming of normal tissue from the mounted tissue slide. Another alternative could be the use of an RNA extraction procedure that allows selection of specific areas of the FFPE slide (only tumoral tissue) for RNA extraction. This methodology has been used to evaluate VEGF A expression in thyroid cancer (Kloos et al. 2009) and in metastatic SCC in the tongue (Okamoto et al. 2002).

Another factor that may have affected our gene expression results was the low quality of our RNA samples from FFPE CSCC tissue, as evidenced by the low RIN number, a parameter recognized to influence gene expression results (Fleige and Pfaffl 2006). RNA quality from FFPE samples of NHS (mean RIN=5.46), which were fixed for 2 days and stored in paraffin from less than a week, yield a quality of RNA similar to that of RNA extracted from placental tissue in RNA later (mean RIN=6.8), but substantially better RNA quality than FFPE samples of CSCC (mean RIN=2.1). Thus, we predict that most likely RNA degradation is not due to the RNA extraction technique, but due to sample storage, sample trim size, and fixation times. These variables have been analyzed before and found to contribute to RNA degradation in FFPE samples (von Ahlfen et al. 2007). Alternately, tumoral microenvironmental factors, such as the presence of inflammatory cytokines, proteases, nucleases, or other molecules may be present in higher concentrations in tumoral samples, thus predisposing these samples to faster RNA degradation before fixation or during storage and RNA extraction. This effect has been noted for RNA extracted from different bovine tissue types (Fleige and Pfaffl 2006). Despite multiple precautions to avoid RNA degradation, we believe that further testing is needed in order to stablish a reliable protocol for quality RNA extraction from tumoral FFPE tissue.

Our results from immunolocalization and quantification of proteins revealed that ligands and receptors of VEGF family were increased in CSCC compared to NHS. Increased PLGF been reported in oral SCC (Cheng et al. 2013; Cheng et al. 2010), and increased VEGF A and KDR in CSCC have also been describe in the dog (Maiolino, De Vico, and Restucci 2000; Al-Dissi et al. 2007). These results contrast with our gene expression results. Possible reasons for these discrepancies are addressed above.

In normal skin samples, VEGF A, Flt-1, and KDR were immunolocalized in blood vessels, apocrine glands, and keratinocites, especialy in the stratum basale of the epidermis, in agreement with other studies in human normal skin (Man et al. 2009; Man et al. 2008; Margaritescu et al. 2009). In CSCC samples, we found an apparent co-localization of VEGF A, KDR, and FLT1 in neoplastic keratinocytes of CSCC, suggesting an autocrine positive regulation to promote tumor growth and invasion, as has been postulated before by Margaritescu et al. (2009) for oral SCC. In addition, we believe that VEGF A and PLGF expression from neoplastic keratinocytes could be targeting endothelial cells to promote angiogenesis in the tumor. Based on these results, the VEGF family could play a crucial role in the tumoral

progression of CSCC. We did not find differences among VEGF A or PLGF variants, leading us to believe that they do not follow a tight regulation in contrast to the placental development in the cat (Chapter 3). We can conclude that the factors measured may play role in CSCC and that their associated paracrine or autocrine signaling cascades may favor tumor progression by increasing vascularity.

Limitations of this study include RNA fragmentation during tissue fixation and block storage and the presence of mixed neoplastic and marginal normal tissue in study samples. Our results encourage further studies in the possible role of VEGF in feline CSCC and can help to determine the implications of specific VEGF- PLGF isoforms in tumoral angiogenesis, proliferation, and metastasis. Of particular interest are the altered relationships between splice variants of Flt1 and perturbed mRNA ratios of panVEGF A: KDR in SCC.

4.6 References

Al-Dissi, A. N., D. M. Haines, B. Singh, and B. A. Kidney. 2007. 'Immunohistochemical expression of vascular endothelial growth factor and vascular endothelial growth factor receptor associated with tumor cell proliferation in canine cutaneous squamous cell carcinomas and trichoepitheliomas', *Vet Pathol*, 44: 823-30.

Alam, Murad, and Désirée Ratner. 2001. 'Cutaneous Squamous-Cell Carcinoma', *New England Journal of Medicine*, 344: 975-83.

Baba, Alecsandru Ioan, and Cornel Câtoi. 2007. 'Epithelial and melanocytic tumors of the skin'.

Behrem, Senija, Kamelija Žarković, Neven Eškinja, and Nives Jonjić. 2005. 'Endoglin is a better marker than CD31 in evaluation of angiogenesis in glioblastoma', *Croatian medical journal*, 46.

Carlile, J, K Harada, R Baillie, Michaelina Macluskey, DM Chisholm, GR Ogden, SL Schor, and AM Schor. 2001. 'Vascular endothelial growth factor (VEGF) expression in oral tissues: possible relevance to angiogenesis, tumour progression and field cancerisation', *Journal of oral pathology & medicine*, 30: 449-57.

Carmeliet, P. 2000. 'Mechanisms of angiogenesis and arteriogenesis', *Nat Med*, 6: 389-95.

Carvalho, T, Helena Vala, C Pinto, M Pinho, and MC Peleteiro. 2005. 'Immunohistochemical studies of epithelial cell proliferation and p53 mutation in bovine ocular squamous cell carcinoma', *Veterinary Pathology*, 42: 66-73.

Chen, Zhong, Conrad W. Smith, Donna Kiel, and Carter Van Waes. 1997. 'Metastastic variants derived following in vivo tumor progression of an in vitro transformed squamous cell carcinoma line acquire a differential growth advantage requiring tumor-host interaction', *Clinical & Experimental Metastasis*, 15: 527-37.

Cheng, S. J., S. L. Cheng, J. J. Lee, H. M. Chen, H. H. Chang, S. H. Kok, M. L. Chiang, and M. Y. Kuo. 2013. 'Increased placenta growth factor mRNA level is significantly associated with progression, recurrence and poor prognosis of oral squamous cell carcinoma', *J Formos Med Assoc*, 112: 253-8.

Cheng, Shih-Jung, Jang-Jaer Lee, Sang-Heng Kok, Chia-Hsuan Chou, Hao-Hueng Chang, Meng Ling Chiang, Hsin-Ming Chen, Mark Yen-Ping Kuo, and Chun-Pin Chiang. 2010. 'Expression of placenta growth factor: An independent factor for prediction of progression and prognosis of oral cancer', *Head & Neck*, 32: 1363-69.

Cullen, John M, and Matthew Breen. 2016. 'An overview of molecular cancer pathogenesis, prognosis, and diagnosis', *Tumors in Domestic Animals*: 1-26.

De Falco, Sandro. 2012. 'The discovery of placenta growth factor and its biological activity', *Exp Mol Med*, 44: 1-9.

De Souza, Jonas A, Iuri A Santana, Gilberto de Castro Jr, Gilberto de Lima Lopes Jr, and Ya-Chen Tina Shih. 2014. 'Economic analyses in squamous cell carcinoma of the head and neck: a review of the literature from a clinical perspective', *International Journal of Radiation Oncology* Biology* Physics*, 89: 989-96.

Demir, R., Y. Seval, and B. Huppertz. 2007. 'Vasculogenesis and angiogenesis in the early human placenta', *Acta Histochemica*, 109: 257-65.

Di Cataldo, S., E. Ficarra, A. Acquaviva, and E. Macii. 2010. 'Automated segmentation of tissue images for computerized IHC analysis', *Computer Methods and Programs in Biomedicine*, 100: 1-15.

Dong, G., T. L. Lee, N. T. Yeh, J. Geoghegan, C. Van Waes, and Z. Chen. 2004. 'Metastatic squamous cell carcinoma cells that overexpress c-Met exhibit enhanced angiogenesis factor expression, scattering and metastasis in response to hepatocyte growth factor', *Oncogene*, 23: 6199-208.

Dong, P. I. N., Kazunari Sakata, Yoshimi Miyajima, Kei-Ichi Chijiwa, Kazunori Mori, and Tadashi Nakashima. 2001. 'The Predictive Value of p53, Ki-67 and Angiogenetic Factors in Primary Hypopharyngeal Carcinoma', *The Kurume medical journal*, 48: 261-66.

Du, Jin-Rong, Ying Jiang, Yan-Mei Zhang, and Hong Fu. 2003. 'Vascular endothelial growth factor and microvascular density in esophageal and gastric carcinomas', *World journal of gastroenterology: WJG*, 9: 1604.

Ferrara, N. 2000. 'Vascular endothelial growth factor and the regulation of angiogenesis', *Recent Progress in Hormone Research, Vol 55*, 55: 15-36.

Fischer, Christian, Massimiliano Mazzone, Bart Jonckx, and Peter Carmeliet. 2008. 'FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy?', *Nature Reviews Cancer*, 8: 942.

Fleige, Simone, and Michael W. Pfaffl. 2006. 'RNA integrity and the effect on the real-time qRT-PCR performance', *Molecular Aspects of Medicine*, 27: 126-39.

Folkman, Judah. 1995. 'Angiogenesis in cancer, vascular, rheumatoid and other disease', *Nat Med*, 1: 27.

Frank Riedel, Karl GÖtte Joachim Schwalb Carsten SchÄfer Karl HÖrmann. 2000. 'Vascular Endothelial Growth Factor Expression Correlates with p53 Mutation and Angiogenesis in Squamous Cell Carcinoma of the Head and Neck', *Acta Oto-Laryngologica*, 120: 105-11.

Fu, R., X. Ma, Z. Bian, and J. Ma. 2015. 'Digital separation of diaminobenzidine-stained tissues via an automatic color-filtering for immunohistochemical quantification', *Biomed Opt Express*, 6: 544-58.

Gallagher, Richard P, Gerry B Hill, Chris D Bajdik, Andrew J Coldman, Shirley Fincham, David I McLean, and William J Threlfall. 1995. 'Sunlight exposure, pigmentation factors, and risk of nonmelanocytic skin cancer: II. Squamous cell carcinoma', *Archives of dermatology*, 131: 164-69.

Gallagher, Richard P, Becky Ma, David I McLean, C Paul Yang, Vincent Ho, J Alastair Carruthers, and Laurence M Warshawski. 1990. 'Trends in basal cell carcinoma, squamous cell carcinoma, and melanoma of the skin from 1973 through 1987', *Journal of the American Academy of Dermatology*, 23: 413-21.

Goldschmidt, Michael H, and Kyle H Goldschmidt. 2017. 'Epithelial and melanocytic tumors of the skin', *Tumors in Domestic Animals*: 88-141.

Gray, Darryl T, Vera J Suman, WP Daniel Su, Ricky P Clay, W Scott Harmsen, and Randall K Roenigk. 1997. 'Trends in the population-based incidence of squamous cell carcinoma of the skin first diagnosed between 1984 and 1992', *Archives of dermatology*, 133: 735-40.

Hanahan, Douglas, and Robert A. Weinberg. 2000. 'The Hallmarks of Cancer', *Cell*, 100: 57-70.

Hanauer, Stephen B., Brian G. Feagan, Gary R. Lichtenstein, Lloyd F. Mayer, S. Schreiber, Jean Frederic Colombel, Daniel Rachmilewitz, Douglas C. Wolf, Allan Olson, Weihang Bao, and Paul Rutgeerts. 2002. 'Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial', *The Lancet*, 359: 1541-49.

Herr, F., N. Baal, and M. Zygmunt. 2009. 'Studies of placental vasculogenesis: a way to understand pregnancy pathology?', *Z Geburtshilfe Neonatol*, 213: 96-100.

Hicklin, Daniel J, and Lee M Ellis. 2005. 'Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis', *Journal of clinical oncology*, 23: 1011-27.

Horak, E. R., N. Klenk, R. Leek, S. LeJeune, K. Smith, N. Stuart, A. L. Harris, M. Greenall, and K. Stepniewska. 1992. 'Angiogenesis, assessed by platelet/endothelial cell adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer', *The Lancet*, 340: 1120-24.

Houck, Keith A, Napoleone Ferrara, Jane Winer, George Cachianes, Bing Li, and David W Leung. 1991. 'The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA', *Molecular endocrinology*, 5: 1806-14.

Jensen, Ellen C. 2013. 'Quantitative analysis of histological staining and fluorescence using ImageJ', *The Anatomical Record*, 296: 378-81.

Johnson, Timothy M, Dan E Rowe, Bruce R Nelson, and Neil A Swanson. 1992. 'Squamous cell carcinoma of the skin (excluding lip and oral mucosa)', *Journal of the American Academy of Dermatology*, 26: 467-84.

Kim, A., M. Im, N. H. Yim, and J. Y. Ma. 2014. 'Reduction of metastatic and angiogenic potency of malignant cancer by Eupatorium fortunei via suppression of MMP-9 activity and VEGF production', *Sci Rep*, 4: 6994.

Kim, Kun, Mayur M Amonkar, Daniel Högberg, and Frida Kasteng. 2011. 'Economic burden of resected squamous cell carcinoma of the head and neck in an incident cohort of patients in the UK', *Head & neck oncology*, 3: 47.

Kim, Seok-Kon, Seung-Goo Park, and Kyung-Wook Kim. 2015. 'Expression of vascular endothelial growth factor in oral squamous cell carcinoma', *Journal of the Korean Association of Oral and Maxillofacial Surgeons*, 41: 11-18.

Klagsbrun, Michael. 1991. 'Regulators of angiogenesis: stimulators, inhibitors, and extracellular matrix', *J Cell Biochem*, 47: 199-200.

Kloos, R. T., M. D. Ringel, M. V. Knopp, N. C. Hall, M. King, R. Stevens, J. Liang, P. E. Wakely, V. V. Vasko, M. Saji, J. Rittenberry, L. Wei, D. Arbogast, M. Collamore, J. J. Wright, M. Grever, and M. H. Shah. 2009. 'Phase II Trial of Sorafenib in Metastatic Thyroid Cancer', *J Clin Oncol*, 27: 1675-84.

Ko, H. H., J. J. Lee, H. M. Chen, S. H. Kok, M. Yen-Ping Kuo, S. J. Cheng, and C. P. Chiang. 2015. 'Upregulation of vascular endothelial growth factor mRNA level is significantly related to progression and prognosis of oral squamous cell carcinomas', *J Formos Med Assoc*, 114: 605-11.

Kumar, Shant, Ashur Ghellal, Cheng Li, Ged Byrne, Najib Haboubi, Ji Min Wang, and Nigel Bundred. 1999. 'Breast Carcinoma', *Vascular Density Determined Using CD105 Antibody Correlates with Tumor Prognosis*, 59: 856-61.

Lacal, P. M., C. M. Failla, E. Pagani, T. Odorisio, C. Schietroma, S. Falcinelli, G. Zambruno, and S. D'Atri. 2000. 'Human melanoma cells secrete and respond to placenta growth factor and vascular endothelial growth factor', *J Invest Dermatol*, 115: 1000-7.

Lee, Sunyoung, Shahla M. Jilani, Ganka V. Nikolova, Darren Carpizo, and M. Luisa Iruela-Arispe. 2005. 'Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors', *The Journal of Cell Biology*, 169: 681-91.

Liotta, Lance A., and Elise C. Kohn. 2001. 'The microenvironment of the tumour–host interface', *Nature*, 411: 375.

Maiolino, P., G. De Vico, and B. Restucci. 2000. 'Expression of Vascular Endothelial Growth Factor in Basal Cell Tumours and in Squamous Cell Carcinomas of Canine Skin', *Journal of Comparative Pathology*, 123: 141-45.

Man, X. Y., X. H. Yang, S. Q. Cai, Z. Y. Bu, X. J. Wu, Z. F. Lu, and M. Zheng. 2009. 'Expression and localization of vascular endothelial growth factor and vascular endothelial growth factor receptor-2 in human epidermal appendages: a comparison study by immunofluorescence', *Clin Exp Dermatol*, 34: 396-401.

Man, X. Y., X. H. Yang, S. Q. Cai, Z. Y. Bu, and M. Zheng. 2008. 'Overexpression of vascular endothelial growth factor (VEGF) receptors on keratinocytes in psoriasis: regulated by calcium independent of VEGF', *J Cell Mol Med*, 12: 649-60.

Marcellini, Marcella, Naomi De Luca, Teresa Riccioni, Alessandro Ciucci, Angela Orecchia, Pedro Miguel Lacal, Federica Ruffini, Maurizio Pesce, Francesca Cianfarani, and Giovanna Zambruno. 2006. 'Increased melanoma growth and metastasis spreading in mice overexpressing placenta growth factor', *The American journal of pathology*, 169: 643-54.

Margaritescu, C., D. Pirici, C. Simionescu, L. Mogoanta, M. Raica, A. Stinga, R. Ciurea, A. Stepan, A. Stinga, and D. Ribatti. 2009. 'VEGF and VEGFRs expression in oral squamous cell carcinoma', *Rom J Morphol Embryol*, 50: 527-48.

Margaritescu, C., D. Pirici, A. Stinga, C. Simionescu, M. Raica, L. Mogoanta, A. Stepan, and D. Ribatti. 2010. 'VEGF expression and angiogenesis in oral squamous cell carcinoma: an immunohistochemical and morphometric study', *Clin Exp Med*, 10: 209-14.

Nakopoulou, Lydia, Kalliopi Stefanaki, Effie Panayotopoulou, Ioanna Giannopoulou, Pauline Athanassiadou, Hariklia Gakiopoulou-Givalou, and Androniki Louvrou. 2002. 'Expression of the vascular endothelial growth factor receptor-2/Flk-1 in breast carcinomas: Correlation with proliferation', *Human Pathology*, 33: 863-70.

Nayak, S., M. M. Goel, S. Chandra, V. Bhatia, D. Mehrotra, S. Kumar, A. Makker, S. K. Rath, and S. P. Agarwal. 2012. 'VEGF-A immunohistochemical and mRNA expression in tissues and its serum levels in potentially malignant oral lesions and oral squamous cell carcinomas', *Oral Oncol*, 48: 233-9.

Okamoto, Masumi, Masayoshi Nishimine, Munehiro Kishi, Tadaaki Kirita, Masahito Sugimura, Mitsutoshi Nakamura, and Noboru Konishi. 2002. 'Prediction of delayed neck metastasis in patients with stage I/II squamous cell carcinoma of the tongue', *Journal of oral pathology & medicine*, 31: 227-33.

Page, David L., and Roy A. Jensen. 1995. 'Angiogenesis in human breast carcinoma: What is the question?', *Human Pathology*, 26: 1173-74.

Park, John E, Gilbert-A Keller, and Napoleone Ferrara. 1993. 'The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial

extracellular matrix and bioactivity of extracellular matrix-bound VEGF', *Molecular biology of the cell*, 4: 1317-26.
Parr, Christian, Gareth Watkins, Mike Boulton, Jun Cai, and Wen G. Jiang. 2005. 'Placenta growth factor is over-expressed and has prognostic value in human breast cancer', *European Journal of Cancer*, 41: 2819-27.
Risau, W., and I. Flamme. 1995. 'Vasculogenesis', *Annu Rev Cell Dev Biol*, 11: 73-91.
Salven, Petri, Päivi Heikkilä, Anu Anttonen, Mikael Kajanti, and Heikki Joensuu. 1997. 'Vascular endothelial growth factor in squamous cell head and neck carcinoma: expression and prognostic significance', *Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc*, 10: 1128-33.
Schimming, Ronald, and Dieter Marmé. 2002. 'Endoglin (CD105) expression in squamous cell carcinoma of the oral cavity', *Head & Neck*, 24: 151-56.
Semenza, G. L. 2007. 'Vasculogenesis, angiogenesis, and arteriogenesis: Mechanisms of blood vessel formation and remodeling', *J Cell Biochem*, 102: 840-47.
Silvan, U., A. Diez-Torre, Z. Bonilla, P. Moreno, M. Diaz-Nunez, and J. Arechaga. 2015. 'Vasculogenesis and angiogenesis in nonseminomatous testicular germ cell tumors', *Urologic Oncology-Seminars and Original Investigations*, 33.
Smith, Conrad W, Zhong Chen, Gang Dong, Elena Loukinova, Michelle Y Pegram, Linda Nicholas-Figueroa, and Carter Van Waes. 1998. 'The host environment promotes the development of primary and metastatic squamous cell carcinomas that constitutively express proinflammatory cytokines IL-1a, IL-6, GM-CSF, and KC', *Clinical & Experimental Metastasis*, 16: 655-64.
Srivastava, Anurag, Peter Laidler, RP Davies, K Horgan, and LE Hughes. 1988. 'The prognostic significance of tumor vascularity in intermediate-thickness (0.76-4.0 mm thick) skin melanoma. A quantitative histologic study', *The American journal of pathology*, 133: 419.
Tae, K., A. K. El-Naggar, E. Yoo, L. Feng, J. J. Lee, W. K. Hong, W. N. Hittelman, and D. M. Shin. 2000. 'Expression of vascular endothelial growth factor and microvessel density in head and neck tumorigenesis', *Clin Cancer Res*, 6: 2821-8.
Tanigawa, N, H Amaya, M Matsumura, and T Shimomatsuya. 1997. 'Association of tumour vasculature with tumour progression and overall survival of patients with non-early gastric carcinomas', *British Journal of Cancer*, 75: 566.
Teifke, J. P., and C. V. Löhr. 1996. 'Immunohistochemical detection of P53 overexpression in paraffin wax-embedded squamous cell carcinomas of cattle, horses, cats and dogs', *Journal of Comparative Pathology*, 114: 205-10.
Tischer, Edmund, R Mitchell, T Hartman, M Silva, D Gospodarowicz, JC Fiddes, and JA Abraham. 1991. 'The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing', *Journal of Biological Chemistry*, 266: 11947-54.
Tse, Gary M. K., Philip C. W. Lui, C. Soon Lee, Fred Y. L. Kung, Richard A. Scolyer, Bonita K. B. Law, Tai-Shing Lau, Rooshdiya Karim, and Thomas C. Putti. 2004. 'Stromal expression of vascular endothelial growth factor correlates with tumor grade and microvessel density in mammary phyllodes tumors: A multicenter study of 185 cases', *Human Pathology*, 35: 1053-57.
Tzoutzos, K., A. Batistatou, G. Kitsos, R. Liasko, and D. Stefanou. 2014. 'Study of microvascular density and expression of vascular endothelial growth factor and its

receptors in cancerous and precancerous lesions of the eyelids', *Anticancer Res*, 34: 4977-83.
Varghese, F., A. B. Bukhari, R. Malhotra, and A. De. 2014. 'IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples', *PLoS One*, 9: e96801.
Vempati, P., A. S. Popel, and F. Mac Gabhann. 2014. 'Extracellular regulation of VEGF: isoforms, proteolysis, and vascular patterning', *Cytokine Growth Factor Rev*, 25: 1-19.
von Ahlfen, Silke, Andreas Missel, Klaus Bendrat, and Martin Schlumpberger. 2007. 'Determinants of RNA Quality from FFPE Samples', *PLoS One*, 2: e1261.
Wei, S. C., P. N. Tsao, S. C. Yu, C. T. Shun, J. J. Tsai-Wu, C. H. H. Wu, Y. N. Su, F. J. Hsieh, and J. M. Wong. 2005. 'Placenta growth factor expression is correlated with survival of patients with colorectal cancer', *Gut*, 54: 666.
Wypij, Jackie M. 2013. 'A Naturally Occurring Feline Model of Head and Neck Squamous Cell Carcinoma', *Pathology Research International*, 2013: 7.
Yao, Yong-Gang, Hoseong S Yang, Zhiming Cao, Jennifer Danielsson, and Elia J Duh. 2005. 'Upregulation of placental growth factor by vascular endothelial growth factor via a post-transcriptional mechanism', *FEBS letters*, 579: 1227-34.
Yao, Yongzhong, Yiming Pan, Jun Chen, Xitai Sun, Yudong Qiu, and Yitao Ding. 2007. 'Endoglin (CD105) Expression in Angiogenesis of Primary Hepatocellular Carcinomas: Analysis using Tissue Microarrays and Comparisons with CD34 and VEGF', *Annals of Clinical & Laboratory Science*, 37: 39-48.
Yoshikawa, H., E. J. Ehrhart, J. B. Charles, D. H. Thamm, and S. M. Larue. 2012. 'Immunohistochemical characterization of feline oral squamous cell carcinoma', *Am J Vet Res*, 73: 1801-6.
Yuspa, Stuart H, Andrzej A Dlugosz, Christina K Cheng, Mitchell F Denning, Tamar Tennenbaum, Adam B Glick, and Wendy C Weinberg. 1994. 'Role of oncogenes and tumor suppressor genes in multistage carcinogenesis', *Journal of Investigative Dermatology*, 103: 90S.
Zhang, Lijian, Jinfeng Chen, Yang Ke, Robert E. Mansel, and Wen G. Jiang. 2005. 'Expression of Placenta growth factor (PlGF) in non-Small cell Lung cancer (NSCLC) and the clinical and prognostic significance', *World Journal of Surgical Oncology*, 3: 68.
Ziegler, Annemarie, Alan S Jonason, David J Leffellt, Jeffrey A Simon, Harsh W Sharma, Jonathan Kimmelman, Lee Remington, Tyler Jacks, and Douglas E Brash. 1994. 'Sunburn and p53 in the onset of skin cancer', *Nature*, 372: 773.