Factores de crecimiento similares a insulina en la placenta de la gata

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Abstract: Placental development involves maternal-fetal signalling events, and it is an under-researched topic in feline reproduction. Few studies of endotheliochorial placentas deal with insulin-like growth factors (IGFs), which are among the principal developmental regulators. While placental expression of IGFs and type 1 IGF receptor (IGF1R) has already been reported in bitches, data regarding the IGF system in queens are limited to uterine tissues. This study aimed to detect IGF1, IGF2, and IGF1R in fetal and maternal placental structures. Samples from twenty-three placentas were classified into one of two groups depending on gestational age (G1: ≤43 d.p.c; G2: ≥44 d.p.c) and processed by indirect immunohistochemistry. Labelling with all the antibodies was stronger in the endometrial glands of earlier placentas than in later ones. Maternal endothelium was moderate to strongly labelled, with a decreasing intensity in the endometrium, while the opposite occurred in the labyrinth. Cytotrophoblast cells were more strongly labelled than the syncytiotrophoblast. IGF1 and IGF1R-positive cells were more abundant in decidual cells of later placentas. These results support that the IGF system plays a central role during gestation and development in cats. As far as we know, this is the first report recording immunohistochemical IGFs/IGF1R detection in fetal regions of the feline placenta.

Keywords: cat, placenta, insulin-like growth factors, trophoblastic cells, immunohistochemistry.

Resumen: La señalización materno-fetal durante el desarrollo placentario es un tema poco investigado en reproducción felina. Los factores de crecimiento similares a insulina (IGF) son importantes reguladores del desarrollo, escasamente estudiados en placentas endotelicoriales. La expresión placentaria de IGFs y del receptor IGF tipo 1 (IGFR1) ya se ha descrito en perras; en gatas solo se han descrito en el útero. El

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objetivo de este estudio fue detectar IGF1, IGF2 e IGFR1 en estructuras placentarias fetales y maternas. Veintitrés muestras de placentas fueron clasificadas en dos grupos, según la edad gestacional (G1: ≤43 d.p.c; G2: ≥44 d.p.c); las mismas se procesaron mediante inmunohistoquímica indirecta. La marcación con todos los anticuerpos fue más intensa en las glándulas endometriales de placentas tempranas que en tardías. El endotelio materno se marcó de manera moderada a fuerte, con intensidad decreciente hacia los vasos del endometrio. Las células del citotrofoblasto se marcaron más que el sincitiotrofoblasto. IGF1 e IGF1R fueron más abundantes en las células deciduales de las placentas tardías. Estos resultados permiten sostener la centralidad del sistema IGF durante el desarrollo placentario felino. Según nuestro conocimiento, este es el primer trabajo que informa y describe la detección inmunohistoquímica de IGFs/IGFR1 en regiones fetales de la placenta felina.

Palabras clave: gata, placenta, factores de crecimiento similares a la insulina, células trofoblásticas, inmunohistoquímica.

1. Introduction

Placental development is a complex process involving signalling events between fetal and maternal cells, yet mechanisms underlying these phenomena during feline placentogenesis have not been studied in depth. Like most carnivores, cats develop a chorioallantoic, belt-shaped placenta with an endotheliochorial interface (Wooding & Burton, 2008). The main component of the placental girdle is the labyrinth, which comprises a body and a front that is included in the maternal-fetal junctional zone. The paraplacenta is formed by the hematophagous organs (less evident than in dogs), which may border the girdle by the free polar zone and the interfetal polar zone. The free polar zone is composed of columnar trophoblastic cells facing the endometrial epithelium. In the body of the labyrinth, maternal components (blood vessels and decidual cells) and fetal ones (blood vessels in the mesenchymal villous core and trophoblastic cells) are arranged to form maternal and fetal parallel lamellae (Leiser & Koob, 1993).

A significant part of the maternal-fetal dialogue is established between decidual cells (DCs) and trophoblastic cells. DCs are the only non-vascular maternal cells that remain in the labyrinth after epithelial degeneration. They are located between maternal vessels and the syncytiotrophoblast (Leiser & Koob, 1993). They have several functions and have been thoroughly studied in experimental animals and humans. The invasion of maternal tissues by trophoblastic cells is modulated by DCs through the balanced secretion of pro- and anti-invasive molecules, leading to a timely and controlled invasion (Sharma *et al.,* 2016). The trophoblast of the cat girdle includes the cytotrophoblast (CTB) and the syncytiotrophoblast (STB). Cytotrophoblast is composed of discrete cells overlaying the mesenchyme. Syncytiotrophoblast is formed as a result of CTB cell fusion and differentiation (Huppertz, 2010; Leiser & Koob, 1993).

Insulin-like growth factors (IGFs) are among the main placental development regulators; they control the placental size, morphology, and functions, consequently influencing fetal growth. The IGF system includes IGF1, IGF2, and insulin, their tyrosine kinase receptors IGF1R, IGF2R, and the insulin receptor, six binding proteins (IGFBPs), and their proteases. Insulin-like growth factors are evolutionarily conserved peptides that induce cell proliferation, protein synthesis, survival, migration, and differentiation in many cell types, mainly by binding to IGF1R. Insulin-like growth factors availability, and thus biological activities, are modulated by IGFBPs, which have a higher affinity for IGFs than IGF1R. Insulin-like growth factors binding proteins may be anchored to the plasma membrane or extracellular matrix elements, accentuating IGFs concentration in the nearby environment of the cells. The downstream IGF1R signalling pathway includes the activation of several intracellular signalling molecules, such as phosphoinositide3-kinase/protein kinase B/mammalian target of rapamycin (LeRoith *et al.,* 2021).

Current knowledge about the expression and functions of the IGF system in placental organogenesis comes mainly from studies dealing with hemochorial placentas. Both *in vivo* and *in vitro* studies in those placentas have shown that IGFs exert endocrine, autocrine, and paracrine actions in regulating placental functions and fetal growth (Roberts *et al.,* 2008; Sferruzzi-Perri *et al.,* 2011). Recently, the association between disturbed trophoblastic IGF signalling and fetal growth idiopathic restriction has been demonstrated in human placentas. In humans, the dysregulation of IGF signalling might lead to an alteration of trophoblastic cell turnover, with an increment in the apoptosis rate (Harris *et al.,* 2019). Besides, the IGF

system participates in myometrial phenotypic and functional changes during gestation, including an initial proliferative phenotype and a subsequent synthetic phase resulting in myocyte hypertrophy (Shynlova *et al.,* 2009). In ewes, which develop a less invasive (synepitheliochorial) placenta, IGF2 mRNA transcripts were demonstrated in maternal cells and chorioallantoic mesenchyme, but not in TB cells (Igwebuike, 2010). In the epitheliochorial placenta of the mare, only TB cells from the endometrial cups (the most invasive TB population) were shown to produce IGF2 (Lennard *et al.,* 1995).

Insulin-like growth factor 1 is paramount to placental growth and development by regulating trophoblast differentiation, proliferation, and survival (Forbes & Westwood, 2008a). Insulin-like growth factor 2 activity has been related to the increasing volume of the placental labyrinth, based mainly on angiogenesis, trophoblastic cell proliferation, and STB formation (Herr *et al.,* 2009; Sferruzzi-Perri, 2018). It has been suggested that IGF2 plays a role in adapting placental transport capacity during development (Sferruzzi-Perri, 2018) and stimulates trophoblast invasion of maternal tissues (Forbes & Westwood, 2008a). The mitogenic, pro-survival, and metabolic actions of IGF2 are thought to be mediated mainly through IGF1R (Sferruzzi-Perri *et al.,* 2017).

A few studies deal with the expression of IGFs/IGFRs in endotheliochorial placentas. In bitches, Kautz described that IGF2 and IGF1R are found in the endometrial glands and the myometrium, even before embryo implantation occurs (Kautz *et al.,* 2014). As we reported previously (Hernández *et al.,* 2020), endometrium, myometrium, and cell populations of the canine placenta (including trophoblastic cells) express IGFs/IGF1R at least in some stages of gestation. Trophoblastic IGF2 transcription was reported in the elephant endotheliochorial placenta (Wooding *et al.,* 2005). Ağaoğlu *et al.* (2016) reported significant differences in IGF2 mRNA concentration between pre- and post-implantation samples of the queen's uterus. More recently, the same research group detected IGFs and IGFRs mRNA and the corresponding proteins from pre-implantation to mid-gestation in uterine tissues (Ağaoğlu *et al.,* 2021).

Numerous studies concerning the IGF system have been conducted in pregnant uterus and placentas, mainly in hemochorial ones; three of them concern the feline uterus (Ağaoğlu *et al.,* 2021; Boomsma *et al.,* 1994). However, to our knowledge, there are no available reports about the IGF system in specific cell populations of the feline placenta. This study aimed to detect IGF1, IGF2, and IGF1R in the cat placenta and to pinpoint the location of these proteins in particular fetal and maternal placental structures.

2. Methods

2.1. PLACENTAL SAMPLES

Samples from 23 mixed-breed queens were obtained by veterinarians after owner-required and authorised ovary hysterectomies. Although most females were going through their first heat, precise data on the queen's age was not available. Samples were formalin-fixed for 24 h and processed using routine histological techniques. Three-micrometer sections were either stained with haematoxylin and eosin or mounted on positively charged slides for further immunohistochemical procedures (Biotraza microscope slides, Cat. #HDA S001A, Huida Medical Instruments Co., Jiangsu, China).

The determination of gestational age was based on the general external characteristics of each embryo-fetus, including the crown-rump length and several developmental features (Evans & Sack, 1973; Knospe, 2002; Pieri *et al.,* 2015). Then, each sample was assigned to one of two groups: group 1 (G1., which included samples from ≤43 days post coitum (d.p.c) placentas, or group 2 (G2), in which ≥44 d.p.c to term placental samples were included. The 44 gestational day corresponds to the beginning of stage 20 of prenatal development in the cat (Knospe, 2002). Placentas were classified in that way to analyse the general tendency in IGF1, IGF2, and IGF1R expression. Fourteen samples were assigned to G1; the remaining 9, belonging to 44 to 55 d.p.c, were included in G2.

2.2. Antibodies

The following antibodies were used: anti-IGF1 (1/600, polyclonal, Biorbyt, orb101730, Saint Luis, USA), anti-IGF2 (1/500, polyclonal, Biorbyt, orb10887, Saint Luis, USA) and anti-IGF1R (1/200, Santa Cruz Biotechnology, sc-712, Dallas, USA). Besides, considering that *IGFs/IGF1R* and their products have high phylogenetic conservation (Cherif-Feildel *et al.,* 2019; Rentería *et al.,* 2008), we conducted a similarity search using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) to infer the homology between target protein sequences of each anti-human antibody (*Homo sapiens*) and the homologous protein of cats. The sequence similarity percentages were: 97.01% for IGF1, 89.19% for IGF2, and 98.10% for IGF1R. This analysis enables further identification of protein variants and regions of the proteins that are highly conserved in orthologous species (Roncador *et al.,* 2015). This search tool was used to identify the peptides that function as antigens and thus are recognised by the antibodies (Liu *et al.,* 2013). Corroboration of the similarity between molecules from other species and from humans using BLAST has been conducted and previously reported by Baravalle *et al.* (2015), Sferruzzi-Perri *et al.* (2017), and Stassi *et al.* (2019). IGF1, IGF2, and IGF1R are homologous and orthologous conserved genes in mammals; between *Homo sapiens* and *Felis catus,* large similarity percentages in nucleotide sequences were confirmed.

2.3. Immunohistochemistry

Sections for immunohistochemical analysis were dewaxed in xylene, rehydrated through a graded series of ethanol, and washed in PBS (2 × 5 min each). Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol for 30 min at room temperature and washed with PBS (2 × 5 min each). Slides were subsequently immersed in citrate buffer (0.01M, pH 6.0) for antigen retrieval, which was performed using microwave irradiation (800W). Samples were irradiated for 3 min at 100% power, then for 6 min at 40% power, and subsequently washed with PBS (2 × 5 min each). Non-specific bindings were blocked with 10% goat non-immune serum (Sigma-Aldrich, Missouri, USA) for 30 min at room temperature. Overnight incubation with primary antibodies was performed at 4°C. Adjacent sections were subjected to the same immunohistochemical method, replacing primary antibodies with rabbit non-immune serum to verify immunoreaction specificity. After washing with PBS (2 × 5 min), samples were subsequently incubated with the universal secondary antibody using the streptavidin-peroxidase method (CytoScan™HRP Detection System, Cell Marque™, Rocklin, USA) for 30 min at room temperature. Liquid 3,3-diaminobenzidine tetrahydrochloride (DAB) was used as the chromogen (Liquid DAB-Plus Substrate Kit; Cell Marque™, Rocklin, USA), for 3 min 10 sec (IGF1), 3 min (IGF2) or 1 min 30 sec (IGF1R). Some sections were incubated with DAB alone to exclude the possibility of non-suppressed endogenous peroxidase

activity. Slices were processed by omitting the primary antibodies for IGF1, IGF2, and IGF1R, as negative controls. The golden-brown DAB-H.O. reaction product was indicative of positively stained structures. Mayer's haematoxylin was used as a nuclear counterstain (Biopur, Rosario, Argentina). Slides were observed using an Olympus BX53 microscope (Olympus, Tokyo, Japan), and representative images were taken with a digital camera (DP73, Olympus) in TIFF format. IGFs and IGF1R localization and distribution in the different placental zones were analysed.

2.4. ANALYSED ZONES AND STRUCTURES

In the region of the placental girdle, three zones were analysed: the maternal zone, the junctional zone, and the labyrinth (Figure 1). Besides, the results of the paraplacental free polar zone epithelia were described. The labelling intensity of connective tissue, endometrial glands, endothelium, vascular smooth muscle, and myometrium was assessed in the maternal zone. As components of the "junctional zone" (JZ) were considered: the junctional CTB (JZ-CTB), the junctional STB (JZ-STB), both forming the front of the labyrinth and the histotroph (Figure 1B3). As the "labyrinth," both the body of the lamellar labyrinth and the fetal zone were described. Thus, we recorded the labelling intensity of fetal endothelia (from chorioallantoic vessels), mesenchymal cells, CTB, STB, DCs, and maternal endothelia (Figures 1B1, B2). Regarding the paraplacenta, trophoblast cells, and the opposite uterine epithelium were evaluated. The qualitative assessment of staining intensity was performed blindly by two independent evaluators. Staining intensity was categorised as being (+), (++), or (+++) if it was mild, moderate, or strong, respectively. This scale was determined for each antibody separately. The absence of labelling was recorded as (-), and the inconsistent labelling as (±).
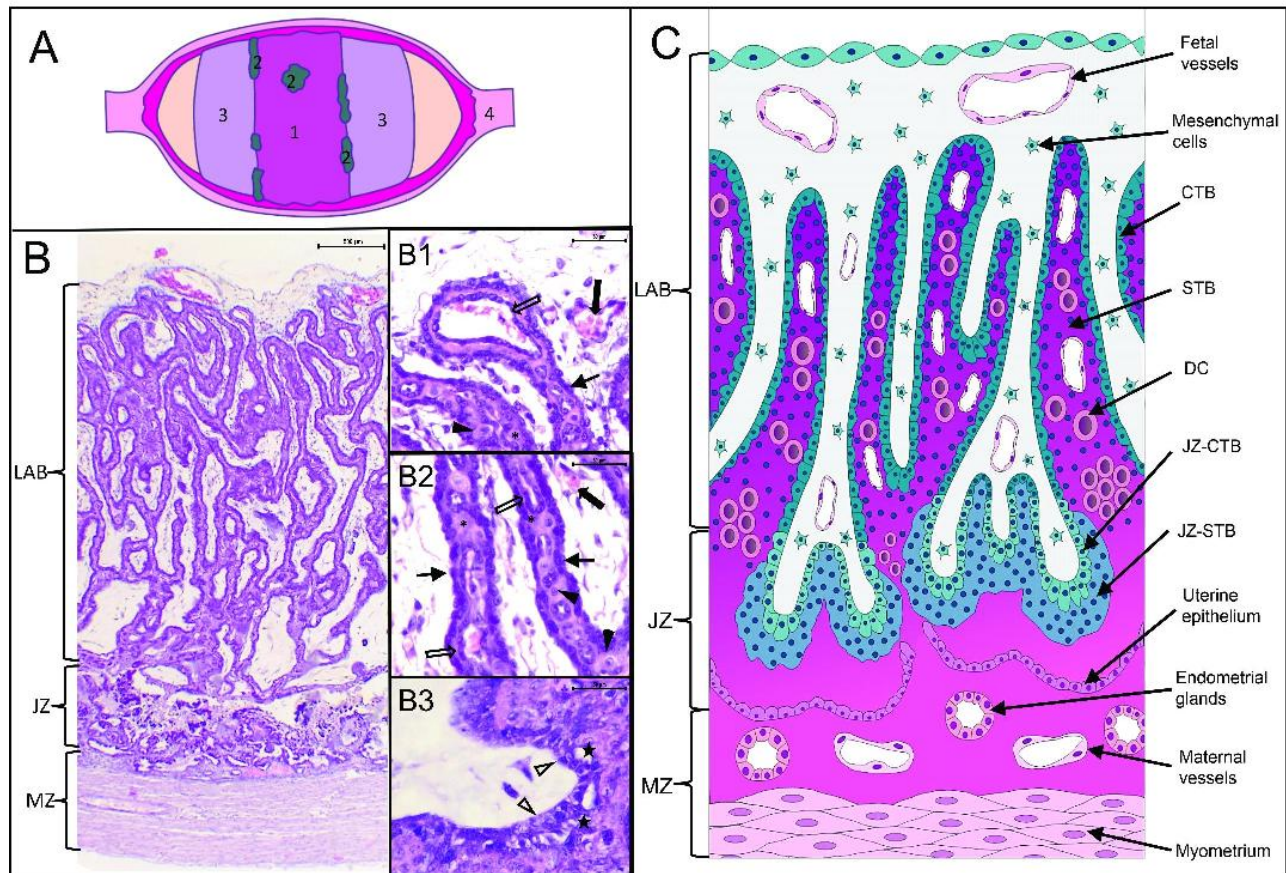
Figure 1

Feline placenta. (**A**) Schematic diagram of the placental gross appearance (cut surface of the uterus).1. Placental girdle; 2. Hematophagous organs; 3. Free polar zones; 4. Interplacental uterine site. (**B**) Structure of the feline placenta. Hematoxylin and eosin (HE). Laminar arrangement of maternal and fetal structures (Bar=500 μm). (B1, B2, B3) magnification of B. (**B1**) Fetal zone (Bar=50 μm). (**B2**) Labyrinth (Bar=50 μm). (**B3**) Junctional zone (Bar=50 μm). LAB: labyrinth; JZ: junctional zone; MZ: maternal zone; thin arrows: cytotrophoblast (CTB); asterisks: syncytiotrophoblast (STB); black arrowheads: decidual cells (DC); thick empty arrows: maternal endothelium; thick black arrows: fetal vessels; empty arrowheads: JZ-CTB; black stars: JZ-STB. (**C**) Schematic representation of the placental girdle showed in (**B**).

In addition, for an overall evaluation, a quantitative analysis of five random placental images (per case) was conducted. Images were taken from the histological sections using an Olympus BX53 microscope (Olympus, Tokyo, Japan) and an Olympus DP73 digital camera (Olympus, Tokyo, Japan). Data from each tissue were analysed and expressed as a percentage of the immunostained area (Diessler *et al.,* 2007), dismissing any eventual non-specific staining. Briefly, the percentage of the immunostained area was calculated using the following formula:

$$\% \ of \ immunostained \ area = \frac{Immunostained \ area}{Total \ area} * 100$$

where, Immunostained area corresponded to the calibrated pixels representing the tissue areas being positive for the immunohistochemical reaction, and Total area was the calibrated area of the pixels representing the entire resolution of the image. The Immunostained area was determined using the image analysis software Image Pro Plus (v3.6 – Media Cybernetics, USA).

2.5. Statistical analysis

The data are presented as mean ± SEM. Mean values were obtained for comparison between two groups using unpaired Student's t test or analysis of variance, followed by the Student–Newman–Keuls (SNK) test for multiple comparisons using Graph Pad Prism 8.0.2 software. For all analyses, the differences were considered significant when $P < 0.05$ (Santos *et al.,* 2021).

2.6. Registration and ethics committee

The Institutional Committee for the Care and Use of Laboratory Animals (CICUAL, Veterinary School, National University of La Plata) approved all the procedures (protocol number Nº 58-3-16T).

3. Results

3.1. Immunohistochemistry

The results for IGF1, IGF2, and IGF1R staining patterns are summarized in Table 1. Briefly, almost all tissue structures showed labelling of both growth factors (GFs) and IGF1R. No staining was observed in slides used as negative controls (Figures 2A, B, C).

Table 1
Insulin-like growth factors (IGF) 1, 2, and 1R staining patterns in cat placentas

			IGF1		IGF2		IGF1R	
			G1	G2	G1	G2	G1	G2
Placental girdle	Maternal zone	Connective tissue	++	+	++	+	+	-
		Glands	+++	++	+++	++	+++	++
		Endothelium	++	++	+++	+	++	+
		Vascular smooth muscle	+	+	+++	++	+++	++
		Myometrium	++	++	++	++	+++	+++
	Junctional zone	Histotroph	+++	++	+++	++	++	++
		JZ-CTB	+++	+++	+++	++	+++	++
		JZ-STB	±	±	±	-	-	-
	Labyrinth	Fetal endothelium	++	++	++	++	+++	+++
		Mesenchymal cells	++	+	++	++	+	+
		CTB	++	+	++	++	++	+
		STB	+	+	+	+	++	+
		Decidual cells	+	++	++	++	+	++
		Maternal endothelium	++	+++	++	+++	+++	+++
Free polar zone		Trophoblast	+++	++	+++	++	+++	++
		Uterine epithelium	+++	++	+++	+++	+++	++

Abbreviations CTB: cytotrophoblast; G1: group 1; G2: group 2; JZ: junctional zone; STB: syncytiotrophoblast. Labelling intensity: (-): no labelling; (±): inconsistent; (+): mild; (++): moderate; (+++): strong.
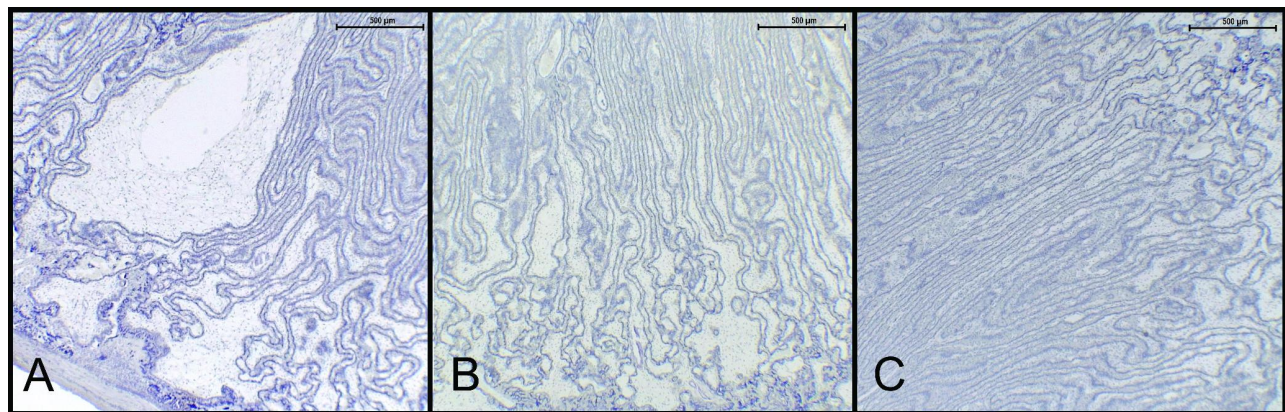

Figure 2
Negative controls for the immunohistochemical detection of (**A**) IGF1R, (**B**) IGF1, and (**C**) IGF2 in the feline placenta. IHC. DAB. (Bar=500 µm).

Most of the components in the maternal zone, but especially the endometrial glands, were positive for IGF1, IGF2, and IGF1R in samples from both groups. The labelling ranged from moderate to strong for the three proteins. Besides, it was distinctly stronger in G1 than in G2, except for the myometrium. That change was observed in the histotroph but only for IGFs (Figures 3A, B, C, D, E). The feline myometrium in different stages of pregnancy was positive for IGF1, 2, and IGF1R, and remained relatively constant (Figure 3F). Maternal endothelium was moderate to strongly labelled, with a general tendency to a decreasing intensity in the endometrial vessels and increased intensity in the labyrinthine ones, from G1 to G2 placentas (Figures 3A, B, C, D; Figure 4).
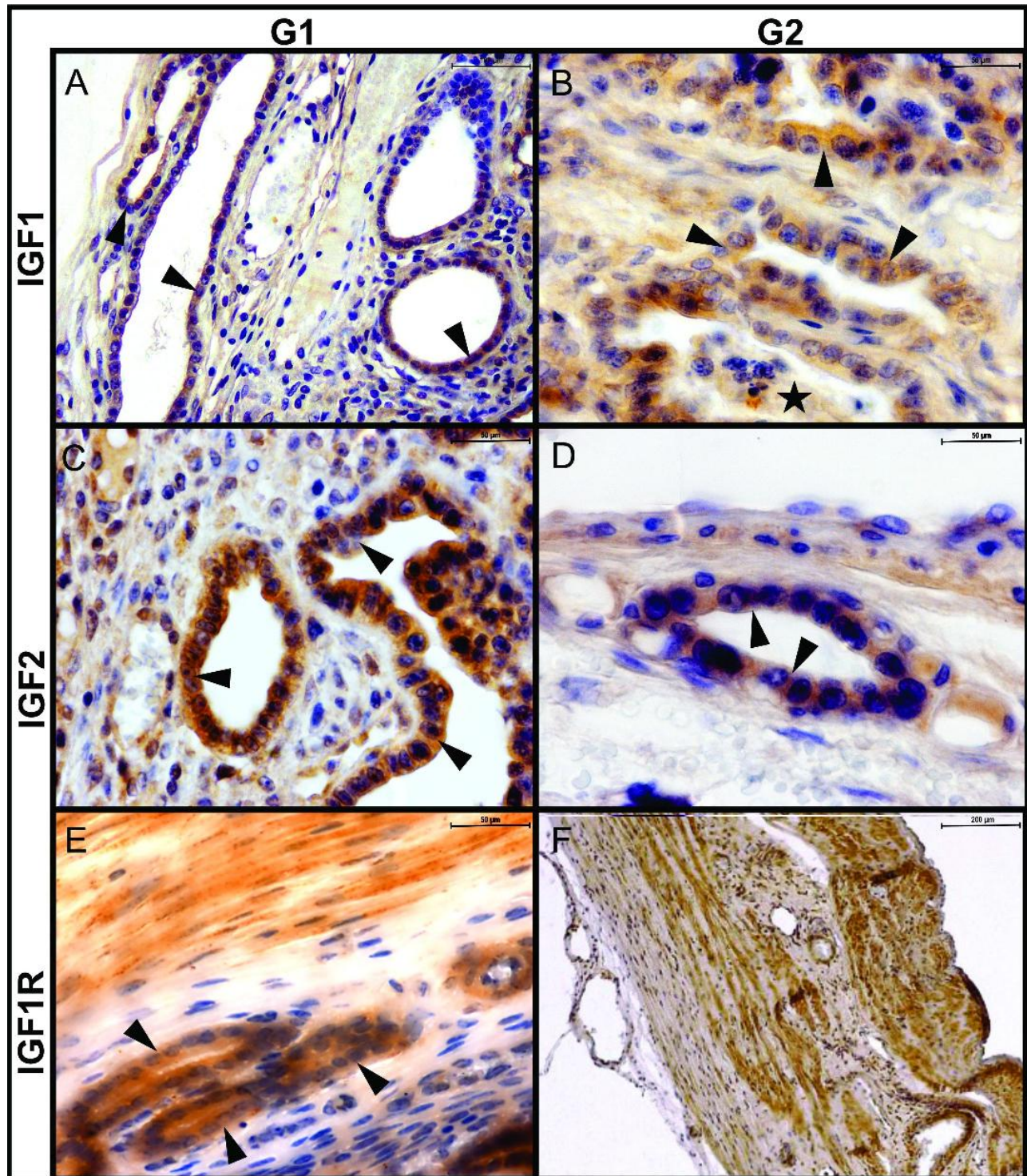
Figure 3

Feline placenta. Immunohistochemical detection of IGF1, IGF2, and IGF1R in the maternal zone, in samples from groups 1 (G1) and 2 (G2). Cytoplasmic labelling of the three molecules (**A**) IGF1, G1. Strong labelling in glandular cells (arrowheads (Bar=100μm). (**B**) IGF1, G2. Histotroph (stars) and glandular cells are moderately labeled (Bar=50μm). (**C**) IGF2, G1. Strong labelling in glandular cells (Bar=50 μm). (**D**) IGF2, G2. Moderate labelling in glandular cells (Bar=50 μm). (**E**) IGF1R, G1. Strong labelling in glandular cells (Bar=50 μm). (**F**) IGF1R, G2. Strong labelling in myometrial myocytes (Bar=200μm). Arrowheads: glandular cells; stars: histotroph. IHC. DAB.

As to the labyrinth, trophoblastic cells were positive for IGFs and IGF1R. The CTB showed stronger labelling of IGFs than the STB, both in the labyrinth and the junctional zone; JZ-STB labelling was inconsistent for the GFs and negative for the receptor (Figure 4; Figures 5A, B, C, D). Signals of IGF1 and IGF1R decreased in the labyrinthine CTB in samples of G2, while IGF2's remained unchanged (Figure 4). Maternal and fetal endothelia in the labyrinth were positive for IGFs and IGF1R (with the abovementioned increase in maternal ones). Concerning decidual cells, IGF1 and IGF1R were more abundant in decidual cells of G2 placentas, whereas IGF2 was equally labelled in DCs from both groups (Figure 4).
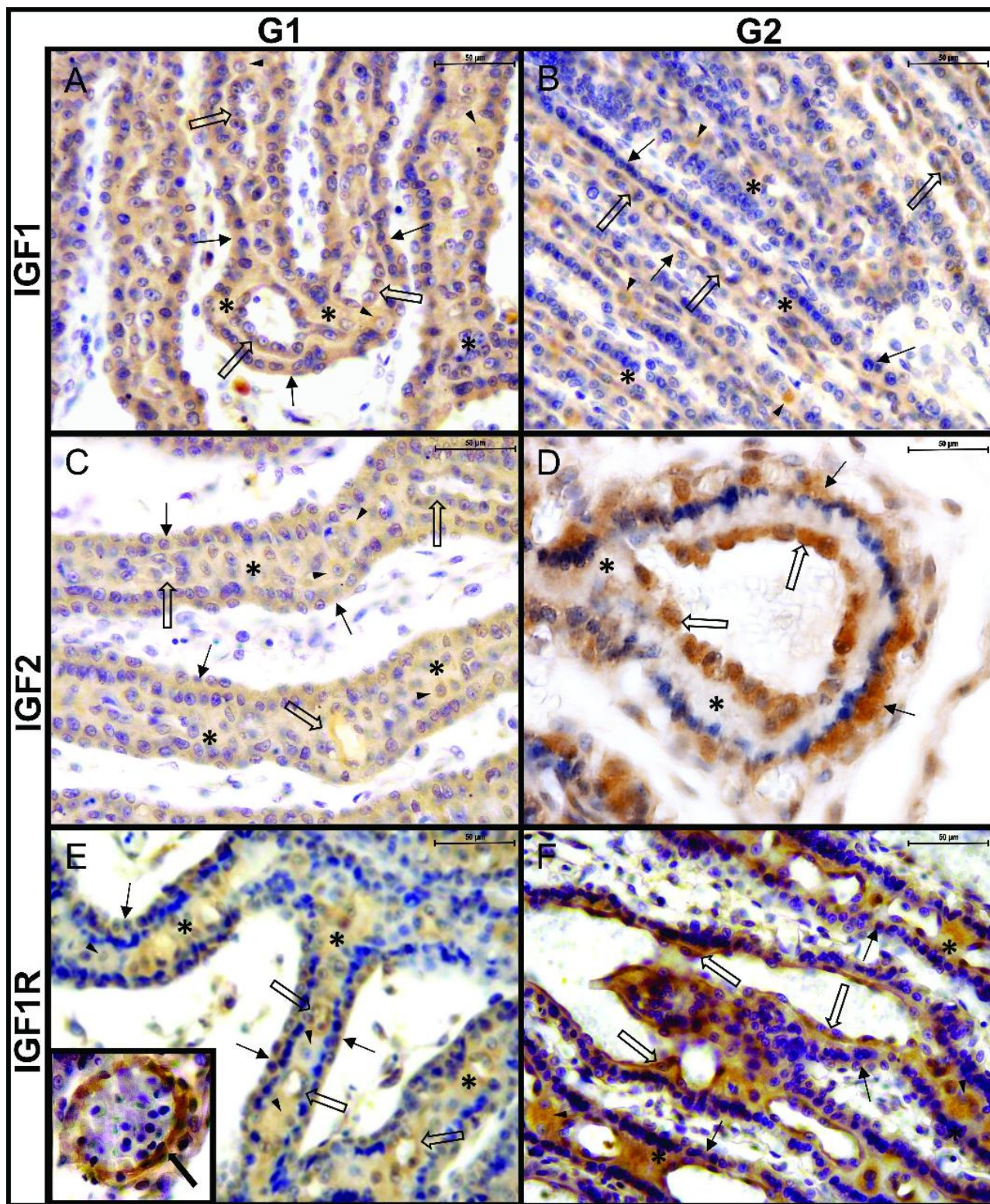
Figure 4

Feline placenta. Immunohistochemical detection ofIGF1, IGF2, and IGF1R in the labyrinth, in samples from groups 1 (G1) and 2 (G2). (**A**) IGF1, G1. Moderate labelling in CTB (thin arrows) and maternal endothelium (thick empty arrows). DC (arrowheads) and STB (asterisks) were mildly labelled. (**B**) IGF1, G2. Mild labelling in CTB and STB, moderate in DC, and strong in the maternal endothelium. (**C**) IGF2, G1. Moderate labelling in CTB, maternal endothelium, and DC. Mild labelling in STB. (**D**) IGF2, G2. Moderate labelling in CTB, mild labelling in STB. Maternal endothelium is strongly labelled. (**E**) IGF1R, G1. Moderate labelling in CTB and STB. Mild labelling in DCs and strong in the maternal endothelium. Inset: strong labelling in the endothelium of a fetal vessel. (**F**) IGF1R, G2. Mild labelling in CTB and STB. The DCs are moderately labelled. Strong labelling in maternal endothelium. Thin arrows: cytotrophoblast (CTB); black arrowheads: decidual cells (DC); asterisks: syncytiotrophoblast (STB); thick empty arrows: maternal endothelium; thick black arrow: fetal vessel. IHC. DAB. (Bar=50 μm).

Concerning the free polar zone, both epithelia were positive for IGF1, IGF2, and IGF1R. While IGF1 and IGF1R decreased in endometrial lining epithelium from samples in G1 to those in G2, IGF2 remained constant (Figures 5E, F).
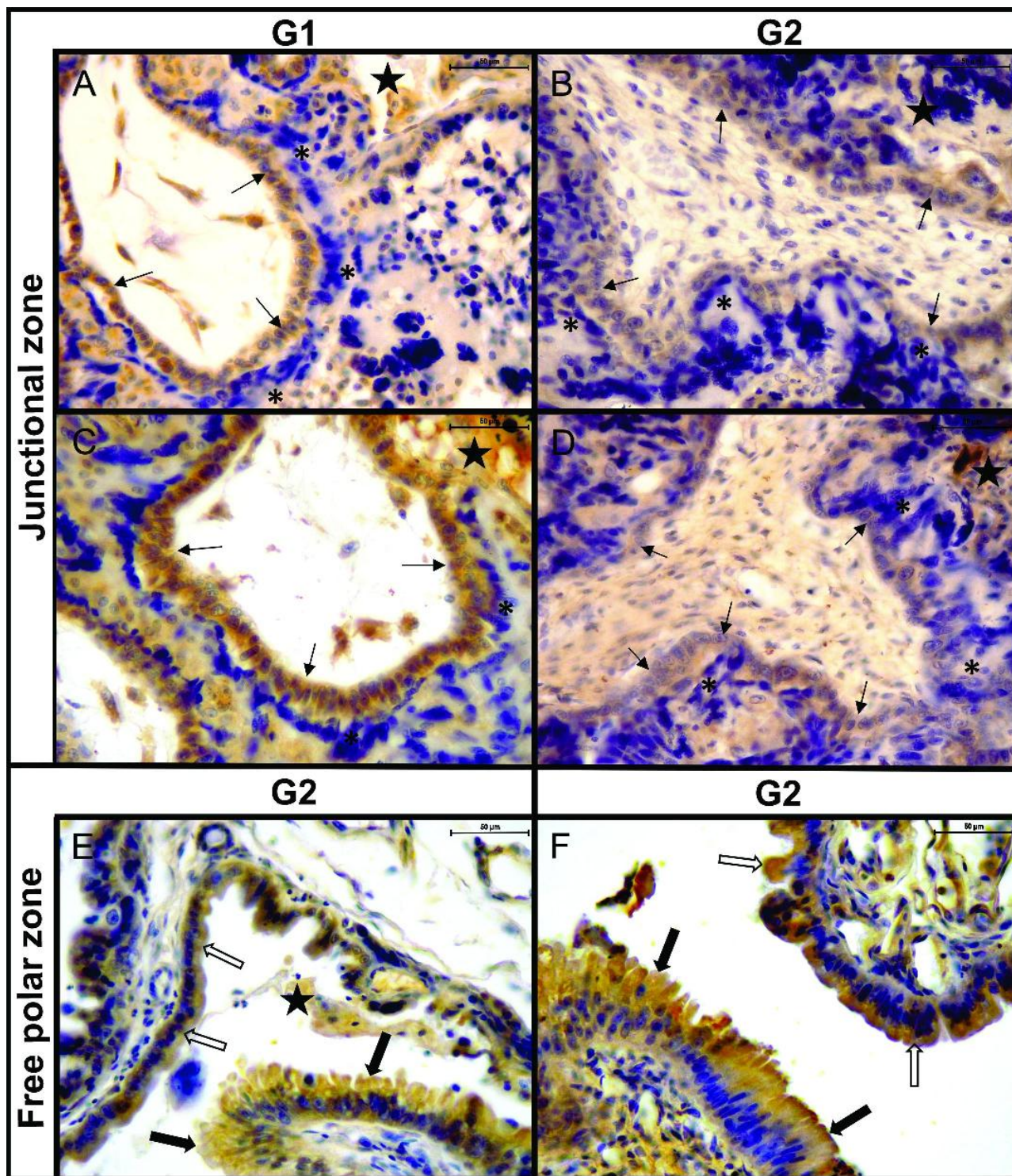
Figure 5

Feline placenta. Immunohistochemical detection of IGF1, IGF2, and IGF1R in the junctional zone (JZ) and the free polar zone. (**A**) JZ. IGF1, G1. Strong labelling in the JZ-CTB (thin arrows) and the histotroph (black stars). The labelling in the JZ-STB (asterisks)was inconsistent (negative in this image). (**B**) JZ. IGF2, G2. Moderate labelling in JZ-CTB and histotroph. The JZ-STB was negative. (**C**) JZ. IGF1R, G1. Strong labelling in JZ-CTB and histotroph. The JZ-STB was negative. (**D**) JZ. IGF1R, G2. Moderate labelling in JZ-CTB and histotroph. The JZ-STB was negative. (**E**) Free polar zone. IGF1, G2. Moderate labelling in the uterine epithelium (thick empty arrows), the trophoblast (thick black arrows), and the histotroph. (**F**) Free polar zone. IGF1R, G2. Moderate labelling in the uterine epithelium and the trophoblast. Thin arrows: cytotrophoblast of junctional zone (JZ-CTB); asterisks: syncytiotrophoblast of junctional zone (JZ-STB); black stars: histotroph; thick empty arrows: uterine epithelium; thick black arrows: trophoblast (TB). IHC. DAB. (Bar=50 μm).

3.2. Immunostained area

The percentage of immunostained area for IGF1 and IGF2 was higher in G1 than in G2 ($p < 0.001$ and $p <= 0.002$, respectively) (Figure 6A, B). In contrast, no differences in IGF1R immunostained area were observed between groups ($p=0.1558$) (Figure 6C).
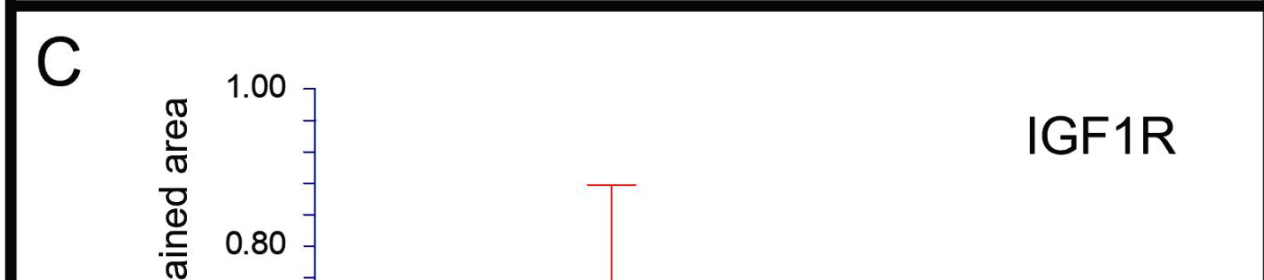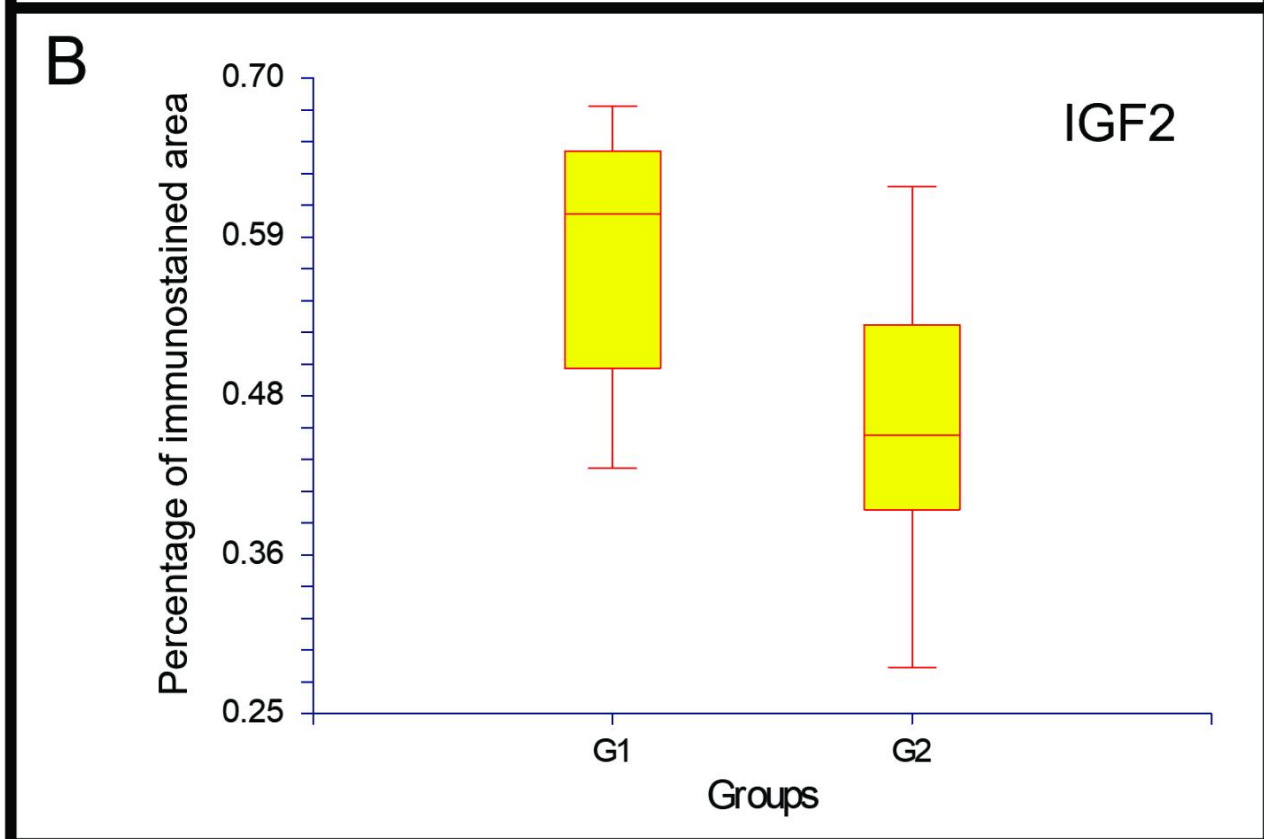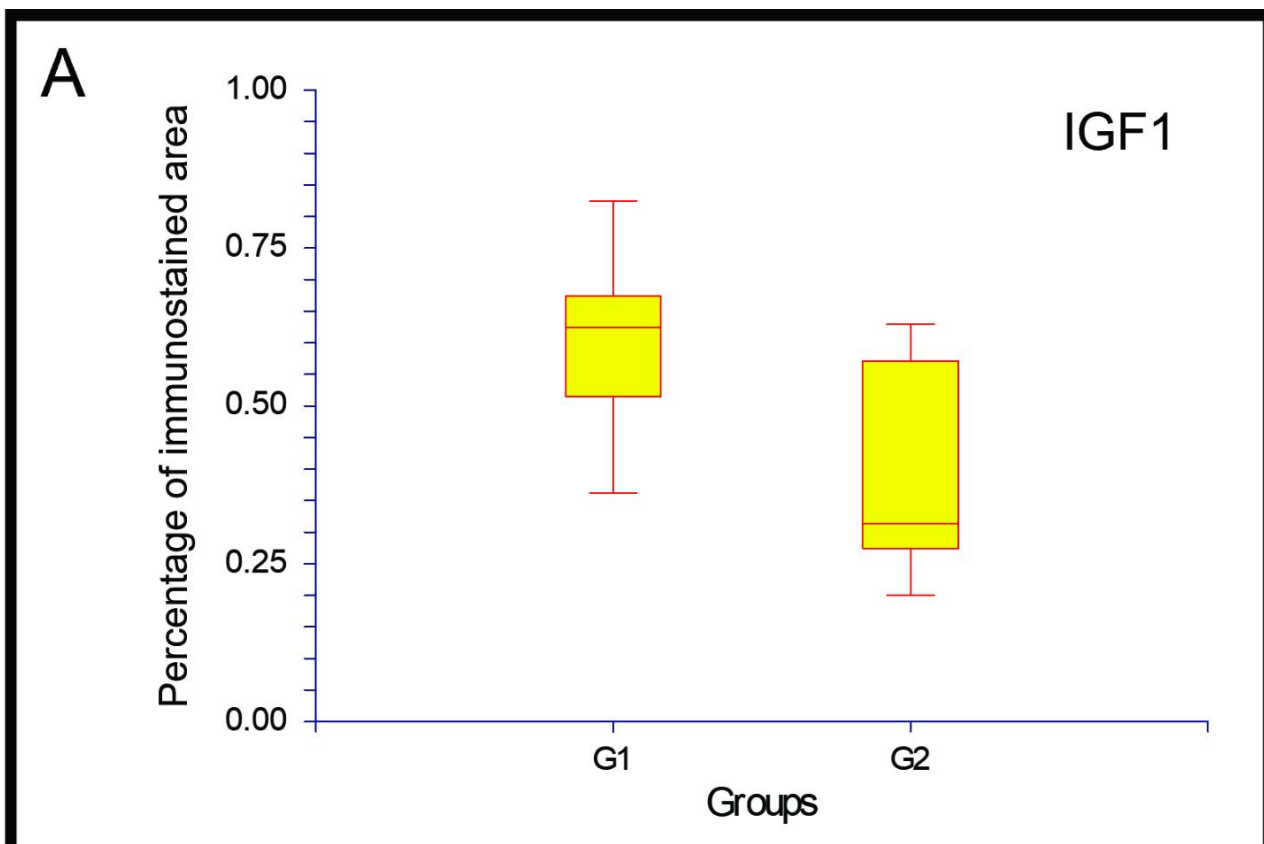
A

IGF1

B

IGF2

C

IGF1R

Figure 6

Percentage of the immunostained area in the placenta and gravid uterus of the cat. P values for the differences between groups 1 and 2 were: p= 0.00000001; n=10 for IGF1 (**A**), p= 0.0002; n=10 for IGF2 (**B**), and p=0.1558; n=10 for IGF1R (**C**).

4. Discussion and conclusions

We investigated the presence and particular localization of IGF1, IGF2, and IGF1R of different gestational age placentas and studied if there was a variation in their expression between placentas in G1 (≤43 d.p.c) and G2 (≥44 d.p.c). As it has been determined for other proteins in the cat placenta (Santos *et al.,* 2021), for most of the structures studied here the qualitative analysis showed either a stronger immunohistochemical marking in early placentas than in later ones, or equal labelling in both groups. This was supported by results regarding the total percentage of immunolabeled area, which were consistently and significatively higher in early placentas for both IGF ligands. Stronger labelling in G1 might reflect the involvement of these molecules in signalling pathways required for early placental development (Guzeloglu-Kayisli *et al.,* 2009; Sferruzzi-Perri *et al.,* 2017). Our finding agrees with Freese *et al.* (2005) in sow gilts; they reported that glandular *IGF1* expression was high during early gestation and decreased as the pregnancy progressed. Ağaoğlu *et al.* (2021) and ourselves (Hernández *et al.,* 2020) observed a similar tendency in ≤ 45 d.p.c feline and from 28 to 62 d.p.c canine uteri, respectively. The similar pattern in uteri from these two species with endotheliochorial placentas strengthens the hypothesis that endometrial IGFs stimulate early placental development through histotrophic signalling, as also occurs in hemochorial placentas, where histotrophic mediated communication is crucial in early pregnancy (Wooding & Burton, 2008).

The IGF system is involved in the proliferation, growth, and differentiation of smooth myometrial myocytes in humans (Tang *et al.,* 1994). Insulin-like growth factor 1 and 2 and IGF1R myometrial expression during pregnancy was also described in other species, such as pigs, rats, and dogs (Hernández *et al.,* 2020; Huynh, 2000; Shynlova *et al.,* 2007; Simmen *et al.,* 1992). Results regarding myometrial staining at different stages of gestation are partially consistent with findings in other species. In rats, it was shown that while *Igf2* and *Igf1r* remained virtually unchanged, *Igf1* expression was higher in the myometrium during the first half of the gestation, concurring with the phase of increased myocyte proliferation (Shynlova *et al.,* 2007). In dogs, IGF2 detection followed that tendency (Hernández *et al.,* 2020), while IGF1 did not. Here, IGF1 and IGF2 remained relatively constant; IGF1R did not vary either, which agrees with results from other studies (Hernández *et al.,* 2020; Shynlova *et al.,* 2007).

The fact that JZ-CTB labelling intensity and intergroup variation have followed those of glands and histotroph might be related to the phagocytic activity of this type of CTB, which was mainly denuded, or at least split from JZ-STB. That activity may also account for JZ-CTB stronger labelling compared to labyrinthine CTB cells. This relation with the absorption of the histotroph may also explain the staining differences between JZ-CTB and JZ-STB. JZ-STB showed a mild and inconsistent reaction to the three antibodies; even being adjacent to the histotroph and the endometrial glands, this trophoblast cell type almost lacks phagocytic activity (Leiser & Koob, 1993).

Insulin-like growth factors exert several functions in trophoblastic cells. As was described above, IGFs/ IGF1R expression has been studied mainly in the trophoblast of hemochorial placentas and, to a lesser extent, in epithelial and synepitheliochorial placentas. Among carnivores, the expression of these growth factors in invasive trophoblast was recently described in canine placentas (Hernández *et al.,* 2020).

The stronger labelling of IGFs in CTB compared to that in STB may obey the central role that these growth factors play in CTB differentiation toward STB; IGFs enhance cytotrophoblast proliferation and syncytial formation and can rescue trophoblastic cells from apoptosis, acting via IGF1R (Forbes *et al.,* 2008b). Syncytiotrophoblast formation depends on the continued proliferation and differentiation of CTB cells, as STB does not proliferate on its own (Forbes & Westwood, 2008a). In mice, signalling through IGF1R promotes STB formation and increases its activity. Expression of IGF1R in both CTB and STB in cats might be related, therefore, to placental development, growth and function, as it is also in women and guinea pigs (Martín-Estal *et al.,* 2021). Whether the origin of syncytial IGFs is entirely cytotrophoblastic or they are partly synthesised in the STB is unknown. Currently, the occurrence of transcriptional and translational activities in the STB is a matter of controversy. While some authors argue that both processes remain active in the STB (Fogarty *et al.,* 2011; Sferruzzi-Perri *et al.,* 2011), others maintain that transcription and translation are downregulated in the syncytium (Ellery *et al.,* 2009; Huppertz, 2010). In human placentas, transcription and translation of *IGF1* in different trophoblastic populations and of *IGF2* only in the extravillous trophoblast were recorded (Han & Carter, 2000; Hidden *et al.,* 2009). Dubova *et al.* (2014) detected IGF1 and IGF2 both in the extravillous trophoblast and STB. Han & Carter *et al.,*(2000) reported IGF2 mRNA in the rhesus monkey in the same localisation. IGF2 was detected in several trophoblastic cells, e.g., those from the highly invasive human extravillous trophoblast or the glycogen cells in mice, which are both related to decidual invasion (Pollheimer *et al.,* 2011; Roberts *et al.,* 2008). Insulin-like growth factor 1 receptors were widely expressed in placental tissues in the first trimester and at term (Martín-Estal *et al.,* 2021).

The distribution of IGFs in placentas of other domestic species has also been studied. In the mare, which develops an epitheliochorial placenta, IGF1 has been localised to the entire trophoblast in the microcotyledons (Arai *et al.,* 2006); however, only the cells from the endometrial cups (the more invasive cell population) produce IGF2 (Lennard *et al.,* 1995). In ewes, IGF2 transcription was restricted to maternal tissues and mesenchyme (Igwebuike, 2010). In invasive placentas, IGFs are initially secreted by the endometrium; then, as pregnancy progresses, both maternal and fetal placental cells produce IGFs (Bowman *et al.,* 2010). In rhesus monkeys, it has been found a higher expression of IGF1 in phagocytic trophoblast facing the histotroph and a higher expression of IGF2 in invasive trophoblastic cells at the first stage of the placentation (Dhara *et al.,* 2001). This contributes to the idea that different functions of IGF1 and IGF2 are evolutionarily conserved among species. Until now, we have not found any further information about the presence of either IGF proteins or transcripts in feline trophoblastic populations.

This different distribution of IGF2 among species and types of placentas might be linked to the function of IGF2 in cell migration and invasion achieved through IGF1R (Sferruzzi-Perri, 2018). Insulin-like growth factor 2 may stimulate invasion by regulating matrix metalloproteinase 2 and 9 availability, exerting a greater effect on these enzymes than IGF1 does (Hills *et al.,* 2004). Those proteinases have been detected in endotheliochorial placentas (Diessler *et al.,* 2017). In pigs around day 60 of gestation, IGF2 expression increases (Freese *et al.,* 2005), coinciding with a time of marked fetal growth rise (Leiser & Koob, 1993). In baboons, *IGF2* mRNA in the syncytiotrophoblast increases markedly as gestation advances (Zollers *et al.,* 2001). Here, a corresponding change in IGF2 protein has not been detected. This may obey either interspecific differences or *IGF2* mRNA′s lower translation rate.

The regulatory role of DCs on trophoblastic populations (Guzeloglu-Kayisli *et al.,* 2009), mainly through IGFBP1, may account for the shortage of IGFs in the feline STB contiguous to DCs. Conversely, in the dog, a species with scarce DCs, the IGF signal in the STB is stronger than in cat samples.

Positivity for the three antibodies observed in the decidual cells agrees with that in bitches (Hernández, unpublished results). *Insulin-like growth factor 1, 2, and 1 receptor* RNA transcripts have been detected in canine stromal decidualized cells in culture (Kautz *et al.,* 2015). Human DCs also express IGF1, IGF2, and IGF1R, as well as IGFBP1 and prolactin. The last two are regarded as decidual markers in this species and have also been detected in feline DCs (Hayati *et al.,* 2007; Hernández *et al.,* 2017, 2019; Hill *et al.,* 1993). Decidual IGFBP1 is involved in IGFs regulation (Guzeloglu-Kayisli *et al.,* 2009). Depending on IGFBP1 posttranslational modifications, mainly phosphorylation, it may have more or less affinity for IGFs. The unphosphorylated form shows more affinity for growth factors, leading to extravillous trophoblast migration; phosphorylated IGFBP1, on the contrary, has less affinity for growth factors (Forbes & Westwood, 2008a; Guzeloglu-Kayisli *et al.,* 2009).

Reciprocal regulation between trophoblastic cells and DCs results in a timed invasion and normal placentation in humans (Hess *et al.,* 2007). Based on our results and those in other species, it is reasonable to postulate that the DCs and the trophoblast at the cat maternal-fetal interface signal through IGFs and IGF1R, among other molecules, by auto and paracrine ways.

The involvement of the IGF system in angiogenesis is well-known. Endothelium responds to IGFs, released by other cells or synthesized locally, mainly by their binding to IGF1R. Signalling transduction downstream is key for vessel formation, as IGFs stimulate hypoxia-inducible factor-dependent and independent VEGF synthesis (Bach, 2015). *In vitro* studies showed that trophoblastic IGF1 stimulates proliferation and inhibits apoptosis in human placental endothelial cells (Troja *et al.,* 2014). Furthermore, IGF2, which is considered a pregnancy-specific angiogenic factor, stimulates endothelial cell migration (Herr *et al.,* 2003). IGF central participation in placental angiogenesis might account for the stronger labelling of maternal endothelia in the labyrinth than in the uterus itself.

In the free polar zone, the trophoblast is composed of vacuolated columnar cells, which phagocytose uterine secretions that keep it separate from the lining endometrial epithelium. This region resembles areolar zones in gilts and mares (Wooding & Burton, 2008). Positivity for IGFs and IGF1R found in trophoblastic cells may obey to phagocytosis of uterine milk rather than to their local expression.

Taken as a whole, our results indicate that IGFs and IGF1R are expressed in almost all the structures forming the placenta, both in early and late placentas, except for JZ-STB. It is a remarkable finding that IGF2 expression tended to be higher in early placentas, which could be due to its role in invasion. Regarding the maternal zone, IGF2 labelling was higher in the earliest samples than IGF1 and IGF1R.

These proteins have been studied in the queen pregnant uteri before, but only in maternal structures. As far as we know, this work constitutes the first one recording immunohistochemical IGFs/IGF1R detection in fetal regions of the placenta. Their high expression and wide distribution coincide with what has been found in another carnivore: the dog. These results may provide insight into the centrality of the IGF system during gestation and prenatal development in species with endotheliochorial placentas.

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Author contributions

Rocío Hernández: conceptualization, formal analysis, investigation, methodology, validation, visualization, roles/writing - original draft. **Gimena Gomez Castro:** investigation, formal analysis, validation, visualization. **Fernanda M. Rodriguez:** investigation, validation, visualization. **Luciano Casas:** investigation, visualization. **Enrique Portiansky:** formal analysis, writing - review & editing. **Claudio Barbeito:** conceptualization, funding acquisition, resources, project administration, supervision, writing - review & editing. **Florencia Rey:** funding acquisition, resources, supervision, writing - review & editing. **Mónica E. Diessler:** conceptualization, methodology, supervision, funding acquisition, writing - review & editing.

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