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Tryptophan degradation enzymes and Angiotensin (1−7) expression in human placenta

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A R T I C L E I N F O

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A B S T R A C T

Indoleamine 2,3-dioxygenase 1 (IDO1) and tryptophan 2,3-dioxygenase (TDO) are key enzymes for tryptophan degradation, regulating immune tolerance during pregnancy. The intrauterine renin-angiotensin system is also involved in the progression of a healthy pregnancy. Angiotensin(1−7) maintains the integrity of fetal membranes via counteracting the pro-inflammatory actions of Angiotensin II. No data are available on placental Angiotensin (1−7) co-expression with TDO. We aimed to characterize TDO mRNA expression and its localization in different areas of the placenta of physiological pregnancies delivered at term; its co-expression with Angiotensin(1−7) and its correlation with the plasma kynurenine/tryptophan (Kyn/Trp) ratio was investigated. This prospective observational study included a nonconsecutive series of 20 singleton uncomplicated pregnancies delivered vaginally. TDO mRNA was expressed in both maternal and fetal sides of the placentas and TDO protein also in the villi and it was co-expressed with IDO1 in almost half of the placental cells at these sites. The percentage of TDO^+ and $IDO1^+$ cells appeared to be influenced by maternal pre-gestational smoking and newborn weight. A strong correlation was found between the percentage of TDO^+ and $IDO1^+$ cells in the villi. TDO^+ cells also expressed Angiotensin(1−7), with a higher percentage on the fetal side and in the villi compared to the maternal one. Kyn/Trp plasma ratio was not correlated with IDO and TDO expression nor with the patient's characteristics. Collectively, our data indicate that TDO is detectable in placental tissue and is co-expressed with IDO and with $Angiotensin(1-7)^+$ on the fetal side and in the villi.

1. Introduction

The placenta is an organ that acts as a selective barrier, regulating exchanges between the maternal and fetal circulations. It allows the transport of nutrients and oxygen from the mother to the fetus and the removal of waste products from fetal blood, and at the same time, it protects the fetus against a wide variety of xenobiotics and infections. Proper placental development and function are therefore essential to maintain pregnancy and preserve fetal health and growth.

There are also many cell types and various pathways of immunity that ensure immunological tolerance (Silvano et al., 2021; Tong and Abrahams, 2020). Among these, the kynurenine pathway (KP) seems to be one of the most important (Munn et al., 1998). This pathway allows tryptophan (Trp) degradation into kynurenine (Kyn), and its metabolites inhibit T-lymphocytes and Natural Killers cells proliferation and activate regulatory T cells (Fallarino et al., 2002).

The two key enzymes of the KP are indoleamine 2,3-dioxygenase 1 (IDO1) and tryptophan 2,3-dioxygenase (TDO) (Badawy, 2018). Studies using immunohistochemical analyses and quantitative Real-Time PCR have suggested the presence of IDO1 in placental tissue from the second trimester of pregnancy onwards and in some studies also in the first trimester (Ban et al., 2013; Chang et al., 2018; Hönig et al., 2004; Sedlmayr and Blaschitz, 2012; Wang et al., 2011). Moreover, IDO1 enzymatic activity seems to increase as pregnancy advances (Karahoda

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et al., 2020; Ligam et al., 2005). However, although its expression and involvement in placental immune tolerance have been documented, the contribution of IDO1 to the normal placental function has been questioned based on the observation that IDO-deficient mice have uncomplicated pregnancies (Munn et al., 1998). Little information is available on TDO expression in the placenta, and studies on placentas from pathological pregnancies were unable to define its precise localization (Keaton et al., 2019; Murthi et al., 2017).

The ratio between maternal plasma levels of Kyn and Trp (Kyn/Trp) is a suggested indicator of the rate of Trp degradation and reflects TDO and IDO1 activities under physiological or pathological conditions (Kudo et al., 2003; Schröcksnadel et al., 2006; Obayashi et al., 2016). The plasmatic Kyn/Trp ratio was found to be significantly higher in women with uncomplicated pregnancy compared to women with pre-eclampsia, in whom the ratio was similar to that of non-pregnant women.

IDO1 and TDO are part of a complex signaling network, that also involves the intrauterine renin-angiotensin system (RAS) in term human decidua, placenta, myometrium, and fetal membranes (Pringle et al., 2011; Wang et al., 2015). This signaling network regulates placental development through processes such as angiogenesis and modulation of placental blood flow (Gao et al., 2017). The pro-inflammatory and pro-hypertensive actions of Angiotensin II (Ang II) are counterbalanced by the Ang(1−7), a protein with anti-inflammatory properties, and this helps to create a favorable niche for embryonic implantation and fetal development (Ghadhanfar et al., 2017; Tamanna et al., 2020). Reduced plasma levels of Ang(1−7) have been found in women with pre-eclampsia compared with normotensive pregnant women (Emanuele et al., 2002; Velloso et al., 2007). Ang(1−7) seems to also modulate immune response, at least in experimental animal models (Khajah et al., 2017). However, no data are available on Ang(1−7) co-expression with TDO in human placenta.

Based on these considerations, the aim of the present study was to better characterize TDO localization in the placenta of physiological pregnancies delivered at term, and to compare TDO, IDO1 and Ang (1−7) expression between the maternal and fetal sides of the placenta. Moreover, TDO co-expression with IDO1 and their correlation with the Kyn/Trp ratio in the maternal venous blood was also investigated.

2. Materials and methods

2.1. Study design

This was a prospective, observational study conducted between March 2020 and February 2021 at Careggi University Hospital in Florence, Italy. A nonconsecutive series of 20 singleton uncomplicated pregnancies delivered vaginally at term was included; the clinical characteristics of the 20 enrolled patients are summarized in Table 1. All women gave written informed consent before inclusion. Fresh placental tissue and maternal blood samples were collected immediately after delivery and sent for analysis to the laboratory of the Department of Health Sciences of the University of Florence.

Table 1
Characteristics of the enrolled patients and their newborns.

Clinical characteristics	Mean ± SD or n (%)
Age (years)	33.1 ± 4.8
Body mass index (Kg/m^2)	21.9 ± 2.5
Gestational age (weeks)	39.5 ± 1.3
Smoking during pregnancy	0
Smoking before pregnancy	3 (15%)
Newborn sex:	9 (45%)
Male	11 (55%)
Female	
Newborn weight (g)	3260 ± 407

2.2. RNA extraction and quantification by Real-Time PCR for TDO placental expression

IDO1 expression has been previously demonstrated in placentas from healthy and pathological pregnancies (Chang et al., 2018). We therefore investigated TDO mRNA (*TDO2*) expression in distinct areas of healthy placentas. Samples of 0.5 × 0.5 × 0.5 cm were obtained from the maternal surface (decidua basalis) and fetal surface (chorion and thin layer of villi) and homogenized in 1 ml Trizol with magnetic beads, using TissueLyser (Quiagen, Hilden, Germany). The RNA was quantified by the NanoDrop spectrophotometer (ThermoFisher Scientific, Waltman, MA, USA) and 1 μg of RNA was used for the reverse transcription reaction with Prime Script RT reagent Kit Takara (Otsu, Japan), after treatment with DNase for 30 min at room temperature; the cDNA samples obtained were amplified with specific primers described below.

PCR amplification was carried out using SYBR Premix Ex Taq, the 18S rRNA housekeeping was used as a normalizer and the difference between CT values of the target gene and 18S gene was used to calculate the delta CT. Quantitative Real-Time PCR (qRT-PCR) was carried out using SYBR Premix Ex Taq (Takara) according to the manufacturer instructions on a Rotorgene RG-3000A cycle system (Qiagen) platform. The primer sequences used were the following: TDO2 fw: 5′-TTCCAGGTGCCTTTTCAGTT-3′ and rev: 5′-TGTCGGGGAATCAGGTATGT-3′; 18S fw: 5′-ATTAAGGGTGTGGGCCGAAG-3′ and rev: 5′-GGTGATCACACGTTCCACCT-3′. The cycle was set at 95 °C for 5 s, 55 °C or 52 °C for 30 s and 72 °C for 30 s, repeated 35 times.

2.3. Immunofluorescence for placental localization of IDO, TDO and Ang (1,7)

Double immunofluorescence analyses were performed on placenta samples (n = 20), that were embedded in paraffin, after fixation with 4% paraformaldehyde. Samples were taken from both the maternal and the fetal surface, including villi (tissue samples of 0.5 ×0.5 ×0.5 cm).

Paraffin section of 5 μm were cut by microtome and collected on adhesive slides, deparaffinized and rehydrated; the sections were soaked in PBS, followed by citrate buffer (pH 6.0) for 20 min at 95 °C to expose antigenic sites and then let to cool to room temperature (RT). Nonspecific binding sites were blocked with 10 mg/ml bovine serum albumin in PBS for 30 min at RT, with 0.2% triton X-100 (Sigma, Darmstadt, Germany), then were treated with a primary antibody, polyclonal rabbit anti-human IDO1 (Abcam, Cambridge, UK, 1:200), monoclonal mouse anti human-TDO (Novus Biologicals, Englewood, CO, USA, 1:200) and monoclonal mouse anti human-Angiotensin 1–7 (Cloud-Clone Corp., Katy, TX, USA 1:50) overnight at 4 °C, and then treated for 2 h at RT with a secondary goat anti-rabbit or anti-mouse antibodies, respectively, conjugated with Alexa Fluor AF594 (red fluorescence) or with fluorescein isothiocyanate FITC AF488 (green fluorescence), all from Life Technology (Thermo Fisher Scientific, Waltham, MA). The signal was amplified with anti-FITC Fluorescein/Oregon green Antibody for 1,30 h (Invitrogen, 1:100). Some samples were treated with the primary antibody anti human CD11c-PE (Immunotools, Katy, TX, 1:20) and CD83 (Immunotools, 1:20) overnight at 4 °C. Nuclei were counterstained with Hoechst 33342 (20 μg/ml; Sigma; blue fluorescence). Omission of primary antibodies was used as negative controls. Slides were mounted with Fluoromount (Sigma) and observed with Leica DMLB microscope (equipped for epifluorescence; Leica Microsystems GmbH, Wetzlar, Germany). For each slide, 5 images were acquired and IDO1$^+$, TDO$^+$, IDO1$^+$/TDO$^+$ and TDO$^+$/Ang(1−7)$^+$ cells were counted. For each placental layer (maternal basal plate, villi, chorion) 1000 cells were counted for IDO1, TDO and Ang(1−7) expression. Images were acquired by Leica DC200 microscope digital colour camera and Leica DC Viewer software. Adobe Photoshop CS8 software (Adobe Systems Incorporated, WA) was used for image processing and figure creating.

2.4. Plasma tryptophan and kynurenine determinations

Blood samples were collected by venipuncture into tubes containing EDTA and centrifuged at a 2000 RPM for 10 min. One ml of plasma was stored at − 80 C°. Tryptophan and kynurenine plasma levels were measured by ELISA immunoassay (ImmuSmol, Bordeaux, France) according to the instructions, and their ratio was calculated and compared.

2.5. Etichs approval

Ethics approval for this study was granted by the local ethics committee (Area Vasta Centro, protocol number 16022_bio).

2.6. Statistical Analysis

Continuous variables were expressed by mean (SD) while categorical ones by absolute and relative frequencies. In order to evaluate the correlation between continuous variables, Pearson's correlation coefficient and its 95% confidence interval was used. To compare the percentage of cells expressing the enzymes studied between different placental areas, a simple Generalized Estimating Equation (GEE) linear regression model was used. Then, a multiple GEE linear regression model was used to adjust for potential confounding factors. Statistical significance was set at $p < 0.05$.

3. Results

3.1. TDO2 placental expression

TDO2 was expressed in all samples, and quantitation of mRNAs showed a similar expression between the maternal and fetal sides of the placenta (Fig. 1). In addition, a strong positive correlation of *TDO2* expression between the two sides was found (Pearson 0.61, $p < 0.01$).

A moderate positive correlation was found between TDO mRNA and TDO protein (TDO$^+$ cells) in the maternal surface of the placenta (Pearson 0.46, $p < 0.05$).

3.2. Placental localization of IDO and TDO

TDO protein was localized both in the fetal and maternal side of the placenta. TDO$^+$ cells were 51% of the total cells in maternal surface, 50% in villi and 55% of the total cells in fetal surface (Fig. 2 and Table 2). No significant difference was found in the percentage of cells expressing IDO1, TDO, or both, between the maternal, fetal and villi surface (Table 2). At the multivariate analysis, the percentage of TDO$^+$ and IDO1$^+$ cells appeared to be influenced by pre-gestational smoking and newborn weight ($p < 0.05$) (Tables 3 and 4). In particular, pre-gestational smoking was associated with a decrease in the percentage of IDO$^+$ cells, and an increase in TDO$^+$ cells.

A significant, very strong correlation was found between the percentage of TDO$^+$ and IDO1$^+$ cells in the villi, as reported in Table 5

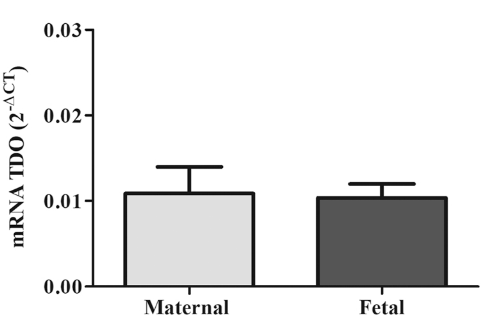

Fig. 1. *TDO2* expression in distinct areas of the placenta by Real-Time PCR. Data are expressed as Mean ± SEM, n = 20.

(Pearson 0.87, $p < 0.01$), but not at the maternal or fetal placental surface.

When we looked at what type of cells expressed IDO1 and TDO, we observed that in the villous tree, which connects to the fetal surface (chorionic plate) and the maternal surface (basal plate), the syncytiotrophoblasts, as well as some mesenchymal and endothelial cells of the villous core, were IDO1$^+$ or TDO$^+$. Double staining revealed that TDO$^+$ cells were also CD83$^+$ and CD11c$^+$ in maternal surface and in the villi (Figs. 3 and 4), suggesting that here TDO was expressed also by antigen presenting cells (APC), such as mature dendritic cells and macrophages. In particular, TDO$^+$/CD83$^+$ cells were 71% of the total TDO$^+$ cells in maternal surface and 53% in villi, and TDO$^+$/CD11c$^+$ cells were 51% of the total TDO$^+$ cells in maternal surface and 47% in the villi. No CD83 or CD11c$^+$ cells were present in the fetal side of the placenta, as expected, and in this area of the placenta the cells that expressed IDO1 and TDO were mesenchymal fibroblast-like chorionic cells (Koo et al., 2012).

3.3. Placental localization of Ang (1,7) and co-expression with TDO

When we assessed the possible co-expression of Ang(1−7) and TDO in the placenta by immufluorescence we observed that TDO$^+$/Ang (1−7)$^+$ cells were localized in all three placental areas, with a higher presence in fetal side and in villi compared to the maternal surface ($p < 0.0001$, Fig. 5). Fig. 5 shows cells expressing both TDO and Ang (1−7) by immunofluorescence.

At the multivariate analysis, the percentage of Ang(1−7)$^+$/TDO$^+$ cells seemed to be influenced by maternal age and pre-gestational smoking ($p < 0.01$), by newborn's weight and sex ($p < 0.001$), and by placental weight ($p = 0.03$). In particular, pre-gestational smoking was associated with an increase Ang(1−7)$^+$/TDO$^+$ cells (Table 6).

3.4. Maternal plasma Kyn/Trp ratio

ELISA assay showed mean Kyn levels of 597.2 ng/ml and mean Trp levels of 8.25 μg/ml in maternal plasma and the Kyn/Trp ratio was 0.08 ± 0.01. This ratio was not significantly correlated with the expression of IDO1 and TDO in the placenta and with the patient's characteristics.

4. Discussion

Our study showed that *TDO2* mRNA and protein were expressed on both the maternal and fetal side of the placenta. In addition, a significant correlation was found between *TDO2* mRNA and the TDO protein expression in the maternal surface. Although IDO1 has been demonstrated in healthy placentas, its distinct localization in all placental areas has not yet been detailed, nor its co-expression with TDO. The number of cells expressing TDO or IDO1 was similar between the three examined areas (maternal and fetal side of the placenta, and villi). However, a significant, very strong correlation was found between the percentage of TDO$^+$ and IDO1$^+$ cells only in the villi. Consistently, this tissue is highly involved in the exchange of oxygen, nutrients, and amino acids necessary for a healthy pregnancy (Broekhuizen et al., 2021).

A high number of TDO$^+$ cells also co-expressed IDO1. Some of the TDO$^+$ cells also co-expressed Ang(1−7), and this co-expression was significantly more represented in the villi and fetal side compared to the maternal side. We found that pre-gestational smoking was associated with a decrease in the percentage of IDO$^+$ cells, and an increase in TDO$^+$ and Ang(1−7)$^+$/TDO$^+$ cells, likely to compensate the IDO$^+$ cells decrease. It is known that nicotine and other components of tobacco smoke are either processed by or transported directly through the placenta (Suter et al., 2019), and that maternal smoking during pregnancy has been found to impair placental structure and function (Niu et al., 2015)(Niu et al., 2015). Although we were not able to study the effect of antenatal smoking, as none of our patients smoked while pregnant, we believe that our results showing an influence of pre-gestational smoking on TDO, IDO, and Ang(1−7) expression may be

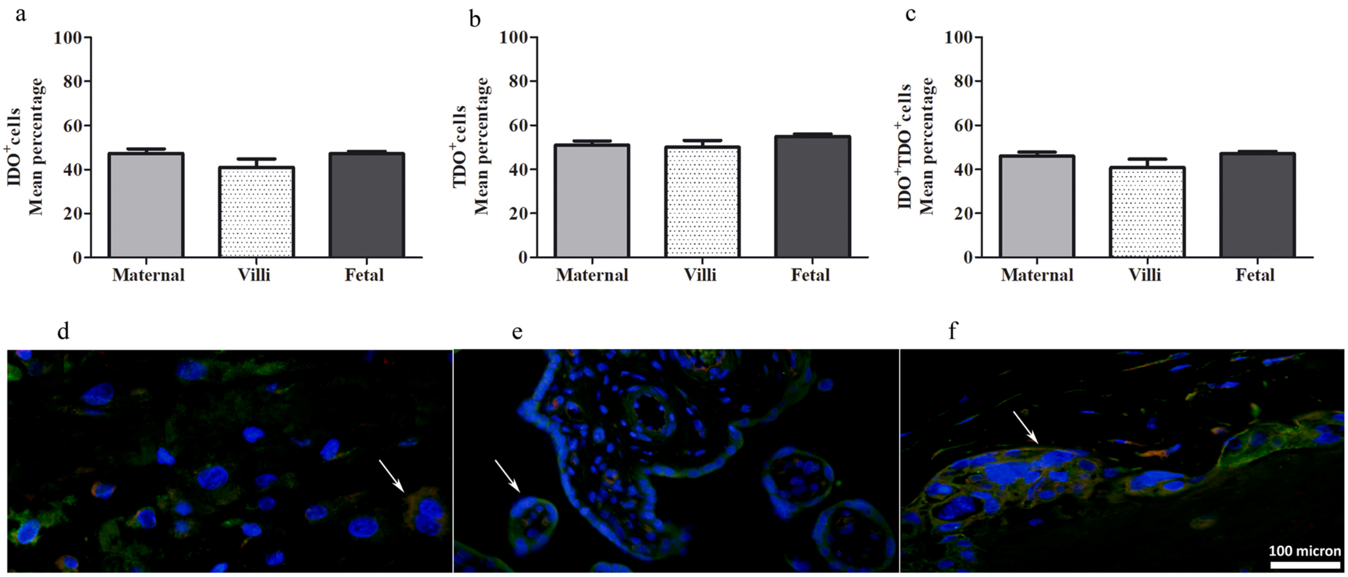

Fig. 2. (a-c) Percentage of IDO1$^+$ (a), TDO$^+$ (b) and IDO$^+$/TDO$^+$ (c) cells in distinct surfaces of the placenta. Mean ± SD, n = 20. $p > 0.05$ for all comparisons. (d-f) Representative pictures: expression of IDO1 (red), TDO (green), nuclei (blue), and merge (yellow-orange, the co-expression of IDO1 and TDO is indicated by the arrow), in maternal (d), villi (e) and fetal (f) areas; magnification 40X. Scale bar 100 µm.

Table 2
Comparison of IDO1 and TDO expression between different areas of the placenta. Data are expressed as percentage (%) of positive cells. Data reported as mean ± SD; n = 20. *Multivariate analysis correcting for possible confounding factors (gestational age at delivery; placental weight; newborn's weight and sex; maternal age, BMI and tobacco use).

Localization	Maternal surface (%)	Villi (%)	Fetal surface (%)	p-value* (villi vs maternal surface)	p-value* (fetal vs maternal surface)
IDO$^+$ cells	47.3 ± 2.10	41.0 ± 3.86	47.2 ± 1.07	0.47	0.73
TDO$^+$ cells	51.0 ± 2.00	50.1 ± 2.99	55.1 ± 1.14	0.97	0.06
IDO$^+$/TDO$^+$cells	46.0 ± 1.93	41.0 ± 3.87	47.0 ± 1.07	0.60	0.30

Table 3
Results of multivariate analysis showing the effect of pre-gestational smoking and newborn weight on the percentage of IDO1$^+$ cells.

Parameter	Estimate (95% CI)	p-value
Pre-gestational smoking	-6.2470 (−9.35 to −3.95)	< 0.0001
Newborn weight	0.0094 (0.0001–0.0187)	0.0466

Table 4
Results of multivariate analysis showing the effect of pre-gestational smoking and newborn weight on the percentage of TDO$^+$ cells.

Parameter	Estimate (95% CI)	p-value
Pre-gestational smoking	5.5759 (1.45–9.70)	0.0081
Newborn weight	0.0075 (0.0007–0.0142)	0.0295

Table 5
Correlation matrix between the percentage of TDO$^+$ cells and IDO$^+$ cells in maternal and fetal surfaces of the placenta, and in the villi, purified by possible confounding factors.

Pearson's CC (CI 95%) p-value	% IDO$^+$ cells in maternal surface	% IDO$^+$ cells in villi	% IDO$^+$ cells in fetal surface
% TDO$^+$ cells in maternal surface	0.59(−0.29 to 0.93) 0.14	/	/
% TDO$^+$ cells in villi	/	0.87(0.35–0.98) < 0.01	/
% TDO$^+$ cells in fetal surface	/	/	-0.54(−0.92 to 0.36) 0.19

of interest, and suggest that future studies should investigate the effects of tobacco use during pregnancy on the expression of these enzymes.

Our multivariate analysis also showed a significant association between percentage of IDO1$^+$ cells (Table 3) and TDO$^+$ cells (Table 4) and newborn weight. To our knowledge, no data are available on TDO/IDO expression and newborn weight. However, it has been recently reported an association between newborn weight and cord blood kyn metabolites (Tan et al., 2022).

The Kyn/Trp ratio measured in our cohort of women was not correlated with the protein expression of IDO1 and TDO in the placenta nor with the patient's characteristics. Currently the plasma Kyn/Trp ratio is used to express the activity of the extrahepatic Trp metabolism. However, the plasma free Trp concentration may depend on its binding to albumin, which can be modified by non-esterified fatty acids levels in pregnancy (Badawy, 2014; Badawy and Guillemin, 2019).

Few information is available on *TDO2* mRNA expression in the placenta of healthy women with physiological pregnancy, as previous studies mainly reported data from pathological pregnancies. *TDO2* mRNA has been previously detected in human placental explants of pregnant women delivered by c-section before labor, and its expression increased following ex vivo exposure to lipopolysaccharide (LPS) (Dharane et al., 2010). In one study, the explanted placental tissues obtained from pregnancies delivered at term or preterm, with or without intrauterine bacterial infection, showed elevated *TDO2* expression in response to LPS, which suggests that inflammatory mediated pathways can stimulate *TDO2* mRNA expression in the placenta (Manuelpillai et al., 2005).

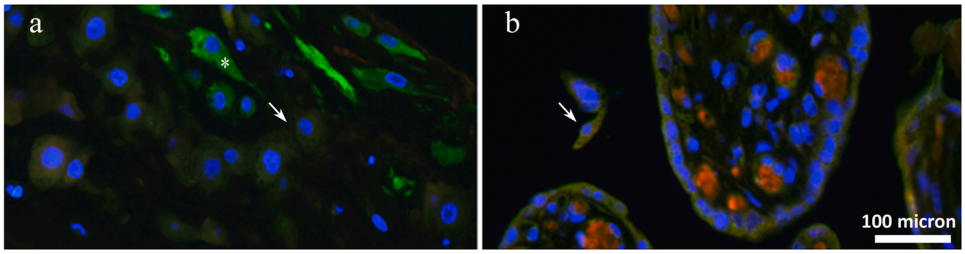

Fig. 3. Representative pictures in immunofluorescence of TDO^{+} $CD11c^{+}$ cells in maternal surface (a) and villi (b): CD11c (red), TDO (green, indicated by asterisk), nuclei (blue) and the merge (yellow-orange, the co-expression of CD11c and TDO is indicated by the arrow); magnification 40X. Scale bar 100 μm.

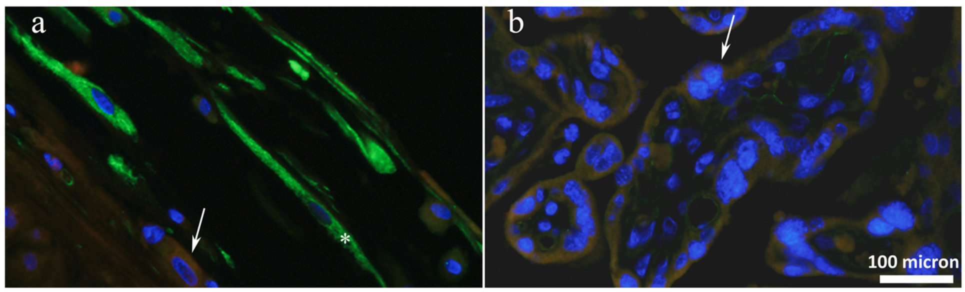

Fig. 4. Representative pictures in immunofluorescence of TDO^{+} $CD83^{+}$ cells in maternal surface (a) and villi (b): $CD83^{+}$ (red), TDO (green, indicated by asterisk), nuclei (blue) and the merge (yellow-orange, the co-expression of CD83 and TDO is indicated by the arrow); magnification 40X. Scale bar 100 μm.

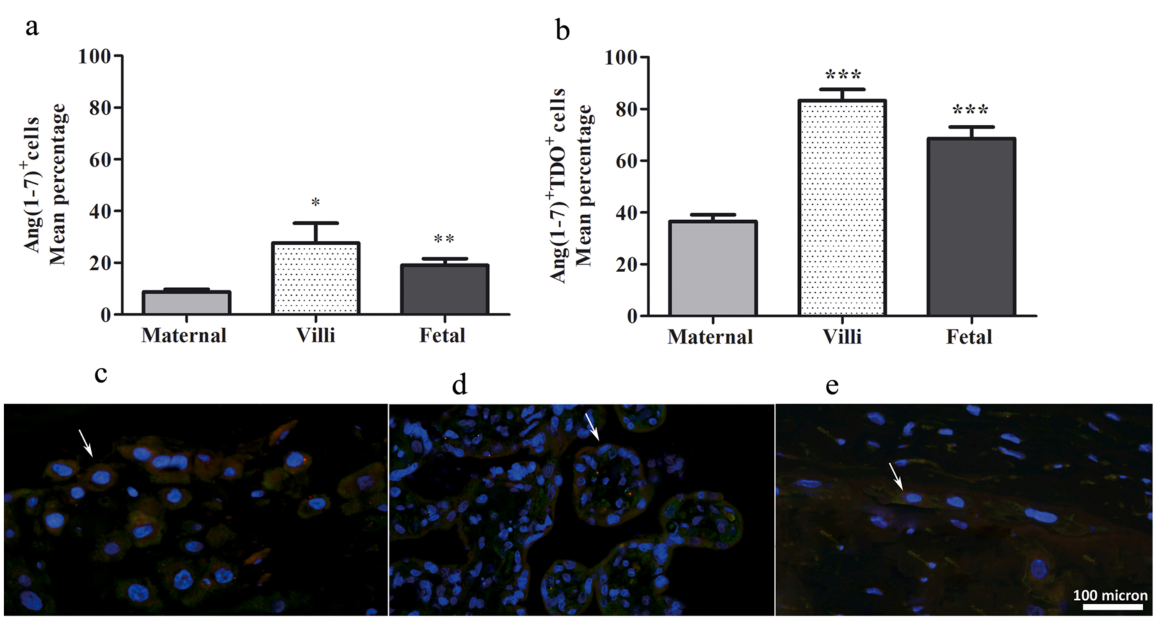

Fig. 5. (a-b) Percentage of $Ang(1-7)^{+}$ cells (a) and of $Ang(1-7)^{+}/TDO^{+}$ cells (b) in distinct surfaces of the placenta. Mean ± SD, n = 20. *$p < 0.05$, * *$p < 0.001$ vs maternal side. (c-e) Representative pictures for $Ang(1-7)^{+}$ (red), TDO^{+} (green), nuclei (blue) and the merge (yellow-orange, the co-expression of $Ang(1-7)^{+}$ and TDO is indicated by the arrow); in maternal (c), villi (d) and fetal side (e), magnification 40X. Scale bar 100 μm.

The comparison of *TDO2* mRNA expression between uncomplicated term pregnancies and pregnancies complicated by pre-eclampsia yielded conflicting results with some authors reporting no difference (Broekhuizen et al., 2020) and others showing a higher expression in placentas from women with pre-eclampsia compared to the healthy controls (Keaton et al., 2019). The latest finding was interpreted by the authors as a compensatory effect to the significant reduction in IDO1 expression in pathological conditions. Conversely, a significant decrease of both *TDO2* mRNA and protein expression was observed in the placentas of pregnancies complicated by fetal growth restriction (FGR) compared to controls (Murthi et al., 2017). One limitation of previous studies is that they evaluated *TDO2* mRNA expression in the whole placenta, while in the present manuscript we have described the expression of *TDO2* mRNA in the two distinct sides of the placenta, the maternal and the fetal surface. TDO protein was localized in the maternal side, in the villi and in the fetal side of the term placentas, specifically in decidual cells, macrophages, dendritic cells, syncytiotrophoblasts, mesenchymal or endothelial cells of the villous core, and chorionic plate's cells, and these cells co-express IDO1. The observed correlation between TDO2 mRNA and TDO protein expression in the maternal surface may depend precisely on the presence of immune system cells, which are actively involved in maintaining immunity.

Table 6
Results of multivariate analysis showing the effect of maternal and neonatal parameters on the percentage of Ang(1–7)$^+$/TDO$^+$ cells.

Parameter	Estimate (95% CI)	p-value
Maternal side	108.1674 (25.3627–190.9722)	0.0105
Fetal side	32.6279 (23.3288–41.9269)	< 0.0001
Villi	47.2843 (35.6161–58.9525)	< 0.0001
Placenta weight	0.0304 (0.0031–0.0578)	0.0294
Maternal age	-0.9436 (−1.4389 to 0.4483)	0.0002
Pre-gestational smoking	14.3158 (9.6868–18.9448)	< 0.0001
Newborn sex	-11.9331 (−181288 to 57374)	0.0002
Newborn weight	-0.0153 (−0.0242 to 0.0063)	0.0008

TDO protein localization in the placenta and its co-expression with IDO1 have not been previously studied. Most studies focused on IDO1 expression because of its major role in the placenta, where it drives the KP, regulating maternal tolerance toward the fetus (Blaschitz et al., 2011; Hönig et al., 2004; Kudo et al., 2020; Sedlmayr et al., 2014). These studies yielded conflicting results, in part due to the different reagents and antibodies used for the research (Broekhuizen et al., 2020).

At the end of pregnancy chorionic vascular endothelial cells are intensely stained for IDO1, which is also localized in the endothelial cells of the intervillous space, while trophoblast cells in term placentas are generally IDO1 negative (Blaschitz et al., 2011). Other studies have shown that the macrophages, the syncytiotrophoblast, the fetal blood vessels endothelial cells in the villous stroma, and the chorionic cells of placentas at the end of pregnancy were IDO1 positive (Hönig et al., 2004; Kudo et al., 2020, 2004, 2003) and this was confirmed in our study.

The renin-angiotensin system (RAS) is classically considered the major regulator of fluid homeostasis and blood pressure (Te Riet et al., 2015) and a tissue-specific RAS has been described in several organs, including the placenta (Cooper et al., 1999; Marques et al., 2011). Ang (1−7) localization in the healthy term placenta has been described in the villi, both in the syncytiotrophoblast and in the cytotrophoblast, and in decidual cells (Valdés et al., 2006). The detection of a co-expression of Ang(1−7) and TDO in healthy placentas, which we found to be more represented in the villi and fetal side of the placenta compared to the maternal side, is a novel finding, and suggests the existence of an interaction between the KP and RAS in pregnancy. All components of the intrauterine RAS have been identified in term human decidua, myometrium, and fetal membranes (Pringle et al., 2017, 2011) and they are involved in placental development through processes such as angiogenesis, modulation of placental blood flow.

The Kyn/Trp ratio measured in our cohort of women immediately after delivery was in agreement with physiologic levels described in healthy subjects (Sakurai et al., 2020). It has been observed that the rate of Trp degradation in maternal plasma increases during pregnancy (Schröcksnadel et al., 1996) and that, after delivery, Trp concentration returns to physiological levels. Kyn concentration, however, remains high, contributing to increased kyn/trp ratio (Schröcksnadel et al., 1996, 2006).

The evaluation of the mRNA expression, protein localization, and activity of IDO1 and TDO in a healthy pregnancy is essential to recognize their variations in pathological conditions and their effects on the maintenance of pregnancy. These enzymes are part of the complex tolerogenic signaling network, interconnected with anti-inflammatory signals, which together provide a favorable niche for maintaining the embryo's development and fetal growth. Therefore, any alterations of their expression or activity might predispose to adverse obstetric outcomes such as miscarriage (Ban et al., 2013; Wei et al., 2020) or hypertensive disorders of pregnancy (Broekhuizen et al., 2020; Iwahashi et al., 2017). Interestingly, in IDO-deficient mice, TDO might compensate for lack of IDO1 activity during gestation (Baban et al., 2004), and such compensatory immunosuppressive mechanism is insensitive to IDO-inhibitors (Baban et al., 2004). TDO activity was reported to contribute to Trp catabolism during the development of the embryo, the fetus and the placenta of mice (Suzuki et al., 2001). However, in late pregnancy, also other mechanisms must operate, the most likely of which is increased flux of free Trp down the KP (Badawy, 2017). This highlights the need to consider the role of TDO in pregnancy. Understanding placental expression and localization of TDO in physiological pregnancy is important to put the basis for investigation of deviations from the normal enzyme expression in pathological conditions and eventually to use it as a target of therapeutic interventions. Moreover, the co-localization of TDO with Ang(1−7) may suggest a link between the two systems studied in both healthy and complicated pregnancies such as in preeclampsia (Chen et al., 2014). It was demonstrated that ACE1 mRNA was higher in the decidual explants from women with a male fetus, and that Ang(1−7) was more expressed in the placenta of female fetus, suggesting that premature births of male newborn, with less Ang(1−7) expression, could be more common than that of female ones and that the maternal decidual RAS is regulated in a sex-specific manner (Wang et al., 2012).

There are open questions about the role of tryptophan degradation for feto-maternal tolerance in the placenta. Further research needs to clarify above all the role of TDO enzyme. Conversely, some preclinical and clinical data report on TDO and IDO1 role in pathologic pregnancies. IDO1 expression was demonstrated to be downregulated in placentas obtained from pregnancy complicated by pre-eclampsia (Santoso et al., 2002). Interestingly, clinical evidence has shown a decrease of Trp concentrations in placentas from women with pre-eclampsia compared with healthy controls after delivery (Keaton et al., 2019), suggesting that there is an increase of its catabolism probably due to an increase of TDO expression and/or activity; this increase compensates the observed IDO1 down-regulation (Kudo et al., 2003), therefore it remains to be clarified how the two enzymes, involved in the functional pathway of kynurenine at the level of the placenta, can undergo changes in expression or activity in different pathological conditions.

Our results suggest that future research on IDO1 and TDO activity or placental expression should examine the different placenta areas separately, and that when studying expression of the enzyme involved in the KP, correction for potential confounders such as maternal pre-gestational smoking and newborn's weight should be applied. Furthermore, we have reported the percentage of cells expressing these enzymes in the placenta as well as the plasmatic Kyn/Trp ratio in physiological conditions, which may be used for comparison in future studies assessing variation of tryptophan metabolism in pathological conditions.

In conclusion, the mechanisms contributing to maternal immune regulation in human placenta are difficult to explore. Further research is needed to strengthen the current findings and to explore potential clinical applications. In particular, additional investigations should focus on the role of molecules such IDO1 and TDO in the maintenance of immune tolerance towards the fetus, ideally through in vivo studies, to evaluate their activity in a complex microenvironment.

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CRediT authorship contribution statement

Angela Silvano: Methodology, Investigation, Data curation, Writing – original draft. **Viola Seravalli:** Methodology, Validation, Writing – original draft, Writing – review & editing. **Noemi Strambi:** Investigation, Data curation. **Enrico Tartarotti:** Methodology, Writing – review & editing. **Lorenzo Tofani:** Formal analysis. **Laura Calosi:** Investigation. **Astrid Parenti:** Conceptualization, Project administration, Resources, Validation, Visualization, Writing – review & editing. **Mariarosaria Di Tommaso:** Conceptualization, Methodology, Project administration, Validation, Visualization, Writing – review & editing.

Decleration of Competing Interest

The authors report no conflict of interest.

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